EVALUATION OF ANTIBACTERIAL ACTIVITIES OF MEDICINAL PLANTS

A
DISSERTATION
SUBMITTED TO THE CENTRAL DEPARTMENT OF MICROBIOLOGY
TRIBHUVAN UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
MASTER OF SCIENCE IN MICROBIOLOGY
(ENVIRONMENT AND PUBLIC HEALTH)

BY
NARESH MAHARJAN

CENTRAL DEPARTMENT OF MICROBIOLOGY
TRIBHUVAN UNIVERSITY
KIRTIPUR, KATHMANDU, NEPAL
2008

RECOMMENDATION

This is to certify that **Mr. Naresh Maharjan** has completed this dissertation work entitled **EVALUATION OF ANTIBACTERIAL ACTIVITIES OF MEDICINAL PLANTS** as a partial fulfillment of M.Sc. degree in Microbiology under our supervision. To our knowledge this work has not been submitted for any other degree.

…….	……	……..
Dr. Anjana Singh, Ph.D.	**Ms. Shaila Basnyat**	**Dr. Mangala Devi Manandhar**
Associate Professor	Lecturer	Professor of Chemistry
Central Department of Microbiology	Central Department of Microbiology	Central Department of Chemistry
Tribhuvan University	Tibhuvan University	Tribhuvan University
Kirtipur, Kathmandu	Kirtipur, Kathmandu	Kirtipur, Kathmandu

Date: …2065-4-31…….

CERTIFICATE OF APPROVAL

On the recommendation of **Dr. Anjana Singh, Ms. Shaila Basnyat** and **Prof**. **Dr. Mangala Devi Manandhar** this dissertation work of **Mr. Naresh Maharjan** is approved for the examination and is submitted to the Tribhuvan University in the partial fulfillment of the requirement of Master of Science Degree in Microbiology.

……

Dr. Anjana Singh, Ph.D.

Head of Department

Central Department of Microbiology

Tribhuvan University

Kirtipur, Kathmandu

Nepal

Date: …2065-4-31……..

BOARD OF EXAMINER

Recommended by:

……..……..……..

Dr. Anjana Singh, Ph.D.

Supervisor

……..……..……..

Ms. Shaila Basnyat

Supervisor

……..……..……..

Prof. Dr. Mangala Devi Manandhar

Supervisor

Approved by:

……..……..……..

Head of Department

Examined by:

……..……..……..

External Examiner

……..……..……..

Internal Examiner

Date: …2065-5-6……..

ACKNOWLEDEMENT

I would like to offer my profound gratitude to my supervisor Dr. Anjana Singh, Associate Professor of Central Department of Microbiology, T.U, for his encouragement, kind support and precious help during this work.

I would like to express my heartiest gratitude and sincerity to my supervisor Prof. Dr. Mangala Devi Manandhar of Central Department of Chemistry and Ms. Shaila Basnyat, Lecturer of Central Department of Microbiology, T.U., for their kind supervision, encouragement and providing me such a golden opportunity to carry out the dissertation work under their guidance. I shall remain indebted for their continuous guidance, valuable direction, useful suggestions, comments, reassurance and inspiration at all time have boon of immense value without which this work would not have been accomplished. Thus, all the credit of this work goes to them.

I would like to offer my heartfully thanks to Dr. Shreekant Adhikari, Associate Professor of Central Department of Microbiology, Dr. Dwij Raj Bhatta, Associate Professor of Central Department of Microbiology, Mr. Binod Lekhak, Assistant Professor of Central Department of Microbiology and Dr. Surya Kant Kalauni, Central Department of Chemistry who help me in every step of this dissertation.

I am also obliged to all the teachers and staffs of Central Department of Microbiology, T.U for their kind cooperation, generosity and motivation.

Specially, thanks to my friends Dinesh Maharjan who helped every step of this dissertation. Also thanks to my friends Shree Krishna Shrestha, Narendra Maden and Sharmila Tandukar for their kind support for this dissertation.

Date…2065-4-31……

……
Naresh Maharjan

ABSTRACT

In this study, antibacterial activities of 9 different medicinal plants were tested against 10 common pathogenic bacterial species.

Medicinal plants were selected on the basis of their common use among the different ethnic groups for common disorder. The selected medicinal plants were *Acorus calamus, Aegle marmelos, Asparagus racemosus, Holarrhena antidysenterica, Mimosa pudica, Terminalia bellirica, Terminalia chebula, Tinospora cordifolia* and *Woodfordia fruticosa*. These medicinal plants were subjected to solvent extraction using hot solvent of increasing polarity into 3 fractions viz: hexane, ethylacetate and methanol using soxhlet apparatus. After removing the solvent under reduced pressure, residues were suspended separately in DMSO. The highest yield was obtained in methanol fraction of *Terminalia chebula* (46.1%) while lowest in hexane fraction of *Asparagus racemosus* (0.7%).

The bacteria selected for the study were *Staphylococcus aureus, Escherichia coli*, *Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis, Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella* Typhi, *Salmonella* Paratyphi A and *Shigella dysenteriae.*

Out of nine tested plants, four plant extracts (44%) showed activity against at least five or more test bacteria and 5 plant extracts (56%) were active against three or less than three bacteria. None of the tested plants extracts were active against all the tested bacteria. *Asparagus racemosus* was the least effective against the tested bacterial species.

Plants extracts showed significant antibacterial activity towards Gram positive bacteria than Gram negative bacteria. *Staphylococcus aureus* was the most susceptible bacteria being sensitive to 18 fractions from 8 medicinal plants. *Proteus vulgaris* was the most resistance bacteria being resistance to all selected plants. Largest ZOI (31 mm) was produced by ethylacetate fraction of *Terminalia bellirica* while lowest MBC of 3.12 mg/ml was shown by ethylacetate fraction of *T. bellirica* against *E. coli*.

Key words: Medicinal plants, Antibacterial activity, Plant extracts

TABLE OF CONTENTS

LIST OF ABBREVIATION

ATCC	-	American Type Culture Collection
DMSO	-	Dimethyl sulfo oxide
EHEC	-	Entero Hemorrhagic *Escherichia* coli
EIEC	-	Entero Invasive *Escherichia coli*
EPEC	-	Entero Pathogenic *Escherichia coli*
ETEC	-	Entero Toxigenic *Escherichia coli*
MBC	-	Minimum Bactericidal Concentration
mcg	-	microgram
MHA	-	Mueller Hinton Agar
MIC	-	Minimum Inhibitory Concentration
NA	-	Nutrient Agar
NB	-	Nutrient Broth
UTI	-	Urinary Tract Infection
ZOI	-	Zone of Inhibition

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPH

LIST OF APPENDICES

CHAPTER-I

1. INTRODUCTION

Nature is extremely discret. Counterpoise is its distinctive feature and justice its governing law. If man was prone to be afflicted by disease, it was only just for nature to provide for balancing factors to set things aright through the shape of herbs and plants (Adly, 1980). Plants and plant products have been a source of foods and medicines from the dawn of human civilization. The plants used by human to cure the illness and to relieve the sufferings are called “Medicinal Plants”.

The use of plants and plant products as medicine can be traced back far as the beginning of human civilization. The earliest mention of medicinal use of plants on the Indian sub-continent has been found in the ‘Rigveda’ which was written between 4500-1600 BC. It is supposed to be the oldest repository of human knowledge. It is Ayurveda, the foundation of medicinal science of Hindu culture, in its eight divisions, deals with specific properties of drugs and various aspects of science of life and the art of healing (HMG, 1993).

After a period of disregard and decline, these traditional systems of “green medicine” are once again back to the center stage of our health programmes. There has been a steady increase in demand for so called natural medicine and these systems i.e. Ayurveda, Unani, Chinease have been now regarded among the scientific community, with respect all the world over (Shivarajan and Balachandran, 1994).

It is difficult to ascertain just how many of earth’s estimated 2, 50,000 species of higher plants (Cowan, 1999) are used in traditional medicines but one estimate puts it somewhere between 35,000 and 70,000 species. Among these, at least 6,500 medicinal plants are found in Asia continent which is one of the largest biodiversity regions of the world (ICS UNIDO, 2006). Nepal Himalaya, globally significant and biologically diverse ecosystem, produce a wide range of unique and valuable medicinal plants and expected to be home of more than 7,000 higher plants (Joshi and Joshi, 2001). Out of which, 701 are medicinal plants (DPR, 2007), whereas Tiwari and Shrestha (2000) published a list of

1630 species having medicinal value. The knowledge about these medicinal plant resources are deeply rooted in the tradition and culture of Nepalese people living in rural areas (Rijal, 1994).

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine. Their impact is particularly large in medicines and the emergence of widespread drug resistance (Okeke et al; 2005). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug resistant pathogens (Bandow et al; 2002). Bacterial and fungal pathogens have evolved numerous defense mechanisms against anti-microbial agents, and resistance to old and newly produced drugs in on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has lead to the screening of several medicinal plants for potential antimicrobial activity (Scazzocchia et al; 2001). Natural products of higher plants may give an new source of anti-microbial agents with possibly novel mechanisms of action (Machado et al; 2003)) contrary to the synthetic drugs, antimicrobial of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu et al; 1999). In addition to this problem, antibiotics are sometimes associated with adverse effects on host which include hypersensitivity, depletion of beneficial gut and mucosal microorganism, immunosuppression and allergic reaction (Idos et al; 1968). So, there is a need to develop alternative antimicrobial drugs.

Most of the plants possess one or more of the medicinal properties, viz: antibacterial, antifungal, antiviral, cardiotonic, anthelmintic, anticancer, sedative, laxative, diuretic and others (Parajuli et al; 2001). Toxic effects of the plants are due to active constituents in various parts such as root, shoot, bark, and fruit etc, and sometime through out whole plant (Nickel, 1959). The main groups of active components are alkaloids, glycosides, saponins, essential oils, mucilage, tannins, bitter principles etc, which inhibit/kill the microorganisms are called antimicrobials. A major part of traditional therapies involve the use of plant extracts (Akerele, 1993).

Generally, these plant extracts are extracted with different kinds of solvent. Such extracts which inhibit and/or kill microorganism are called antimicrobials. Different kinds of medicinal plants may posses such antimicrobial substances to different extent. (Cox and

Balick, 1994). Various surveys carried out in USA, UK and other countries showed that antibiotics can be obtained from higher plants too (Dixit and Tripati, 1982).

Innumerable biologically active compounds that are found in plants (Alade and Irobe, 1993) posses antibacterial properties (Branter and Grein, 1994). World widely evaluation of plants possessing antibacterial activity for various diseases is growing in recent years (Clark and Hufford, 1993). This present research is done for the evaluation of antibacterial activities of some medicinal plants which are used for treatment of some infectious diseases. Nevertheless demonstrating activity in a bioassay is a necessary first step in the drug development process (Cox and Balick, 1994)

From the time immemorial many medicinal plants are well known in Nepal. The history of medicine and medicinal plants in the country can be traced to the Vedic period, where Nepal-Himalaya was mentioned as a sacred heaven of potent medicinal and Aromatic plants. These medicinal systems, which have evolved over centuries, are increasing recognized for their complementary and sometimes superior role over western medical systems. In addition to Ayurveda, the medical knowledge practiced in Nepalese village based on their experiences is also valuable knowledge of man kind (Baral and Kurmi, 2006).

The use of medicinal plants in traditional medicine is widespread in Nepal, with majority of the population relying on it. This can be explained by such factors as the lack of sufficient health post, doctors, medications, road facilities and the high expenses associated with such treatments (Taylor et al; 1996). In Nepal, about 75-80% of the rural population are said to use these traditional remedies (Manandhar, 1980). Although the use of herbal medicine is widespread in Nepal, only few species have actually been screened for their biological activity. It is therefore worthwhile to further investigate the activity of Nepalese medicinal plants.

CHAPTER-II

2. OBJECTIVES

2.1 General objective

Evaluation of antibacterial activities of some medicinal plants (*Acorus calamus, Aegle marmelos, Asparagus racemosus, Holarrhena antidysenterica, Mimosa pudica, Terminalia bellirica, Terminalia chebula, Tinospora cordifolia* and *Woodfordia fruticosa*).

2.2 Specific objective

1. To extract crude compound from the above selected medicinal plants using different solvent (Hexane, Ethylacetate and Methanol).
2. To determine antibacterial activities of different extracted fraction of the plants against Gram positive and Gram negative bacteria.
3. To determine minimum bactericidal concentration (MBC) value of different active extracted fraction of plant.

CHAPTER-III

3. LITERATURE REVIEW

According to WHO, a medicinal plant is any plant containing substances in one or more of it's organs that can be used for therapeupeutic purpose or service as impetus for chemopharmaceutical semi-synthesis (PCONS and RIDA, 1995).

World is endowed with a rich wealth medicinal plants. Man cannot survive on this earth for long time without the plant kingdom because the plant products and their active constituents played an important role. Herbs have always been the principal form of medicine in Indian subcontinent and presently they are becoming popular throughout the world, as people strive to stay health in the face of chronic stress, chronic diseases and pollution, and to treat illness with medicines that work in count with the body's own defense (Perumalsamy et al; 1998). There is a widespread belief that green medicines are healthier and more harmless or safer than synthetic ones.

It is found that plant materials are present in or have provided the models for 50% western drugs (Robbers, 1996). An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as for traditionally used rural remedies (UNESCO, 1998).

Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, much recent attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine (Essawi and Srour, 2000). Research on medicinal plants has found that they posses anti-microbial activities (Glosh et al; 1993). The substance that can either inhibit the growth of pathogens or kill them and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs (Ahmad and Beg, 2001). It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant

microbial pathogens (Barbour et al; 2004). Plant based antimicrobial represent a vast untapped source for medicines and further exploration of plant antimicrobials needs to occur (Kokoska et al; 2002).

Historical Overview

Medicinal plants are used in therapy as sources of certain substances and drugs having remedial effects since prehistoric ages. The beneficially and medical properties of plants have been known and used by human beings in some form or other (Adly, 1980).

The earliest mention of the medicinal use of plants has been found in "Rigveda" sometimes between 4500-1600 B.C which is considered as the oldest repository of human knowledge on plant usage. Rigveda mention 67 plants having therapeutic effects. Yajurveda lists 81 plants and Atharveda 290 plants. Later "Charak Samhita" (600 B.C) lists a total of 341 plants and their use. "Susruta Samhita" also dealt with plants related to medicine (Sharma, 1981).

Medicinal plants of Nepal

Nepal falls neither within Tropical nor temperate latitudes. However, the position of the himalaya has made it possible to create life zones that are tropical, sub-tropical, warm-temperate, cool temperate, subalpine, alpine and arctic (HMG/N, 2002). Because of such unique geographical setting and topographical variations, Nepal has remained a metting place of species of the various bioclimatic zones of the world highlighting the country's richness of richness of plant diversity.

In Nepal, the list of medicinal plant diversity recorded 700 species from 7000 species of higher plant. Distribution pattern of these plants are found approximately 49.2% in the tropical zone, 53.96% on the subtropical zone, 35.7% in temperate zone, 18.09% in the subalpine zone and 7.14% in the alpine zone (Malla and Shakya, 1984-85).

3.1 Brief description of plants species included in this study

3.1.1 *Acorus calamus* (Nep: Bojho, Eng: sweet flag)

Family: - Araceae

Description:-Perennial, semiaquatic, erect, aromatic herb, root stock thick, creeping, 5-5.4 x 0.6-2 cm wide. Leaves distichous, nerves parallel, spathe 4-8 cm, tapering, covered with small, yellow-green, two sexual flowers (HMG, 1993).

Use: - rhizome is pungent, carminative, emetic, laxative, diuretic, helminthiasis, used in dyspepsia, remitted fevers, cough, bronchitis, dysentery, gout, odontalgia, epilepsy, depression and other mental disorder (Baral and Kurmi, 2006).

Chemical constituents: - Acoradin together wih 2, 4, 5-trimethoxy-benzaldehyde; 2, 5 dimethoxybenzoquinone, galangin and sitosterol were isolated from rhizomes. Dry rhizomes contain a yellow aromatic volatile oil, Glucoside acorin. Leaves contain oxalic acid and calcium (Joshi, 2006).

3.1.2 *Aegle marmelos* (Nep: Bel; Eng: Bael fruit tree)

Family: - Rutaceae

Description :- A small tree, 10m high, leaves alternate, leatlets 3-5 ovate-lanceolate, lateral sessile, terminal long petioled; flowers 2- sexual, greenish white, 3cm in diameter, in short panicles, sweet-scented, fruit 5-12cm in diameter, globose, oblong or pyriform, rind grey or yellow pulp sweet, thick and orange coloured (HMG/N, 1993).

Medicinal uses: - Pulp of ripe fruit is aromatic, cooling and laxative, unripe or half-ripe fruit is used as astringent, digestive, stomachic and in diarrhea (HMG/N, 1993). Root bark is useful in hypochondriasis, melancholia and palpitation of heart. Leaf juice is applied in abscess (Baral and Kurmi, 2006).

Chemical constituents: - Umbelliform, skimmianine, marmin, β-sitosterol from immature barks and roots. Fruit contains psoralein and tannic acid; aegelinol, furocoumarlin, marmelosin, marmelide. Ripe fruit, xanthotoxol, marmesin etc. The pulp contains mucilage, pectin, reducing sugars, tannin, a volatile oil, bitter principle. Fresh leaves yield yellowish green oil (Joshi, 2006).

3.1.3 *Asparagus racemosus* (Nepali: Kurilo; Eng: Asparagus)

Family: - Liliaceae

Description: - An extensively scandent, much-branched spinous under- shrub (Joshi, 2006). Root tuberous 5-13cm long, 5-8cm wide, pointed at both ends longitudinally striated. Leaves or flattened branchlets (Cladodes) 12-25mm long, spreading. Flowers bisexual, racemes, very slender, 4mm long and perianth petaloed (HMG/N, 1993).

Medicinal uses: - It is galactogogue, diuretic, antidysenteric and demulcent. It cures swelling, diseases. It is a good remedy for vaginal disorders like leucorrhoea, uterine disorders, excess of bleeding and colicky pain. It is used against ulcerated tongue. In Ayurveda, it is used in diabetes, jaundice and other urinary disorders, roots are used to cure diarrhea as well as in dysentery. It is also used in fevers.

Chemical constituents: - Fresh leaves yield diosgenin. Plant yields Shatavarin I to IV. Fruits and flowers contain glycosides of quercetin, rutin and hyperoside, sitosterol, stigmasterol and their glucosides (Joshi, 2006).

3.1.4 Terminalia chebula (Nep: Harro, Eng: Myrobalan)

Family: - Combretaceae

Description: - Large deciduous tree, 24-30m high; leaves 7.6-15.2cm long, ovate or elliptic, acute, petioled. Flowers all hermaphrodite, sessile, dull-white or yellow. Fruit 108-3.3cm ellipsoidal or obvoid or obovoid from a broad base and glabrous, 5-ribben when dry (Baral and Kurmi, 2006).

Medicinal uses: - Fruit is light digestive, astringent, antiseptic, diuretic and carminative. On local application, it heals wounds and ulcers, cures local swelling. It is also used in skin and eye diseases, chronic and recurrent fever, diarrhea and dysentery, cough and dyspnoea (Joshi, 2006). Finally powdered fruit used as a dentifrice and considered useful in carious teeth and bleeding and ulceration of the gum (HMG/N, 1993).

Chemical constituents: - Fruit contains hydrolysable tannins, chebulagic acid, Chebulagic acid and corulagin. Also contains chebulic acid, 3:6- digalloglycose, ellagic acid, and β-D- glucogallin. The carbohydrates present in myrobalan are: glucose and sorbitol (major constituents), about 1% each of fructose and sucrose, a small amount of gentibiose and traces of Arabinose, maltose, rhamnose and xylose. Eighteen typical aminoacids were reported besides small quantities of phosphoric, succinic, quinic, and dihydro- and dehydro-shikimic acids (The Wealth of India, 1976).

3.1.5 *Terminalia bellirica* (Nep: Barro; Eng: Belliric Myrobalan)

Family:-Combretaceae

Description :-Large tree, 18-24m tall, leaves alternate, 7.6-20cm long, petioled; upper flower of the spike male, lower one hermaphrodite, male flower sessile, greenish yellow; fruit 12-18.7 mm in diameter, globular, suddenly narrowed into a short stalk, smooth, covered by a close fulvous tomentum and when dried obscurely 5-angled (HMG/N 1993).

Medical uses: - Fruit is bitter, astringent, tonic, laxative, antipyretic and is used in piles, dropsy, diarrhea, leprosy, biliousness, dyspepsia and headache. Half ripe fruit is purgative and fully ripe is astringent (HMG/N, 1993).

Chemical constituents: - Fruit contain about 17% tannin and β-sitosterol, gallic acid, ellagic acid, ethylgalate, galloyl glucose and chebulagic acid. Heart wood and bark were found to contains ellagic acid. Seed-coat of the fruit were found to contains Gallic acids (Joshi, 2006).

3.1.6 *Holarrhena antidysenterica* (Nep: Kutaj; Eng: Kurchi)

Family: - Apocynaceae

Morphology: - A small deciduous tree, bark pale. Leaves opposite, membranous 15-30x 3-12cm, nearly sessile, ovate, oblong. Flowers white in terminal corymbose cymes. Seeds elongated and light yellow.

Uses: - Bark is powerful antidysenteric, antidiarrhoea, anthelmintic. It is used in amoebic dysentery and also in piles, leprosy. Seeds are used as astringent, carminative, febrifuge, antidysenteric and anthelmintic (IUCN, 2000). Seeds are also used in diarrhea and for eczema. Leaves are used in chronic bronchitis, boils, ulcers and dysentery (Baral and Kurmi, 2006).

Chemical constituent: - Bark contains a large numbers of alkaloids, the chief amongst them are conessine, nor-conessine, conesimine, iso-conessimine, kurchine, conimine, holarrhimine, holarrhine, kurchinine and lettocine. It addition to the alkoloids it contains gum, resin and tannin. A triterpene alcohol, lupeol and β- sitosterol from the bark. Seeds contain a drying oil. Latex contains caoutchouc and two resinols. It was found to be useful in the synthesis of steroid hormone (Joshi, 2006).

3.1.7 *Woodfordia fruticosa* (Nep: Dhaero; Eng: Fireflame)

Family: - Lythraceae

Description :- Pubescent shrub, leaves opposite, sometimes in whorls of three, sessile, lanceolate, 5-10cm long, entire, under surface white and with black glandular dots. Flowers clustered, numerous, shortly stalked and red corolla.

Medicinal uses: - Dried flowers are astringent, styptic, uterine sedative, anthelmintic, antibacterial, vulnerary and febrifuge. It is used in diarrhea, dysentery, menorrhoea, ulcers, hepatopathy, haemorrhoids, leprosy, skin diseases and foul ulcers (Baral and Kurmi, 2006).

Chemical constituents: - Extracts of flowers reported to have ellagic acid, β-sitosterol polystachoside, octacosonol, myricetin-3-galactoside, cyanidin-3, 5-diglucoside, pelargonidin-3, 5-diglucoside, chryophanol-8-O-β-D-glucopyranoside (Joshi, 2006).

3.1.8 *Mimosa pudica* (Nep: Lazzabati; Eng: Sensitive plant)

Family: - Leguminosae

Description :- A widely spreading diffused under shrub, leaves very sensitive, leaflets 12-29 pairs, oblique, narrows- oblong, acute; flowers in small peduncled heads, pink.

Medicinal uses: - Decoction of root is useful in gravellish complaints, leaves rubbed into a paste is applied to hydrocele. Leaves and root used in piles and fistula (HMG/N, 1993). Lajjalu is astringent, cooling, antiseptic, alterative and blood purifier. It is resolved, alterative and carminative. It is used in burning sensation of body, diarrhea, dysentery, haemophilic conditions, leucorrhoea, morbid condition of vagina (Joshi, 2006).

Chemical constitution: - Plant contains β-amyrin, β-sitosterol and friedelin. Mucilage of seeds contains galactose and mannose. An adrenaline like substance identified in extracts of leaves were reported (Joshi, 2006).

3.1.9 *Tinospora cordifolia* (Nep: Gurjo; Eng: Gulancha tinospora)

Family:-Menispermaceae

Description: - Glabrous, climbing, succulent shrub; stem 1-5cm thick, outer surface rough yellow, inner surface smooth. Leaves petioled, 5-10cm in diameter, cordate, alterative; Flowers axillary, terminal raceme yellow, males fascicled and females usually solitary.

Medicinal uses :- Stem is bitter, astringent, anodyne, anthelmintic, alterant, antiperodic, antispasmodic, antipyretic, cardiotonic, hematinic, expectorant, aphrodisiac and tonic, it is also used in intermittent fevers gout, vomiting, skin diseases, leprosy, cough, asthma, jaundice, seminal weakness, uropathy and splenopathy. Leaf juice is useful in gonorrhoea (Baral and Kurmi, 2006). Starch from root and stem is nutrient, used in chronic diarrhea and chronic dysentery (Joshi, 2006).

Chemical constituents: - Furanoid, bitter principle tinosporine and a furanoid diterpere tinosporide and tinosporidine, β-sitosterol from stems. Cordifol, hepatocosanol and octacosanol from leaves were reported (Joshi, 2006).

3.2 Antimicrobial agents

Antimicrobial agents are the greatest contribution of the 20th century to the therapeutics. Their advent changed the outlook of the physician about the power drugs can have on disease.

Antimicrobial agent is a chemical substance that either kills microorganism or prevent their growth. The action of antimicrobial agents upon microorganism may be either bacteriostatic i.e. inhibiting growth and reproduction or bactericidal i.e. actually killing the microorganism (Tripathi, 2003). In general antimicrobial agent act in one or more of the following ways:-

A) Damaging the bacterial cell membrane leading to loss of cell contents and so to cell death.
B) Inhibiting cell wall formation leading to cell lysis.
C) Inhibiting protein biosynthesis.
D) Inhibiting nucleic acid production so preventing bacteria from reproducing (Cheesbrough, 1993).

Herbal medicine represents one of the most important fields of traditional medicine all over the world. To promote the proper use of herbal medicine and to determine their potential as sources of new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way (Awadh et al; 2001).

Over the past 20 years, there been an increased interest in the investigation of natural compounds as sources of new antimicrobial agents. Different extracts from traditional medicinal plants have been tested to identify the sources of the therapeutic effects. As a result some natural products have been approved as new antimicrobial drugs, but there is still an urgent need to identify novel substances that are active towards pathogens with high resistance (Recio, 1989). In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plant.

Some of the notable works done in world wide in the view to determine antimicrobial activities of plant includes:-

In-vitro antibacterial activity of *Camellia sinensis*, *Azadhirachta indica* and *Acorus calamus* against enteropathogenic *Escherichia coli*, *Shigella sonni*, *Salmonella* Typhi, and *Klebsilla pneumonia* and *Vibro cholera* by disc diffusion and tube dilution methods found that ether extracts appeared to have potent antibacterial activity than aqueous extracts (Vijaya and Ananthan, 1994).

A studied done on methanol extracts from twenty one plant species of selected medicinal plants in Nepal for activity against eight strains of bacteria and five strains of fungi shown that all plants extracts produced activity against at least two bacterial strains, and twenty showed activity against at least two fungi (Taylor et al; 1995).

Miranda et al. (1996) studied antimicrobial activity of petroleum ether extracts of *Punica granatum* fruit rind, bark and seed of *Holarrhena antidysenterica*; root of *Berberis asiatica;* root and latex of *Bombax malabaricum*, leaves of *Gossypium herbaceum* against

four species of *Shigella* and *E. coli*. *Punica granatum* extracts showed significant activity compared to other extracts.

In-vitro antimicrobial activity of fifty four extracts from twenty one South African medicinal Plants against *Staphylococcous aureus, Stapylococcous epidermidis, Bacillus subtilis, K. pneumoniae* and *E. coli* found that antimicrobial activity of the extracts were mainly active against Gram positive bacteria. None of the extracts showed any antimicrobial activity against *K. pneumoniae* (Rabe and Staden, 1996).

Determination of antimicrobial activity of seventy eight selected medicinal plants from the Indian traditional medicines against *B. subtilis, S. aureus, E. coli* and *Pseudomanas aeruginosa* found that at the concentration of 1.6 mg/ml, only 10% of the plant extracts were active. Similarly, at the concentration of 6.25%, only 44% of the plant extracts were active; at the concentration of 25 mg/ml 90% of the plant extract were active against at least at least two bacteria (Valsraj et al; 1997). The plants looked promising were *Terminalia bellirica, Aegle marmelos, Zizyphus jujube* and *Alangium saluiifolium.*

Antimicrobial activity of *Adhatoda vasica* leaves against various bacteria shown that significant antibacterial activity was given against Gram positive bacteria *Steptococcus faecalis, S. aureus, S. epidermidis* and the Gram negative *E. coli* (Branter and Chakrabory, 1998).

In-vitro antimicrobial activity against a wide range of Gram positive and Gram negative organisms by tested ethanol extract, different fractions obtained by partitioning method from ethanol extract and a pure compound of the root bark of *Holarrhena antidysenterica* shown that carbon tetrachloride and chloroform fractions were found to be relatively more active while Ethylacetate fraction was least potent in the series (Choudhary, 1998).

Ethanol extracts of *Syzygium andamariicum* and *Syzygium cumin* stem bark inhibited the growth of all tested Gram positive and Gram negative bacteria (Chattopadhyay et al;

1998). *Syzgium* cumin was found to be more potent than *Syzygium andamanicum* and *Syzygium samarangense*.

Zaiba et al. (1998) screened a total of twenty eight ethanol extracts of twenty seven plants for their *in-vitro* antimicrobial activity against four bacteria and six soil borne fungi. It was shown that antibacterial activity against at least one or two test bacteria (*E. coli, B. subtilis, S. aureus* and *Steptococcus pneumoniae*) was detected among 96% of the extract. Fruit of *Hemidesmus indicus*, fruit of *Terminalia bellirica*, fruit of *Terminalia chebula* and bud of a *Syzygium aromticum* shown significant broad spectrum activity. Extract of *Embilica officinalis* and *Punica granatum* showed strong antibacterial but moderate antifungal activity. *Acorus calamus* shows strong antifungal but moderate antibacterial activity.

Ethno medical information gathered from traditional healers and rural dwellers in the Eastern Cape Province, South Africa found that *Grewia occidentalis, Polystichum pungens, Cheilanthes viridis* and *Malva paruifolia* are the mostly commonly used plants for the treatment of wound (Grierson and Afolayan, 1999). They found that methanol extracts of *G. occidentalis, P. pungens* and *C. viridis* showed significant inhibition against tested Gram positive and Gram negative bacteria. Extracts from *M. parvifolia* did not show any antibacterial activity at the concentration of 5.0 mg/ml.

A test done by Mustafa et al. (1999) for *in-vitro* antimicrobial activity of prepared water, ethanol, n-hexane and chloroform extracts from dried powdered of *Acacia nilotica* fruit against human pathogenic bacteria and *Candida albicans* using agar dilution method found that extracts in water and ethanol were generally more active than those in hexane and chloroform. It was also found that the extracts were more active against Gram positive cocci than Gram negative bacilli.

Evaluation of the effect of the aqueous extracts of two drugs viz: Triphala churna and Chyavanprash and two drug plants viz: *Adhatoda vasica* and *Azadirachta indica* against *Sarcina* spp, *Bacillus* spp, *E. coli* and *B. megaterium*. It was found that Triphala churna was profoundly effective against all tested organisms (Prasad et al, 1999).

Antimicrobial activity of 60% methanolic extract from *Bryophyllum pinnatum* leaf against various bacterial species showed that the extract inhibited the growth of five out of eight bacteria used, at a concentration of 25 mg/ml (Akinpelu, 2000) and also found that *K. pneumoniae, P. aeruginosa* and *C. albicans* were found to resist the action of the test extract.

The screening of antimicrobial activity of methanolic extract from *Solanum torvum* fruit showed zone of inhibition at the range of 6 to 29 mm against the tested human and animal pathogens (Chah et al; 2000) with maximum Inhibition zone diameter shown against *Streptococcus pyogens* and Minimum inhibitory concentration (MIC) value ranges from 0.3125 to 1.25 mg/ml against the tested organism.

Essawi and Srour (2000) studied antibacterial activity of organic and aqueous extracts of fifteen Palestinian medicinal plants against eight different species of bacteria: *B.subtilis*, two *E. coli*, *S. aureus* (methicillin resistant), two *S. aureus* (methicillin sensitive) spp, *Psedomanas aeruginosa* and *Enterococcus fecalis*. Of the fifteen plants tested, eight showed antibacterial activity with most active antibacterial plants against both Gram positive and Gram negative bacteria were *Thymus vulgaris* and *Thymus organism*. In general, organic extract showed the same or greater activity than the aqueous extract.

The screening for antibacterial activity of aqueous, methanol and ethyl acetate extracts of fourteen plants used in traditional Zulu treatment for treatment of ailments of an infectious nature found that most of the activity detected was against Gram-positive bacteria. In general, methanol extracts exhibited higher activity than aqueous and ethyl acetate extracts (Kelmanson et al; 2000).

Ethanol extracts of 45 Indian medicinal plants traditionally used in medicine were studied by Ahmad and Beg (2001) for their antimicrobial activity against certain drug-resistant bacteria and a yeast *Candida albicans*. Experiment found that extracts of 40 plants showed antimicrobial activity against one or more test bacteria whereas antifungal activity was detected in 24 plant extracts. Overall, broad antimicrobial activity was

observed in twelve plants viz: *Holarrhena antidysenterica, Terminalia bellirica, Terminalia chebula, Punica granatum etc.*

Srinivasan et al. (2001) screened fifty medicinal plants belonging to twenty six families for potential antimicrobial activity. Result shown that 72% plant extracts shown activity against both Gram positive and Gram negative bacteria. Only nine plant extracts showed antifungal activity. The maximum zone of inhibition (38 mm) was observed with the crude extract of *Allium sativum* against Gram positive organism *B. subtilis*.

Antimicrobial activity of crude ethanol extracts of sixteen Siberian medicinal plants tested against five species of microorganism found that out of sixteen plants tested, twelve showed antimicrobial activity against one or more species of microorganism (Kokoska et al; 2002).

The evaluation of antibacterial activity of aqueous and ethyl acetate extract from leaves of *Piper regnelli* shown that aqueous extract was weakly positive against *S. aureus, B. subtilis* (Pessine et al; 2003). They also found that ethyl acetate showed good activity against them with MIC and MBC value at 15.67 µg/ml.

Ram et al. (2004) studied antimicrobial properties of certain medicinal plants used by adivasi tribes of the Eastern Ghats of Andhra Pradesh, India. Ethanol extracts of twenty three crude drug samples used for various skin diseases were assayed for antimicrobial activity against four bacterial and one fungal human pathogens. The highest inhibitory zones (32 mm) were observed in the extracts of *Pentanema indicum* against *Micrococcus luteus.*

The screening of antimicrobial activity of the ethanol extracts and some other fractions from ten medicinal plants reported by Aqil et al. (2005) against beta lactamase producing MRSA and MSSA has shown that extracts of plants from *Punica granatum, Terminalia chebula, Terminalia bellirica, Holarrhena antidysenterica, Delonix regia* and *Camellia sinensis* showed a broad spectrum activity.

Study on antimicrobial activity of crude and methanol extract of dry fruit of *Terminalia bellirica* against nine human microbial pathogens by disc diffusion method found that crude aqueous extract of dry fruit at 4 mg concentration showed zone of inhibition ranging from 15.5-28 mm (Elizabeth, 2005). He also found that both crude and methanol extracts of *T. bellirica* were strongly inhibition to *S. aureus*, forming larger zone of inhibition 28 mm and 30 mm respectively.

Preliminary antimicrobial screening of the methanol extracts from *Zingiber officinale, Asteracantha longifolia, Citrus acida, Salacia microsperma* and *Tinospora cordifolia* found that methanol extracts of the plants were significantly active against both Gram positive and Gram negative bacteria. *T. cordifolia* and *Zingiber officinale* extracts were less active against all the tested bacteria (Samy, 2005).

The study done by Balakrishnan et al. (2006) on antimicrobial activity of *Mimosa pudica, Aegle marmelos* and *Sida cordifolia* against *B. subtilis, S. aureus, K. pneumoniae, P. aeruginosa, E. coli* and *S.* Typhi. They found that the maximum inhibitory zone of inhibition was shown by *Sida cordifolia* against *B. subtilis* (35 mm) and *S.* Typhi (26 mm). *Mimosa pudica* and *Aegle marmelos* were found to be active against the entire microorganism tested.

Study on antibacterial activity of methanol, ethanol acetone and hot water extract from twenty one plant species against *Vibrio cholera, E. coli, S. aureus, Shigella, S.* Typhi by agar well diffusion method shown that the most active extracts were those obtained from *Punica granatum* and *Indigofera daleoides* (Mathabe et al; 2006).

The study on *in-vitro* and *in-vivo* antidiarrhoeal potential of chloroform extract of the root of *Aegle marmelos* by agar dilution and agar disc diffusion technique against 35 tested pathogenic diarrhea causing strains shown that, the extract was found to be mostly active against the strains of *V. cholerae* followed by *E. coli* and *Shigella* (Mazumder et al; 2006).

To determine the antimicrobial activity of medicinal plants, Nair and Chanda (2006) selected a total of 20 plant species and tested antimicrobial activity against seven Gram negative strains and five Gram positive strains. It was found that maximum antibacterial activity was shown by the aqueous extracts of *Parthenium hysterophorous*. In general, aqueous extracts showed less activity than ethanol extracts. The antibacterial activity of the extracts is more pronounced on Gram positive than on Gram negative bacteria.

The evaluation of antimicrobial activity of aqueous, hexane and ethanol extracts from black myrobalan (fruit of *Terminalia chebula*) by agar well diffusion method against uropathogen *E.coli* observed that both aqueous and ethanol extracts showed strong zone of inhibition (21-24 mm) while hexane extract showed mild zone of inhibition (9mm) against the strain evaluated. MIC value of aqueous and ethanol extracts was found to be 6.25 mg/ml and 9.12 mg/ml respectively. Minimum bacterial concentration (MBC) values were two fold four fold higher than the corresponding MIC value (Chattopadhyay, 2007).

Nair and Chanda (2007) studied antibacterial activity of aqueous and ethanol extracts of 10 medicinal plants using both agar disc diffusion and agar well diffusion methods against medically important bacterial strains *P. aeruginosa* and *S.* Typhi were the most resistant strains while the most susceptible *were B. cereus* and *Proteus* mirabilis. The ethanol extracts were more potent than aqueous extract. *Emblica officinalis* showed strong activity against all the tested bacterial strains.

Oliveria et al. (2007) extracted methanol extracts and their fraction hexane, ethylacetate and methanol extract from 27 plants from Brazilian Southeast region to identify extracts with antibacterial properties against *B. subtilis, Pseudomonas aeruginosa, Aeromonas hydrophila* and *S. aureus* by agar diffusion method. Thirteen of the tested crude extracts showed antibacterial activity. MIC value range from 5.0 to 0.625 mg /ml, while MBC in the range of 5.0 to 1.25 mg/ml.

The test done by Parekh and Chanda (2007 a) on antimicrobial activity of the 34 Indian plants against seven members of Enterobacteriaceae by agar disc diffusion and agar well

diffusion methods found that ethanol/methanol extracts were more active than aqueous extracts for all the plant studied. The most susceptible bacterium was *K. pneumoniae* while the most resistant bacteria were *Salmonella* Typhimurium and *E. coli. Woodfordia fruticosa* showed best antibacterial activity.

Study on antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* flower at two different concentrations by the agar well diffusion method shown that the antibacterial activity at 5.0 mg/ml concentration was more than that at 2.5 mg/ml concentration against all tested microorganism (Parekh and Chanda, 2007 b) and also found that Gram negative bacteria were more susceptible to the plant extract than Gram positive bacteria.

In Nepal, study on the antimicrobial activity of medicinal plants is limited. Very few researches which have done nationally are:-

Rijal (1994) studied antimicrobial activities of 32 indigenous plants by filter paper disk diffusion method on the dried extracts obtained from 50% alcohol. The test organisms were *S. aureus. B. pumilis, B. subtilis, E. coli, S.* Typhi*, Shigella dysenteriae, Candida albican* and *S. cerevisae.*

Methanol extracts from selected 21 medicinal species found in Nepal were assayed for their antimicrobial activity against 8 strains of bacteria and five strains of fungi by Taylor et al. (1995). All of the extracts showed activity against at least two bacterial strains, and twenty showed activity against at least two fungi.

Methanol extracts from twenty plant species were evaluated for their antimicrobial activity against 11 species of bacteria and four species of fungi, studied by Taylor et al. (1996). Fifteen of the extracts showed activity against bacteria and fourteen showed activity against fungi. The extracts which showed broadest spectrum of activity were *Eupatorium odoratum, Terminalia alata* and *Rumex hastatus*. None of the extract was active against *E. coli, K. pneumoniae, Enterobacter aerogens* and *S.* Typhimurium.

Antimicrobial properties of crude extract from the rind of fruit of *Punica granatum* done by Bhatta (1998) against 14 known local isolates of bacteria found that *P. granatum* shown antimicrobial properties against 13 test organism.

Crude Ethanol extract of 9 medicinal plants of Nepal viz*: Azadirachta indica, Swertia chirayita, Acorus calamus, Withania somnitera, Terminalia chebula, Berberis aristata, Paranassia nubicola, Curcuma angustifolia* and *Glycyrrhiza glabra* were studied by Devkota et al.(1999) for their antimicrobial activity against *P. aeruginosa, S. aureus, E. coli*, MRSA, *V.cholerae, S.* Typhi, *Shigella dysenteriae* and *Shigella flexerni.G. glabra* showed the best antibacterial activity among all tested herbal plant extract.

Pokharel (2000) screened and evaluated the antimicrobial activity of some medicinal plants of Nepal and isolation of pure antimicrobial compound from *Bauhinia variegata*. It was found that ethanol extracts from five were found to have relatively higher antimicrobial activities. Gram positive bacteria were found to be more sensitive to medicinal plants than Gram negative bacteria.

The screening and evaluation of antimicrobial activity of 20 different medicinal plants of Nepal against 8 pathogenic organisms found that extract from *Rubus ellipticus* was most effective (Baidya, 2001). The smallest MBC values of 6.25 mg/ ml were obtained with *Adhatoda vasica* against *P. aeruginosa*.

Evaluation on antimicrobial activity of twenty nine medicinal plants used to treat skin ailment of Kaski district, Nepal found that eleven plants produced zone of inhibition (ZOI) with all tested bacteria while seven plant species were unable to produce any ZOI against tested bacteria (Parajuli et al; 2001).

Parajuli (2001) tested antibacterial activity of 29 plant species against four organisms viz: *S aureus, B. subtilis, E. coli* and *P. aeruginosa*. It was found that 12 species showed distinct ZOI with all types of test organisms

Evaluation for antibacterial property of eight different medicinal plants against isolated enteric bacteria from the drinking water of Kathmandu valley found that all plants were

ineffective against *P. aeruginosa*. The largest ZOI was obtained with *Rhododendr*on *arboreum* against *V. cholerae* (19 mm), while smallest MBC value of 25 mg/ml was obtained with *Alnus nepalensis* and *Myrica esculenta* against *S.* Typhi (Prasai, 2002).

Study done by Radha (2004) for antimicrobial activity of six Lichen species and three higher plants species against ten bacteria and one fungus found that Gram positive organisms were most sensitive to inhibition by ethanol and methanol suspensions of plant extracts than Gram- negative bacteria.

Mahato and Chaudhary (2005) studied altogether 25 plant species of Palpa district, Nepal according to Ethnomedical uses and screened for their antibacterial activity by disc diffusion method. Four strains of bacteria employed in test were two Gram positive viz: *Bacillus subtilis, S. aureus* and two Gram negative *E. coli, P. aeruginosa*. Out of the 25 plant species, the extract of 13 species (52%) showed positive response against at least one of the tested bacteria, while the extracts of 11 species (44%) showed positive response against at least two bacteria. The extracts from 12 plant species showed no antibacterial activity against any of the four strains of tested bacteria.

Evaluation of antibacterial activity of 16 different medicinal plants was tested by Thapa (2006) against 14 bacterial species. It was found that *Syzygium aromaticum* was the most active plant as it was effective for all the tested bacterial species. *Acorus calamus* was the least effective against the tested organisms. Gram positive organisms were more sensitive to medicinal plants extracts than Gram negative bacteria while the lowest MIC (0.097 mg/ml) was given by dimethyl sulpho oxide suspension of *Syzgium aromaticum* against *K. pneumoniae.*

Eight different medicinal plants were screened and evaluated for their antibacterial activity against the enteric bacteria isolated from water on the basis of their common use among different ethnic groups for common disorders. Maximum zone of inhibition was observed in case of *Proteus mirabilis* (31 mm) due to *Syzygium cumini* and minimum was against *Pseudomonas* spp (10 mm) by *Psidium guajava* (Bajracharya, 2007).

3.3 Selection of the organisms

Plants and different plants extracts under this study were found to be used in treatment of various diseases in one way or other in traditional medicines. So the organism was selected randomly to determine the potential antimicrobial activity of selected plants and plants extracts.

3.4 Short description of microorganisms involved in this study with their pathogenicity

3.4.1 ***Staphylococcus aureus*** :- This Gram positive spherical cocci causes boils, pustules, impetigo, infections of wounds, ulcers, osteomyelities, mastitis, septicaemia, meningitis, Pneumonia and Pleural empyema. It also causes toxic food poisoning, toxic shock syndrome and toxic exfoliation (Cheesbrough, 2002).

3.4.2 ***Escherichia coli*****: -** This Gram negative straight rod is part of the normal microbial flora of the intestinal tract of humans and animals often associated with opportunistic infection. The virulent strains of *E. coli* act as specific pathogens of the gut (enteritis) and of extra-intestinal sites (UTI, wound infection). Clinical infections caused by *E. coli* include: UTI, septic infections of wound, diarrheas, dysentery, septicaemia, pneumonia, neonatal meningitis, and abscesses in a variety of organs. Three main virulent types of *E. coli* causing gastrointestinal disease include ETEC, EPEC, and EHEC (Chakraborty, 2000).

.3.4.3 ***Klebsiella pneumoniaea and Klebsiella oxytoca***: - Theses Gram negative non motile capsulated rods causes pneumonia, urinary tract infection, septicaemia and rarely diarrhea. It also causes pyogenic infections such as abscesses, meningitis and septicaemia (Cheesbrough, 1993; Chakraborty, 2000).

3.4.4 ***Proteus mirabilis***: - This actively motile Gram negative aerobic bacilli commonly causes urinary infection in the elderly and young males. It also causes

abdominal and wound infections, septicaemia and occasionally meningitis and chest infection (Chakraborty, 2000).

3.4.5 ***Proteus vulgaris***: - It occasionally causes wound and burn infection, urinary infection (Cheesbrough, 2000).

3.4.6 ***Pseudomonas aeruginosa***: - This slender Gram negative bacilli is an etiological agent of community acquired pneumonia which is rare but fatal. Most of the infections are opportunistic and hospital acquired. It also causes urinary tract infections after instrumentation or catherization, wound and burn infection, chronic otitis media and chronic otitis externa (Collee et al; 1996). It is one of the important agents responsible for infantile diarrhea and sepsis (Ananthanarayan and Paniker, 2001).

3.4.7 ***Salmonella* Typhi**: - This Gram negative rod is responsible for Enteric fever (Typhoid), gastrointestinal tract infection, osteomyelitis in children, nephrotyhoid in those with urinary schistosomiasis (Cheestrough, 1993; Chakraborty, 2000).

3.4.8 ***Salmonella* Paratyphi A**: - It causes paratyphoid fever, diarrhea and inflammation of entire intestinal tract (Chakraborty, 2000).

3.4.9 ***Shigella dysenteriae***: - It causes bacillary dysentery or Shigellosis. The main clinical features are frequent passage of loose scanty feces containing blood and mucus, along with abdominal cramps and tenasmus (Cheesbrough, 2002).

3.5 Screening and evaluation of antibacterial activity

First step in assessment of new antibiotic is screening step and in this step effectiveness of antimicrobial substance is evaluated by determination of zone of inhibition (ZOI), minimum inhibitory concentration (MIC) of the antimicrobial agent (WHO, 1991), and or minimum bacterial concentration (MBC), for bacteria and minimum fungicidal concentration (NFC) for fungi (Carpinella et al., 1991)

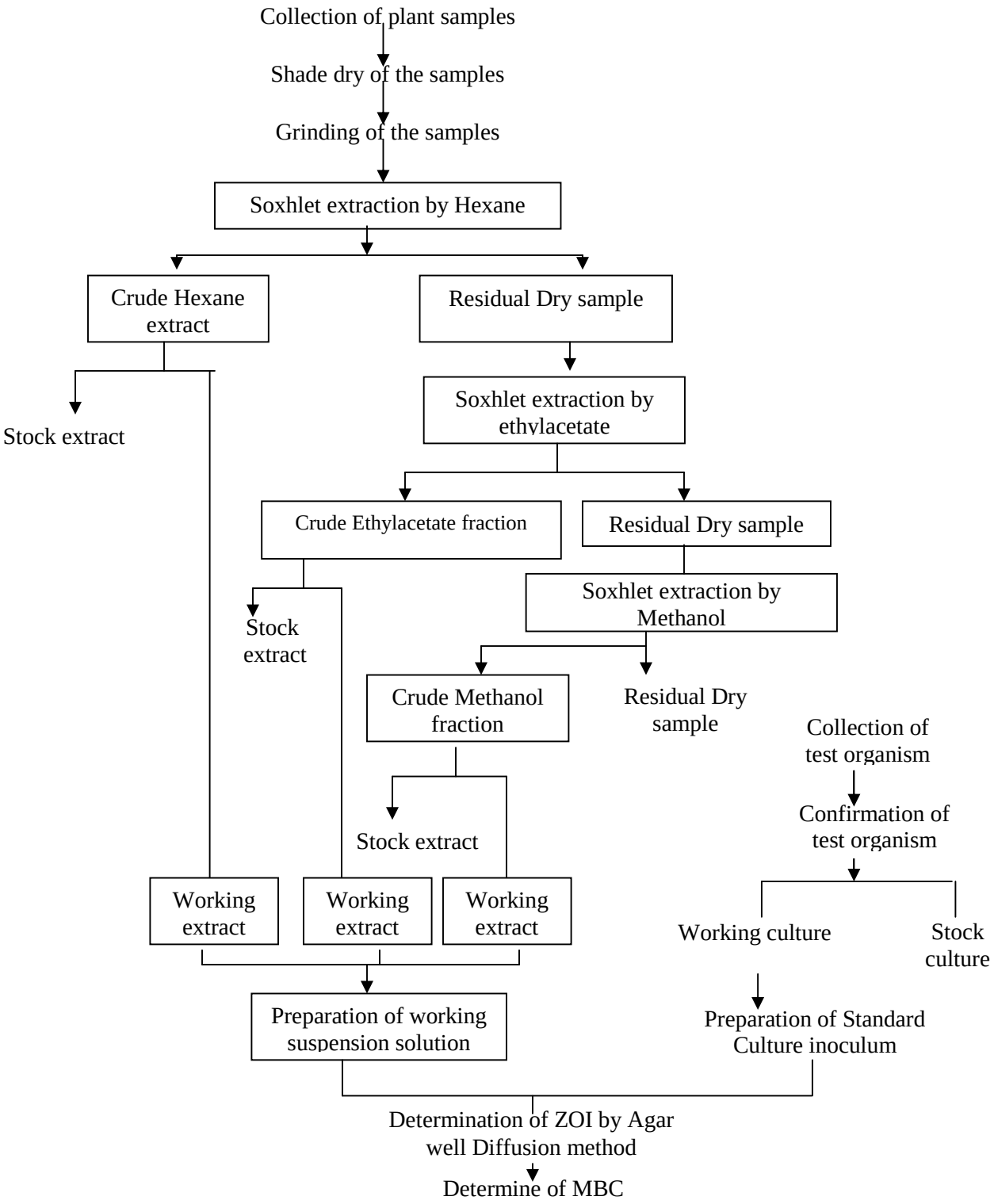
MIC is the lowest concentration of antibacterial agent that will inhibit visible growth of an organism after overnight incubation (WHO, 1991). MBC is the amount of the agents that will prevent growth after subculture of the organisms to antibacterial agent free medium (Collee et al; 1996).

There are several techniques to determine in-vitro antimicrobial activity of compounds. Some of these are:

3.5.1 Agar well diffusion (cup plate) technique: This method was originally determined by Dingle et al. (1953), for the evaluation of enzymatic activity for the degradation of pectin and other polysaccharides. Later this method has been modified for the evaluation of antimicrobial activity of the solvent extracted fraction of the plant material (Perez et al; 1990).

3.5.2 Two fold dilution method: This technique has been recommended by WHO (1991), for quantitative estimation of antibiotic activity, in which test substances are incorporated into broth by two-fold serial dilution method. Then the tubes are inoculated with the test organisms. The point at which no growth occurs after overnight incubation is taken as MIC, if required MBC can be determined. (Cheesbrough, 1993).

There are also other techniques which are used for screening of in-vitro antimicrobial activity of a compound. These are 1. Micro broth two-fold serial dilution (TFSQ) 2. Mictotitre (MT) technique (Khan, 1997) and 3. Agar Disc Diffusion (DD).



Flow chart of method of evaluation of antimicrobial activity of hexane, ethylacetate and methanol fraction of medicinal plants

CHAPTER-IV

4. MATERIALS AND METHODS

This study was conducted from June, 2007 to July, 2008. During this period, antibacterial activity of extracts from selected 9 medicinal plants were studied against 10 common bacterial pathogens. The extraction of crude hexane, ethylacetate and methanol fraction were done at lab of Central Department of Chemistry while antibacterial activity was done at lab of Central Department of Microbiology, T.U, Kirtipur.

4.1 Materials

All the materials used in this study are given in the Appendix –A

4.2 Method

4.2.1 Collection of medicinal plants

The selected medicinal plants were collected from different parts of Nepal. Some medicinal plants samples were also collected from market (Ason market). The list of medicinal plant, their corresponding parts used under this study, month of sample collection and location/place of the sample plants are given in Appendix - B.

4.2.2 Identification and documentation of sample plants

Collected medicinal plants were identified accourding to the description given on different book viz. Flora of Kathmandu Valley by HMG/N (1986), Medicinal plants of Nepal by HMG/N (1970, 1993), Flora of British India (1992) and other pertinent taxonomic literature.

4.2.3 Processing of the sample

a) Washing and chopping

Root, bark and other underground parts were washed thoroughly to remove soil and extraneous matter such as other parts of the same plants or grasses herbs or any other unwanted matter. Collected samples were then chopped into small fragments. Stem, roots, were chopped into 3-5 cm pieces. For marketing samples, after it were collected from market (Ason bazaar), it was fragmented into small pieces with the help of clean knife.

b) Drying of the sample

After chopped, the samples were spread under the shade at room temperature until completely dried. Samples were turned up and down at least twice a day to hasten drying.

c) Grinding

The dried samples then proceed to grinding by means of machine to obtain fine sample powder.

4.2.4 Extraction of plant material

4.2.4.1 Soxhlet extraction with different solvent (hexane, ethylacetate, and methanol)

Shade-dried finely powdered various parts of medicinal plants were subjected to continuous extraction with three different solvent viz. hexane, ethylacetate and methanol in the order of increasing polarity, by using soxhlet apparatus to obtain crude extract viz. hexane extract, ethylacetate fraction and methanol extract respectively. After complete extraction, the solvent will be totally removed by using rotary evaporator these fractions were separately assayed for their antibacterial activity. This fractionation method may help in order to find the active fraction (s) and to trace the active antimicrobial chemical

constituent(s) for bioassay guided isolation (Shrestha, 2005). The detail process of extraction is explained below:

Known weight (approx.70-90 gm) of dried plant powder was packed in sterile filter paper and it was loaded in a clean and dried thimble of soxhlet apparatus carefully. It was then fitted with pre-dried round bottom flask having a capacity of 1000 ml. Soxhlet apparatus was then set up with the help of stands. Then about 400 ml of solvent hexane was slowly poured from the upper mouth of the soxhlet extractor. Then upper mouth part was fitted with condensor having cold water running through tap. The flask was constantly heated using electric heating mantle with controlled temperature. The process was continued until the colourless solvent appeared from plant material on soxhlet apparatus.

The marc of the hexane extracts was used for the extraction of ethylacetate extracts. Ethylacetate was taken in round bottom flask and heated in controlled temperature. After complete extraction by solvent ethylacetate, the marc of the ethylacetate extracts was used for the extraction by solvent methanol. For this, methanol was taken in round bottom flask and heated in controlled temperature until the coloured solvent appeared from the marc of the ethylacetate extracts.

Three fractions (solution) were obtained and each fraction was collected in a separate round bottom flask.

- The hexane extractive solution
- The ethylacetate extractive solution
- The methanol extractive solution.

For each extracted solution, solvent was completely removed by rotary vacuum evaporator separately.

4.2.4.2 Removal of solvent

After complete extraction, the round bottom flask containing extract was fitted with rotary vacuum evaporator under negative pressure. The flask was constantly heated in rotating condition by using water both below 55^{o}C. Solvent was completely removed and was collected in separate round bottom flask which was then stored in sterile bottle. The extract obtained in round bottom flask was then weighted till constant weight appeared and result was noted. The crude extract was then transferred. In a bottle by sterile spatula and was labeled and stored in a refrigerator.

4.2.5 Preparation of stock/working solution

100 mg/ml of each crude extract was made by taking 1 gm of the extract in 10 ml of DMSO in clean and capped test tubes. The extract was dissolved in DMSO by vortexing. After making stock/working solution the test tubes were capped, sealed and stored in refrigerator (2-8^{o}C) until use (Thapa, 2006).

4.2.6 Collection of standard cultures

The standard cultures of bacteria under study were collected from different labs.

After obtaining the culture, the test organisms were streaked on nutrient agar plates and incubated at 37^{0}C for 24 hours. From the isolated colony Gram staining was performed. The organism thus obtained were tested for their purity and confirmed by their morphological, cultural and biochemical characteristics. The organisms in the study include:

1) *Staphylococcus aureus* (ATCC 25923)
2) *Escherichia coli* (ATCC 25922)
3) *Klebseilla pueumoniae* (ATCC 20063)
4) *Klebseilla oxytoca*
5) *Proteus mirabilis* (ATCC 49132)

6) *Proteus vulgaris*
7) *Pseudomonas aeruginosa* (ATCC 27853)
8) *Salmonella* Typhi
9) *Salmonella* Paratyphi A
10) *Shigella dysenteriae*

4.2.7 Preparation of standard culture inoculum

Two to three similar appearance colonies from NA plate were touched with the inoculating loop aseptically. It was then transferred to a tube containing 2 ml of sterile nutrient broth. The tube was then compared with turbidity standard (McFarland Nephelometer standard tube 0.5) recommended by WHO (1991) for antimicrobial susceptibility test.

4.2.8 Preparation of media

The media used in the study were prepared according to the manufacturer's recommendation. The detailed procedure for preparation of media is given in the Appendix- D.

4.2.9 Screening and evaluation of antibacterial activity

Agar well diffusion method and two fold broth dilution method were used in this study for screening and evaluation of antibacterial activity of medicinal plant extracts. In agar well diffusion method, the diameter of zone of inhibition (ZOI) produced by plant extract on particular microorganism was measured for the estimation of antibacterial activity of that medicinal plant extract. Similarly, two fold broth dilution method was applied for the determination of minimum bactericidal concentration (MBC).

4.2.9.1 Qualitative screening and determination of antibacterial activity

The antibacterial activity of crude extracts of medicinal plants was screened against the test organisms by agar well diffusion method as given by Dingle et al. (1953).

Sterile Mueller-Hinton agar (MHA) plates of approximately 4 mm thickness were prepared. Before using the plates, they were dried under hot air over at appropriate temperature to remove excess of moisture from the surface of the media. The fresh inoculum comparable with turbidity standard was prepared as given in section 4.2.7.

Then, sterile cotton swabs were taken out and were dipped into the prepared inoculum. The excess of inoculum was removed by pressing and rotating against the upper inside side wall of the tube above the liquid level and then swabbed carefully all over the plates. The plate was rotated through an angle of 60^{o}C after each swabbing. Finally the swab was passed round the edges of the agar surface. The inoculated plates were left to dry for few minutes at room temperature with the lid closed. (WHO, 1991).

Four wells were made in each plate with the help of sterile cork borer no.8. So, the diameter of a well was 8 mm. 100 µl of hexane, ethylacetate and methanol extract (100 mg/ml) from the selected plants and DMSO as negative control were added in each well with the help of micropippete. The plates were then left for half and hour with the lid closed so that the extract diffused into media. The plates were incubated overnight at 37^{o}C. (Parek and Chand 2007 a).

After proper incubation (18-24 hours), the plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the zone of inhibition in millimeters. The absence of a zone of inhibition was interpreted as the absence of activity.The triplicate assay was performed in the case of presence of zone of inhibition. The ZOI were measured using scale and mean was recorded.

4.2.9.2 Determination of minimum bactericidal concentration (MBC)

The crude extract of different fraction of medicinal plants, which showed antibacterial activity were then subjected to two fold serial dilution method as described by Baron et al. (1994) to determine minimum bactericidal concentration (MBC). The process is as follows:

- A set of 12 test tubes were taken and labeled as positive control, negative control and tube no. 1 to 10.
- With the help of sterile pipette 1 ml NB was added into positive control and tube no. 1 to 10.
- Then 1 ml of the crude extract from particular medicinal plants was added aseptically to each tube labeled as negative growth control and 1 no. labeled test tube.
- The tube no. 1 now contains 1 ml of broth and 1 ml of extract. It was then homogenized in shaker. Then, 1 ml of its content was transferred aseptically to tube no. 2 with the help of micropipette.
- After complete homogenization of the content in tube no.2, 1 ml of its content was transferred to tube no.3. In the same way, two fold dilution was prepared up to the tube no. 10.
- From the 10 no. tube 1 ml of its content was discarded. Hence all the tubes from negative control to tube No. 10 contained equal volume i.e. 1 ml with gradually decreasing concentration. No plant extract was added to the tube labeled as positive control.
- Now, with the help of micropipette, 50 µl of inoculums (turbidity equal to a McFarland standard 0.5) was added to all tubes except labeled as negative control.
- Then all the tubes were incubated at $37^{o}C$ for 24 hours and observed for turbidity by comparing with +ve and –ve controls.
- The results were interpreted on the basis of the fact that growth occurs in the positive control and any other tube in which the concentration of the extract is not sufficient to inhibit growth and the lowest concentration of the agent that inhibits growth of the organisms as detected by lack of visible turbidity is designated the minimum inhibitory concentration (MIC). However, in some cases it was difficult to identify whether the turbidity was due to the growth of bacteria or due to the turbidity of plant extract itself. So MBC was done to determine the lowest concentration of antimicrobial that is required to kill the bacteria and hence produce a sterile culture.

- To determine MBC, NA plate was taken and labeled from 1 to 10 and +ve and –ve control with the help of marker. The tubes were subculture on nutrient agar plate with same labeled number and then plates was incubated at 37^0C for 24 hours.
- Then they were observed for the growth of bacteria. The labeled tube no. where no growth of organism after incubation of 24 hours is called as MBC.

4.2.10 Antibiotic susceptibility test

Antibiotic susceptibility of collected bacterial cultures was determined by the standard disc diffusion method of Baur et al. (1966).

For the determination of antibiotic sensitivity test, sterile Mueller-Hinton Agar plates were prepared as mentioned in Appendix D. The inoculum of bacteria was prepared as given in section 4.2.7. Sterile cotton swab was dipped into the prepared inoculum culture and excess inoculum was removed by pressing against the inside wall of the tube and then swabbing was done carefully all over the surface of the agar plate three times, rotating the plates through an angle of 60^0 after each swabbing. Then the plates were left for 5-10 minutes at room temperature.

Then antibiotics discs were taken from their respective vials with the help of sterile forceps and placed carefully on the plate, at least 15 mm away from the edge and sufficiently separated from each other to avoid the overlapping of the zones of inhibition. Then each disc was slightly pressed with the help of forceps to make proper contact with the surface of the media. All the plates were incubated at 37^0C for 18-24 hours.

After proper incubation, the zone of inhibition in each disc was measured with the help of ruler. The zone of inhibition given by antibiotics disc was then interpreted with the help of standard chart provided by Hi-media (Appendix E).

CHAPTER –V

5. RESULT

5.1 Percentage yield of crude extracts of medicinal plants

Shade dried powder of various parts of medicinal plants were subjected to continuous extraction with the solvent: hexane, ethylacetate, and methanol in the order of increasing polarity for 10-15 hours by using soxhlet extractor to obtain crude hexane, ethylacetate and methanol extracts of the respective plants. The Percentage yield of the crude extracts is as shown in the Table no.1.

$$\text{Percentage yield} = \frac{\text{weight of extract}}{\text{weight of sample}} \times 100$$

Table 1: Percentage yield of extracts

S.N.	Name of Plant used	Method of extraction	Solvent used for extraction	% yield
1.	*Acorus calamus*	Soxhlet	Hexane	12.08
			Ethylacetate	2.01
			Methanol	22.35
2.	*Holarrhena antidysenterica*	Soxhlet	Hexane	3.28
			Ethyacetate	1.63
			Methanol	9.43
3.	*Woodfordia fruticosa*	Soxhlet	Hexane	2.14
			Ethylacetate	1.52
			Methanol	21.60
4.	*Terminalia bellirica*	Soxhlet	Hexane	0.8
			Ethylacetate	1.44
			Methanol	40.46
5.	*Terminalia chebula*	Soxhlet	Hexane	1.03
			Ethylacetate	2.13
			Methanol	46.1
6.	*Tinospora cordifolia*	Soxhlet	Hexane	0.82
			Ethylacetate	0.9
			Methanol	24.2
7.	*Mimosa pudica*	Soxhlet	Hexane	1.92
			Ethylacetate	2.38
			Methanol	26.64
8.	*Asparagus racemosus*	Soxhlet	Hexane	0.7
			Ethylacetate	1.34
			Methanol	18.08
9.	*Aegle marmelos*	Soxhlet	Hexane	1.9
			Ethylacetate	2.83
			Methanol	23.6

Table no.1 shows that highest yield was observed from methanol fraction of *T. chebula* (46.1%) followed by methanol fraction of *T. bellirica* (40.46%). The least yield were from the hexane fraction of *A. racemosus* (0.7%) followed by hexane fraction of *T. bellirica* (0.8%).

5.2 Qualitative screening for antibacterial activity

Antimicrobial activities of hexane, ethylacetate and methanol extracted fraction of selected medicinal plants under this study were assayed for their possible antibacterial activity by agar well diffusion method described by Dingle et al. (1953). In the case zone of inhibition seen, three reading of the diameter of zone of inhibition were taken and their mean was recorded.

Table 2 Antibacterial property of hexane, ethylacetate and methanol extracts of different plants against tested bacteria

S.N	Plant material	Extract	Test organism									
			S. aureus	*E. coli*	*K. pneumoniae*	*K. oxytoca*	S. Typhi	*S.* Paratyphi A	*Sh. dysentriae*	*P. mirabilis*	*P. vulgaris*	*Ps. aeruginosa*
1	*T. chebula*	Hexane	+	-	-	-	-	-	-	-	-	-
		Ethylacetate	+	+	-	+	-	+	+	-	-	-
		Methanol	+	-	-	-	-	-	+	-	-	-
2	*T. bellirica*	Hexane	-	-	-	-	-	-	-	-	-	-
		Ethylacetate	+	+	-	+	+	-	+	-	-	+
		Methanol	+	+	-	-	-		+	-	-	-
3	*A. calamus*	Hexane	+	-	-	-	-	-	-	-	-	-
		Ethylacetate	+	+	-	-	-	-	+	-	-	-
		Methanol	+	-	-	-	-	-	-	-	-	-
4	*T. cordifolia*	Hexane	-	-	-	-	-	-	-	-	-	+
		Ethylacetate	-	-	-	-	-	-	-	-	-	-
		Methanol	-	-	-	+	-	-	-	+	-	-
5	*A. racemosa*	Hexane	-	-	-	-	-	-	-	-	-	-
		Ethylacetate	+	-	-	-	-	-	-	-	-	-
		Methanol	-	-	-	-	-	-	-	+	-	-
6	*H. antidysenterica*	Hexane	-	+	-	-	-	-	-	-	-	-
		Ethylacetate	-	-	-	-	-	-	+	+	-	-
		Methanol	+	-	-	+	-	-	-	-	-	+
7	*A. marmelos*	Hexane	+	-	-	-	-	-	-	-	-	-
		Ethylacetate	+	-	+	-	-	-	+	-	-	-
		Methanol	+	-	+	-	-	-	+	-	-	-
8	*W. fruticosa*	Hexane	-	-	-	-	-	-	+	-	-	-
		Ethylacetate	+	+	-	-	+	+	+	-	-	-
		Methanol	+	-	+	-	-	-	+	-	-	-
9	*M. pudica*	Hexane	+	-	-	-	-	-	-	-	-	-
		Ethylacetate	+	-	-	-	-	-	-	-	-	-
		Methanol	+	-	-	+	-	+	-	-	-	-

Notes: Positive (+) sign indicates that the extracts of medicinal plants inhibited the growth of bacteria and thus showed antibacterial activity.

Absence of zone of inhibition was denoted as negative (-).

5.3 Evaluation of antibacterial activity

The mean diameter of zone of inhibition and minimum bactericidal concentration of hexane, ethylacetate and methanol fraction of different medicinal plants which showed zone of inhibition during qualitative screening process (as indicated by + sign in table) are shown in the Tables 3 to 11.

5.3.1 ZOI and MBC given by *Acorus calamus* against tested bacteria

Table 3: ZOI and MBC given by *Acorus calamus* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	13	50
		Methanol	-	-
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	17	25
		Methanol	-	-
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	13	25.0
		Ethylacetate	18	12.5
		Methanol	12	25.0

Extracts of *Acorus calamus* was effective against *E. coli, Sh. dysentriae* and *S. aureus*. Highest ZOI was observed with ethylacetate fraction against *S. dysenteriae* and smallest with methanol fraction against *S. aureus.*

5.3.2 ZOI and MBC given by *Aegle marmelos* against tested bacteria

Table 4: ZOI and MBC given by *Aegle marmelos* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	12	50
		Methanol	12	50
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	18	3.12
		Methanol	20	6.25
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	13	25
		Ethylacetate	16	25
		Methanol	12	50

Extracts of *Aegle marmelos* was effective against *K. pneumoniae, S. dysenteriae* and *S. aureus*. The highest ZOI was shown by methanol fraction against *S. dysenteriae*.

5.3.3 ZOI and MBC given by *Asparagus racemosus* against tested Bacteria

Table 5: ZOI and MBC given by *Asparagus racemosus* against tested Bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	13	50
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate		
		Methanol	-	-
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	-	-
		Ethylacetate	13	50
		Methanol	-	-

Extracts of *Asparagus racemosus* showed very narrow antibacterial activity. Only *P. mirabilis* and *S. aureus* were sensitive. The plant extract was bactericidal only at high concentration of 50 mg/ml.

5.3.4 ZOI and MBC given by *Holarrhena antidysenterica* against tested bacteria

Table 6: ZOI and MBC given by *Holarrhena antidysenterica* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	11	25
		Ethylacetate	-	-
		Methanol	-	-
2	Klebsiella pneumoniae (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	10	25
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate	11	50
		Methanol	-	-
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	11	25
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	17	6.25
		Methanol	-	-
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	12	50

Table no. 6 showed that only hexane fraction inhibited *E. coli*. Ethylacetate fraction inhibited 2 bacterial species viz*: P. mirabilis* and *S. dysenteriae* while methanol fraction inhibited *K. oxytoca.* Highest ZOI (17 mm) was observed by ethylacetate fraction against *S. dysenteriae* while smallest (10 mm) was shown by methanol fraction against *K. oxytoca.*

5.3.5 ZOI and MBC given by *Mimosa pudica* against tested Bacteria

Table 7: ZOI and MBC given by *Mimosa pudica* against tested Bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane		
		Ethylacetate	-	-
		Methanol	-	-
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	16	25
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate		
		Methanol	-	-
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	17	50
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	10	50
		Ethylacetate	19	25
		Methanol	21	25

Table no. 7 shows that hexane fraction *of M. pudica* only inhibited *S. aureus*. Ethylacetate fraction inhibited only one bacterial species while methanol fraction inhibited 3 bacterial species. High MBC against *S.* Paratyphi shown that methanol extract of the plant may be primarily bacteriostatic.

5.3.6 ZOI and MBC given by *Terminalia bellirica* against tested bacteria

Table 8: ZOI and MBC given by *Terminalia bellirica* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	31	3.12
		Methanol	21	12.5
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	13	25
		Methanol	-	-
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate		
		Methanol	-	-
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate	15	50
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	18	6.25
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	17	25
		Methanol	19	12.5
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	-	-
		Ethylacetate	18	6.25
		Methanol	19	12.5

Five out of ten bacteria were sensitive to the antibacterial action of *T. bellirica*. Large ZOI (31 mm) along with low MBC (3.12 mg/ml) shown by ethylacetate fraction in Table no. 8 shown that the fraction may have bactericidal action against *E. coli*.

5.3.7 ZOI and MBC given by *Terminalia chebula* against tested bacteria

Table 9: ZOI and MBC given by *Terminalia chebula* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	13	25
		Methanol		
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	12	50
		Methanol	-	-
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate		
		Methanol	-	-
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	-	-
		Ethylacetate		
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate		
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	18	12.5
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	21	12.5
		Methanol	22	12.5
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	11	>50
		Ethylacetate	19	12.5
		Methanol	21	6.25

Table 9 shows that out of 10 bacterial species tested, only 5 bacteria were inhibited by *T. chebula* extracts. Maximum ZOI was shown by methanol fraction against *S. dysenteriae* while minimum ZOI was shown by hexane fraction against *S. aureus.* Activity of hexane fraction of *T. chebula* against *S. aureus* was weak as evident by MBC >50 mg/ml.

5.3.8 ZOI and MBC given by *Tinospora cordifolia* against tested bacteria

Table 10: ZOI and MBC given by *Tinospora cordifolia* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	14	25
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	13	25
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane	13	50
		Ethylacetate	-	-
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-

Table no. 10 shows that only one bacterial strain was inhibited by hexane fraction while two bacterial species were inhibited by methanol fraction. None of the organism was inhibited by ethylacetate fraction. None of the bacteria was killed by concentration of plant extract below 25 mg/ml.

5.3.9 ZOI and MBC given by *Woodfordia fruticosa* against tested bacteria

Table 11: ZOI and MBC given by *Woodfordia fruticosa* against tested bacteria

S.N	Test Organisms	Extract	Antimicrobial activity	
			ZOI (mm)	MBC (mg/ml)
1	*Escherichia coli* (ATCC 25922)	Hexane	-	-
		Ethylacetate	26	12.5
		Methanol	-	-
2	*Klebsiella pneumoniae* (ATCC 20063)	Hexane	-	-
		Ethylacetate	-	-
		Methanol	12	50
3	*Klebsiella oxytoca*	Hexane	-	-
		Ethylacetate	-	-
		Methanol		
4	*Proteus mirabilis* (ATCC 49132)	Hexane	-	-
		Ethylacetate	-	-
		Methanol		
5	*Proteus vulgaris*	Hexane	-	-
		Ethylacetate	-	-
		Methanol	-	-
6	*Pseudomonas aeruginosa* (ATCC 27853)	Hexane		
		Ethylacetate	-	-
		Methanol	-	-
7	*Salmonella* Typhi	Hexane	-	-
		Ethylacetate	15	25
		Methanol	-	-
8	*Salmonella* Paratyphi A	Hexane	-	-
		Ethylacetate	14	25
		Methanol	-	-
9	*Shigella dysenteriae*	Hexane	14	50
		Ethylacetate	26	3.12
		Methanol	23	6.25
10	*Staphylococcus aureus* (ATCC 25923)	Hexane	-	-
		Ethylacetate	20	12.5
		Methanol	16	25

As shows in table 11 only 6 bacteria were inhibited by *W. fruticosa*. Low MBC against *S. dysenteriae* shown by ethylacetate fraction of *W. fruticosa* shows that fraction may be bactericidal even at low concentration.

5.4 Antimicrobial susceptibility pattern

Table12: Antibiotic sensitivity pattern of selected bacteria

Organism	Antibiotic used												
	Amikacin	Ampicillin	Cephalexin	Chloramphenicol	Ciprofloxacin	Claxacillin	Co-trimoxazole	Erythromycin	Gentamycin	Nalidixic acid	Norfloxacin	Ofloxacin	Penicillin G
S. aureus (ATCC 25923)	-	-	S	-	-	R	S	S	-	-	-	S	R
E. coli (ATCC 25922)	-	-	-	S	S	-	S	-	-	-	S	S	R
K. pneumoniae (ATCC 20063)	R	R	R	-	S	-	I	-	R	-	-	-	-
K. oxytoca	R	S	S	I	-	-	S	-	S	-	-	-	-
P. mirabilis (ATCC 49132)	-	-	-	R	S	-	I	-	-	-	S	S	R
P. vulgaris	-	-	-	S	I	-	S	-	-	-	S	S	R
Ps. aeruginosa (ATCC 27853)	S	-	R	R	S	-	-	-	S	-	S	-	-
S. Paratyphi A	-	I	-	S	R	-	-	-	-	R	S	R	-
S. Typhi	-	S	-	S	S	-	-	-	-	R	S	S	-
Sh. dysenteriae	S	-	-	I	R	-	I	I	S	-	-	-	-

Note: R = resistance S = sensitive I = Intermediate

(-) = antibiotic sensitivity test not done

Modified Kirby-Bauer disc diffusion assay method was applied to determine Antibiotic susceptibility test of selected bacteria which is given in table 12. *K. pnemuoniae* was the most resistant bacteria among selected bacterial species.

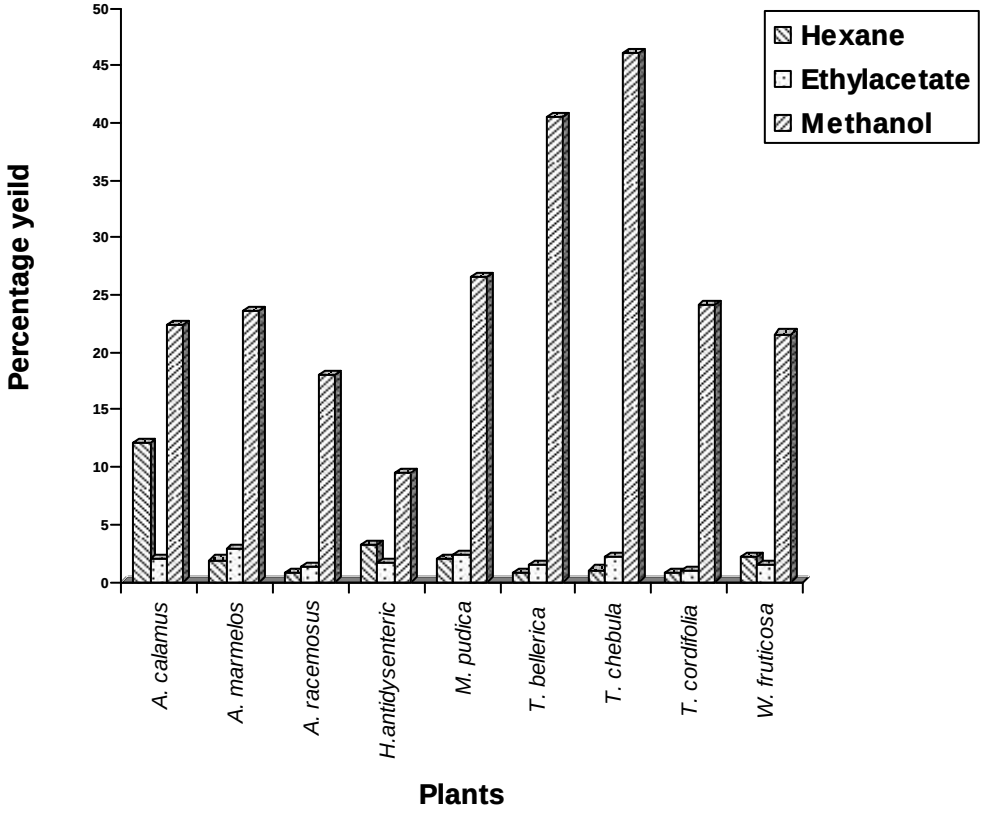


Figure 1: Percentage yield of Hexane, Ethylacetate, Methanol fraction from different Plant species.

The figure 1 shows that maximum percentage yield was given by methanol fraction of *T. chebula* followed by *T. bellirica*. The lowest yield was given by hexane fraction of *A. racemosus* followed by *T. bellirica*.

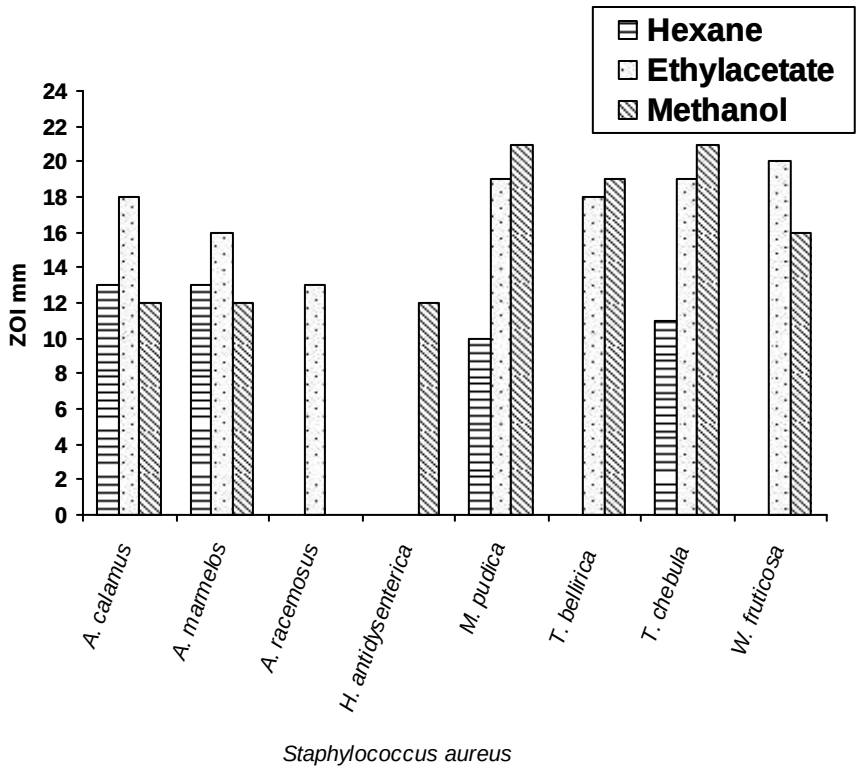


Figure 2: ZOI exhibited by different plant extracts against *Staphylococcus aureus.*

Figure 2 shows that methanol fraction of *T. chebula* and *M. pudica* showed the maximum zone of inhibition (ZOI) against *S. aureus.*

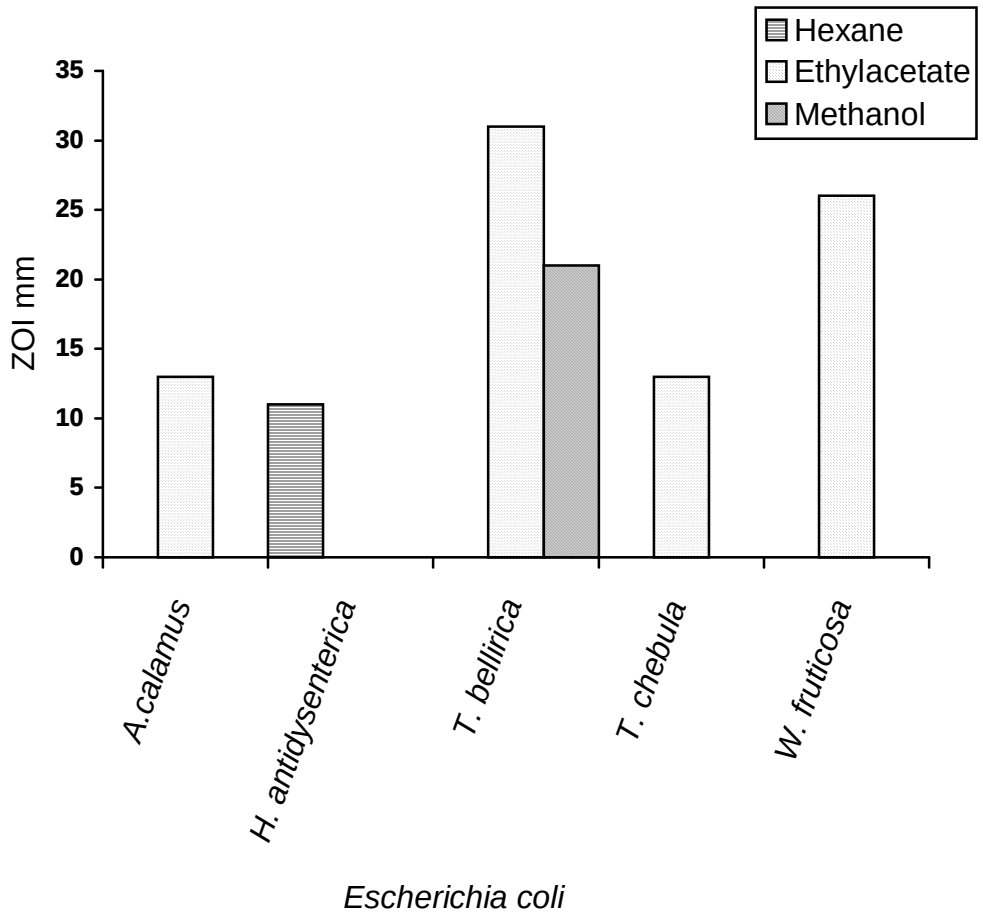


Figure 3: ZOI exhibited by different plant extracts against *Escherichia coli.*

Figure 3 shows that maximum ZOI was given by ethylacetate fraction of *T. bellerica* while lowest was given by hexane fraction of *H. antidysenterica*. Out of 10 tested medicinal plants only 5 showed antimicrobial activity against *E. coli.*

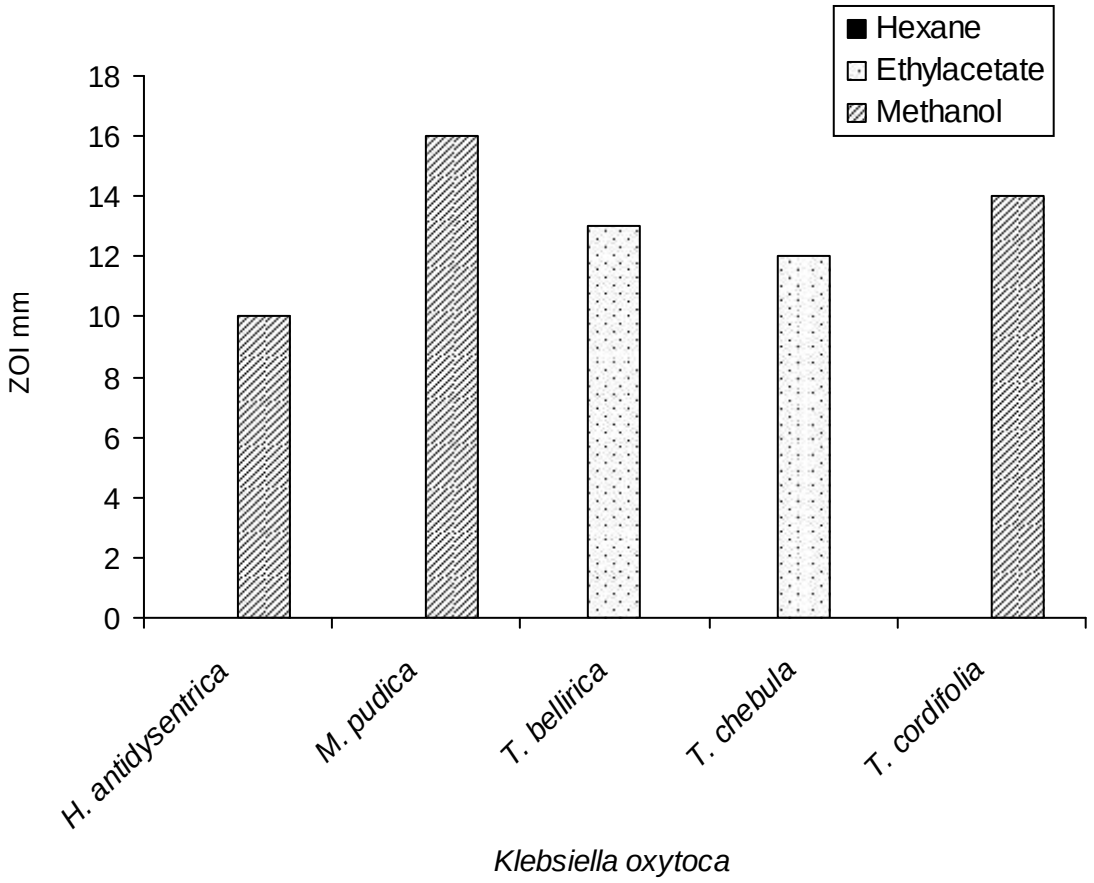


Figure 4: ZOI exhibited by different plant extract against *Klebsiella oxytoca*.

Figure 4 shows that only 5 plants showed antibacterial activity against *K. oxytoca*. Among 5 plants, methanol fraction of *M. pudica* showed maximum zone of inhibition while methanol fraction of *H. antidysenterica* showed minimum zone of inhibition

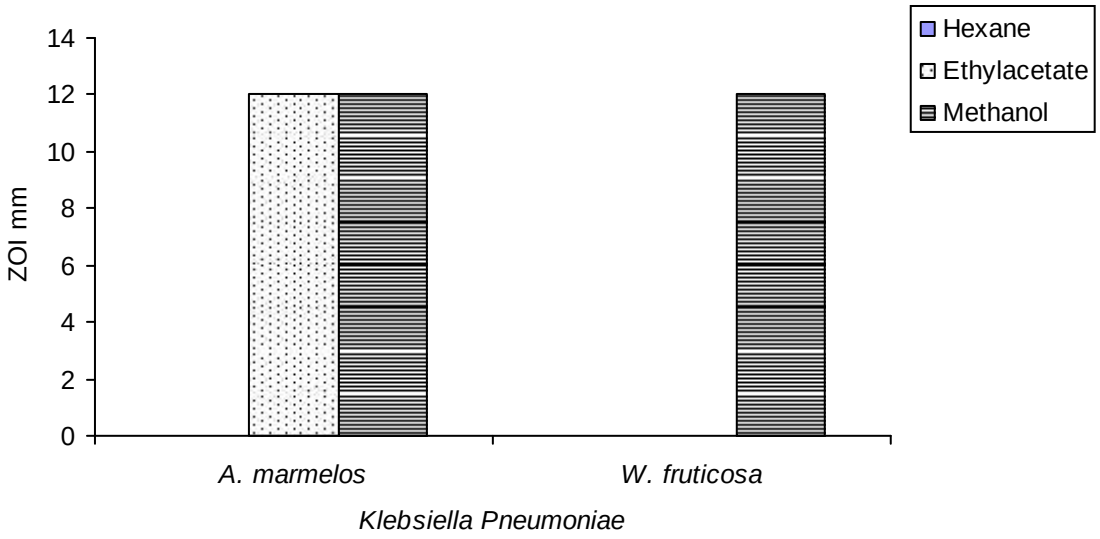


Figure 5: ZOI exhibited by different plant extract against *Klebsiella pneumoniae*.

Figure 5 shows that out of 27 plant extracts, only 3 extracts from 2 plants had antimicrobial activity against *K. pneumoniae*. However, hexane extracts showed no ZOI.

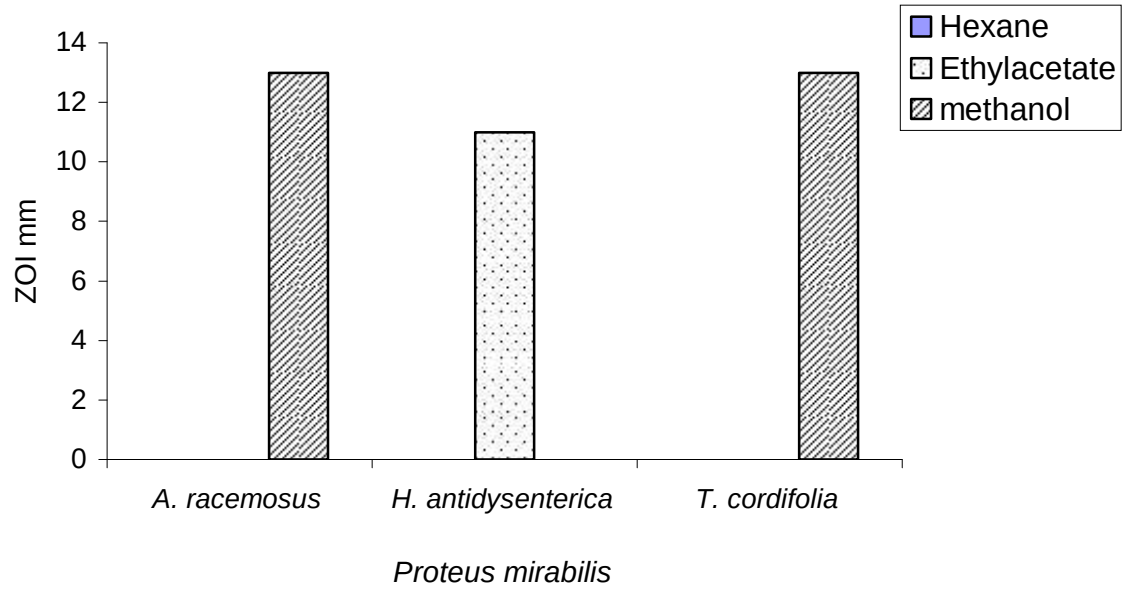


Figure 6: ZOI exhibited by different plant extracts against *Proteus mirabilis*.

Only 3 plant extracts exhibited zone of inhibition against *P. mirabilis*. Among this methanol fraction of *A. racemosus* and *T. cordifolia* produced maximum ZOI.

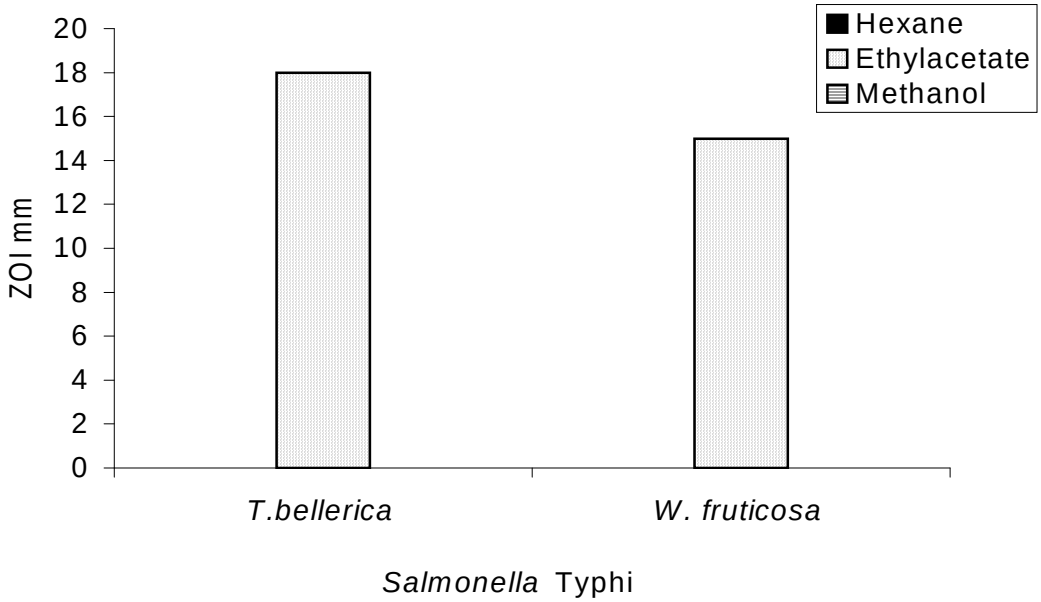


Figure 7: ZOI exhibited by different plant extract against *Salmonella* Typhi.

Only two plant extracts showed antimicrobial activity against *Salmonella* Typhi. Among these two plants ethylacetate fraction of *T. bellirica* showed maximum zone of inhibition.

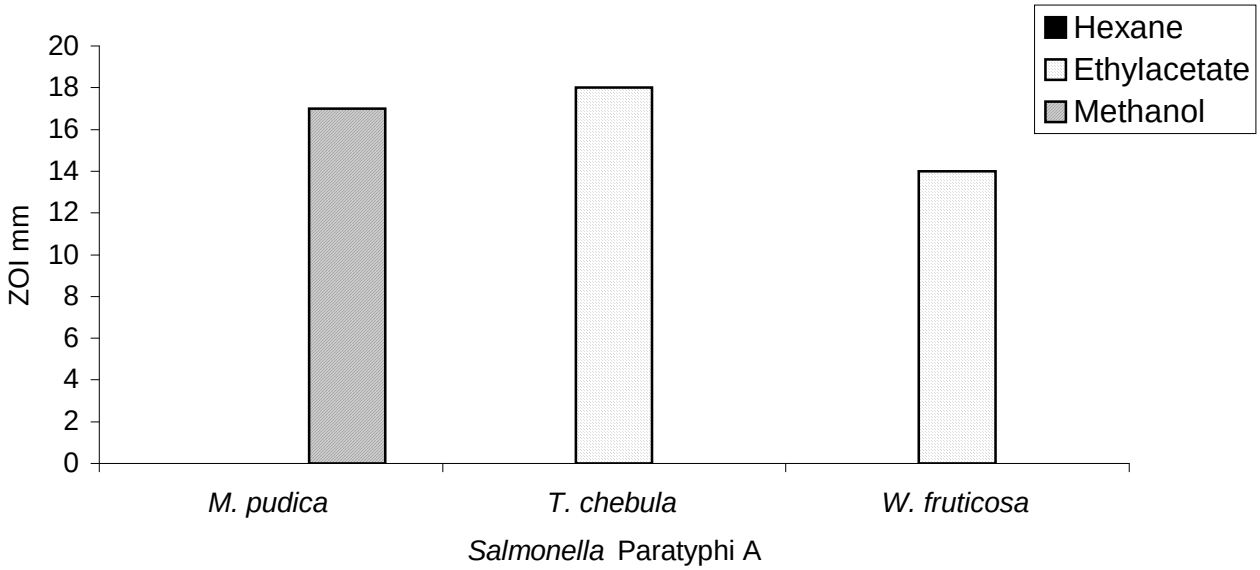


Figure 8: ZOI exhibited by different plant extract against *Salmonella* Paratyphi A.
Only 3 extracts showed antibacterial activity against *S.* Paratyphi A. Ethylacetate fraction of *T. chebula* produced the maximum zone of inhibition.

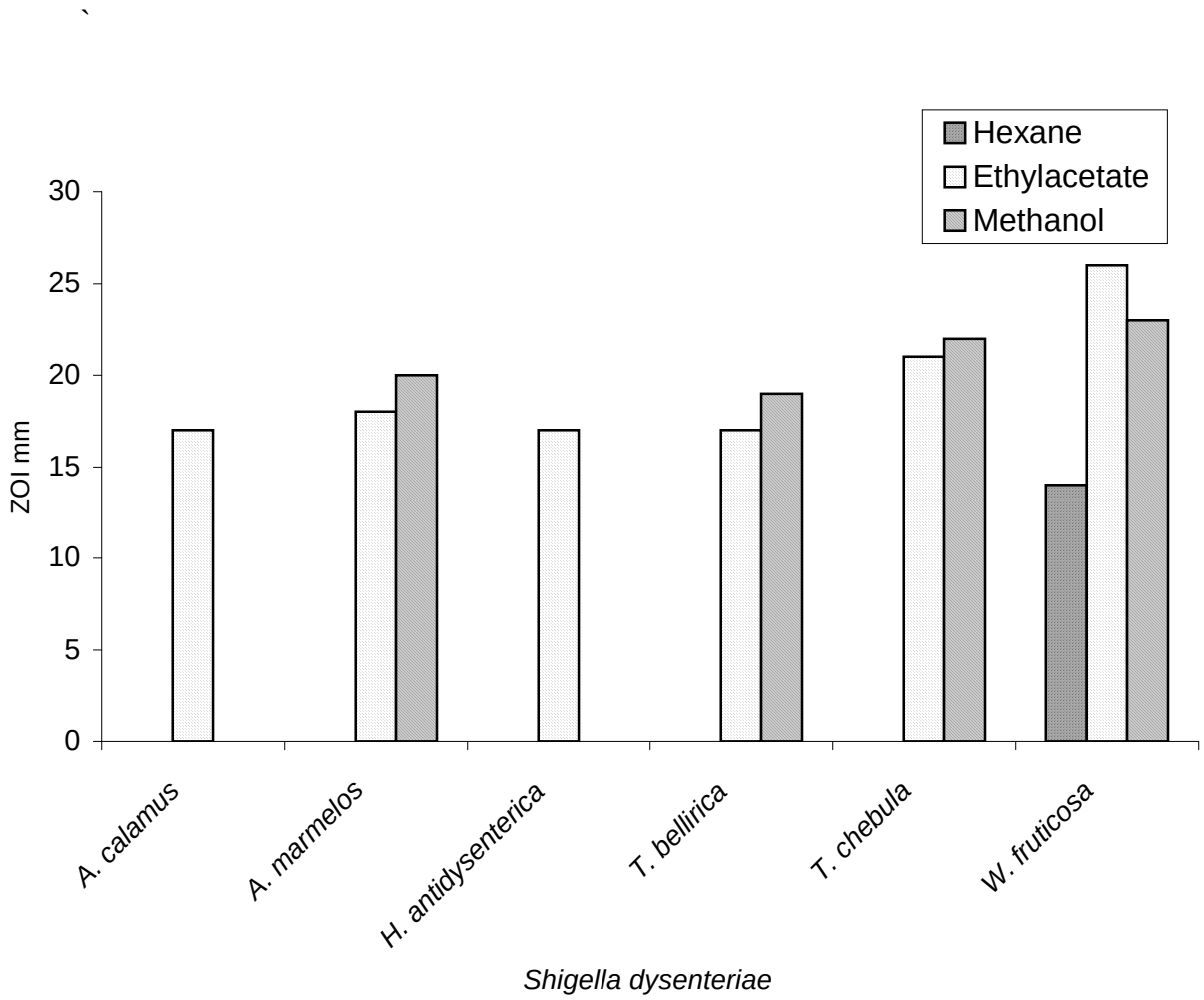


Figure 9: ZOI exhibited by different plant extract against *Shigella dysenteriae*.

Ethylacetate fraction of *W. fruticosa* exhibited maximum zone of inhibition against *S. dysenteriae* whereas hexane fraction of *W. fruticosa* exhibited minimum zone of inhibition against *S. dysenteriae.*

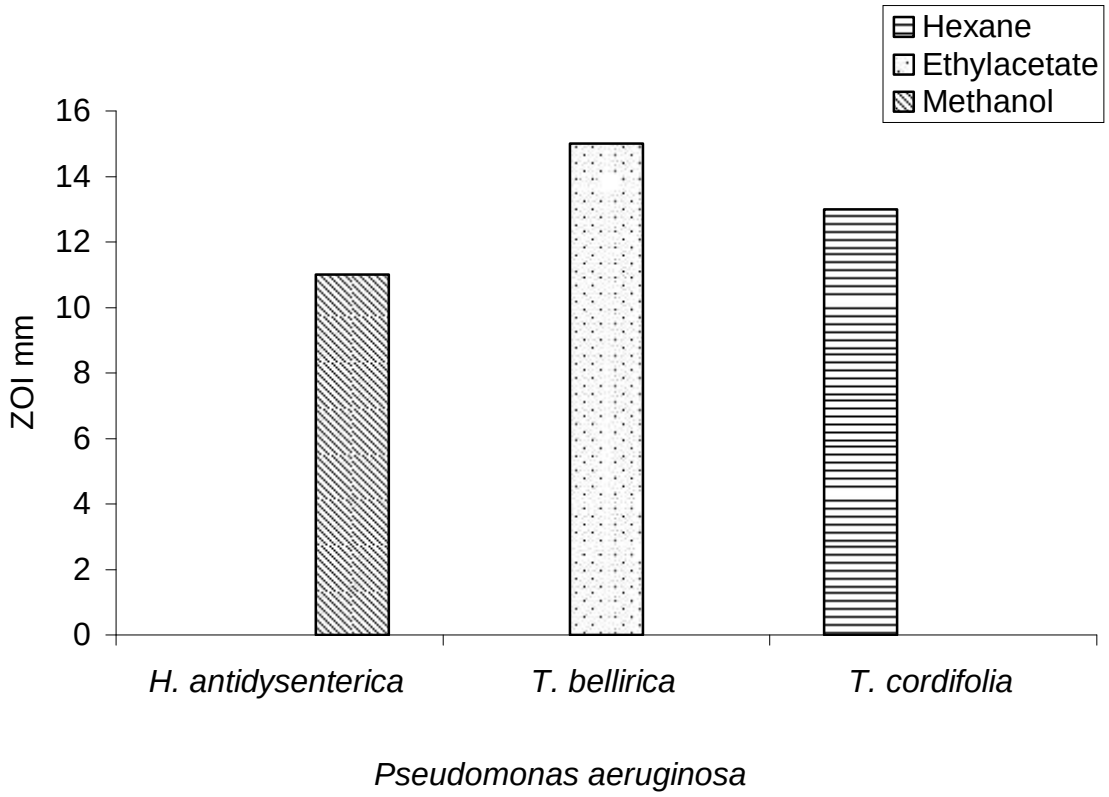
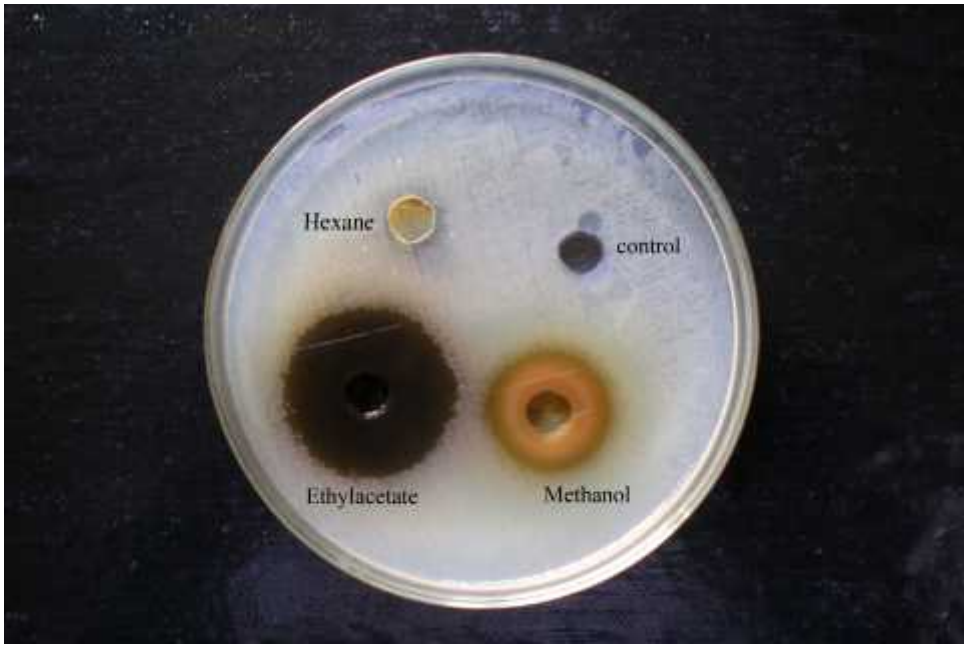


Figure 10: ZOI exhibited by different plant extracts against *Pseudomonas aeruginosa*

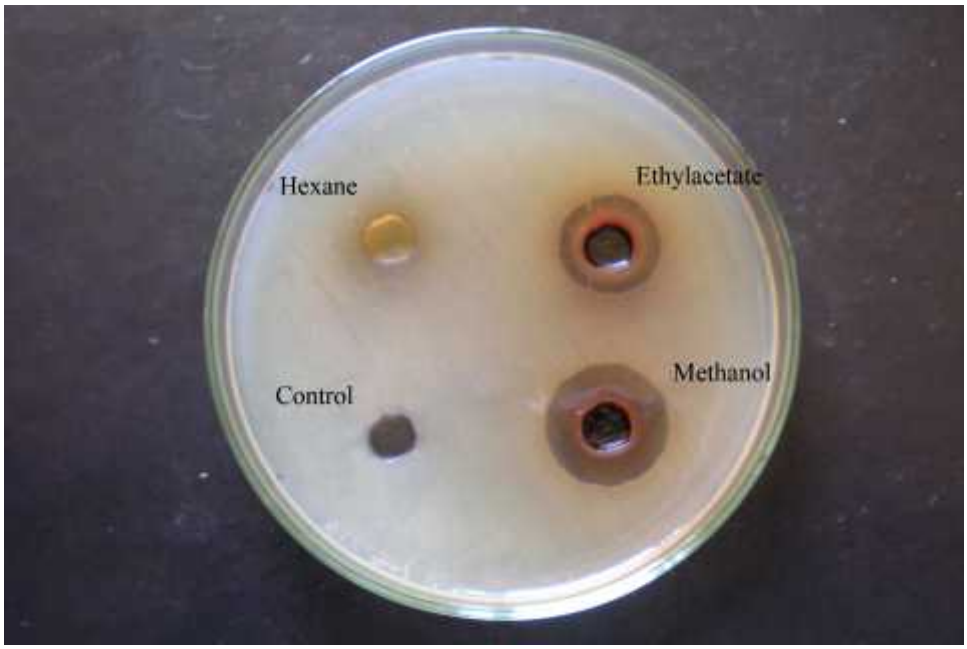
Figure 10 shows that only 3 extracts showed ZOI with maximum zone of inhibition by ethylacetate fraction of *T. bellirica* against *Ps. aeruginosa*.

Photograph 1: Soxhlet extractor showing extraction of medicinal plants

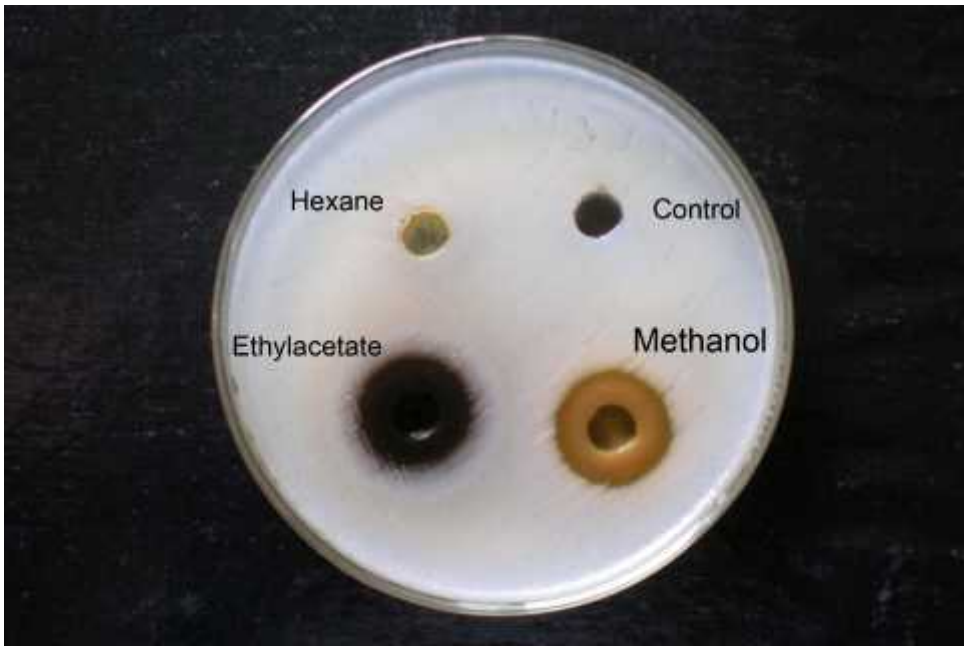
Photograph 2: Rotary vacuum evaporator showing the recovery of extracting solvent



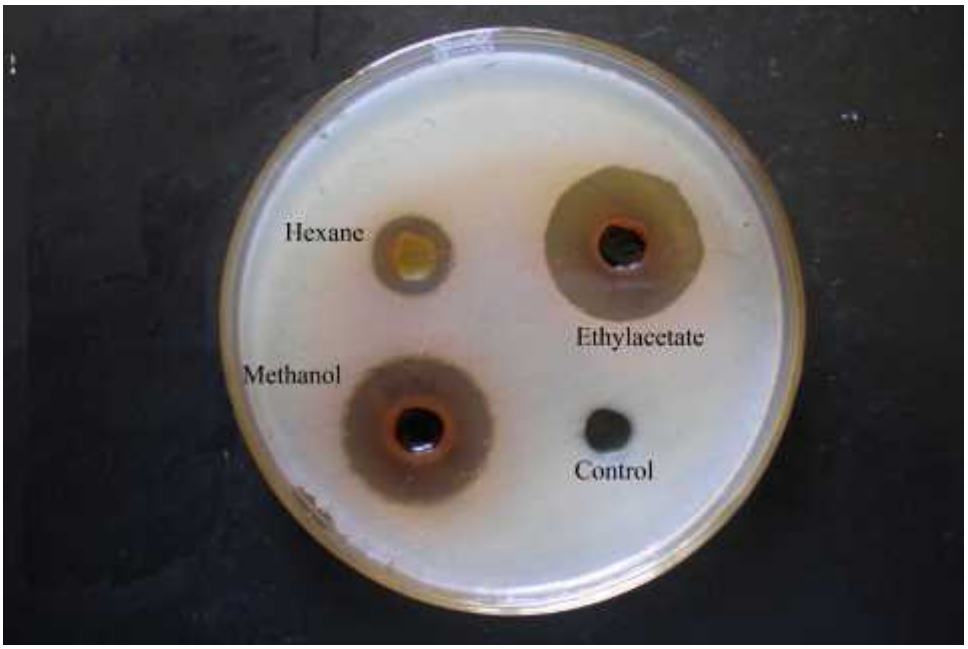
Photograph 3 Zone of inhibition produced by hexane, ethylacetate and methanol fraction of *Terminalia bellirica* against *Escherichia coli*



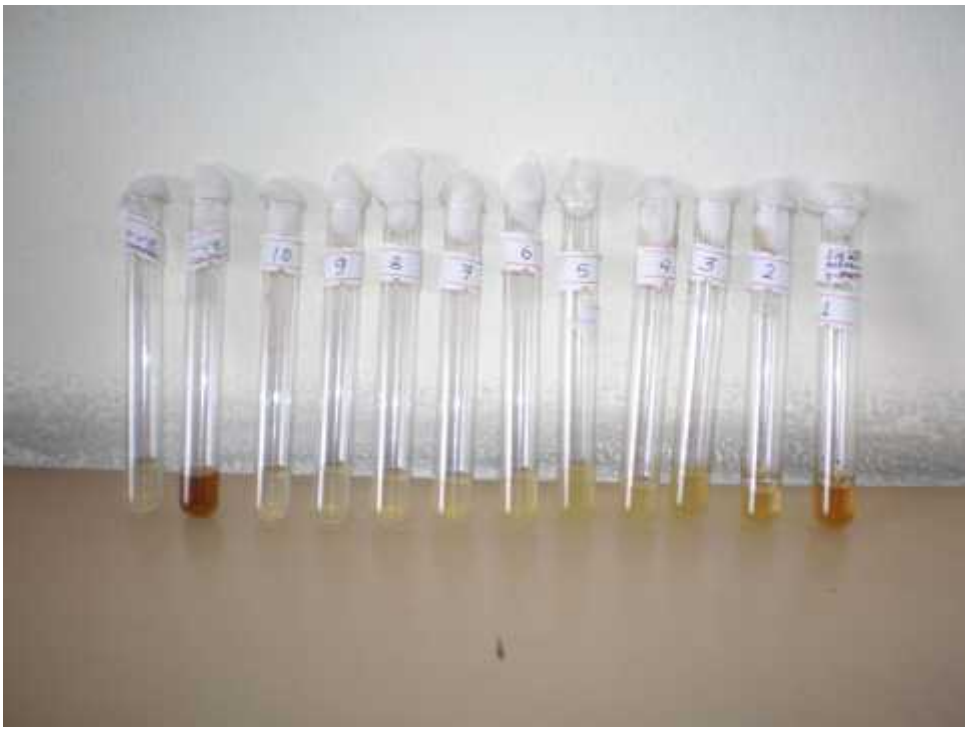
Photograph 4 Zone of inhibition produced by hexane, ethylacetate and methanol fraction of *Aegle marmelos* against *Shigella dysenteriae*



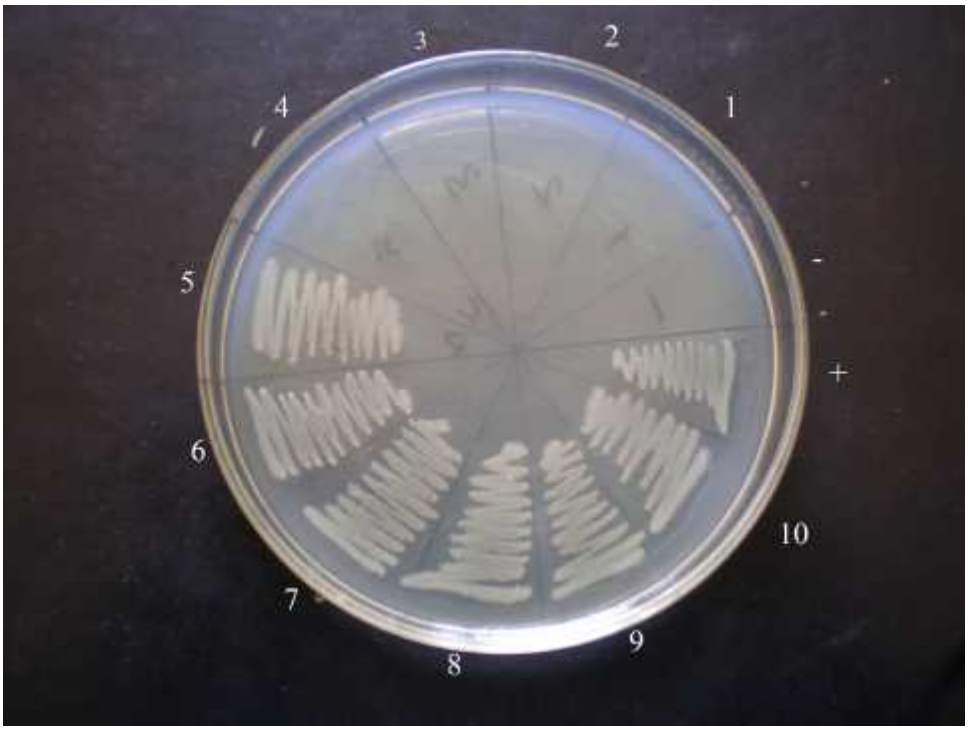
Photograph 5 Zone of inhibition produced by hexane, ethylacetate and methanol fraction of *Terminalia chebula* against *Shigella dysenteriae*



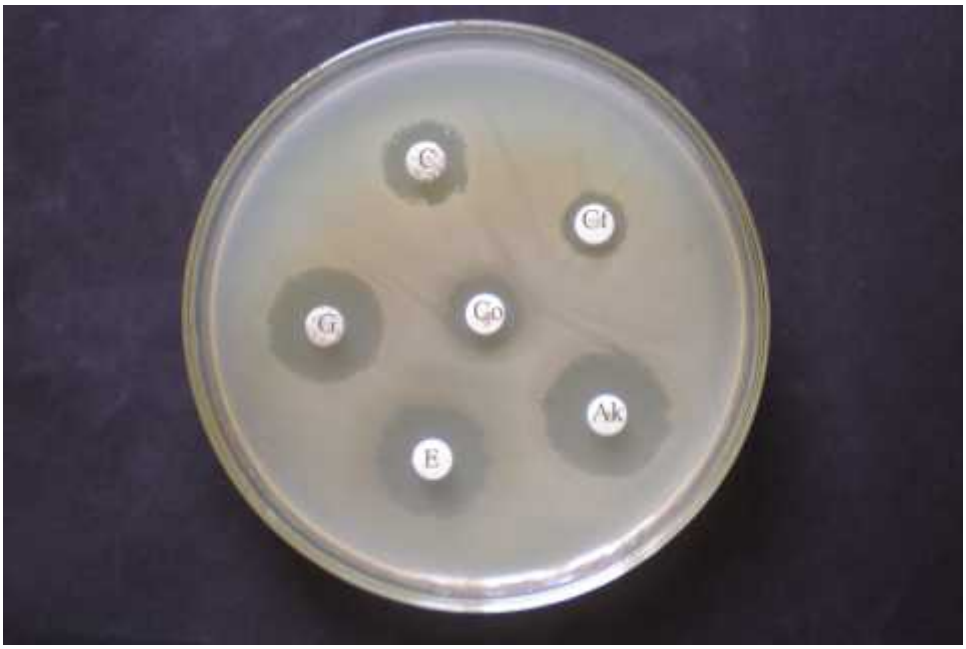
Photograph 6 Zone of inhibition produced by hexane, ethylacetate and methanol fraction of *Woodfordia fruticosa* against *Shigella dysenteriae*



Photograph 7 Determination of MIC value of ethylacetate fraction of *Terminalia bellirica*



Photograph 8 Determination of MBC value of methanol fraction of *Aegle marmelos* against *Shigella dysenterica*



Photograph 9 Determination of antibiotic susceptibility pattern of *Shigella dysenteriae*

Photograph 10 Different medicinal plant sample

CHAPTER–VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

In this study, nine medicinal plants were selected randomly and then collected from various parts of the country. Among nine medicinal plants, three were collected from market 'Ason bazaar' Kathmandu. These medicinal plants were selected on the basis of their useful as cited in literature and folklore for diseases like cough, fever, wounds, diarrhea, dysentery, sore throat etc.

Identification of the collected medicinal plants was done by referring various literature and herbarium found in Ayurveda Campus, Kirtipur and Central Department of Botany, Kirtipur. The name of the plant, place of collection and parts used are given in Appendix B.

Samples were properly labeled and then left for shade drying as drying in sunlight may cause loss of some active compounds. Large plant if extracted without grinding results in poor percentage yield on extraction, also large were chopped before drying and then subjected to grinding to facilate complete and easy extraction of the active compounds.

Upon going through various literatures, it was found that there is no specific process for the extraction of compounds from the plants. Among percolation and soxhlet extraction process, mostly preferred soxhlet extraction process for the easy and better extraction of compounds from the plants.

Various types of solvent were used for the extraction of compounds from the plant sample. Commonly, methanol, ethanol and other alcoholic solvent were used. Ironically, water, the universal solvent wasn't used frequently as it is less effective for non polar organic compound also removal of water from solution needs high temperature or sophiscated equipment. The crude extract of a plant may consist of several substances

widely differing in their chemical properties and polarities. Therefore it is essential at first to separate them into groups of compounds with similar polarities before identifying the active compounds.

In this study, three solvent viz: hexane, ethylacetate and methanol were used in the order of increasing polarity to obtain hexane, ethylacetate and methanol extract (fraction) by using soxhlet extraction method. Thapa (2006) and Baidya (2001) used hexane, chloroform, n-butanol and aqueous solvent in the order of increasing polarity for the extraction of compounds from the plant. This study supports our study purpose.

It was found that for the same plant, continuous extraction with different solvent viz: Hexane, ethylacetate and methanol in the increasing order of polarity gave different percentage yield. Hexane solvent takes less time for complete extraction than ethylacetate and methanol solvent.

In the present study, highest yield was observed from methanol fraction of *T. chebula* (46.1%) followed by methanol fraction of *T. bellirica* (40.46%). The least yield were from the hexane fraction of *A. racemosa* (0.7%) followed by hexane fraction of *T. bellirica* (0.8%). The range of % yield from hexane solvent is 0.7-3.28% exception being 12.08% from the *A. calamus*. Range of % yield from for ethylacetate fraction be 0.9-2.83 % while for methanol yield ranges of % was 9.43-46.

There is a distinct difference in the % yield of extracts of different solvent even for same sample. The different in yield might be due to plants contain more polar compounds than non-polar compounds. There are various factors which influences the % yield from plants are: - extraction type, part of plant materials, finless of powder, extent of dryness and nature of solvent used for extraction etc. The old and dried plant material yield lesser than the fresh ones. Similarly, incomplete extraction results in lesser yield.

Each fraction after removal of solvent was tested for antimicrobial activity against selected ten bacterial species, by agar well diffusion method of Dingle et al. (1953).

In this study 27 extracts obtained by continuous extraction from 9 plants using hexane, ethylactate and methanol solvent in the order of increasing polarity were studied for their antimicrobial property. These extracts were dissolve in 100% DMSO and tested against 10 different bacterial species using agar well diffusion method.

In the study conducted by Abascal and Yamal (2002) showed that "Acorenone, from *Acorus calamus* rhizome, strongly inhibited a strain of *S. aureus* that was resistant to ten common antibiotics". Souwalak et al. (2005) reported that β-asarone from methanol extract of it exhibited inhibitory effect against *Staphylococcus aureus* ATCC 25923. In our study also, methanol fraction of *Acorus calamus* exhibited inhibitory effect against *S. aureus* 25923 which supports our result. During the study it was found that *A. calamus* inhibited only 3 bacterial species viz: *E. coli*, *Sh. dysenteriae* and *S. aureus*. It was ineffective in all other organism included in this study. Ethylacetate fraction shows inhibitory activity against all 3 bacteria while hexane and methanol fraction shows inhibitory activity only against *S. aureus*. This result is in accordance with Ahmad (2001) where the alcoholic extracts of *A. calamus* demonstrate only moderate antibacterial activity viz: *S. aureus, Sh. dysenteriae* and *E. coli*.

Asparagus racemosus gave ZOI against only 2 bacteria viz*: P. mirabilis* and *S. aureus.* Methanol fraction inhibited *P. mirabilis* only, while ethylacetate fraction inhibited *S. aureus* only. Hexane fraction did not inhibit growth of bacteria tested. The study done by Baidya (2001) also found that 95 % ethanol extract from *A. racemosus* produced the similar results.

H. antidysenterica showed relatively broad spectrum activity since it produced ZOI against 6 bacterial strain among tested strain. Hexane fraction was active only against *E. coli*. ethylacetate was active against *P. mirabilis* and *Sh. dysenterica*. Similarly methanol fraction was inhibitory towards against *Ps. aeruginosa* and *S. aureus*. From this study it is clear that plant may contain more polar active compound than non-polar compound. Baidya (2001) showed similar results with ethanolic extracts which support our result.

Meanwhile, *M. pudica* produced ZOI against only to 3 bacteria viz: *S. aureus*, *K. oxytoca a*nd *S.* Paratyphi. Methanol fraction inhibited all 3 positive bacterial species while ethylacetate and hexane fraction inhibit *S. aureus* only. This result did not support the study done by Bajracharya (2007) and Srinivasan (2007) where none of the tested bacteria were inhibited by *M. pudica*.

Terminalia bellerica proved to have relatively wide antibacterial activity, being effective against 6 bacteria out of 10 test bacteria. Ethylacetate fraction inhibited growth of all 6 bacteria viz: *E. coli*, *K. oxytoca*, *Ps. aeruginosa*, *S.* Typhi, *Sh. dysenteriae* and *S. aureus* while methanol fraction was found to be inhibitory towards 3 bacteria viz:E. *coli, Sh. dysenteriae* and *S. aureus*. None of the bacterial species tested was inhibitory to hexane fraction. From this study it was found that active compounds might be polar compounds. Similar type of results was shown by ethanolic extracts of *T. bellirica* (Ahmad, 2001). Similarly, Ahmad (1998) found no antibacterial activity to tested bacterial species by hexane fraction which further supports our result.

Terminalia chebula was inhibitory to 5 bacterial species (50%) out of 10 bacterial species tested. Its methanol and ethylacetate fraction showed more inhibitory effect than hexane fraction. It inhibited *E. coli, K. oxytoca, S. paratyphi, Sh. dysenteriae* and *S. aureus*. Hexane fraction only inhibited *S. aureus*. This result shown that *T. chebula* contains more active compounds than non-polar compounds.

Similarly, *Tinospora cordifolia* produced ZOI against 3 bacterial species viz: *K. oxytoca*, *P. mirabilis* and *Ps. aeruginosa*. No antimicrobial activity produced by any solvent used against *S. aureus*. Ethylacetate fraction did not give any activity against tested bacterial species. Sami (2005) found that methanolic extracts from *T. cordifolia* inhibited growth of *P. mirabilis*.

W. fruticosa was effective against 6 bacteria out of 10 bacterial species tested. The most effective fraction was found to be ethylacetate fraction against *E. coli* and *Sh. dysenteriae*. All three fraction produced ZOI against *S. dysenteriae*. No antimicrobial activity was produced by *W. fruticosa* against *K. oxytoca, P. mirabilis, P. vulgaris* and

Ps. aeruginosa. Bajaracharya (2007) also found that all the tested bacterial species was inhibited by *Woodfordia fruticosa*. This result also supports our result as the result showed antibacterial activity towards 6 bacteria.

Meanwhile, out of 10 bacterial species, *Aegle marmelos* inhibited 3 bacterial species viz: K. *pneumoniae, Sh. dysenteriae* and *S. aureus.* All three fractions show antibacterial activity with *S. aureus* whereas Bajracharacharya (2007) found that no antibacterial activity at all by *A. marmelos* against tested bacterial species.

Qualitative screening of antimicrobial substance does not indicate the extent of the potency of a sample. A substance may demonstrate a wide spectrum of activity but have minuscule potency. Such substance has no utility practically and development of such product is not economically feasible. So, the antimicrobial activity must also be quantitatively assayed. For this, two methods: Agar well diffusion and two fold serial dilution were employed.

In Agar well diffusion method, the material under test diffuses from agar well into the surrounding agar in a concentric circle and inhibits or kills the microorganisms that are susceptible. This effect manifested by a clear zone around the well is measured as zone of inhibition (ZOI).

In this study for *S. aureus*, 18 test suspensions out of 27 suspension of hexane, ethylacetate and methanol fraction produced zone of inhibition and 8 out of 9 plant extracts were effective. Largest ZOI was observed with methanol fraction of *T. chebula* and *M. pudica* with ZOI 21 mm respectively. The smallest ZOI of 10 mm was observed with hexane fraction of *M. pudica*. The ZOI value for other test suspension of hexane, ethylacetate and methanol fraction of studied plants was in the range of 12-20 mm.

K. pneumoniae was the second most resistant bacteria among the tested bacteria. It was inhibited only by ethylacetate fraction of *A. marmelos*, and methanol fraction of *A. marmelos* and *W. fruticosa*. All three active fractions showed ZOI of 12mm.

In case of *E. coli*, six test suspension out of 27 test suspension of hexane, ethylacetate and methanol fraction of 9 plant shown inhibitory activity. Only five plants showed ZOI. The highest antibacterial activity was shown by ethylacetate fraction of *T. bellirica* and lowest of 11mm by hexane fraction of *H. antidysenterica*. The bacteria were totally resistant to *A. marmelos, A. racemosus, T. cordifolia* and *M. pudica*.

For *K. oxytoca*, five test suspensions from nine plants under study exhibited the inhibitory effect and produced ZOI. Methanol fraction of *M. pudica* was most potent with 16mm zone of inhibition while minimum potency was given by methanol fraction of *H. antidysenterica*. None of the hexane fraction of studied nine plant produced zone of inhibition against *K. oxytoca*.

S. Typhi showed less antimicrobial effect. Only 2 plants extract viz: ethylacetate fraction of *T. bellirica* and *W. fruticosa* showed antibacterial effects with ZOI value 18mm and 15mm respectively.

In the case of *S.* Paratyphi, only ethylacetate fraction of *T. chebula* and *W. fruticosa*; and methanol fraction of *M. pudica* showed significant zone of inhibition. The largest ZOI 18mm was produced by *T. chebula* while lowest (14mm) was produced by *W. fruticosa*.

Antibiotic susceptibility pattern against *Sh. dysenteriae* shown that only 11 extracts from 6 plants showed antimicrobial activity. The highest ZOI was shown by ethylacetate fraction of methanol while lowest ZOI shown by hexane fraction of *W. fruticosa*.

For *Ps. aeruginosa*, ethylacetate fraction of *T. bellirica* was most potent with 15 mm ZOI while lowest inhibitory zone 11mm was exhibited by methanol fraction of H. antidysenterica. Hexane fraction of *T. cordifolia* produced ZOI 12mm. All other plant extracts were resistant to *Ps. aeruginosa*.

For *P. mirabilis*, methanol fraction of *T. cordifolia*, methanol fraction of *A. racemosus* and ethylacetate fraction of *H. antidysenterica* produced zone of inhibition. These three fraction produced ZOI at the range of 11mm to 13mm.

P. vulgaris was the most resistance bacterium. None of the plant extracts studied gave any inhibitory zone.

However, all the plant extracts used for this study were active against both Gram positive and Gram negative bacteria except *T. cordifolia* which were active only against Gram negative. The largest ZOI was 31mm produced by ethylacetate fraction of *T. bellirica* while lowest of 10mm by hexane fraction of *T. bellirica*.

The diameter of zone of inhibition largely depends upon the diffusibility of the antimicrobial substance. Hence sometimes the ZOI demonstrated the antimicrobial substance doesn't commentate the efficiency of the substance. This necessitates the determination of MIC and MBC to give correct picture of antimicrobial potency of the compound. Since the extracts were colored, hence MBC was done in this study by two fold serial dilution method.

For *S. aureus*, lowest MBC of 6.25 mg/ml was observed in ethylacetate fraction of *T. chebula* and methanol fraction of *T. bellirica*. The highest MBC (>50 mg/ml) was found in hexane fraction of *T. chebula*. Most of the active fraction against *S. aureus* gave MBC at the range of 12.5 to 50 mg/ml.

For *E. coli*, most effective plant extracts was found to be ethylacetate fraction of *T. bellirica* with MBC value of 3.12 mg/ml and highest MBC value of 50 mg/ml was observed in ethylacetate fraction of *A. calamus*.

In the case of *K. pneumoniae*, all three fractions viz: ethylacetate and methanol fraction of *A. marmelos*; and methanol fraction of *W. fruticosa* showed same MBC value of 50 mg/ml.

For *K. oxtoca*, least MBC value was showed by two plant extracts only. The most bactericidal and least MBC value of 6.25 mg/ml was observed in ethylacetate fraction of *T. bellirica* while 25 mg/ml MBC was observed in ethylacetate fraction of *W. fruticosa*.

Among two plant extracts that inhibited *S.* Typhi, the most lethal and least MBC value of 6.25 mg/ml was observed in ethylacetate fraction of *T. bellirica* while 25 mg/ml MBC was observed in ethylacetate fraction of *W. fruticosa*.

For *S.* Paratyphi, among three active extracts, the most bactericidal plant extract was found to be ethylacetate fraction of *T. chebula* which gave MBC value of 12.5 mg/ml. High MBC value of 50 mg/ml was observed in methanol fraction of *M. pudica*.

Only three fraction viz: hexane fraction of *T. cordifolia*, ethylacetate fraction of *T. bellirica* and methanol fraction of *H. antidysenterica* produced MBC in the range of 12.5-25 mg/ml against *Ps. aeruginosa*.

In the case of *P. mirabilis*, methanol fraction of *T. cordifolia* found to produce MBC of value 25 mg/ml while methanol fraction of *A. racemosus* and *H. antidysenterica* found to produce MBC 50 mg/ml.

In over all analysis, most bactericidal and lowest MBC value of 3.12 mg/ml was shown by *ethylacetate* fraction of *T. bellirica* against *E. coli and* ethylacetate *fraction of W. fruticosa* against *Sh. dysenteriae*. Most of the MBC value of other plant extracts were in the ranges of 6.25 to 25 mg/ml. Least potent i.e highest MBC value of >50 mg/ml was shown by hexane fraction of *T. chebula* against *S. aureus*.

The extracts from medicinal plants showing large ZOI and small MBC value, may contain those compound, which are able to inhibit or kill the microbial population of tested bacteria. Conversely, the extracts, from medicinal plants showing large ZOI and large MBC value may contain those compounds which diffuse through the medium readily and inhibit the growth but could not kill the organism at the same time. For instance, the linear relationship between ZOI and MBC value was not observed.

Medicinal plants showing large ZOI as well as large MBC are that they are able to diffuse well but primarily inhibitory for e.g. methanol fraction of *M. pudica* having ZOI of 21

mm and MBC value of 25 mg/ml against *S. aureus*. Similarly ethylacetate fraction of *M. pudica* having ZOI of 17 mm and MBC value of 50 mg/ml against *S.* Paratyphi.

Large ZOI and small MBC value indicate that the extract diffuses properly through the agar as well as have excellent antimicrobial property. These are the most potent ones and promise good prospects. The ethylacetate fraction of *T. bellirica* demonstrated a ZOI of 31 mm and of MBC of 3.12 mg/ml against *E. coli*. Similarly, ethylacetate fraction of *W. fruticosa* produced a ZOI of 26 mm and MBC of 3.12 mg/ml against *Sh. dysenteriae*.

Small ZOI and large MBC values are given by plants which have poor antimicrobial activity and poor diffusibility. In this experiment, hexane fraction of *M. pudica* produced ZOI of 10 mm and MBC of 50 mg/ml against *S. aureus*. Similarly hexane fraction *of T. chebula* exhibited 11 mm ZOI and >50 mm MBC against *S.* aureus.

Of the hexane fractions from 9 medicinal plants, hexane fraction from 7 plants showed antibacterial activity against the tested bacterial species. Out of 7 plants hexane fractions, four plant showed activity against *S. aureus* (represents Gram positive bacteria). Maximum antibacterial activity was shown by *A. marmelos* and *A. calamus* (13 mm). Only three hexane fractions showed antibacterial activity against Gram negative bacteria (*Sh. dysenteriae, Ps. aeruginosa* and *E. coli*). Other Gram negative bacteria were resistant to the hexane fractions of plants. For Gram negative bacteria, maximum inhibition zone (14 mm) was shown by *W. fruticosa* against *Sh. dysenteriae* while minimum activity was shown by *H. antidysenterica* against *E. coli*.

Out of 9 medicinal plants studied, ethylacetate fractions from 8 plants showed antibacterial activity against the tested bacterial species. *T. cordifolia* did not show any antibacterial activity against the tested bacteria. Out of 8 ethylacetate fractions, seven showed activity against *S*. aureus, with maximum ZOI produced by *W. fruticosa* (20 mm) followed by *T. chebula* and *M. pudica* (19 mm). Ethylacetate fraction of *H. antidysenterica* did not show any activity against *S. aureus*.

In the case of tested Gram negative bacteria, all was inhibited by ethylacetate fractions except *P. vulgaris*. Of this, *E. coli* was inhibited by 4 plants extracts, *K. pneumoniae* by only one plant extracts, *K. oxytoca* by 2 plants extracts, *P. mirabilis* by only one plant extracts, *Ps. aeruginosa* by also one plant extracts, *S.* Typhi by 2 plants extracts, *S.* Paratyhi by 2 plants extracts, *Sh. dysenteriae* by 6 plants extracts.

Among ethylacetate fraction, maximum zone of inhibition was produced by *T.bellirica* against *E. coli* (31 mm) followed by *W. fruticosa* against *E. coli* and *Sh. dysenteriae* (26 mm) while lowest ZOI was produced by *H. antidysenterica* against *P. mirabilis* (11 mm).

In the case of methanol fraction, all plants showed antibacterial activity with at least one tested bacteria. Out of 9 methanol fractions, seven showed antibacterial activity against *S. aureus* with maximum inhibition zone produced by *T. chebula* and *M. pudica* (21 mm) while lowest by *H. antidysenterica, A. marmelos* and *A. calamus* (12 mm).

Similarly in the case of Gram negative bacteria, eight plants methanol fractions showed antibacterial activity except *A. calamus. E. coli* was inhibited by only one plant extracts, *K.* pneumoniae by two plants extracts, *K. oxytoca* by 3 plants extracts, *P. mirabilis* by two plants extracts, *Ps. aeruginosa* by only one plant extracts, *S.* Typhi by none, *S.* Paratyphi by only one plant and *Sh. dysenteriae* by four plants extracts.

Among methanol fraction, maximum zone of inhibition was produced by *W. fruticosa* against *Sh. dysenteriae* (23 mm) followed by *T. bellirica* against E. coli (21 mm) while lowest by *H. antidysenterica* against *K. oxytoca* (10 mm).None of the methanol extracts shown antibacterial activity against *S.* Typhi and *P. vulgaris*.

In classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria (Cutcheon et al; 1992). However, in this study, a large number of the extracts (11 fraction from 7 plants) were active against both Gram positive and Gram negative bacterium, similarly 7 fraction from 5 plants were active against only

Gram-positive bacteria, while only 5 fraction from 4 plants were active against Gram-negative bacteria. The activity against both the types of bacteria may be indicative of the presence of the broad spectrum antibiotic compounds.

6.2 Conclusion

From this study we can conclude that selected medicinal plants under this study had antibacterial activity against common bacterial species. *Holarrhena antidysenterica, Terminalia bellirica* and *Woodfordia fruticosa* had relatively broad spectrum antibacterial activity (inhibited 6 out of 10 bacterial species tested). Gram positive bacteria was more sensitive to selected medicinal plants than Gram negative bacteria.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

- All together 9 medicinal plants were selected and evaluated for their antibacterial activity against 10 bacterial species.
- Three solvents viz: hexane, ethylacetate and methanol were used in the order of increasing polarity to extract crude hexane, ethylacetate and methanol fraction by using soxhlet extractor.
- The highest yield was obtained in methanol fraction of *Terminalia chebula* (46.1%) while lowest in hexane fraction of *Asparagus racemosus* (0.7%).
- Agar well diffusion method and two fold dilution method were used for the evaluation of antibacterial activity.
- *Holarrhena antidysenterica, Terminalia bellirica* and *Woodfordia fruticosa* showed relatively broad spectrum antibacterial activity (inhibited 6 out of 10 bacterial species tested) while *Asparagus racemosus* showed least antibacterial activity (inhibited only 2 bacterial species).
- Among all the tested bacteria, *Staphylococcus aureus* was the most susceptible bacteria being sensitive to 18 fractions from 8 medicinal plants.
- Among tested Gram-negative bacteria, *Shigella dysenteriae* was the most susceptible bacteria being sensitive to 11 fractions from 6 medicinal plants.
- *Proteus vulgaris* was the most resistance bacteria being resistance to all selected plants.
- Largest ZOI (31 mm) was produced by ethylacetate fraction of *Terminalia bellirica* against *E. coli* while lowest ZOI of 10 mm by hexane fraction of *Mimosa pudica* against *Staphylococcus aureus*.
- In this study the plants extracts showed significant antibacterial activity towards Gram positive bacteria than Gram negative bacteria.

7.2 Recommendations

Based on the study, the following recommendations are made:-

1. Out of 701 medicinal plants found in Nepal, antibacterial activity of only 9 medicinal plants extracts have been evaluated in the present study. Antibacterial activity of other medicinal plants should be studied. .

2. Same plants from different areas may give different yields and activity based on variation in ecological factors. These should be studied.

3. *In vivo*-tests should be carried out for further investigation.

4. Extracts which showed higher antibacterial activity during evaluation, should be analyzed in detail to identify the active antibacterial constituents and also their mode of action.

6. Extracts of the plants need to be screened for cytotoxicity activity.

CHAPTER-VIII

8.REFERENCES

Abascal K and Yamal E (2002) Herbs and drug resistance: Part I- herbs and microbial resistance to antibiotics. Alternative and Complementary Therapies 8(2): 237-241.

Adly A (1980) The history of medicinal and aromatic plants. Hamdard Foundation Press, Pakistan pp: 10-20.

Ahmad I and Beg AZ (2001) Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology 74: 113-123.

Akerele O (1993) Summary of WHO Guide lines for the assessment of herbal medicines. Herbal Gram 28: 13-28.

Akinpelu DA. (2000) Antimicrobial activity of *Bryophyllum Pinnatum* leaves. Fitoterapia 71: 193-194.

Alade PI and Irobe ON (1993) Antibacterial activities of crude extracts of *Acalypha wilkesinan* from Manna Nigeria. Journal of Ethnopharmacology 39: 235-236.

Ananthanarayan R and Panikar CKJ (2001) Text book of microbiology. 6th Edition. Reprint, Orient Longman Pvt, Ltd pp 250-298.

Aquil F, Sajjad M, Khan A, Owais M and Ahmad I (2005) Effect of certain bioactive plant extracts on clinical isolates of β-lactamase producing methicillin resistant *Staphylococcus aureus*. Journal of Basic Microbiology 45(2): 106-114.

Awadh Ali NA, Juelich WD, Kusnick C and Lindequist U (2001) Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. Journal of Ethnopharmacology 74: 173-179.

Baidya MR (2001) Screening and evaluation of *in-vitro* antimicrobial activity of some medicinal plants of Nepal. M.Sc. Thesis Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Bajarachra AM (2007) Study of drinking water quality of Kathmandu metropolitian area and evaluation of antibacterial property of some medicinal plants against isolated enteric bacteria. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Balakrishnan N, Bhaskar VH, Jayakar B and Sangameswaran B (2006) Antibacterial activity of *Mimosa pudica*, *Aegle marmelos*, and *Sida cordifolia*. Pharmacognosy Magazine 2(7): 1-5.

Bandow JE, Brotz H, Leichert LIO, Labischinski H and Hecker M (2002) Proteomic approach to under standing antibiotic action. Antimicrobial Agents Chemotherapy 47: 948-955.

Baral SR and Kurmi PR (2006) A compendium of medicinal plants in Nepal. IUCN. The World Conservation Union. Mass Printing Press, Chhauni, Kathmandu pp 52-392.

Barbour E.K, Al Sharif M, Sagherian VK, Habre AN, Talhouk RS and Talhouk SN (2004) Screening of selected indigenous plants of Lebanon for antimicrobial activity. Journal of Ethnopharmacology 93: 1-7.

Baron EJ, Peterson LR and Finegold SM (1994) Bailey and Scott's diagnostic Microbiology, 9th Edition , Mosby year Book, Inc. USA pp 166-177.

Baur AW, Kirby WMM, Sherris JC and Turch M (1966) Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical pathology 45: 494-496.

Bhatta L (1998) Study of antmicrobial properties of *Punica granatum*. M.Sc Thesis Central Department of Microbiology, Kirtipur, Kathmandu, Nepal.

Bhattarai NK (1989) Traditional phytotherapy among the sherpas of the Helambu, central Nepal, Journal of Ethnopharmacology 27: 45-54.

Branter A and Grein E (1994) Antibacterial activity of plant extracts used externally in traditional medicine. Journal of Ethnopharmacology 44: 35-40.

Branter AH and Chakraborty A (1998) *In vitro* antibacterial activity of alkaloids isolated from *Adatoda vasica* NEES. Pharmaceutical and Pharmacological Letters 8(3): 137-139.

Carpinella MC, Herrero GG, Alonso RA and Palacios SM (1999) Antifungal activity of *Melia azadirachta* fruit extract. Fitoterapia 70(3): 296-298.

Chah KF, Muko KN and Oboegbulem SI (2000) Antimicrobial activity of methanolic extract of *Solanum torvum* fruit. Fitoterapia 71: 187-189.

Chakraborty P (2000) A textbook of microbiology. 1st edition. Reprinted Vikas Publishing House Pvt. Ltd.

Chattopadhyay D, Sinha BK and Vaid LK (1998) Antibacterial activity of *Syzygium* species. Fitoterapia 69(4): 365-367.

Chattopadhyay RR, Bhattacharya SK, Medda C, Chanda S, Datta S and Pal NK (2007). antibacterial activity of black myrobalan (fruit of *Terminalia chebula* Retz) against uropathogen *Escherichia coli*. Pharmacognosy Magazine 11: 973-1296.

Cheesbrough M (1993) Medical laboratory manual for tropical countries. Vol. II, ELBS, pp 196-200.

Cheesbrough M (2002) District laboratories practices in tropical countries. Cambridge University Press. U.K 2: 124-130.

Choudhary MH (1998) Antimicrobial activity of the root bark of *Holarrhena antidysenterica.* Bangladesh Journal of Microbiology 15(2): 67-70.

Clark AM and Hufford CD (1993) Discco and development of novel prototype antibiotics for opportunistic infections related to the acquired immunodeficiency syndrome. In: human medical agents from plants. America Chemical Society 534: 228-241.

Collee JG, Duguid JP, Fraser AG, Marmion BP and Simmons A (1996) Laboratory strategy in the diagnosis of infective syndrome. In: Collee JG, Marnion BP, Fraser AG and Sinnons A (eds) Mackie and MC Cartney practical medical microbiology, 14th edition Churchill, Livingstone , New York, pp 245-413.

Cowan MM (1999) Plant product as antimicrobial agents. Clinical Microbiology Reviews 12(4): 564-582.

Cox PA and Balick MJ (1994) The ethnobotanical approach to drug discovery. Scientific American 270: 82-87.

Cutcheon AR, Ellis SM, Hancock REW and Towers GHN (1992) Antibiotic screening of medicinal plants of the British Columbian native people. Journal of Ethnopharmacology 37: 213-223.

Devkota KP, Acharya R, Baral MP and Adhikari RP (1999) Antimicrobial activities of some herbal plants used in traditional medicine in Nepal. Proceedings of 3rd National conference on Science and Technology March 8-11, Vol. II. 1311-1317.

Dingle J, Red WW and Solomons GL (1953) The enzymatic degradation of pectin and other polysacchrides, applications of the cup assay method to the estimation of enzyme. Journal of Science, Food and Agriculture 4: 149-153.

Dixit SN and Tripati SC (1982) Antifungal antibiotics from higher plant. Recent Advances in the Biology of Microorganism 2: 519-523.

DPR (2007) Medicinal plants of Nepal, Bulletin of Department of Plant Resources No. 28 Medicinal Plants of Nepal (Revised) Government of Nepal. Ministry of Forest and Soil Conservation .Department of Plant Resources (DPR), Kathmandu, Nepal.

Elizabeth KM (2005) Antimicrobial activity of *Terminalia bellerica*. Indian Journal of Clinical Biochemistry 20(2): 150-153.

Essawi T and Srour M (2000) Screening of some Palestinian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology 70: 343-349.

Glosh TR, Sen T, Dutta AS and Nagchandhary AK (1993) Anti-diarrhoeal activity of methanol fraction of extracts of unripe fruits of *Psidium guajawa* Linn. Phytotherapy Research 22: 15-28.

Grierson DS and Afolayan AJ (1999) Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. Journal of Ethnopharmacology 66: 103-106.

HMG/N (1970) Medicinal plants of Nepal. Ministry of Forest Department of Medicinal Plants. Kathmandu, Nepal pp 50-75.

HMG/N (1986) Standards of ayurvedic crude drugs. Ministry of Forest Department of Medicinal Plants. Kathmandu, Nepal pp 25-160.

HMG/N (1993) Medicinal plants of Nepal. Ministry of Forest and soil conservation, Department of Medicinal Plants. Kathmandu, Nepal pp 72-150.

HMG/N 2002 Forest and vegetation types of Nepal tree improvement and silviculture component , Department of Forest and Soil Conservation , Kathmandu, Nepal, pp 170.

Hussain SAM, Barakat HH, Merfort I and Nawwar MA (1997) Tanins from the leaves of *Punica granatum*. Phytochemistry 45: 819-823.

ICS-UNIDO (2006) South-East Asian (SEA) regional workshop on "extraction on technologies for medicinal and aromatic plants" Lucknow, India pp 1-2.

Idos O, Guthe T, Willeox R and Deweck AL (1968) Nature and extent of penicillin side reaction with particular references to fatalities from anaphylactic shock. Bulletin of WHO 38: 159-188.

IUCN (2000) National register of medicinal plants. IUCN/ Nepal 13: 200-202.

Iwu MM, Duncan AR and Okunji CO (1999) Prospective on new crops and new uses. In: Janick J (ed) New Antimicrobial of Plants Origin, ASHA, Press Alexandrial, U.A pp 457-462.

Joshi AR and Joshi SD (2001) Genetic heritage of medicinal and aromatic plants of Nepal Himalayas. Buddha Acadenmic Publishers and Distributors Pvt. Ltd. Kathmandu, Nepal pp 239

Joshi SG (2006) Medicinal plants. Oxford and IBH Publishing Co.PVT. Ltd. New Delhi

Kelmanson JE, Jager AK and Staden JV (2000) Zulu medicinal plants with antibacterial activity. Journal of Ethnopharmacology 69: 241-246.

Khan ZK (1997) *In vitro* screening techniques for antibacterial and antifungal activity of medicinal plants. International Workshop on Medicinal Plants. Their Bioactivity Screening and Evaluation (CDRI), Lucknow, India pp 4.

Kokoska L, Polesny Z, Rada V, Nepovim A and Vanek T (2002) Screening of some Siberian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology 82: 51-53.

Machado TB, Pinto AV, Pinto MCFR, Leal ICR, Silva MG, Amaral ACF, Kuster RM and Netto KR (2003*). In vitro* activity of Brazilian medicinal plants, Naturally occurring naphthoquinones and their analoques, against methicilin-resistant *Staphylococcus aureus*. International Journal of Antimicrobiology 21: 279-284.

Mahoto RB and Chaudhary RP (2005) Ethnomedicinal study and antibacterial activities of selected plants of Palpa districts, Nepal. Scientific World 3(3): 5-8.

Malla SB and Shakya PR (1984/85) Medicinal plants of Nepal nature's paradise. Edited by J.C Majpuria. White Lotus Ltd. Bangkok. pp 261-297.

Manandhar NP (1980) Medicinal plants of Nepal Himalaya. Ratna Pustak Bhandar, Kathmandu. Nepal pp 85.

Mathabe MC, Nikolova RV, Lall N and Nyazema NZ (2006) Antibacterial activities of medicinal plants used for the treatment of diarrhea in Limpopo Province, South Africa. Journal of Ethnopharmacology 105: 286-293.

Mazumber R, Bhattacharya S, Mazumber A, Pattnik K, Tiwary PM and Chaudhary S (2005) Antidiarrhoeal evaluation of *Aegle marmelos* (correa) Linn. Root Extract. Phytotherapy Research 20(1): 82-84.

Miranda D, Pereira L, Sirsat SM, Antarkar DS and Vaidya AB (1995) *In vitro* action of selected medicinal plants against microorganisms involed in human gastrointestinal Infections. Journal of Research in Ayurveda and Siddha 14(3-4): 149-159.

Mustafa NK, Tanira MOM, Dar FK and Sanze HN (1999) Antimicrobial activity of *Acacia Nilotica* sub spp Nilotica fruit extracts. Pharmacy and pharmcology communications 5(9): 583-586.

Nair R and Chanda S (2007) *In vitro* antimicrobial activity of *Psidium guajova* leaf extracts against clinically important pathogenic microbial strains. Brazilian Journal of Microbiology 38: 452-458.

Nair R and Chanda SV (2007) Antibacterial activities of some medicinal plants of the western region of India. Tukish Journal of Biology 31: 231-236.

Nickel LG (1959) An applied microbiology. Iowa state University Press 4: 281.

Okeke LN, Laxmininarayan R, Bhutta ZA, Duse AG, Jenkins PO , Brien TF, Pablos-Mendez A and Klugman KP (2005). Antimicrobial resistance in developing countries. Recent Trend and Current Lancet Infectious Diseases 5(1): 481-493.

Oliveira DF, Pereira AC, Figueiredo HCP, Carvalho DA, Silva G, Nunes AS, Alves DS and Carvalho HWP (2007) Antibacterial activity of plant extracts from Brazilian southeast region. Fitoterapia 76: 697-699.

Parajuli S (2001) Medicinal plants used in cuts and wounds in Kaski district (Western Nepal) and their antibacterial activities. M.Sc. thesis, Central department of Botany, Tribhuvan University , Kirtipur, Kathmandu, Nepal.

Parajuli S, Chaudhary RP and Taylor RSL (2001) Antibacterial activity of medicinal plants used to treat skin aliments in Kaski district, Nepal. In: Jha PK, Baral SR, Karmacharya SB, Lekhnak HB, Lacoul P and Bainya CB (eds)environment and agriculture. Biodiversity, Agriculture and Pollution in South Asia, pp 230-237.

Parekh J and Chanda S (2007 a) *In vitro* screening of antibacterial activity of aqueous and alcoholic extracts of various Indian plant species against selected pathogens from Enterobacteriaceae. African Journal of Microbial Research 1(6): 092-099.

Parekh J and Chanda S (2007 b) *In vitro* antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz flower (Lythraceae). Brazilian Journal of microbiology 38: 204-207.

Parvathi S and Brindha R (2003) Ethnobotanical medicines of animal union. Ancient Science of Life Vol. XXII.

Perez C, Paul M and Bazerque P (1990) Antibiotic assay by agar well diffusion method. Acta Bio Medicine Experimentalis 15: 113-115

Perumalsamy R, Ignacimuthus and Sem A (1998) Screening of 34 Indian medicinal plants for antibacterial properties. Journal of Ethnopharmacology 62: 173-182.

Pessini LG, Filho DPB, Nakamura CV and Cortez DGA (2003) Antibacterial activity of extracts and neolignans from *Piper regnelli* (miq) C.D.C var. palescens 98(2): 21-28.

Pokhrel NR (2000) Screening and evaluation of the antimicrobial activity of some medicinal plants of Nepal and isolation of pure antimicrobial compound from *Baubinia variegate*. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Poole K, Krebes K, McNally C and Neshat S (1993) Multiple antibiotic resistance in *Pseudomonas aeruginosa*: Evidence for involvement of and efflux operon. Journal of Bacteriology 171: 7363-7372.

Prasad G, Pandey S and Gupta KK (1999) Antimicrobial activity of certain indigenous drug and drugs plants against certain bacterial species. Hamdard Medicius 42(3): 53-57.

Prasai T (2002) Drinking water Quality Assessment of Kathmandu Valley and Antibacterial property of medicinal plants against enteric bacteria isolated from water. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Professional consultancy service (PCONS) and rural industrial development associates (RIDA) (1995), "Feasibility study report on integrated medicinal and herbal cultivation and processing plants in SAARC Countries ", Council for regional project, Kathmandu, Nepal pp 35.

Rabe T and Staden JV (1997) Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 56: 81-87.

Radha D (2004) Evaluation of antimicrobial activities of some medicinal plants. M.Sc Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Ram A.J, Bhakshu L.M and Raju RRV (2004) *In vitro* antimicrobial activity of certain medicinal plants from Eastern Ghats, India, used for skin diseases. Journal of Ethnopharmacology 90: 353-357.

Rawat RBS and Uniyal RC (2003) National medicinal plants board committed for overall development of the sector. Agro Bioscience Medicinal Plants 1: 12-16.

Recio MC (1983) A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978-1988. Phytotherapy Research 3: 1445-1453.

Rijal K (1994) Preliminary study on some medicinal plants and essential oils for their antimicrobial activities. In: proceedings of national conference on science and technology. RONAST, Kathmandu, Nepal pp 390-393.

Robbers J, Speedie M and Tyler V (1996) Pharmacognosy and Pharmabiotechnology . Willins, Bartimore pp 1-14.

Robin EH, Anril W, Alexender M, Loeto M and Keith K (1998) Nasopharyngeal carriage and antimicrobial resistance in isolates of *Steptococcus pneumoniae* and *Haemophilus influenza* type b in children under 5 years of age in Botswana. International Journal of Infectious Diseases 3(1): 18-25.

Samy RP (2005) Antimicrobial activity of some medicinal plants from India. Fitoterapia 76: 697-699.

Scazzocchio F, Comets MF, Tomassini L and Palmery M (2001) Antibacterial Activity of *Hydastis canadensi* extract and its major isolated alkaloids. Planta Medica 67: 561-563.

Sharma LN and Sherpa MT (1996) Technoeconomic feasibility study of medicinal and aromatic herbs in SAARC countries. Rural industrial Development also cites (RIDA). Project Report, Kathmandu, Nepal.

Sharma P. (1981) Ayurveda ko baigyanik ittihas. 2nd Edition, Chaukhamba Orintalia, Baranasi pp 37-122.

Shrestha AR (2005) Biological and phytochemical studies on root and rhizome of *Rheum imodi* of Nepalese origin. M.Sc Thesis, Central Department of Chemistry, Tribhuvan University , Kirtipur, Kathmandu, Nepal.

Sivaranjan VV and Balchandran I (1994) Ayurvedic drugs and their plant sources. Oxford and IBH Publishing Company, New Delhi, India pp 570.

Souwalak P, Nongyao P, Vatcharin R and Metto O (2005) Antimicrobial activities of the crude methanol extracts of *Acorus calamus* Linn. Songklanakarin. Journal of Science and Technology 27(2): 517-523.

Srinivasan D, Nathan S, Suresh T and Perumalsamy PL (2001) Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of Ethnopharmacology 74: 217-220.

Taylor RSL, Edel F, Manandhar NP and Towers GHN (1996) Antimicrobial activities of Southern Nepalese medicinal plants. Journal of Ethnopharmacology 50: 97-102.

Taylor RSL, Manandhar NP and Towers GHN (1995) Screening of selected medicinal plants of Nepal for antimicrobial activities. Journal of Ethnopharmacology 46: 153-159.

Thapa (2006) Evaluation of antibacterial activity of some medicinal plants frequently used in respiratory and gastrointestinal diseases in Nepal. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

The Wealth of India (1976) A Dictionary of Indian raw material. Council of Scientific and Industrial Research, New Delhi X(Sp-W): 165-173.

Tiwari NN and Shrestha KK (2000) MAPDON- Medicinal and aromatic plant database of Nepal-Japan joint symposium on conservation and utilization of Himalayan medical resources. Department of Plant Resources, Kathmandu pp 53-74.

Tripathi KD (2003) Essential of medical pharmacology. 5th Edition Jaypee Brothers Medical Publishers (P) LTD. New Delhi pp 623-630.

UNESCO (1998) Terminal report: Promotion of ethnobotany and the sustainable use of plant Resources in Africa, Paris.

Valsaraj R, Pushpangadan P, Smitt UW, Adsersen A and Nyman V (1997) Antimicrobial screening of selected medicinal plants from India. Journal of Ethnopharmacology 58(2): 75-83.

Vijaya K and Ananthan S (1994) Effect of three medicinal plants on enteropathogens an in-vitro study. Biomedicine 14: 21-23.

WHO (1991) Basic laboratory procedure in clinical bacteriology. World Health Organization, Geneva.

Zaiba IA, Beg AZ and Mahmood Z (1998) Antimicrobial potency of selected medicinal plants with special interest in activity against phytopathogenic fungi. India Veterinary Medical Journal 24(4): 299-306.

APPENDIX -A

List of materials used for the study

Soxhlet extraction

- Condenser
- Grinder
- Heating mantle
- Rotatory vacuum evaporator
- Round bottom flask
- Sieve
- Soxhlet extractor stands

Media for culture

- Mannitol salt agar (MSA)
- MacConkey's agar
- Muller Hinton Agar (MHA)
- Nutrient agar (NA)
- Nutrient broth (NB)
- Triple sugar iron agar (TSI)
- Sulphide indole motility (SIM)
- MRVP (Glucose, Phosphate, Peptone broth)
- Simmon's citrate medium
- Urease medium

Chemical Reagents :

DMSO (Dimethyl sulpho oxide), Crystal violet, Gram's iodine, Kovac's reagent, Methyl red, a-napthol solution, Barium chloride, Conc. H_2SO_4, Hexane, Ethylacetate, Methanol, Safranin, Blood plasma.

Antibiotic Discs : Antibiotic discs from Hi-Media laboratory

Apparatus and Equipments:

- Aluminium foils
- Autoclave
- Compound Microscope
- Cork borer no 8
- Cotton roles
- Electric paper
- Forceps
- Hair drier
- Incubator
- Inoculating loop
- Micropipette
- Measuring scale
- Plant cutter
- Refrigerator
- Sticker
- Water distillation plant
- Vortex shaker

Glass ware

- Beakers
- Conical flask
- Glass rods
- Measuring cylinders
- Microscopic slides
- Petri dishes
- Pipettes
- Screw capped test tubes
- Test tubes

APPENDIX – B

List of medicinal plants used in the evaluation of antimicrobial activities

Botanical Name	Family	Parts used	Place of collection	Month of collection	Local Name
Acorus calamus	Araceae	Rhizome	Thankot, Kathmandu	Shrawan	Bojho
Aegle marmelos	Rutaceae	Fruit	Ason Bazaar,kathmandu	Magh	Bel
Asparagus racemosa	Liliaceae	Tuberous Root	Chitwan	Manshir	Kurilo
Mimosa pudica	Leguminosae	Root	Nawalparasi	Bhadra	Lazzawati
Terminalia bellerica	Combretaceae	Fruit	Ason Bazaar,kathmandu	Jestha	Barro
Terminalia chebula	Combretaceae	Fruit	Ason Bazaar,kathmandu	Jestha	Harro
Tinospora cordifolia	Menispermaceae	Stem	Chitwan	Kartik	Guduchi
Woodfordia fruticosa	Lythraceae	Flower	Machhegaun, Kirtipur	Jestha	Dhaero
Holarrhena antidysenterica	Apocynaceae	Seed	Ason Bazaar,kathmandu	Kartik	Indrajau, Kutaj

APPENDIX – C

Lists of test organisms and their collection

S.N	Name of Test Organisms	Collection
1	*Staphylococcus aureus* (ATCC 25923)	National Public Health Laboratory, Teku
2	*Escherichia coli* (ATCC 25923)	National Public Health Laboratory, Teku
3	*Klebsiella pneumoniae* (ATCC 200603)	National Public Health Laboratory, Teku
4	*Klebsiella oxytoca*	Central Department of Microbiology, Kirtipur
5	*Proteus mirabilis* (ATCC 49132)	National Public Health Laboratory, Teku
6	*Proteus vulgaris*	Central Department of Microbiology, Kirtipur
7	*Pseudomonas aeruginosa* (ATCC 27853)	National Public Health Laboratory, Teku
8	*Salmonella* Paratyphi A	National Public Health Laboratory, Teku
9	*Salmonella* Typhi	National Public Health Laboratory, Teku
10	*Shigella dysenteriae*	National Public Health Laboratory, Teku

APPENDIX – D

Composition of media

1. Nutrient Agar (NA)

Ingredients gm/litre

Peptone 5.0
Sodium Chloride 5.0
Beef extract 1.5
Yeast extract 1.5
Agar 15.0
Final pH (at 25^0C) 12.4+/- 0.2
Procedure: 28 gms of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^0C for 15 minutes.

2. Nutrient Broth (NB)

Ingredients gm/litre

Peptone 5.0
Sodium Chloride 5.0
Beef extract 1.5
Yeast extract 1.5
Final pH (at 25^0C) 7.4+/- 0.2
Sterilized by autoclaving at 15lbs pressured (121^0C) for 15 minutes.

2. Mueller Hinton Agar (MHA)

Ingredients gm/litre

Beef Infusion Broth 300.0
Casein Acid Hydrolysate 17.0
Starch 1.0
Agar 17.0
Final pH 7.0+/-0.2
Procedure: 3.8gms of media was suspended in 100 ml distilled water, boiled to dissolve and sterized by autoclaving at 121^0 for 15 minutes. It was poured while at 45-55^0C in sterile 9cm diameter plates in 25ml quanties. To ensure the uniformity in depth of medium, the plates were placed over level surface and the medium was poured into it.

APPENDIX-E

Turbidity standard

Turbidity standard is prepared by pouring 0.6 ml of 1% (10 g/litre) solution of barium chloride dehydrate into a 100 ml graduated cylinder, and filling to 100 ml with 1% (10 ml/litre) sulphuric acid. The turbidity standard solution should be placed in a tube identical to the one used for the broth sample. It can be stored in the dark at room temperature for 6 months, provided it is sealed to prevent evaporation.

Source: - Basic Laboratory Procedure in Clinical Bacteriology, World Health Organization, Geneva (1991).

Actual concentration of the extracts during two folds dilution:

Crude name of test tube	Concentration	
	mg/ml	µg/ml
-ve control	100.00	100,000.0000
1st	50.00	50,000.0000
2nd	25.00	25,000.0000
3rd	12.50	12,500.0000
4th	6.25	6250.0000
5th	3.125	3125.0000
6th	1.5625	1562.5000
7th	0.78125	781.2500
8th	0.390.625	390.6250
9th	0.1953125	195.3125
10th	0.09765625	97.6565
+ve contral	0.00	0.0000

APPENDIX –F

Zone size interpretation chart for antibiotic sensitivity test

Antibiotic or chemotherapeutic agent	Strength	Diameter of zone of inhibition (mm)		
		Resistant	Intermediate	Sensitive
Amikacin	30mcg	<14	15-16	>17
Ampicillin	10mcg			
Gram –ve enteric organisms		13	14-16	17
Staphylococci		28	-	29
Cephalexin	30	14	15-17	18
Chloramphenicol	30	12	13-17	18
Ciprofloxacin	5	15	16-20	21
Cloxacillin	5	11	12-13	14
Co-trimoxazole	25	10	11-15	16
Erythromycin	15	13	14-22	23
Gentamycin	10	12	13-14	15
Nalidixic acid	30	13	14-18	19
Norfloxacin	10	12	13-16	17
Ofloxacin	5	12	13-15	16
Penicillin G	10 unit			
Staphylcocci		28	-	29
Enterococci		14	-	15

Note:mcg = micro-gram

Source: Product Information Guide, Hi Media Laboratories Pvt. Limited, Mumbai, India.

CHAPTER–VI

7. DISCUSSION AND CONCLUSION

6.1 Discussion

In this study, nine medicinal plants were selected randomly and then collected from various parts of the country. Among nine medicinal plants, three were collected from market 'Ason bazaar' Kathmandu. These medicinal plants were selected on the basis of their useful as cited in literature and folklore for diseases like cough, fever, wounds, diarrhea, dysentery, sore throat etc.

Identification of the collected medicinal plants was done by referring various literature and herbarium found in Ayurveda Campus, Kirtipur and Central Department of Botany, Kirtipur. The name of the plant, place of collection and parts used are given in Appendix B.

Samples were properly labeled and then left for shade drying as drying in sunlight may cause loss of some active compounds. Large plant if extracted without grinding results in poor percentage yield on extraction, also large were chopped before drying and then subjected to grinding to facilate complete and easy extraction of the active compounds.

Upon going through various literatures, it was found that there is no specific process for the extraction of compounds from the plants. Among percolation and soxhlet extraction process, mostly preferred soxhlet extraction process for the easy and better extraction of compounds from the plants.

Various types of solvent were used for the extraction of compounds from the plant sample. Commonly, methanol, ethanol and other alcoholic solvent were used. Ironically, water, the universal solvent wasn't used frequently as it is less effective for non polar organic compound also removal of water from solution needs high temperature or sophiscated equipment. The crude extract of a plant may consist of several substances

widely differing in their chemical properties and polarities. Therefore it is essential at first to separate them into groups of compounds with similar polarities before identifying the active compounds.

In this study, three solvent viz: hexane, ethylacetate and methanol were used in the order of increasing polarity to obtain hexane, ethylacetate and methanol extract (fraction) by using soxhlet extraction method. Thapa (2006) and Baidya (2001) used hexane, chloroform, n-butanol and aqueous solvent in the order of increasing polarity for the extraction of compounds from the plant. This study supports our study purpose.

It was found that for the same plant, continuous extraction with different solvent viz: Hexane, ethylacetate and methanol in the increasing order of polarity gave different percentage yield. Hexane solvent takes less time for complete extraction than ethylacetate and methanol solvent.

In the present study, highest yield was observed from methanol fraction of *T. chebula* (46.1%) followed by methanol fraction of *T. bellirica* (40.46%). The least yield were from the hexane fraction of *A. racemosa* (0.7%) followed by hexane fraction of *T. bellirica* (0.8%). The range of % yield from hexane solvent is 0.7-3.28% exception being 12.08% from the *A. calamus*. Range of % yield from for ethylacetate fraction be 0.9-2.83 % while for methanol yield ranges of % was 9.43-46.

There is a distinct difference in the % yield of extracts of different solvent even for same sample. The different in yield might be due to plants contain more polar compounds than non-polar compounds. There are various factors which influences the % yield from plants are: - extraction type, part of plant materials, finless of powder, extent of dryness and nature of solvent used for extraction etc. The old and dried plant material yield lesser than the fresh ones. Similarly, incomplete extraction results in lesser yield.

Each fraction after removal of solvent was tested for antimicrobial activity against selected ten bacterial species, by agar well diffusion method of Dingle et al. (1953).

In this study 27 extracts obtained by continuous extraction from 9 plants using hexane, ethylactate and methanol solvent in the order of increasing polarity were studied for their antimicrobial property. These extracts were dissolve in 100% DMSO and tested against 10 different bacterial species using agar well diffusion method.

In the study conducted by Abascal and Yamal (2002) showed that "Acorenone, from *Acorus calamus* rhizome, strongly inhibited a strain of *S. aureus* that was resistant to ten common antibiotics". Souwalak et al. (2005) reported that β-asarone from methanol extract of it exhibited inhibitory effect against *Staphylococcus aureus* ATCC 25923. In our study also, methanol fraction of *Acorus calamus* exhibited inhibitory effect against *S. aureus* 25923 which supports our result. During the study it was found that *A. calamus* inhibited only 3 bacterial species viz: *E. coli*, *Sh. dysenteriae* and *S. aureus*. It was ineffective in all other organism included in this study. Ethylacetate fraction shows inhibitory activity against all 3 bacteria while hexane and methanol fraction shows inhibitory activity only against *S. aureus*. This result is in accordance with Ahmad (2001) where the alcoholic extracts of *A. calamus* demonstrate only moderate antibacterial activity viz: *S. aureus, Sh. dysenteriae* and *E. coli*.

Asparagus racemosus gave ZOI against only 2 bacteria viz*: P. mirabilis* and *S. aureus.* Methanol fraction inhibited *P. mirabilis* only, while ethylacetate fraction inhibited *S. aureus* only. Hexane fraction did not inhibit growth of bacteria tested. The study done by Baidya (2001) also found that 95 % ethanol extract from *A. racemosus* produced the similar results.

H. antidysenterica showed relatively broad spectrum activity since it produced ZOI against 6 bacterial strain among tested strain. Hexane fraction was active only against *E. coli*. ethylacetate was active against *P. mirabilis* and *Sh. dysenterica*. Similarly methanol fraction was inhibitory towards against *Ps. aeruginosa* and *S. aureus*. From this study it is clear that plant may contain more polar active compound than non-polar compound. Baidya (2001) showed similar results with ethanolic extracts which support our result.

Meanwhile, *M. pudica* produced ZOI against only to 3 bacteria viz: *S. aureus*, *K. oxytoca a*nd *S.* Paratyphi. Methanol fraction inhibited all 3 positive bacterial species while ethylacetate and hexane fraction inhibit *S. aureus* only. This result did not support the study done by Bajracharya (2007) and Srinivasan (2007) where none of the tested bacteria were inhibited by *M. pudica*.

Terminalia bellerica proved to have relatively wide antibacterial activity, being effective against 6 bacteria out of 10 test bacteria. Ethylacetate fraction inhibited growth of all 6 bacteria viz: *E. coli*, *K. oxytoca*, *Ps. aeruginosa*, *S.* Typhi, *Sh. dysenteriae* and *S. aureus* while methanol fraction was found to be inhibitory towards 3 bacteria viz:E. *coli*, *Sh. dysenteriae* and *S. aureus*. None of the bacterial species tested was inhibitory to hexane fraction. From this study it was found that active compounds might be polar compounds. Similar type of results was shown by ethanolic extracts of *T. bellirica* (Ahmad, 2001). Similarly, Ahmad (1998) found no antibacterial activity to tested bacterial species by hexane fraction which further supports our result.

Terminalia chebula was inhibitory to 5 bacterial species (50%) out of 10 bacterial species tested. Its methanol and ethylacetate fraction showed more inhibitory effect than hexane fraction. It inhibited *E. coli, K. oxytoca, S. paratyphi, Sh. dysenteriae* and *S. aureus*. Hexane fraction only inhibited *S. aureus*. This result shown that *T. chebula* contains more active compounds than non-polar compounds.

Similarly, *Tinospora cordifolia* produced ZOI against 3 bacterial species viz: *K. oxytoca, P. mirabilis* and *Ps. aeruginosa*. No antimicrobial activity produced by any solvent used against *S. aureus*. Ethylacetate fraction did not give any activity against tested bacterial species. Sami (2005) found that methanolic extracts from *T. cordifolia* inhibited growth of *P. mirabilis*.

W. fruticosa was effective against 6 bacteria out of 10 bacterial species tested. The most effective fraction was found to be ethylacetate fraction against *E. coli* and *Sh. dysenteriae*. All three fraction produced ZOI against *S. dysenteriae*. No antimicrobial activity was produced by *W. fruticosa* against *K. oxytoca, P. mirabilis, P. vulgaris* and

Ps. aeruginosa. Bajaracharya (2007) also found that all the tested bacterial species was inhibited by *Woodfordia fruticosa*. This result also supports our result as the result showed antibacterial activity towards 6 bacteria.

Meanwhile, out of 10 bacterial species, *Aegle marmelos* inhibited 3 bacterial species viz: K. *pneumoniae, Sh. dysenteriae* and *S. aureus.* All three fractions show antibacterial activity with *S. aureus* whereas Bajracharacharya (2007) found that no antibacterial activity at all by *A. marmelos* against tested bacterial species.

Qualitative screening of antimicrobial substance does not indicate the extent of the potency of a sample. A substance may demonstrate a wide spectrum of activity but have minuscule potency. Such substance has no utility practically and development of such product is not economically feasible. So, the antimicrobial activity must also be quantitatively assayed. For this, two methods: Agar well diffusion and two fold serial dilution were employed.

In Agar well diffusion method, the material under test diffuses from agar well into the surrounding agar in a concentric circle and inhibits or kills the microorganisms that are susceptible. This effect manifested by a clear zone around the well is measured as zone of inhibition (ZOI).

In this study for *S. aureus*, 18 test suspensions out of 27 suspension of hexane, ethylacetate and methanol fraction produced zone of inhibition and 8 out of 9 plant extracts were effective. Largest ZOI was observed with methanol fraction of *T. chebula* and *M. pudica* with ZOI 21 mm respectively. The smallest ZOI of 10 mm was observed with hexane fraction of *M. pudica*. The ZOI value for other test suspension of hexane, ethylacetate and methanol fraction of studied plants was in the range of 12-20 mm.

K. pneumoniae was the second most resistant bacteria among the tested bacteria. It was inhibited only by ethylacetate fraction of *A. marmelos*, and methanol fraction of *A. marmelos* and *W. fruticosa*. All three active fractions showed ZOI of 12mm.

In case of *E. coli*, six test suspension out of 27 test suspension of hexane, ethylacetate and methanol fraction of 9 plant shown inhibitory activity. Only five plants showed ZOI. The highest antibacterial activity was shown by ethylacetate fraction of *T. bellirica* and lowest of 11mm by hexane fraction of *H. antidysenterica*. The bacteria were totally resistant to *A. marmelos, A. racemosus, T. cordifolia* and *M. pudica*.

For *K. oxytoca*, five test suspensions from nine plants under study exhibited the inhibitory effect and produced ZOI. Methanol fraction of *M. pudica* was most potent with 16mm zone of inhibition while minimum potency was given by methanol fraction of *H. antidysenterica*. None of the hexane fraction of studied nine plant produced zone of inhibition against *K. oxytoca*.

S. Typhi showed less antimicrobial effect. Only 2 plants extract viz: ethylacetate fraction of *T. bellirica* and *W. fruticosa* showed antibacterial effects with ZOI value 18mm and 15mm respectively.

In the case of *S.* Paratyphi, only ethylacetate fraction of *T. chebula* and *W. fruticosa*; and methanol fraction of *M. pudica* showed significant zone of inhibition. The largest ZOI 18mm was produced by *T. chebula* while lowest (14mm) was produced by *W. fruticosa*.

Antibiotic susceptibility pattern against *Sh. dysenteriae* shown that only 11 extracts from 6 plants showed antimicrobial activity. The highest ZOI was shown by ethylacetate fraction of methanol while lowest ZOI shown by hexane fraction of *W. fruticosa*.

For *Ps. aeruginosa*, ethylacetate fraction of *T. bellirica* was most potent with 15 mm ZOI while lowest inhibitory zone 11mm was exhibited by methanol fraction of H. antidysenterica. Hexane fraction of *T. cordifolia* produced ZOI 12mm. All other plant extracts were resistant to *Ps. aeruginosa*.

For *P. mirabilis*, methanol fraction of *T. cordifolia*, methanol fraction of *A. racemosus* and ethylacetate fraction of *H. antidysenterica* produced zone of inhibition. These three fraction produced ZOI at the range of 11mm to 13mm.

P. vulgaris was the most resistance bacterium. None of the plant extracts studied gave any inhibitory zone.

However, all the plant extracts used for this study were active against both Gram positive and Gram negative bacteria except *T. cordifolia* which were active only against Gram negative. The largest ZOI was 31mm produced by ethylacetate fraction of *T. bellirica* while lowest of 10mm by hexane fraction of *T. bellirica*.

The diameter of zone of inhibition largely depends upon the diffusibility of the antimicrobial substance. Hence sometimes the ZOI demonstrated the antimicrobial substance doesn't commentate the efficiency of the substance. This necessitates the determination of MIC and MBC to give correct picture of antimicrobial potency of the compound. Since the extracts were colored, hence MBC was done in this study by two fold serial dilution method.

For *S. aureus*, lowest MBC of 6.25 mg/ml was observed in ethylacetate fraction of *T. chebula* and methanol fraction of *T. bellirica*. The highest MBC (>50 mg/ml) was found in hexane fraction of *T. chebula*. Most of the active fraction against *S. aureus* gave MBC at the range of 12.5 to 50 mg/ml.

For *E. coli*, most effective plant extracts was found to be ethylacetate fraction of *T. bellirica* with MBC value of 3.12 mg/ml and highest MBC value of 50 mg/ml was observed in ethylacetate fraction of *A. calamus*.

In the case of *K. pneumoniae*, all three fractions viz: ethylacetate and methanol fraction of *A. marmelos*; and methanol fraction of *W. fruticosa* showed same MBC value of 50 mg/ml.

For *K. oxtoca*, least MBC value was showed by two plant extracts only. The most bactericidal and least MBC value of 6.25 mg/ml was observed in ethylacetate fraction of *T. bellirica* while 25 mg/ml MBC was observed in ethylacetate fraction of *W. fruticosa*.

Among two plant extracts that inhibited *S.* Typhi, the most lethal and least MBC value of 6.25 mg/ml was observed in ethylacetate fraction of *T. bellirica* while 25 mg/ml MBC was observed in ethylacetate fraction of *W. fruticosa*.

For *S.* Paratyphi, among three active extracts, the most bactericidal plant extract was found to be ethylacetate fraction of *T. chebula* which gave MBC value of 12.5 mg/ml. High MBC value of 50 mg/ml was observed in methanol fraction of *M. pudica*.

Only three fraction viz: hexane fraction of *T. cordifolia*, ethylacetate fraction of *T. bellirica* and methanol fraction of *H. antidysenterica* produced MBC in the range of 12.5-25 mg/ml against *Ps. aeruginosa*.

In the case of *P. mirabilis*, methanol fraction of *T. cordifolia* found to produce MBC of value 25 mg/ml while methanol fraction of *A. racemosus* and *H. antidysenterica* found to produce MBC 50 mg/ml.

In over all analysis, most bactericidal and lowest MBC value of 3.12 mg/ml was shown by *ethylacetate* fraction of *T. bellirica* against *E. coli and* ethylacetate *fraction of W. fruticosa* against *Sh. dysenteriae*. Most of the MBC value of other plant extracts were in the ranges of 6.25 to 25 mg/ml. Least potent i.e highest MBC value of >50 mg/ml was shown by hexane fraction of *T. chebula* against *S. aureus*.

The extracts from medicinal plants showing large ZOI and small MBC value, may contain those compound, which are able to inhibit or kill the microbial population of tested bacteria. Conversely, the extracts, from medicinal plants showing large ZOI and large MBC value may contain those compounds which diffuse through the medium readily and inhibit the growth but could not kill the organism at the same time. For instance, the linear relationship between ZOI and MBC value was not observed.

Medicinal plants showing large ZOI as well as large MBC are that they are able to diffuse well but primarily inhibitory for e.g. methanol fraction of *M. pudica* having ZOI of 21

mm and MBC value of 25 mg/ml against *S. aureus*. Similarly ethylacetate fraction of *M. pudica* having ZOI of 17 mm and MBC value of 50 mg/ml against *S.* Paratyphi.

Large ZOI and small MBC value indicate that the extract diffuses properly through the agar as well as have excellent antimicrobial property. These are the most potent ones and promise good prospects. The ethylacetate fraction of *T. bellirica* demonstrated a ZOI of 31 mm and of MBC of 3.12 mg/ml against *E. coli*. Similarly, ethylacetate fraction of *W. fruticosa* produced a ZOI of 26 mm and MBC of 3.12 mg/ml against *Sh. dysenteriae*.

Small ZOI and large MBC values are given by plants which have poor antimicrobial activity and poor diffusibility. In this experiment, hexane fraction of *M. pudica* produced ZOI of 10 mm and MBC of 50 mg/ml against *S. aureus*. Similarly hexane fraction *of T. chebula* exhibited 11 mm ZOI and >50 mm MBC against *S.* aureus.

Of the hexane fractions from 9 medicinal plants, hexane fraction from 7 plants showed antibacterial activity against the tested bacterial species. Out of 7 plants hexane fractions, four plant showed activity against *S. aureus* (represents Gram positive bacteria). Maximum antibacterial activity was shown by *A. marmelos* and *A. calamus* (13 mm). Only three hexane fractions showed antibacterial activity against Gram negative bacteria (*Sh. dysenteriae, Ps. aeruginosa* and *E. coli*). Other Gram negative bacteria were resistant to the hexane fractions of plants. For Gram negative bacteria, maximum inhibition zone (14 mm) was shown by *W. fruticosa* against *Sh. dysenteriae* while minimum activity was shown by *H. antidysenterica* against *E. coli*.

Out of 9 medicinal plants studied, ethylacetate fractions from 8 plants showed antibacterial activity against the tested bacterial species. *T. cordifolia* did not show any antibacterial activity against the tested bacteria. Out of 8 ethylacetate fractions, seven showed activity against *S*. aureus, with maximum ZOI produced by *W. fruticosa* (20 mm) followed by *T. chebula* and *M. pudica* (19 mm). Ethylacetate fraction of *H. antidysenterica* did not show any activity against *S. aureus*.

In the case of tested Gram negative bacteria, all was inhibited by ethylacetate fractions except *P. vulgaris*. Of this, *E. coli* was inhibited by 4 plants extracts, *K. pneumoniae* by only one plant extracts, *K. oxytoca* by 2 plants extracts, *P. mirabilis* by only one plant extracts, *Ps. aeruginosa* by also one plant extracts, *S.* Typhi by 2 plants extracts, *S.* Paratyhi by 2 plants extracts, *Sh. dysenteriae* by 6 plants extracts.

Among ethylacetate fraction, maximum zone of inhibition was produced by *T.bellirica* against *E. coli* (31 mm) followed by *W. fruticosa* against *E. coli* and *Sh. dysenteriae* (26 mm) while lowest ZOI was produced by *H. antidysenterica* against *P. mirabilis* (11 mm).

In the case of methanol fraction, all plants showed antibacterial activity with at least one tested bacteria. Out of 9 methanol fractions, seven showed antibacterial activity against *S. aureus* with maximum inhibition zone produced by *T. chebula* and *M. pudica* (21 mm) while lowest by *H. antidysenterica, A. marmelos* and *A. calamus* (12 mm).

Similarly in the case of Gram negative bacteria, eight plants methanol fractions showed antibacterial activity except *A. calamus. E. coli* was inhibited by only one plant extracts, *K.* pneumoniae by two plants extracts, *K. oxytoca* by 3 plants extracts, *P. mirabilis* by two plants extracts, *Ps. aeruginosa* by only one plant extracts, *S.* Typhi by none, *S.* Paratyphi by only one plant and *Sh. dysenteriae* by four plants extracts.

Among methanol fraction, maximum zone of inhibition was produced by *W. fruticosa* against *Sh. dysenteriae* (23 mm) followed by *T. bellirica* against E. coli (21 mm) while lowest by *H. antidysenterica* against *K. oxytoca* (10 mm).None of the methanol extracts shown antibacterial activity against *S.* Typhi and *P. vulgaris*.

In classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria (Cutcheon et al; 1992). However, in this study, a large number of the extracts (11 fraction from 7 plants) were active against both Gram positive and Gram negative bacterium, similarly 7 fraction from 5 plants were active against only

Gram-positive bacteria, while only 5 fraction from 4 plants were active against Gram-negative bacteria. The activity against both the types of bacteria may be indicative of the presence of the broad spectrum antibiotic compounds.

6.2 Conclusion

From this study we can conclude that selected medicinal plants under this study had antibacterial activity against common bacterial species. *Holarrhena antidysenterica, Terminalia bellirica* and *Woodfordia fruticosa* had relatively broad spectrum antibacterial activity (inhibited 6 out of 10 bacterial species tested). Gram positive bacteria was more sensitive to selected medicinal plants than Gram negative bacteria.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

- All together 9 medicinal plants were selected and evaluated for their antibacterial activity against 10 bacterial species.
- Three solvents viz: hexane, ethylacetate and methanol were used in the order of increasing polarity to extract crude hexane, ethylacetate and methanol fraction by using soxhlet extractor.
- The highest yield was obtained in methanol fraction of *Terminalia chebula* (46.1%) while lowest in hexane fraction of *Asparagus racemosus* (0.7%).
- Agar well diffusion method and two fold dilution method were used for the evaluation of antibacterial activity.
- *Holarrhena antidysenterica, Terminalia bellirica* and *Woodfordia fruticosa* showed relatively broad spectrum antibacterial activity (inhibited 6 out of 10 bacterial species tested) while *Asparagus racemosus* showed least antibacterial activity (inhibited only 2 bacterial species).
- Among all the tested bacteria, *Staphylococcus aureus* was the most susceptible bacteria being sensitive to 18 fractions from 8 medicinal plants.
- Among tested Gram-negative bacteria, *Shigella dysenteriae* was the most susceptible bacteria being sensitive to 11 fractions from 6 medicinal plants.
- *Proteus vulgaris* was the most resistance bacteria being resistance to all selected plants.
- Largest ZOI (31 mm) was produced by ethylacetate fraction of *Terminalia bellirica* against *E. coli* while lowest ZOI of 10 mm by hexane fraction of *Mimosa pudica* against *Staphylococcus aureus*.
- In this study the plants extracts showed significant antibacterial activity towards Gram positive bacteria than Gram negative bacteria.

7.2 Recommendations

Based on the study, the following recommendations are made:-

2. Out of 701 medicinal plants found in Nepal, antibacterial activity of only 9 medicinal plants extracts have been evaluated in the present study. Antibacterial activity of other medicinal plants should be studied. .

2. Same plants from different areas may give different yields and activity based on variation in ecological factors. These should be studied.

3. *In vivo*-tests should be carried out for further investigation.

4. Extracts which showed higher antibacterial activity during evaluation, should be analyzed in detail to identify the active antibacterial constituents and also their mode of action.

6. Extracts of the plants need to be screened for cytotoxicity activity.

CHAPTER-VIII

8.REFERENCES

Abascal K and Yamal E (2002) Herbs and drug resistance: Part I- herbs and microbial resistance to antibiotics. Alternative and Complementary Therapies 8(2): 237-241.

Adly A (1980) The history of medicinal and aromatic plants. Hamdard Foundation Press, Pakistan pp: 10-20.

Ahmad I and Beg AZ (2001) Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology 74: 113-123.

Akerele O (1993) Summary of WHO Guide lines for the assessment of herbal medicines. Herbal Gram 28: 13-28.

Akinpelu DA. (2000) Antimicrobial activity of *Bryophyllum Pinnatum* leaves. Fitoterapia 71: 193-194.

Alade PI and Irobe ON (1993) Antibacterial activities of crude extracts of *Acalypha wilkesinan* from Manna Nigeria. Journal of Ethnopharmacology 39: 235-236.

Ananthanarayan R and Panikar CKJ (2001) Text book of microbiology. 6th Edition. Reprint, Orient Longman Pvt, Ltd pp 250-298.

Aquil F, Sajjad M, Khan A, Owais M and Ahmad I (2005) Effect of certain bioactive plant extracts on clinical isolates of β-lactamase producing methicillin resistant *Staphylococcus aureus*. Journal of Basic Microbiology 45(2): 106-114.

Awadh Ali NA, Juelich WD, Kusnick C and Lindequist U (2001) Screening of Yemeni medicinal plants for antibacterial and cytotoxic activities. Journal of Ethnopharmacology 74: 173-179.

Baidya MR (2001) Screening and evaluation of *in-vitro* antimicrobial activity of some medicinal plants of Nepal. M.Sc. Thesis Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Bajarachra AM (2007) Study of drinking water quality of Kathmandu metropolitian area and evaluation of antibacterial property of some medicinal plants against isolated enteric bacteria. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Balakrishnan N, Bhaskar VH, Jayakar B and Sangameswaran B (2006) Antibacterial activity of *Mimosa pudica*, *Aegle marmelos*, and *Sida cordifolia*. Pharmacognosy Magazine 2(7): 1-5.

Bandow JE, Brotz H, Leichert LIO, Labischinski H and Hecker M (2002) Proteomic approach to under standing antibiotic action. Antimicrobial Agents Chemotherapy 47: 948-955.

Baral SR and Kurmi PR (2006) A compendium of medicinal plants in Nepal. IUCN. The World Conservation Union. Mass Printing Press, Chhauni, Kathmandu pp 52-392.

Barbour E.K, Al Sharif M, Sagherian VK, Habre AN, Talhouk RS and Talhouk SN (2004) Screening of selected indigenous plants of Lebanon for antimicrobial activity. Journal of Ethnopharmacology 93: 1-7.

Baron EJ, Peterson LR and Finegold SM (1994) Bailey and Scott's diagnostic Microbiology, 9th Edition , Mosby year Book, Inc. USA pp 166-177.

Baur AW, Kirby WMM, Sherris JC and Turch M (1966) Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical pathology 45: 494-496.

Bhatta L (1998) Study of antmicrobial properties of *Punica granatum*. M.Sc Thesis Central Department of Microbiology, Kirtipur, Kathmandu, Nepal.

Bhattarai NK (1989) Traditional phytotherapy among the sherpas of the Helambu, central Nepal, Journal of Ethnopharmacology 27: 45-54.

Branter A and Grein E (1994) Antibacterial activity of plant extracts used externally in traditional medicine. Journal of Ethnopharmacology 44: 35-40.

Branter AH and Chakraborty A (1998) *In vitro* antibacterial activity of alkaloids isolated from *Adatoda vasica* NEES. Pharmaceutical and Pharmacological Letters 8(3): 137-139.

Carpinella MC, Herrero GG, Alonso RA and Palacios SM (1999) Antifungal activity of *Melia azadirachta* fruit extract. Fitoterapia 70(3): 296-298.

Chah KF, Muko KN and Oboegbulem SI (2000) Antimicrobial activity of methanolic extract of *Solanum torvum* fruit. Fitoterapia 71: 187-189.

Chakraborty P (2000) A textbook of microbiology. 1st edition. Reprinted Vikas Publishing House Pvt. Ltd.

Chattopadhyay D, Sinha BK and Vaid LK (1998) Antibacterial activity of *Syzygium* species. Fitoterapia 69(4): 365-367.

Chattopadhyay RR, Bhattacharya SK, Medda C, Chanda S, Datta S and Pal NK (2007). antibacterial activity of black myrobalan (fruit of *Terminalia chebula* Retz) against uropathogen *Escherichia coli*. Pharmacognosy Magazine 11: 973-1296.

Cheesbrough M (1993) Medical laboratory manual for tropical countries. Vol. II, ELBS, pp 196-200.

Cheesbrough M (2002) District laboratories practices in tropical countries. Cambridge University Press. U.K 2: 124-130.

Choudhary MH (1998) Antimicrobial activity of the root bark of *Holarrhena antidysenterica.* Bangladesh Journal of Microbiology 15(2): 67-70.

Clark AM and Hufford CD (1993) Discco and development of novel prototype antibiotics for opportunistic infections related to the acquired immunodeficiency syndrome. In: human medical agents from plants. America Chemical Society 534: 228-241.

Collee JG, Duguid JP, Fraser AG, Marmion BP and Simmons A (1996) Laboratory strategy in the diagnosis of infective syndrome. In: Collee JG, Marnion BP, Fraser AG and Sinnons A (eds) Mackie and MC Cartney practical medical microbiology, 14th edition Churchill, Livingstone , New York, pp 245-413.

Cowan MM (1999) Plant product as antimicrobial agents. Clinical Microbiology Reviews 12(4): 564-582.

Cox PA and Balick MJ (1994) The ethnobotanical approach to drug discovery. Scientific American 270: 82-87.

Cutcheon AR, Ellis SM, Hancock REW and Towers GHN (1992) Antibiotic screening of medicinal plants of the British Columbian native people. Journal of Ethnopharmacology 37: 213-223.

Devkota KP, Acharya R, Baral MP and Adhikari RP (1999) Antimicrobial activities of some herbal plants used in traditional medicine in Nepal. Proceedings of 3rd National conference on Science and Technology March 8-11, Vol. II. 1311-1317.

Dingle J, Red WW and Solomons GL (1953) The enzymatic degradation of pectin and other polysacchrides, applications of the cup assay method to the estimation of enzyme. Journal of Science, Food and Agriculture 4: 149-153.

Dixit SN and Tripati SC (1982) Antifungal antibiotics from higher plant. Recent Advances in the Biology of Microorganism 2: 519-523.

DPR (2007) Medicinal plants of Nepal, Bulletin of Department of Plant Resources No. 28 Medicinal Plants of Nepal (Revised) Government of Nepal. Ministry of Forest and Soil Conservation .Department of Plant Resources (DPR), Kathmandu, Nepal.

Elizabeth KM (2005) Antimicrobial activity of *Terminalia bellerica*. Indian Journal of Clinical Biochemistry 20(2): 150-153.

Essawi T and Srour M (2000) Screening of some Palestinian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology 70: 343-349.

Glosh TR, Sen T, Dutta AS and Nagchandhary AK (1993) Anti-diarrhoeal activity of methanol fraction of extracts of unripe fruits of *Psidium guajawa* Linn. Phytotherapy Research 22: 15-28.

Grierson DS and Afolayan AJ (1999) Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. Journal of Ethnopharmacology 66: 103-106.

HMG/N (1970) Medicinal plants of Nepal. Ministry of Forest Department of Medicinal Plants. Kathmandu, Nepal pp 50-75.

HMG/N (1986) Standards of ayurvedic crude drugs. Ministry of Forest Department of Medicinal Plants. Kathmandu, Nepal pp 25-160.

HMG/N (1993) Medicinal plants of Nepal. Ministry of Forest and soil conservation, Department of Medicinal Plants. Kathmandu, Nepal pp 72-150.

HMG/N 2002 Forest and vegetation types of Nepal tree improvement and silviculture component , Department of Forest and Soil Conservation , Kathmandu, Nepal, pp 170.

Hussain SAM, Barakat HH, Merfort I and Nawwar MA (1997) Tanins from the leaves of *Punica granatum*. Phytochemistry 45: 819-823.

ICS-UNIDO (2006) South-East Asian (SEA) regional workshop on "extraction on technologies for medicinal and aromatic plants" Lucknow, India pp 1-2.

Idos O, Guthe T, Willeox R and Deweck AL (1968) Nature and extent of penicillin side reaction with particular references to fatalities from anaphylactic shock. Bulletin of WHO 38: 159-188.

IUCN (2000) National register of medicinal plants. IUCN/ Nepal 13: 200-202.

Iwu MM, Duncan AR and Okunji CO (1999) Prospective on new crops and new uses. In: Janick J (ed) New Antimicrobial of Plants Origin, ASHA, Press Alexandrial, U.A pp 457-462.

Joshi AR and Joshi SD (2001) Genetic heritage of medicinal and aromatic plants of Nepal Himalayas. Buddha Acadenmic Publishers and Distributors Pvt. Ltd. Kathmandu, Nepal pp 239

Joshi SG (2006) Medicinal plants. Oxford and IBH Publishing Co.PVT. Ltd. New Delhi

Kelmanson JE, Jager AK and Staden JV (2000) Zulu medicinal plants with antibacterial activity. Journal of Ethnopharmacology 69: 241-246.

Khan ZK (1997) *In vitro* screening techniques for antibacterial and antifungal activity of medicinal plants. International Workshop on Medicinal Plants. Their Bioactivity Screening and Evaluation (CDRI), Lucknow, India pp 4.

Kokoska L, Polesny Z, Rada V, Nepovim A and Vanek T (2002) Screening of some Siberian medicinal plants for antimicrobial activity. Journal of Ethnopharmacology 82: 51-53.

Machado TB, Pinto AV, Pinto MCFR, Leal ICR, Silva MG, Amaral ACF, Kuster RM and Netto KR (2003*). In vitro* activity of Brazilian medicinal plants, Naturally occurring naphthoquinones and their analoques, against methicilin-resistant *Staphylococcus aureus*. International Journal of Antimicrobiology 21: 279-284.

Mahoto RB and Chaudhary RP (2005) Ethnomedicinal study and antibacterial activities of selected plants of Palpa districts, Nepal. Scientific World 3(3): 5-8.

Malla SB and Shakya PR (1984/85) Medicinal plants of Nepal nature's paradise. Edited by J.C Majpuria. White Lotus Ltd. Bangkok. pp 261-297.

Manandhar NP (1980) Medicinal plants of Nepal Himalaya. Ratna Pustak Bhandar, Kathmandu. Nepal pp 85.

Mathabe MC, Nikolova RV, Lall N and Nyazema NZ (2006) Antibacterial activities of medicinal plants used for the treatment of diarrhea in Limpopo Province, South Africa. Journal of Ethnopharmacology 105: 286-293.

Mazumber R, Bhattacharya S, Mazumber A, Pattnik K, Tiwary PM and Chaudhary S (2005) Antidiarrhoeal evaluation of *Aegle marmelos* (correa) Linn. Root Extract. Phytotherapy Research 20(1): 82-84.

Miranda D, Pereira L, Sirsat SM, Antarkar DS and Vaidya AB (1995) *In vitro* action of selected medicinal plants against microorganisms involed in human gastrointestinal Infections. Journal of Research in Ayurveda and Siddha 14(3-4): 149-159.

Mustafa NK, Tanira MOM, Dar FK and Sanze HN (1999) Antimicrobial activity of *Acacia Nilotica* sub spp Nilotica fruit extracts. Pharmacy and pharmcology communications 5(9): 583-586.

Nair R and Chanda S (2007) *In vitro* antimicrobial activity of *Psidium guajova* leaf extracts against clinically important pathogenic microbial strains. Brazilian Journal of Microbiology 38: 452-458.

Nair R and Chanda SV (2007) Antibacterial activities of some medicinal plants of the western region of India. Tukish Journal of Biology 31: 231-236.

Nickel LG (1959) An applied microbiology. Iowa state University Press 4: 281.

Okeke LN, Laxmininarayan R, Bhutta ZA, Duse AG, Jenkins PO , Brien TF, Pablos-Mendez A and Klugman KP (2005). Antimicrobial resistance in developing countries. Recent Trend and Current Lancet Infectious Diseases 5(1): 481-493.

Oliveira DF, Pereira AC, Figueiredo HCP, Carvalho DA, Silva G, Nunes AS, Alves DS and Carvalho HWP (2007) Antibacterial activity of plant extracts from Brazilian southeast region. Fitoterapia 76: 697-699.

Parajuli S (2001) Medicinal plants used in cuts and wounds in Kaski district (Western Nepal) and their antibacterial activities. M.Sc. thesis, Central department of Botany, Tribhuvan University , Kirtipur, Kathmandu, Nepal.

Parajuli S, Chaudhary RP and Taylor RSL (2001) Antibacterial activity of medicinal plants used to treat skin aliments in Kaski district, Nepal. In: Jha PK, Baral SR, Karmacharya SB, Lekhnak HB, Lacoul P and Bainya CB (eds)environment and agriculture. Biodiversity, Agriculture and Pollution in South Asia, pp 230-237.

Parekh J and Chanda S (2007 a) *In vitro* screening of antibacterial activity of aqueous and alcoholic extracts of various Indian plant species against selected pathogens from Enterobacteriaceae. African Journal of Microbial Research 1(6): 092-099.

Parekh J and Chanda S (2007 b) *In vitro* antibacterial activity of the crude methanol extract of *Woodfordia fruticosa* Kurz flower (Lythraceae). Brazilian Journal of microbiology 38: 204-207.

Parvathi S and Brindha R (2003) Ethnobotanical medicines of animal union. Ancient Science of Life Vol. XXII.

Perez C, Paul M and Bazerque P (1990) Antibiotic assay by agar well diffusion method. Acta Bio Medicine Experimentalis 15: 113-115

Perumalsamy R, Ignacimuthus and Sem A (1998) Screening of 34 Indian medicinal plants for antibacterial properties. Journal of Ethnopharmacology 62: 173-182.

Pessini LG, Filho DPB, Nakamura CV and Cortez DGA (2003) Antibacterial activity of extracts and neolignans from *Piper regnelli* (miq) C.D.C var. palescens 98(2): 21-28.

Pokhrel NR (2000) Screening and evaluation of the antimicrobial activity of some medicinal plants of Nepal and isolation of pure antimicrobial compound from *Baubinia variegate*. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Poole K, Krebes K, McNally C and Neshat S (1993) Multiple antibiotic resistance in *Pseudomonas aeruginosa*: Evidence for involvement of and efflux operon. Journal of Bacteriology 171: 7363-7372.

Prasad G, Pandey S and Gupta KK (1999) Antimicrobial activity of certain indigenous drug and drugs plants against certain bacterial species. Hamdard Medicius 42(3): 53-57.

Prasai T (2002) Drinking water Quality Assessment of Kathmandu Valley and Antibacterial property of medicinal plants against enteric bacteria isolated from water. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Professional consultancy service (PCONS) and rural industrial development associates (RIDA) (1995), "Feasibility study report on integrated medicinal and herbal cultivation and processing plants in SAARC Countries ", Council for regional project, Kathmandu, Nepal pp 35.

Rabe T and Staden JV (1997) Antibacterial activity of South African plants used for medicinal purposes. Journal of Ethnopharmacology 56: 81-87.

Radha D (2004) Evaluation of antimicrobial activities of some medicinal plants. M.Sc Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Ram A.J, Bhakshu L.M and Raju RRV (2004) *In vitro* antimicrobial activity of certain medicinal plants from Eastern Ghats, India, used for skin diseases. Journal of Ethnopharmacology 90: 353-357.

Rawat RBS and Uniyal RC (2003) National medicinal plants board committed for overall development of the sector. Agro Bioscience Medicinal Plants 1: 12-16.

Recio MC (1983) A review of some antimicrobial compounds isolated from medicinal plants reported in the literature 1978-1988. Phytotherapy Research 3: 1445-1453.

Rijal K (1994) Preliminary study on some medicinal plants and essential oils for their antimicrobial activities. In: proceedings of national conference on science and technology. RONAST, Kathmandu, Nepal pp 390-393.

Robbers J, Speedie M and Tyler V (1996) Pharmacognosy and Pharmabiotechnology . Willins, Bartimore pp 1-14.

Robin EH, Anril W, Alexender M, Loeto M and Keith K (1998) Nasopharyngeal carriage and antimicrobial resistance in isolates of *Steptococcus pneumoniae* and *Haemophilus influenza* type b in children under 5 years of age in Botswana. International Journal of Infectious Diseases 3(1): 18-25.

Samy RP (2005) Antimicrobial activity of some medicinal plants from India. Fitoterapia 76: 697-699.

Scazzocchio F, Comets MF, Tomassini L and Palmery M (2001) Antibacterial Activity of *Hydastis canadensi* extract and its major isolated alkaloids. Planta Medica 67: 561-563.

Sharma LN and Sherpa MT (1996) Technoeconomic feasibility study of medicinal and aromatic herbs in SAARC countries. Rural industrial Development also cites (RIDA). Project Report, Kathmandu, Nepal.

Sharma P. (1981) Ayurveda ko baigyanik ittihas. 2nd Edition, Chaukhamba Orintalia, Baranasi pp 37-122.

Shrestha AR (2005) Biological and phytochemical studies on root and rhizome of *Rheum imodi* of Nepalese origin. M.Sc Thesis, Central Department of Chemistry, Tribhuvan University , Kirtipur, Kathmandu, Nepal.

Sivaranjan VV and Balchandran I (1994) Ayurvedic drugs and their plant sources. Oxford and IBH Publishing Company, New Delhi, India pp 570.

Souwalak P, Nongyao P, Vatcharin R and Metto O (2005) Antimicrobial activities of the crude methanol extracts of *Acorus calamus* Linn. Songklanakarin. Journal of Science and Technology 27(2): 517-523.

Srinivasan D, Nathan S, Suresh T and Perumalsamy PL (2001) Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of Ethnopharmacology 74: 217-220.

Taylor RSL, Edel F, Manandhar NP and Towers GHN (1996) Antimicrobial activities of Southern Nepalese medicinal plants. Journal of Ethnopharmacology 50: 97-102.

Taylor RSL, Manandhar NP and Towers GHN (1995) Screening of selected medicinal plants of Nepal for antimicrobial activities. Journal of Ethnopharmacology 46: 153-159.

Thapa (2006) Evaluation of antibacterial activity of some medicinal plants frequently used in respiratory and gastrointestinal diseases in Nepal. M.Sc. Thesis, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

The Wealth of India (1976) A Dictionary of Indian raw material. Council of Scientific and Industrial Research, New Delhi X(Sp-W): 165-173.

Tiwari NN and Shrestha KK (2000) MAPDON- Medicinal and aromatic plant database of Nepal-Japan joint symposium on conservation and utilization of Himalayan medical resources. Department of Plant Resources, Kathmandu pp 53-74.

Tripathi KD (2003) Essential of medical pharmacology. 5th Edition Jaypee Brothers Medical Publishers (P) LTD. New Delhi pp 623-630.

UNESCO (1998) Terminal report: Promotion of ethnobotany and the sustainable use of plant Resources in Africa, Paris.

Valsaraj R, Pushpangadan P, Smitt UW, Adsersen A and Nyman V (1997) Antimicrobial screening of selected medicinal plants from India. Journal of Ethnopharmacology 58(2): 75-83.

Vijaya K and Ananthan S (1994) Effect of three medicinal plants on enteropathogens an in-vitro study. Biomedicine 14: 21-23.

WHO (1991) Basic laboratory procedure in clinical bacteriology. World Health Organization, Geneva.

Zaiba IA, Beg AZ and Mahmood Z (1998) Antimicrobial potency of selected medicinal plants with special interest in activity against phytopathogenic fungi. India Veterinary Medical Journal 24(4): 299-306.

APPENDIX -A

List of materials used for the study

Soxhlet extraction

- Condenser
- Grinder
- Heating mantle
- Rotatory vacuum evaporator
- Round bottom flask
- Sieve
- Soxhlet extractor stands

Media for culture

- Mannitol salt agar (MSA)
- MacConkey's agar
- Muller Hinton Agar (MHA)
- Nutrient agar (NA)
- Nutrient broth (NB)
- Triple sugar iron agar (TSI)
- Sulphide indole motility (SIM)
- MRVP (Glucose, Phosphate, Peptone broth)
- Simmon's citrate medium
- Urease medium

Chemical Reagents :

DMSO (Dimethyl sulpho oxide), Crystal violet, Gram's iodine, Kovac's reagent, Methyl red, a-napthol solution, Barium chloride, Conc. H_2SO_4, Hexane, Ethylacetate, Methanol, Safranin, Blood plasma.

Antibiotic Discs : Antibiotic discs from Hi-Media laboratory

Apparatus and Equipments:

- Aluminium foils
- Autoclave
- Compound Microscope
- Cork borer no 8
- Cotton roles
- Electric paper
- Forceps
- Hair drier
- Incubator
- Inoculating loop
- Micropipette
- Measuring scale
- Plant cutter
- Refrigerator
- Sticker
- Water distillation plant
- Vortex shaker

Glass ware

- Beakers
- Conical flask
- Glass rods
- Measuring cylinders
- Microscopic slides
- Petri dishes
- Pipettes
- Screw capped test tubes
- Test tubes

APPENDIX – B

List of medicinal plants used in the evaluation of antimicrobial activities

Botanical Name	Family	Parts used	Place of collection	Month of collection	Local Name
Acorus calamus	Araceae	Rhizome	Thankot, Kathmandu	Shrawan	Bojho
Aegle marmelos	Rutaceae	Fruit	Ason Bazaar,kathmandu	Magh	Bel
Asparagus racemosa	Liliaceae	Tuberous Root	Chitwan	Manshir	Kurilo
Mimosa pudica	Leguminosae	Root	Nawalparasi	Bhadra	Lazzawati
Terminalia bellerica	Combretaceae	Fruit	Ason Bazaar,kathmandu	Jestha	Barro
Terminalia chebula	Combretaceae	Fruit	Ason Bazaar,kathmandu	Jestha	Harro
Tinospora cordifolia	Menispermaceae	Stem	Chitwan	Kartik	Guduchi
Woodfordia fruticosa	Lythraceae	Flower	Machhegaun, Kirtipur	Jestha	Dhaero
Holarrhena antidysenterica	Apocynaceae	Seed	Ason Bazaar,kathmandu	Kartik	Indrajau, Kutaj

APPENDIX – C

Lists of test organisms and their collection

S.N	Name of Test Organisms	Collection
1	*Staphylococcus aureus* (ATCC 25923)	National Public Health Laboratory, Teku
2	*Escherichia coli* (ATCC 25923)	National Public Health Laboratory, Teku
3	*Klebsiella pneumoniae* (ATCC 200603)	National Public Health Laboratory, Teku
4	*Klebsiella oxytoca*	Central Department of Microbiology, Kirtipur
5	*Proteus mirabilis* (ATCC 49132)	National Public Health Laboratory, Teku
6	*Proteus vulgaris*	Central Department of Microbiology, Kirtipur
7	*Pseudomonas aeruginosa* (ATCC 27853)	National Public Health Laboratory, Teku
8	*Salmonella* Paratyphi A	National Public Health Laboratory, Teku
9	*Salmonella* Typhi	National Public Health Laboratory, Teku
10	*Shigella dysenteriae*	National Public Health Laboratory, Teku

APPENDIX – D

Composition of media

3. Nutrient Agar (NA)

Ingredients gm/litre

Peptone 5.0
Sodium Chloride 5.0
Beef extract 1.5
Yeast extract 1.5
Agar 15.0
Final pH (at 25^0C) 12.4+/- 0.2
Procedure: 28 gms of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^0C for 15 minutes.

2. Nutrient Broth (NB)

Ingredients gm/litre

Peptone 5.0
Sodium Chloride 5.0
Beef extract 1.5
Yeast extract 1.5
Final pH (at 25^0C) 7.4+/- 0.2
Sterilized by autoclaving at 15lbs pressured (121^0C) for 15 minutes.

4. Mueller Hinton Agar (MHA)

Ingredients gm/litre

Beef Infusion Broth 300.0
Casein Acid Hydrolysate 17.0
Starch 1.0
Agar 17.0
Final pH 7.0+/-0.2
Procedure: 3.8gms of media was suspended in 100 ml distilled water, boiled to dissolve and sterized by autoclaving at 121^0 for 15 minutes. It was poured while at 45-55^0C in sterile 9cm diameter plates in 25ml quanties. To ensure the uniformity in depth of medium, the plates were placed over level surface and the medium was poured into it.

APPENDIX-E

Turbidity standard

Turbidity standard is prepared by pouring 0.6 ml of 1% (10 g/litre) solution of barium chloride dehydrate into a 100 ml graduated cylinder, and filling to 100 ml with 1% (10 ml/litre) sulphuric acid. The turbidity standard solution should be placed in a tube identical to the one used for the broth sample. It can be stored in the dark at room temperature for 6 months, provided it is sealed to prevent evaporation.

Source: - Basic Laboratory Procedure in Clinical Bacteriology, World Health Organization, Geneva (1991).

Actual concentration of the extracts during two folds dilution:

Crude name of test tube	Concentration	
	mg/ml	µg/ml
-ve control	100.00	100,000.0000
1st	50.00	50,000.0000
2nd	25.00	25,000.0000
3rd	12.50	12,500.0000
4th	6.25	6250.0000
5th	3.125	3125.0000
6th	1.5625	1562.5000
7th	0.78125	781.2500
8th	0.390.625	390.6250
9th	0.1953125	195.3125
10th	0.09765625	97.6565
+ve contral	0.00	0.0000

APPENDIX –F

Zone size interpretation chart for antibiotic sensitivity test

Antibiotic or chemotherapeutic agent	Strength	Diameter of zone of inhibition (mm)		
		Resistant	Intermediate	Sensitive
Amikacin	30mcg	<14	15-16	>17
Ampicillin	10mcg			
Gram –ve enteric organisms		13	14-16	17
Staphylococci		28	-	29
Cephalexin	30	14	15-17	18
Chloramphenicol	30	12	13-17	18
Ciprofloxacin	5	15	16-20	21
Cloxacillin	5	11	12-13	14
Co-trimoxazole	25	10	11-15	16
Erythromycin	15	13	14-22	23
Gentamycin	10	12	13-14	15
Nalidixic acid	30	13	14-18	19
Norfloxacin	10	12	13-16	17
Ofloxacin	5	12	13-15	16
Penicillin G	10 unit	28	-	29
Staphylcocci				
Enterococci		14	-	15

Note:mcg = micro-gram

Source: Product Information Guide, Hi Media Laboratories Pvt. Limited, Mumbai, India.