CHAPTER - I

1. INTRODUCTION

Plants have been in use for medicinal purposes from the beginning of human civilization. Antiquities of medicinal herbs are to be traced back as far as the vedic period, 4500 B.C. to 1600 B.C. Ayureveda, the science of life in Hinduism, remains to be the main source of medical knowledge and skill in most part of South Asia including Nepal. Vaidhyas and Kabirajs followed Ayurveda in their pursuit of knowledge and practice in medicine (IUCN, 2000). After a period of decline of these traditional system "green medicine" are once again back to the centre stage of our health programs (Sivaranjan and Balchandran,1994).

More than 35,000 plants species are being used in various human culture around the world for medical purposes (Lewinton,1993). WHO (1989) identifies four main reasons for this widespread acceptance:

1) Medicinal plants have been in use for untold centuries and have proved reliable and effective in treating and preventing diseases.
2) Most species of medicinal plants are not toxic and therefore give rise to few, if any, side effect; even when adverse effects do occur, they are much less serious than those caused by chemically synthesized medicines.
3) People living in rural and mountainous areas have easy access to local medicinal plants, so that their use in preventing and controlling diseases cost much less than if western medicine were used and is thus economically beneficial to developing countries.
4) Medicinal plants are an important source of practical and inexpensive new drugs for people throughout the world.

WHO has estimated that 80% of the world’s population rely chiefly on traditional medicine. A major part of traditional therapies involve the use of plant extracts or their active constituents (Akerele,1993). The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years. Enthnopharmacologists, Botanists, Mirobiologists and Natural products chemists are combing the earth for phytochemicals and “leads” which could be developed for treatment of infectious diseases.

The resistance to antimicrobial agents is a world wide public health problem, which is responsible for the growing number of infection becoming untreatable in both hospital and community setting. Furthermost in spite of emergence of many new drugs from the synthetic field the problem of senescence and so called civilization diseases eg:- immunodeficiency syndrome, arthritis, mental disorder and cancer cannot be tackled and therefore there is a greater demand for natural medicines and "Health foods" than ever before in the world (Sharma, 1995).

Plants have been a rich source of medicines, because they produce a host of bioactive molecules, most of which probably evolved as chemical defences against predation or interaction. Most of the plant possess one or more of the medicinal properties *viz*, antibacterial, antifungal, antiviral, antihelmintic, anticancer, sedative, laxative, cardiotonic, diuretic and others (Parajuli *et al.*, 1998). The crude extracts of herbs, commonly used in traditional systems of medicine, are crucial in the treatment of disease to human beings. Different types of bacterial and fungal infections such as diarrhea, dysentery, cough, fevers etc. are treated by traditional medicines in the Himalaya under various systems chiefly Ayurvedic, Unani, Tibetan and Shiddha.

Medicinal properties of plants are due to the active constituents present in different parts of the plants (Mitscher *et al.,* 1980). Plants have been a rich source of medicine because they produce a host of bioactive molecules, most of which probably evolved as chemical defenses against predation or infection (Cox and Balick, 1994). There are many groups of substances occurring in plants and they are responsible for their medicinal as well as toxic properties (Sundaresan and Britto, 2000).The main group of active components are alkaloids, glycosides, saponins, essential oil etc. These active components of plants which inhibit /or kill the microorganisms are called antimicrobials. These antimicrobials are extractable with different kinds of solvents and could be used to treat illness (Kruger, 1992).

The one quarter to one half of all pharmaceuticals dispensed having higher plant origins, very few are intended for use as antimicrobials since we have relied on bacterial and fungal sources. Since the advent of antibiotics in the 1950s, the use of plant derivates as antimicrobials has been virtually nonexistent. However, modern definition of antibiotics, has inspired many workers to explore plants for these properties and various surveys carried out in U.S.A. and U.K. also showed that antibiotics can be obtained from higher plants too (Dixit and Tripathi, 1982).

The use of plants, as well as other alternative form of medical treatment is enjoying great popularity in the late 1900s. The ascendancy of the human immunodeficiency virus (HIV) has spurred intensive investigation into the plant derivatives which may be effective, especially for use in underdeveloped nations with less access to expensive western medicines (Declercq, 1995). The effort is continuously running for the development of potent antimicrobial or immunomodulators from plant (Khan, 1997).

Since, Himalayas are considered the big store house of enormous important plants and Nepal Himalayas representing the central himalaya provide shelters,to a large number of species distributed from few meters to around 5000m above sea level.Great range of bioclimatic variation from tropical to alpine zones brings richness in biological diversity in Nepal (Joshi and Joshi, 2001). According to estimate approximately 85% people of Nepal, particularly living in the rural areas, depend directly or indirectly on traditional medicine based on herbal drugs (Mills, 1994). Majority of the population reside in remote areas where treatment of disease through local drug is common health care practice. Since, there are many barriers to accessing health posts,doctors and allopathic medicines, such as lack of sufficient facilities, inaccessibility and expense people in these area use traditional remedies for cultural and social reasons too (Taylor *et al.,*1996).

Nepal contains a wealth of both plant diversity and knowledge regarding the medicinal applications of those plants. However, few studies have followed upon these ethnobotanical investigation with laboratory work verify the actual therapeutic value of these plants (Taylor and Towers, 1998).

Respiratory and gastrointestinal diseases are common diseases/aliments among the people living in rural as well as urban areas of Nepal. People suffer from these infections one or more in their lifetime. They usually treat these disease by use of traditional herbal remedies for cultural and social reasons, as well as due to lack of facilities of modern allopathic medicines.

Enteric bacterial infections causing diarrhea, dysentery and enteric fevers are important health problems throughout the world. Diarrhoeal infections are secondary only to cardiovascular diseases as a cause of death, and they are the leading cause of childhood death. In developing countries, diarrhoeal diseases account for 1.5 million

death each year among children aged 1-4 years. The risk of children in this age group dying from diarrhoeal disease is 600 times greater in developing countries than in developed countries (WHO, 2004). The etiological agents for these gastrointestinal problems are bacterial, viral, protozoal and fungal for eg; *Salmonella* sps, *Shigella* sps, EIEC, EHEC, *Vibrio cholerae*, *Bacillus* sps, *Enterobacter* sps, rotavirus, enteroviruses, *Entamoeba histolytica* etc.

Respiratory tract infections are one of the most frequently encountered diseases. They are very important cause of sickness and account for 50% cases of general practioner's consultations. The infections are common during winter seasons, i.e. between October to March (Nagoba, 2005).

Since, respiratory tract infection are the most common human ailments, the proper research in this area is of high importance. About 30%of the morbidity (range 27-40%) is accounted for by upper respiratory tracts infections while 20-24% of deaths are due to lower respiratory tract infections, out of total acute respiratory disease. The etiological agents for the respiratory tract infections are mostly due to bacterial, viral infections for eg*: Streptococcus pyogens, Hemophilus influenzae*, *Staphylococcus aureus*, Pneumococci*, Pseudomonas aeruginosa*, Rhino virus, Para influzea virus etc.

This present research is focused on the evaluation of antimicrobial activities of some medicinal plants traditionally used for treatment of some disease. Nevertheless, demonstrating activity in a bioassay is a necessary first step in the drug development process (Cox and Balick, 1994). So, this study will be the first step to investigate the antimicrobial activity of highly important medicinal plants of Nepal and also helpful in extraction of the active constituents of the plants, which will give information in preparation of antimicrobials to be used in the modern allopathic system of treatment.

Ethnopharmacological research has led to the discovery of many pharmaceuticals in the world (Cox and Balick, 1994). Hence, it is very important to carryout such research on medicinal plants to fulfill the demand of alternatives medicinal treatment in large part of our country. If local knowledge of medicinal herbs is thoroughly and scientifically explored, this would undoubtedly be significant in finding the way of medications and cost of medicine may become cheaper, which could be sold within the country and possibly exported, which may be helpful to raise socioeconomic conditions of the people and nation.

CHAPTER-II

2. OBJECTIVES

2.1 General objective

Evaluation of antibacterial activity of some medicinal plants frequently used in respiratory and gastrointestinal diseases in Nepal.

2.2 Specific objective

1. Collection and identification of the plants.
2. Extraction of crude extracts as active organic derivative from the plants.
3. To screen and evaluate antimicrobial activity of crude extracts.
4. Comparative evaluation of activities of extracts in traditional use and their effectiveness in some disease specific microbial agents.
5. To determine the minimum bactericidal concentration (MBC) value of crude extract.
6. To study the antimicrobial activity of different fractions of *Glycyrrhiza glabra*.

CHAPTER-III

3. LITERARURE REVIEW

3.1 Medicinal Plants

3.1.1 Definition and Importance

"A medicinal plant is any plant which, in one or more of its organ, contains substances, that can be used for therapeutic purpose or which is precursor for synthesis of useful drugs" (WHO, 1999). The plants that posses therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "medicinal plants".

The importance of medicinal plants for providing basic health needs of developing countries needs no emphasis. Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people.Plants are a valuable source of a vast array of bioactive lead structure from which more potent and less toxic drugs may be synthesized. For these reasons, many researchers have tried to screen new biological components from plants sources. Several drugs sold today are simple synthetic modifications, or copies of naturally obtained substances (Cho *et al.*, 2001).

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 from traditionally used as rural herbal remedies (UNESCO, 1998).

Many commercially proven drugs used in modern medicines were initially used in crude from in traditional or folk healing practices or for other purposes that suggested potentially useful biological activity. Many drugs currently used clinically or in the process of development are based on natural products from higher plants.At present, there are 125 different drugs of known structure that are clinically used and derived from about 100 species of higher plants. These drugs are used for treatment of human disease. Nearly 60% of the anti-tumor and anti-infective agents, commercially available, are of natural products origin (Koba *et al.,* 1994).

Thousands of plants extracts of all continents are being screened for activity against HIV and cancer in various laboratories of U.S. The renewed interest in plant antimicrobials in the past 20yrs has been at a rapid rate since, they are relatively safer

than synthetic alternatives (Iwu, 1999). Plants based antimicrobials, represents a vast untapped source for medicine continued, exploration of plant antimicrobials, needs to occur. Clinical microbiologist have two reasons to be interested in the topic of antimicrobial plant extract.

1) It is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs since several are already tested in human and the effective life span of antimicrobial derived from microorganisms is limited
2) The public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics (Alper, 1998).

Plant based antimicrobial are effective in treatment of infectious diseases, which simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials, they are effective yet gentle (Murry, 1995). The potential for developing antimicrobials into medicines appears rewarding from both the perspective of drugs development and that of phytomedicines.

A large number of plants of established medicinal and aromatic value grow wild in Nepal. The diverse geographical terrains and climatic conditions of the country, ranging from tropical to alpine zones, offer tremendous possibilities for the introduction and cultivation of many more medicinal and other plants of economic value (Malla, 1991).

High dependency of majority of population in Nepal on plants as a source of medicine proves the importance of medicinal plants in the rural Nepalese society. Since modern health services, trained man power and facilities, have not been provided to the greater part of the rural areas of our country where the majority, of the population lives (Bhattarai, 1989).

This indicates that it is very much necessary to carryout various research activities on medicinal plants to fulfill the demand of alternative medicinal treatment in large part our, country.

3.1.2 Historical overview

Human beings have been using plants in every aspects of life since the dawn of human civilization. The beneficiality and medicinal properties of plants have been known and used by human beings in some form or other (Jain and Saklani, 1991). The earliest mention of the medicinal use of plants has been found in “Rig Veda” some

times between 4500 and 1600 B.C., which is considered as the oldest repository of human knowledge on plant usage (DPR, 1970).

“Rig-Veda” has described 67 plants (Malla and Shakya, 1984-85). The “Ayurveda” a subsidiary text of “Atharvaveda,” has also mentioned 290 herbal drugs, written around 2500 B.C. describes further in detail the therapeutic uses of medicinal plants (Malla and Shakya, 1984-85). “Charak Samhitia” is another earliest treatise on “Ayurveda”(600 B.C.), which lists a total of 341 plants and plants products for use in health management, “SusrutaSamhita” also dealt with plants related to medicine. Subsequent authors of later treatises have extended the lists of Ayurvedic single plant drugs to six hundred species of plants (Bhattacharjee, 1998).

3.1.3 Medicinal Plants of Nepal

Nepal is a land of topographic contrasts and floristic diversity. The physiographic diversity caused by altitudinal variations has contributed to occurrence of various types of vegetation. In Nepal the lists of medicinal plants so far recorded by DPR comprises over 700 different species that constitute a little over 12% of the vascular flora of 5400 species reported to date, but at present number of medicinal and aromatic plants of Nepal is about 1463 . Still we are not able to know the total number of species, which are used as medicine in our country. The lists includes traditional rural remedies and Ayurvedic medicine many more of them are included as allopathic pharmacopoeias (Malla, 1991).

3.2 Brief description of Plants species under study

3.2.1 *Achyranthes bidentata* Blume (C.N:Datiwan)

Morphological Features: This genus belongs to the family Amaranthaceae. It is a Pubescent herb of around 30cm in height. Leaves opposite, simple petiolate, estipulate, elliptic to ovate, acute, 2-8cm long. Flowers in slender spike, 4-6cm long, sepals 4, lanceolate and shinning. Bracts usually reduced to spine, 2-aurculated at the base. Stamens 5, staminodes toothed. Ovary sub-compressed, oblong. Style filiform , stigma capitellate (Malla *et al.,* 1997)

Medicinal significance: The plant is used as diuretic and astringent. White variety bitter, pungent, heating, laxative stomachic itching, pain in the abdomen, ascites, dyspepsia, dysentery. Seeds used in piles. Roots are used in sore throat, hypertension amenorrhea, retention of placenta, carbuncles, traumatic injury, asthenia of liver and kidney, tiredness in lower part of body (IUCN, 2004).

3.2.2 *Acorus calamus* L.(C.N.: Bojho)

Morphological Features: This genus belongs to family Araceae. It is an erect herb with aromatic rootstock. Leaves 15-35cm long and 0.5-1cm broad, ensiform, with distinct midrib and wavy margins. Peduncle 0.2-0.5cm broad. Spathe 3.5-4cm long and 0.3-0.5cm in diameter, cylindrical, slightly curved. Flowers bisexual, each with a perianth of 6 orbicular concave segments, stamens6, ovary conical, 2-3 celled. Fruits oblong berries 4cm long.

Medicinal significance: Rhizomes, are used as carminative, stimulant and tonic, it is anti-spasmodic, carminative, antihelmintic and used in sore throat and voice disorders, treatment of epilepsy and other mental ailments, chronic diarrhea and dysentery, bronchial catarrh, intermittent fever and glandular and abdominal tumors.

Important biochemical constituent(s): Volatile oil “Asaryl aldehyde” and bitter glycoside “Acorin”(Joshi and Joshi, 2001)

3.2.3 *Azadirachta indica* A. Juss.(C.N.:Neem)

Morphological Features: This genus belong to the family Meliaceae. It is an evergreen tree of 12m high. Leaves to30cm long, pinnate, leaflets 7cm, lanceolate, acuminates, serrated, 12-17 in numbers. Flowers small, white, with pleasant odors, in loose clusters. Fruits round, oblong, 1.7cm diameter, greenish yellow when ripe.

Medicinal significance: Bark of the root and stem and leaves have antibiotic activity, relieves cough, vomiting, burning sensation near heart. The leaves are antihelminthic, insecticidal, good in ophthalimia, skin diseases. The tender leaves are astringent, good for cough, asthma, piles etc (IUCN, 2004).

Important biochemical constituent(s): Nimbin molecule with an acetoxy, alactone, an ester methoxy and an aldehyde group.

3.2.4 *Cuminum cyminum* Linn.(C.N.: Jeera).

Morphological Features: This genus belongs to family Umbelliferae. It is a small, slender, annual herb about 1ft high, with a much branched angular or striated stem, bearing 2or 3 partite linear leaves, bluish green in colour and having sheathing bases. The flowers are white or rose coloured borne in compound umbels. The fruits are grayish, about ¼ inch long, tapering towards both base and apex and compressed laterally with ridges (The Wealth of India, 1950).

Medicinal significance: Seeds are stimulant, carminative, astringent, and useful in diarrhea and dyspepsia as spices and in veterinary medicine (HMG/N, 1997). The volatile oil of cumin is also used as carminative (BPC, 1968).

3.2.5 *Glycyrrhiza glabra* Linn. (C.N.: Jethi madhu)

Morphological Features: This genus belongs to family Leguminosae. It is a tall, perennial plant, 50cm to 1m high, rhizome and roots 0.6-2cm long, outer surface grey, longitudinally striated, inner surface yellow; leaflets 4-7 pairs, oblong to elliptical lanceolate, acute or obtuse flower in racemes, 1cm long

Medicinal significance: Root and rhizome are tonic,expectorant,demulecent and mild laxative, it is also used for allaying cough catarrh, bronchitis, sore throat, cold and flu. It is also used to treat gastritis and peptic ulcer diseases (HMG/N, 1997).

Important biochemical constituent(s): Glycyrrhizin (2-14%), which gives it sweet pleasant taste. Its active constituents include glycyrrhizin, glycyrrhetinic acid, flavonoids, asparagines, iso-flavonoids and chalcones.

3.2.6 *Jasminium humile* Linn.(C.N.: Jai)

Morphological Features: This genus belongs to family Oleaceae. It is a shrub of 4m, tall; leaves alternate, petiolate, 3-foliate of pinnate, leaflets 1-4x 0.22cm; lanceolate, acute, obtuse or acuminate, entire, glabrous, flowers in cymes, yellow in colour.

Medicinal significance: Leaves are used to treat cough, bronchitis, sinusitis and asthma. Flowers, are astringent, tonic to the heart and bowels, root is used in ring worm. Milky juice of the plants is used for destroying the unhealthy lining walls of chronic sinuses and fistulas (HMG/N, 1997).

3.2.7 *Juniperus indica* Bertol.(C.N.: Dhupi)

Morphological Features: This genus belongs to family Cupressacece. It is a large gregarious shrub, or small tree to 20m tall, with a stout trunk. Leaves two kinds; those on lower branches awlshaped,3-6mm long, spreading those on terminal branches scale like 1.5mm long, a depressed, overlapping in four ranks giving a smooth cord like appearance to the branches. Fruit 1-seed, at first brown then shinning blue, to 13mm long (Joshi and Joshi, 2000).

Medicinal significance: The plant is bitter, pungent, acrid, heating, appetizer, carminative, antihelminthic, alexipharmic, laxative, useful in diarrhea, abdominal

pains, diseases of the spleen and abdominal, as cities, tumors, bronchitis, indigestion etc. The fruit is useful in asthma, chronic bronchitis etc (IUCN, 2004).

Important biochemical constituent(s): Oil of the plant contains sabinine, terpen-4-ol and pinene as major constituents.Lignans,terpenoids, flavonoids, sesquiterpenes etc. are also its components.

3.2.8 *Justicia adhatoda* L. (SYN.:*Adhatoda vasica*) (C.N.: Asuro)

Morphological Features: This genus belongs to family Acanthaceae. It is a large strong-smelling gregarious shrub of 1-2m, leaves25x8cm, elliptic, entire, acuminate, petiolate. Flowers in long terminal spikes. Bracts broadly ovate(2x1.5 cm), corolla, white, calyx 3cm blipped, fruit capsule, 3-4cm seed compressed (HMG/N, 1997)

Medicinal significance: Leaves and roots used in cough, chronic bronchitis, asthma and rheumatism. It is also insecticidal, expectorants, anti-helmintic, anti-inflammatory and antispasmodic

Important biochemical constituent(s): Vasicine, essential oil, crystalline acid and a white crystalline alkaloid (Joshi and Joshi, 2001).

3.2.9 *Mentha piperita* Linn.(C.N.: Peppermint)

Morphological Features: This genus belongs to family labiatae. It is a herb, with leaves of 0.6-2.5x0.20.8cm; opposite, sessile lanceolate, crenate, glabrous, flowers in terminal spike, purplish.

Medicinal significance: The volatile oil from plant is antiseptic and antibacterial. The whole plant stimulant, carminative used for allaying nausea sickness, diarrhea-vomiting and gastric colic. Helps to relieve stuffiness and catarrh and analgesic effect. It is also used in menstrual pain, asthma and insomnia,cold,flu, arthritis, gout, headaches, neuralgia, sciatica and general aches and pain. Diluted oil is used as an inhalant and chest rub for respiratory problems. In digestive tract it relaxes smooth muscles and reduces inflammation relieving pain, asthma, and spasms, flatulence, ache, heartburn and indigestion (HMG/N, 1997).

Important biochemical constituent(s): The volatile oil contains up to 1.5% menthol, flavoniods, rosmarinic acid, monoterpenes and sesquiterpenes.

3.2.10 *Myrica esculenta* Buch-Ham. Ex D.Don.(C.N.: Kaphal)

Morphological Features: This genus belongs to family Myricaceae. It is a medium sized tree with nodules in the roots capable of fixing atmospheric nitrogen due to the

presence of Frankia. Leaves short petiolate, acute or obtuse, entire, minutely gland dotted beneath. Male flowers in drooping spikes; female flowers in axillary's, erect spikes. Fruits globose, red succulent (Joshi and Joshi, 2001).

Medicinal significance: Bark is astringent, carminative and antiseptic ,useful in fever, cough, asthma, and also used in sinusitis (IUCN, 2004).

Important biochemical's constituent(s): Myricetin (coloring matter in a from of glycoside), a glycone (a second glycoside in traces) in bark.

3.2.11 *Ocimum sanctum* L. (SYN.: *Ocimum sanctum*) (C.N.: Tulsi)

Morphological Features: This genus belongs to family Labitate. It is an erect biennial or triennial herb of about 75cm in height, profusely branched and hairy. Stem usually quadrangular. Leaves opposite, decussate, to 5cm long, hairy, entire or toothed, dotted with minute glands. Bracts sessile flowers small, less in small conspicuous purplish or reddish, on slender spikes in small compact clusters. Fruits small seeds or yellowish, sub-globose (Joshi and Joshi, 2001).

Medicinal significance: Decoction of leaves is used to treat common cold. Decoction of roots is given in malarial fever. Juices of leaves is used in ear ache, digestive complaints, bronchitis, catarrh, skin diseases, expectorant and to control ringworm. Oil extracted from leaves has anti bacterial properties (Bhattacharjee, 1998).

Important biochemical constituent(s): Essential oil of leaves and shoots contains eugenol as the major component. Other constituents are nerol, terpinene, pinene, and cavacrol. Leaves also contain ursalic acid, apigenin, luteolin and orientin.

3.2.12 *Piper nigrum* Linn.(C.N.: Marich, Black pepper)

Morphological Features: This genus belongs to family Piperaceae. Black pepper is considered as the "King of Spices". The pepper plant is a perennial woody vine growing to 4m in height on supporting trees, poles or trellises. The leaves are large, ovate, leathery, smooth without marginal serration and dark green. The flowers are small whitish and borne on hanging catkins or more properly, spikes. The fruits, commonly called berries or pepper corns, are small nearly spherical and dull green in colour when immature, turning yellowish and finally red as they ripen.

Medicinal significance: Pepper is used for a variety of purposes. It is prescribed for dyspepsia, malaria, delirium, tremens, hemorrhoids, flatulence, as in paraplegia and arthritic diseases. It is also used in local applications for relaxed sore throat, piles and some skin diseases (Kirtikar and Basu, 1935, Snell and Snell, 1952).

3.2.13 *Spilanthes acmella* Murr. (SYN.: *Spilanthes calava*) (C.N.: Marahaththi)

Morphological Features: This genus belongs to the family Asteraceae. *Spilanthes acmella* is also known as toothache plant. An annual ascending erect stout herbs, 20-50cm high or 15-40cm high. Leaves opposite, petiolate, broadly ovate, narrowed at base, acute or obtuse at apex irregularly crenate 3 nerved from the base petiole 0.5-1cm pubescent. Flower(yellow and red cone shaped), heads yellow, 0.5-1cm solitary, conical, peduncle up to 10cm pubescent. Ray florets absent, disc florets yellow, achene's dark brown.

Medicinal significance: The flower heads are chewed to relieve the toothache and other mouth related trouble. Leaves are used externally in treatment of skin diseases. Roots decoction is used as purgative also used in lithotrophic, diuretic, whole plant used in treatment of dysentery. Powder decoction of fresh head is given to get relieve from cough and sore throat (HMG/N, 1997).

3.2.14 *Syzygium aromaticum* Linn.(C.N.: Laung, Clove)

Morphological Features: This genus belongs to family: Myrtaceae. The clove tree is the pyramidal or conical ever green tree, 9-12m high and some times taller. The main stem is erect and often forking at a height of 1.5-1.8m. The bark is smooth and grey, leaves are lanceloate, in pairs, acute at both ends 7.5-12.3x2.5-3.75cm in size gland dotted and fragrant (due to oil glands). Flower buds borne in small clusters at the ends if the branches, greenish, turning pink at the time of maturity and aromatic. The fruit is a purple drupe about one inch long and half it is called ("Mother of cloves").Seed is oblong, soft, grooved on one side and 1.5cm long (Pruthi, 1976).

Medicinal significance: It is aromatic, stimulant and carminative. It is used in various forms of gastric irritation, and dyspepsia to relieve nausea and vomiting, to cure flatulence and antiseptic and antibacterial (B.P.C, 1968).

Important biochemical constituent(s): The clove bud oil contains free eugenol as its main constituents.

3.2.15 *Trachyspermum ammi* (L.) Sprague(C.N.: Jwano, Ajowan)

Morphological Features: This genus belongs to the family Umbelliferae. It is an erect, glabrous pubescent, branched annual herb, to 90cm tall with striate stems. Leaves 2-4-pinnately divided with linear segments. Flowers white, small in terminal or seemingly lateral pedunculate, compound umbels. Fruits ovoid, muricate, aromatic;

cremocarps, 2-3mm long, grayish brown; mericarps compressed , with distinct ridges and tubercular surface, 1-seeded.

Medicinal significance: Fruits are antispasmodic, stomachic, carminative, stimulant and tonic. Used in diarrhea ,dyspepsia, colic, flatulence, indigestion and cholera (Joshi and Joshi, 2001).

Important biochemical constituent(s): Fruits have aromatic essential oil containing cumene, thymol and thymine.

3.2.16 *Zanthoxylum armatum* D.C.(C.N.: Timur)

Morphological Features: This genus belongs to the family Rutaceae. It is a shrub or rarely small tree, with corky bark and numerous long straight leaf stalks. Leaves pinnate; leaflets 2-6 pairs, lanceolate, 8cm long , toothed, sparsely gland-dotted. Flowers 1mm, one sexed; than calyx with 6-8 acute lobes; petals absent; stamens 6-8, much longer than calyx in male flower. Ripe capsules 3-4mm diameter., globular, red wrinkled, aromatic; seed shining black (Joshi and Joshi, 2001)

Medicinal significance: Stem and bark are used as aromatic, tonic in fever, dyspepsia or cholera. Fruits are used as fish poisoning and as remedy for toothache, carminative and stomachic (HMG/N, 1997).

Important biochemical's constituent(s): Tannic acid and Gallic acid, starch, mineral salts, mucilage and albumen have been isolated from rhizome.

3.3 Antimicrobial agents

a) **Definition of Antimicrobial agents:-** Antimicrobial agents is a chemical substance that either kills microorganisms or prevents their growth.

b) **Mode of action**: The antimicrobials can be either bacteriostatic or bactericidal. The bacteriostatic antimicrobials are those which prevents the active multiplication of bacteria at usual dosages and bacterostatic antimicrobials are those which kill bacteria at usual dosages. Some bacterostatic agents became bactericidal when used at higher concentration. In general antimicrobial agents act in one or more of the following ways. They may act by (a) inhibiting cell wall formation leading to cell lysis. (b) damaging the bacterial cell membrane leading to loss of cell contents and so to cell death. (c) inhibiting protein production and therefore arresting bacterial growth and (d) inhibiting the production of nucleic acids and therefore preventing bacteria from reproducing (Cheesbrough, 1993).

3.4 Antimicrobial activity

World wide infectious diseases is the number one cause of death accounting for approximately one-half of the deaths in tropical countries. Death from infectious diseases ranked fifth in 1981 has become the third leading cause of death in 1992, an increase of 58% (Pinner *et al*., 1996). Some infectious disease once thought to be all but conquered have returned with a vengeance others have developed stubborn resistance to antibiotic drugs. Resistance by disease causing organisms to antimicrobial drugs is a major public health problem world wide (Park, 2005).

The incidence of microbial infection is increasing alarmingly with the increase of sizeable susceptible population of immunocompromised patients (ICP), including AIDS. That is why there is growing world wide concern regarding the problem of infectious diseases. To solve this serious problem there has been reawakening of interest in the development of antimicrobial drugs. The effort is continuously running for the development of potent antimicrobial and immunomoudulator(s) from medicinal plants. Screening for new drugs in plant implies the screening of extracts for the presence of novel compounds and review has shown that there are numerous plants which has good antimicrobial activity which can be used for curing different types of disease (Khan, 1997).

It was after the discovery of microorganisms as the causative agents for many infections and septic diseases of human beings and animals that more interest was created in plant substance which were toxic to those microorganisms. Since then a large numbers of studies have been carried out throught the world with respect to antimicrobial, activities of medicinal plants used in traditional medicine. Some of the notable works conducted from the Indian subcontinent and worldwide includes:

Sampurna and Nigam (1980) found that one mixture containing the oils of *Eucalyptus citriodra*, *Cinnamomum zylanium*, *Cuminum cyminum*, *Mentha arvensis*, *Mentha spicata*, and *Mentha piperita* and other containing *M. piperita*, *E citricodora*, *C zeylanicum, Trachyspermum ammi*, *Carum carvi* and *C cyminum* posses potent antibacterial efficacy against *Salmonella typhi*.

Baslas and Kumar (1980) reported the oil obtained from *Cuminum cyminum* seeds exhibited moderare antibacterial activity against *Streptococcus faecalis*, whereas Cumin aldehyde and cuminol inhibited the growth of *S faecalis* as well as *Staphylococcus* sps, *E coli* and *Klebsiella pneumoniae* significantly.

Huhtanen (1980) reported inhibition of *Clostridium botulinum* by spice extracts. He found *Syzygium aromaticum* had MIC of 500ppm and *Myristic fragans* had MIC of 125pppm against it. White and Black pepper(*Piper nigrum*) had MIC of 125ppm against it.

Mehta *et al.,* (1981) in their research found that the essential oil of *Zanthoxylum alatum* fruits exhibited fairly good antibacterial activity but less antifungal activity. The antihelminthic activity was better than that of piperazine phosphate.

Syed *et al.,* (1986) studied antimicrobial activity of the essential oils of the umberlliferae family. Cumin showed remarkable activity against the pathogens like *S. aureus*, *E coli*, *Salmonella typhi*, *Shigella dysentery*, *Vibrio cholerae* at quite low concentrations (400-800 ppm).

Joshi and Edington (1990) reported that plant species including *Jasminum humile*, *Artemisa dubia, Gaultheria fragrantissima* were used to treat cough, bronchitis, sinusitis and asthma in two village communities in Central Nepal.

Jain and Kar (1992) dealt with the antibacterial activity of *Piper nigrum* oil, following zones of inhibition were seen *E coli* 8mm; *B subtilis* 8mm; *S aureus* 8mm and *S typhi* 8mm where filter discs used were of 6mm.

Vijaya and Ananthan (1994) tested in-vitro antibacterial activity of three indigenous plant *viz. Camellia sinensis* (tea), *Azadhirachta indica* (Neem), and *Acorus calamus* (sweet flag) against enteropathogenic *E coli*, *Shigella sonni*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Vibro cholera*. The inhibition studies were performed by using disc diffusion and tube dilution methods. Ether extracts appeared to be more potent than the aqueous extracts and tea showed a higher antibacterial activity than the other two plants.

Tassou *et al*., (1995) studied the effects of essential oil from mint *M. pipertia* on *Salmonella enteridis* and *Listeria monocytogens* in model food systems at 4º C and 10º C.Results obtained showed that the oil showed antibacterial activity on the model foods and no growth was seen on them.

Deans *et al.,* (1995) found that the essential oil from *S. aromaticum* exhibited significant antimicrobial activity against collection of 25 different genera of test bacteria and 25 different isolates of *Listera monocytogens*. The oil was also tested against three fungi strains; a plant pathogen, a spoilage type and a mycotoxigenic

strain. This resulted in high yield of growth inhibition at both concentration of 1 and 10 µg/ml growth medium.

Cai and Wu (1996) found that a crude methanol extract of *S. aromaticum* exhibited preferential growth inhibitory activity against Gram negative periodontal growth inhibitory activity against Gram negative periodontal oral pathogens including *Porphyromonas gingivalis* and *Prevotella intermedia*.

Raman *et al.,* (1996) reported that ethanol extract of *Achryanthes aspera* stems and alkaloids AM-1 and AM-2 isolated from the plant exhibited antibacterial activity against *B. subtilis, S. aureus*, *Pseudomonas aeruginosa* and *Shigella dysenteriae*.

Saroja *et al.,* (1997) found that both aqueous and organic extracts of *Azadirachta indica*, *Ocimum sanctum*, *Adhatoda vasica*, *Solanum tritobatum*, *Withania somnifera Euphorbia pilulifera*, *Emblica officinalis*, and *Allium sativum* had bactericidial activity against Mycobacterium tuberculosis.

Farooq and Pathak (1998) observed antimicrobial activity of total plant extract obtained from *Ocimum sanctum*, *Adhatoda vasica*, *Glycyrrhiza glabra*, *Piper nigrum Piper longum*, *Onosma bracteatum*, *Tinospora cordifolia*, *Fagonica cretica, Emblica officinals*, *Saussurea lappa* and *Terminalia chebula.*

Mahato (1998) studied about use of plants like *Myrica esculenta*, *Acorus calamus*, *Artemisia dubia* in respiratory disorder and found its antibacterial properties also.

Brantner and Chakraborty (1998) investigated *Adhatoda vasica* Nees leaves and found that strong activity of alkaloids fraction against against *P. aeruginosa* (MIC= 164µg/ml), significant antibacterial activity against the Gram positive bacteria *Streptococcus faecalis*, *S. aureus*, *S. epidermidis* and the Gram negative *E.coli* were also evident. Only moderate antibacterial effect could be observed using *B. subtilis*, *Campylobacter jejuni* and *Salmonella enteritidis* as test organisms.

Ozguven *et al.,* (1998) selected 15 different species or origins from the 25 *Mentha* species or ecotypes, and tested their essential oils for the effects of three microorganisms, *S aureus, E coli*, *Enterococcus faecalis* in order to find out their antimicrobial activities. The essential oils of *Mentha* species exhibited strong antimicrobial activities. Antimicrobials effects were determined even at low concentrations.

Rajendhran *et al.,* (1998) analyzed the antimicrobial activites of some selected medicinal plants, they found extracts from *Acrous calamus*, *Ocimum sanctum*, *Zingiber officinale*, *Cinnamomum zeylanicum*, *Moringa oleifera*, and *Piper bettle* exhibited activity against. *S. aureus*, *E. coli, P. aeruginosa* and *Klebsiella sps*. *E. coli* exhibited higher resistant to antibiotics and plants extracts. *Ocimum sanctum* was most effective against four microorganisms.

Nimri *et al.,* (1999) also reported the amtibacterial activity of ethanol extracts of traditionally used medicinal plants species of Jordan and other middle east countries. Three plant species *Punica granatum*, *Qurecus infectoria* and *Rhus coriana* showed the broad spectrum activity and the susceptible bacteria were *Streptococcus pyogens*.

Zaiba *et al.,* (1999) investigated 28 ethanolic extracts of 27 plants for their in-vitro antimicrobial activity against 4 bacteria and 6 soil borne fungi. Antibacterial activity against one or two test bacteria (*E. coli*, *B. subtilis*, *S. aureus* and *Streptococcus pneumoniae* was detected among 96% of the plant extract. Significant effect was shown by *Hemidesmus indicus*. *Terminalia bellerica*, *Terminial chebula*, and *S. aromaticum*. Extracts of *Emblica officinalis* and *P grantum* showed strong antibacterial but moderate antifungal activity. While *A calamus* gave strong antifungal but moderate antibacterial activity. Maximum potency (low MIC value) was detected in *S. aromaticum* followd by *H indicus*, *T chebula*, *T bellerica*, *A calamus* and *E. officinalis*.

Pradhan *et al.,* (1999) studied antibacterial activity of *Piper nigrum* against bacteria like *E,coli*, *S. aureus*, *B. cereus*, *Salmonella typhimurium*. It was found that the active compound present 3,4-dihyroxyphenlethanol glycoside was effective ay 2.25 mmol/l against *E.coli*, *S. aureus*, and *B. cereus* but inactive against *S. typhimurium*.

Kelmanson *et al.,* (2000) also found greater activity of medicinal plants used in traditional zulu medicine against Gram positive bacteria. Tuber and bark extracts of *Dioscorea sylvatica* had activity against Gram negative *E. coli* and extracts from *Dioscorea dregeona, Cheilanthes viridis* and *Vernonia colorate* were active against *P. aeruginosa.*

Samy and Ignacimuthu (2000) studied the antimicrobial activity of 30 Indian floklore medicinal plants used by tribal healers to treat infections by using disk diffusion method against *B.subtilis, E.coli*, *Klebisella aerogenes* and *S. aureus*. Twenty plants showed activity against one or more species of bacteria used in this assay. *Cassia*

occidentalis, and *Cassia auricularia* exhibited significant broad spectrum activity against *B.subtilis* and *S. aureus*. Eight plant extracts exhibited both antibacterial and antifungal activities.

Shigeharu *et al.,* (2001) studied the antibacterial activity of 14 essential oil and their major constituents against respiratory tract pathogens like *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Streptoccuus pyogens* , *S. aureus*. Penicillin suspectibile and resistant *S. pneumoniae* were comparable in susceptibility. Among 14 essential oil, Cinnamon bark, lemon grass and thyme oil showed the lowest MID followed by essential oil containing terpene alcohol as major constituents.

Imai *et al.,* (2001) studied the effects of essential oils of peppermint(*M.piperita*), spearmint(*M.spicata*), and Japanese mint(*M.arvensis*) of the four major constituent of the essential oil of peppermint and three major constituent of the essential oil of spearmint; in the proliferation of *Helicobacter pylori*, *Salmonella entritidis*, *E.coli*, MRSA and MSSA were examined. The antibacterial effect of the mint oil was potent and bactericidal.

Tsukiyama *et al*., (2002) investigated the antibacterial activity of Licochalcone A against spore forming bacteria. Licochalcone A isolated from roots of licorice(*Glycyrrhiza inflata)*. The activity of the extract showed that growth of *B. subtilis* was inhibited and MIC value of 2to 3µg/ml, but it was not effective against Gram negative bacteria at 50µg/ml.

Ohno *et al.,* (2003) studied, the antimicrobial activity of essential oils against *Helicobacter pylori.*Threteen essential oils used were *Cupresseus sempervirens*, *Juniperus communis*, *Melalenca alternifolia, Lippa citriodora*, *Ocimum basilicum M.piperita Cymbopgon citrates* and *Lippia citriodora* , completely inhibited it's growth in-vitro at a concentration of 0.1%(V/V). Among them Lemon grass(*Cymbopgon citrates*) and Lemon verbena(*Lippia citriodora*) were most potent.

Pessini *et al.,* (2003) studied antibacterial effect of extracts of *Piper regnelli.* The evaluation of aqueous and ethyl acetate extract of leaves of *P.regnelli* showed that aqueous extract was weakly positive to *S.aureus, B.subtilis* while ethyl acetate showed good activity against them with MIC and MBC at 15.62µg/ml. In contrast Gram-negative bacteria were not inhibited by the extract at concentration 1000mg/ml.

Bonjar (2004) screened 50 methanolic extracts of plants used in Iranian folkloric medicines for antibacterial activity 30 samples had antibacterial activity against at least on one of the bacteria. Among the active plants, 32.6% were active against Gram negative, 62% against Gram positive and 47% against Gram negative.

Aqil *et al.,* (2005) examined the ethanolic extracts and some fractions from 10 medicinal plants, known for it's antibacterial activity against , beta lactamase producing MRSA and MSSA.The extracts of plants from *Camellia sinesis*, *Delonix regia*, *Holarrhena antidysentrica, P grantum*, *T. chebula* and *T.belerica* showed a broad spectrum activity. The extracts of leaves of *O sanctum* showed better activity against three MRSA strains while *Allium sativum* and *Citrus sineusis* exhibited no activity against MRSA.

Attia and Samar (2005) selected seven plant extracts for its antidiarrhoeal effect on castor oil induced diarrhea, gastrointestinal movements in rats and freshly slaughtered rabbits. A significant effect of test plant was achieved, the plant extracts decreased the gastrointestinal movement as indicated by the significantly($P<0.005$ to 0.001).

Mathabe *et al., (*2006) studied antibacterial activities of 21 plants species used in traditional medical practices in Limpopo Province, South Africa, for treatment of diarrhea. Among 21 plant extracts, *P grantum* and *Indigo feradaleoides* showed highest antibacterial activity against *E.coli*, *S aureus*, *Shigella, S.typhi, V.cholerae* with inhibition zones ranging between 10 and 31mm and MIC values ranged from 0.0392 and 0.69 mg/ml.

Kumar *et al.,* (2006) investigated the antibacterial and antifungal activities of 61 Indian medicinal plants used in various infectious disorder. On the basis of result obtained. It was concluded that the crude extracts of 28 plants showed activity against at least one of test organisms used in screening and most significant antimicrobial activity was given by *Dorema ammoniacum*, *Sphaeranthus indicus*, *Dracaena cinnabari*, *Mallotus Philippinensis, Nardostachys jatamansi* etc.

Salari *et al.,* (2006) studied the antibacterial effects of *Eucalyptus globulus* leaf extract on 56 isolates of *S aureus*, 25 isolates of *S. pyogens*, 12 isolates of *S pneumoniae* and 7 isolates of *Haemophilus influenzae* clinical isolates of respiratory tracts disorder. The result suggests that leaf extract of *E. globulus* can be used to treat respiratory tract infection.

Rojas *et al.,* (2006) screened the antimicrobial activity of ten medicinal plants of Clombian folkloric medicine against *S. aureus*, Beta haemolitic Streptococcus, *B. cerus, P. aeruginosa* and *E.coli* and *C.albicans*. All plants showed antimicrobial activity in regards to at least three microorganisms tested. The ethanolic extract of *B orellana*, *G. sepium*, *J mimosifolia* and *P pulchrum* were most active. *E.coli, B.cereus*, *S aureus*, were the most suspceptible bacteria toall plants while Beta haemolytic Streptococcus ,*P. aeruginosa* and *C.albicans* were the most resistant microorganisms.

Indu *et al.,* (2006) evaluated the antibacterial activity of the aqueous extracts of *A sativum*, *M.fragrans, Z officinals, A.cepa, P.nigrum*, against serotypes of *E. coli*, 8 serotype of *Salmonella, Listeria monocytogenns* and *Aeromonas hydrophila*. Garlic extract showed excellent activity against all test organisms, except *L monocytogens*. Nutmeg showed good anti-listerial activity although *E.coli* and *Salmonella* were serotype dependent. Both garlic and nutmeg extracts were effective against *A. hydrophila*. Extracts of ginger showed inhibitory activity against two serotypes of *E.coli* as 08 (ETEC) and 088 only. Extracts of onion and pepper did not show any activity against test organisms.

Study about the antimicrobial activities of medicinal plants and essential oils is limited in Nepal. Even though regular study about antimicrobial activities of medicinal plants extracts is carried out by Department of Forestry and Plant Research. Some of the notable work done are as follows;

Adhikari and Sharma (1988) carried out an experiment to determine MIC values of *Allium sativum*, *Azadirachta indica*, A.vulgaris, and *H asiatica* against *E.coli*, *P. aerugonisa*, *Streptococcus* sps and *S.aureus*.

Shrestha and Sharma (1988) observed the antimicrobial activities of some plant products *viz. Mentha arvensis*, *Acorus calamus, Zanthoxylum oxyphyllum* and turpentine oil against some Gram positive and Gram negative bacteria by MIC techniques.

Risal (1994) studied antimicrobial activities of 32 indigenous plants by filter paper disk diffusion method on the dired extracts obtained from 50% alcohol. The test organisms employed were *S. aureus*, *B.pumilis*, *B subtilis, E.coli, S. typhi, Sdysenteriae, c.albicans* and *S. cerevisiae*. Some extracts showed inhibition of bacterial growth by tuber plant of *Stephania grandulifera* and flower bud of *Sphaeranthus senegalensis* and aerial part of *Chenopodium ambrosoides* showed

encouraging activities in fungal growth. *Perilla frutescence* oil from leaf of the plants showed strong inhibition zone for fungal growth and weak inhibition activities for bacteria.

Taylor *et al.,* (1996) tested 20 species of plants belonging to thirteen different families against eleven strains of bacteria and 4 strains of fungi. Only four were active against *P. aeruginosa*. None of the extract were active against the other Gram negative bacteria tested such as *E.coli, K. pneumoniae, Enterobacter arogenes*, and *S. typhmurium*. The extracts which showed broadest spectra of activity were *Eupatorium odoratum*, *Terminalia alata, M. philippensis and Rumex hastatus.*

Devkota *et al., (*1999) studied antimicrobial activities of nine medicinal plants of Nepal *viz. Glycyrrhiza glabra*, *Azadirachta indica, Swertia chirayita*, *A. calamus*, *Withania somnifera, T,chebula*, *Berberis aristata*, *Parnassia nubicola* and *Crcuma angustifolia* for antimicrobial activity against *P.aeruginosa*, *S. aureus*, *E.coli*, MRSA, *V. cholerae*, *S.typhi*, *S. dysenteriae* and *Sh. fexneri* were carried out. *G. glabra* showed best antimicrobial activity among all tested herbal plant extract.

Parajuli *et al.*, (2001) studied antibacterial activity of 29 medicinal plants used to treat skins ailments of kaski district, Nepal. Eleven plants species including *Zanthoxylum armatum* were able to produce ZOI with all tested bacteria while seven plant species including *Ficus bengalensis*, *Maesa chisia*, were unable to produce inhibition zone with any of the test bacteria.

Parajuli (2001) tested antibacterial activity of 29 plants species against four test organisms, *viz, S. aureus*, *B.subtilis*, *E.coli* and *P. aeruginosa*. Of them 12 species showed distinct ZOI with all types of test organisms.

Gautum (2002) selected 33 plants species used for curing respiratory disease in traditional society in Nawalparasi district of Nepal. Among them only 13 plants were able to produce ZOI with all three test organisms like *S.aureus*, *K. pneumoniae*, and *P.aeruginosa*. two plant species *Brassica rapa* and *Vitex negundo* didn't show ZOI with any of test bacteria.

Timisina (2003), investigated 20 different medicinal plants for their antimicrobial properties against ten microorganisms. Among 20 tested plant seven (35%) were found active against at least 6 or more test organisms and 4 plants (20%) were active against 4 test microorganisms. *Rhododendron anthopogon* and *Rhus Javanica* were

the most active plants as they were effective for all microorganisms. *Boerhavia diffusa* was ineffective to all of the test microorganisms.

Few studies have been done in dissertation works in Central Department of Microbiology.

Thapa (1997) determined MIC values of *Moringa oleifera*, *Azadirachta indica*, *Momordica charantia* and *T. chebula*

Bhatta (1998) studied the antimicrobial activity of rind of *P.grantum* and found that the extract was effective against 13 out of 14 tested bacteria.

Pokhrel (2000) screened and evaluated antimicrobial activity of 20 different medicinal plants of Nepal against 8 different microorganisms and reported that extracts were more effective in Gram positive than Gram negative.

Sharma (2000) studied the antimicrobial activity of some spices used in Nepal and found that essential oil from Cinnamon, Clove, and Timur showed high degree of inhibition whereas others like black pepper and coriander oil were found comparatively les inhibitory.

Similarly, Baidya (2001) screened and evaluated antimicrobial activity of 20 different medicinal plants of Nepal against 8 pathogenic organisms and found that extract from *Rubus ellipticus* was most effective.

Prasai (2002) tested the antibacterial activity of eight different medicinal plants against seven different Gram negative bacteria. Among 8 plants, four plants viz. *Alnus nepalensis* , *Ficus religisoa*, *Myrica esculenta* and *Rhododendron arboretum* were effective against *E.coli*, *Klebsiella sps, Proteus vulgaris*, *S typhi*, *Shigella* spp. and *V. cholerae*. All plants were ineffective against *P. aeruginosa*.

Radha (2004) investigated antimicrobial activity of 6 species of lichens and 3higher plants against ten bacteria and one fungi. The Gram positive organisms were most sensitive to inhibition by ethanolic and methnolic suspensions of plant extracts where as *C.albicans* and *E.coli* were found tomost resistant one. Simiarly, Ethanolic suspension of extract from *Parmelia tinctorum* and *P. sanctiangeli* inhibited 9 out of 11 micro organisms while extract of *V. negundo* has narrowest antimicrobial activity.

3.5 Solvent extraction of natural products

There are various solvent extraction methods for the extraction of natural products. The process of solvent extraction is generally employed for the isolation of dissolved substance from solution or from the removal of undesired soluble impurities from solid mixtures (Chatwal and Anand, 1998). Mainly three methods are well established.

a) **Steam distillation:** This method is applicable to those substances which (i) have non-volitile impurities (ii) are insoluble in water (ii) have a high molecular weight and (iv) posses a fairly high vapour pressure at above boiling point of water (Tewari *et al*., 1992). The steam volatile natural products (eg, those in the essential oil) such as alcohol, esters and carbonyl compounds of aliphatic (both cyclic and acyclic) and simpler aromatic systems are removed by steam distillation.

b) **Batch extraction:** This process, which is also called cold extraction, is the most common type of extraction process. In this method, solvent is added to the solution to be extracted in a separating funnel, which is conical or pear shaped with short stem and fitted with ground glass and interchangeable stopper. The extracting solvent and material to be extracted are taken in a separating funnel in the ratio 3:1 respectively. After a definite period of time just turning the stopcock can collect the extract. The compound to be extracted is again covered with extracting solvent. This process is repeated for several time to make complete extraction. This method is useful for the detection of heat sensitive compounds from natural products. It is reported that the batch extraction process is less effective than continuous process (Furnis *et al.,* 1996).

c) **Continuous extraction:** In this process same solvent is recycled and used for dissolving the soluble compound present in the solid substances to be extracted. For the continuous extraction of the solid by hot solvent, it is better to use soxhlet extraction apparatus. Generally for the analysis of antimicrobial activity or other biological activity, plants extracts are prepared by soxhlet apparatus (Furnis *et al*., 1996). This method is used for the extraction of fats and oil from seeds and alkolids and essential oils from flowers and leaves of plants in analyzing biological and other samples (Chatwal and Anand, 1998).

Adhikari and Sharma (1988) used soxhlet apparatus for continuous extraction of different medicinal plants *viz*, *Allium sativum*, *Artemesia vulgaris*, *Hydrocotyl asiatica* and *Azadirachta indica*. These extracts were used for the analysis of antimicrobial activity. Devkota *et al.,* (1999) used soxhelt appararus for the extraction of different medicinal plants such as *Swerita chiratyita*, *Acorus calamus*, *Withania somnifera*, *Terminalia chebula* etc. for analysis of antimicrobial activity. Similarly, Pokhrel (2000), Baidya (2001), Timisina (2003), Radha (2004) also used Soxhlet extraction apparatus for the extraction of different medicinal plants in their separate study. These extracts were used for analysis of antimicrobial activity.

3.6 Selection of the organisms.

Most of the plant materials under study were found to be used in respiratory tract infections and intestinal disorder in one way or other in traditional medicines. So the antimicrobial screening was targeted to those organisms related with respiratory complaints and intestinal disorder.

a) Respiratory tract infection:

The warm moist surfaces of human respiratory tract provide ideal conditions for the growth of pathogens. Since, the human respiratory tract is divided into upper respiratory tract infection and lower respiratory tract infection.

The upper respiratory tract can be the site of several types of relevant infection like:-

- Pharyngitis, rhinitis; sometimes involving tonsillitis, and giving rise to "sore throat".
- Nasopharyngitis in nasopharynx
- Otits media in ear
- Sinusitis in nose
- Epiglottis
- Of all of those infections, pharyngitis is by far the most frequent, in addition, the untreated infection may have serious sequel. The most common pathogenic bacteria encountered are both Gram positive and Gram negative ; *E.coli*, *Haemophilus* sps, *Klebsiella* sps, *Enterobacter* sps, *Pseudomonas aeruginosa*, coagulase positive *Staphylococcus*, *Streptococcus pneumoniae*, and beta haemolytic Streptococcus Group A.

Lower respiratory tract infection are infection occurring below the level of the larynx, i.e. in the trachea, the bronchi, or in the lung tissues; tracheitis (lung abscess), bronchitis (pneumonia). The organisms encountered in the lower respiratory tract infection are, *Haemophilus influenzae*, *Streptoccus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Mycobacterium tuberculosis* etc (WHO, 2004).

b) Intestinal disorder/gastroenteritis

Gastroenteritis is a very common disorder. It has many causes, can range from mild to severe, and usually manifests with symptoms of vomiting, diarrhea, and abdominal discomfort. Other causes of some of these symptoms include viral infections, improper diet, malabsorbation syndromes, various enteropathies, and inflammatory bowel diseases. Bacterial gastroenteritis usually is self limited, but improper management of an acute infection can lead to a protracted course. It may be infective or noninfective. Infective gastroenteritis is commonly caused by *Salmonella* sps, *Shigella dysenteriae*, EPEC, ETEC, EHEC, EIEC, *Vibro cholerae*, *Staphylococcus aureus, Bacillus cereus* and *Campylobacter jejuni* etc. (Chakraborty, 2000, Nagoba, 2005).

3.7 Short description of microorganisms involved in this study with their pathogenecity

3.7.1. *Bacillus subtilis*

It is a Gram positive, rod, spore former, spore are very resistant ellipsoidal, spores are centrally located in the vegetative cell, very resistant to heat. They can tolerate 100ºC for several hours. It is commonest saprophytes found as contaminants in foods, clinical specimens and laboratory culture. It is facultative thermopiles, capable of growth over the range 12-55 ºC, grows well on a ordinary media forming large colonies that are circular or irregular, gray yellow, granular and difficult to emulsify. It fails to grow anaerobically, it produces acid from glucose, xylose, sucrose and mannitol.

It is less commonly found opportunistic pathogen. It sometimes cause food poisoning (Collee *et al.*, 1996).

3.7.2. Staphylococcus aureus

It is Gram positive, coagulase positive, highly adaptable, non-sporing, non-motile pathogen. They are aerobic and facultative anaerobic cocci. In nutrient agar at 37ºC

they produce colonies of 1-3mm diameter, smooth low convex, opaque, butyrous consistency within 18-24hrs. In MSA, the form 1mm diameter yellow colonies surrounded by yellow medium due to acid fermentation. They are catalase, and DNase positive (Collee *et al.*, 1996).

It is a common etiologic organisms in soft tissues infections and may be found on the skins of nearly 20% healthy people eg:- folliculitis, impetigo, carbuncles, furuncles etc. Staphylococcal pneumonia should be suspected in any severe pneumonia case if response to anti-pneumococcal antibiotics is unsatisfactory, evidence of progressive sepsis or radiological evidence of cavitations, abscess formation or if there is metastatic abscess. It also causes toxic food poisoning toxic shock syndrome and toxic skin exfoliation (Cheesbrough, 2000).

3.7.3 *Streptococcus pneumoniae*

It is a Gram positive, oval or lancent shaped diplococcus that occurs in pairs or short chains. They are non-motile and non-sporing and all freshly isolated strains are capsulated. It is aerobic and facultative anaerobe. It grows best in air or hydrogen with 5-10% CO_2. Grows at 37 ºC on ordinary media, but better on media with serum, blood or heated blood. Colonies on blood agar as a small, smooth and transparent low convex, while tiny they show draughtsman form as they grow to a diameter of about 1mm. haemolysis or partial clearing of blood is produced underneath in a narrow zone around the colonies. The organisms tend to autolyse quickly, important character is solubility of culture in bile.

It is the most common cause of bacterial pneumonia. It causes pneumonia, meningitis, otitis media, sinusitis and complications from pneumococcal pneumonia include pleural effusion, pneumothorax and empyema (Collee *et al.*, 1996)

3.7.4 *Streptococcus pyogens*

It is Gram positive cocci, 0.7-0.9 µm in diameter occurring in chains of varying length. Capsules may be seen in very young cultures, they are non-motile and non-sporing, facultative anaerobic. They grow best on nutrient agar with blood or serum at 37ºC colonies are small 0.5-1mm after 24hrs, semitransparent, low convex, discrete and with matt or glossy surface when freshly isolated. Beta haemolysis can be seen surrounding colonies on horse or sheep blood agar. It ferments different kinds of sugar producing acid no gas. It is catalase negative and bacitracin sensitive.

It causes a variety of inflammatory and suppurative conditions such as sore throat, scarlet fever. Cellulites, erysipelas, impetigo, puerperal fever etc. Indirectly associated with rheumatic fever, glomerulonephritis. Also found in throat or nasal cavity in a proportion of apparently healthy persons (Collee *et al.*, 1996).

3.7.5. *Escherichia coli*

It is a Gram negative, non-sporing, non-capsulated bacilli, grow well on ordinary media both aerobically and anaerobically. Most of them are lactose fermenter and thus grows as smooth glossy, pink colonies on MacConkey's agar. It produces greenish metallic sheen on EMB and yellow colonies on XLD agar. The optimum growth temperature is 36-37ºC. It is catalase, methyl red, ONPG and indole positive, but oxidase, Voges-Proskauer, citrate, urease, H_2 S negative.

E. coli forms apart of normal intestinal flora of man and animal. The virulent strains of *E. coli* causes UTI, gall bladder and wound infection, appendicitis, peritonitis, bacteraemia and meningitis especially in new borne. Diarrhoeal disease especially in infants and also in adults. Certain strains of *E coli* are recognized as gastrointestinal pathogens which are classified as Enteropathogenic(EPEC), Enteroinvasive (EIEC), Enterotoxigenic(ETEC), and Enterohaemorrhagic(EHEC). These strains contains one or other virulence factors, causing diarrhoeal diseases (Collee *et al.,* 1996)

3.7.6 *Enterobacter aerogenes*

Enterobacter aerogens are motile rod shaped cells, some of which are encapsulated, they also posses peritrichous flagella , it is facultative anaerobe, some ferment both glucose and lactose as carbon source, H_2S negative. It is Voges-Proskauer positive, urease negative, indole negative, citrate positive and urease negative.

It is widely distributed in nature like water, sewage, soil, and in faeces of healthy persons. They cause bacteremia, lower respiratory tract infection, skin infections, soft tissues infection, UTI, endocarditis, intraabdominial infections, septic arthritis, oestomyelitis and ophthalmic infection (Hoffman and Andreas, 2003)

3.7.7 *Klebsiella pneumoniae*

It is Gram negative, non motile, non flagellated and facultative anaerobic enteric bacilli with a prominent polysaccharide capsule, as its virulence factor. They ferment (glucose, lactose, mannitol), with production of acid and gas, split urea by means of

ureases.They grow well in blood agar and MacConkey agar producing large mucoid colonies (Cheesbrough, 2000).

It is traditionally been associated with lobar pneumonia, but it causes pneumonia, bacteraemia, wound infections. The clinical syndrome produced in *Klebisella pneumoniae* may be indistinguishable from that produced by other bacterial pneumonias, except in severe infection but the fatality rate is about 68% in untreated cases (Collee *et al.*, 1996).

3.7.8 *Proteus mirabilis*

They are Gram negative, actively motile, non capsulated, aerobic bacilli. They grow in ordinary media and culture emits characteristics putrefactive fishy or seminal odour. In nutrient agar and blood agar, colonies spread or swarm over the surface of the medium. They form pale colonies in MacConkey agar or DCA medium and do not swarm on these media. They don't ferment lactose, hydrolyze urea rapidly. Phenlyalaninediaminase positive, ONPG negative, indole negative.

It is common cause of UTI, it may also be associated with gastroenteritis, diarrhea, septicemia and occasionally meningitis and chest infection as well as respiratory tract infection (Cheesbrough, 2000, Collee *et al.,* 1996)

3.7.9. *Proteus vulgaris*

It is Gram negative, actively motile aerobic bacillus with characteristics swarming growth on many, even well dried and solid laboratory media. It forms pale or colorless colonies in MacConkeys agar or DCA medium and don't swarm on surface of these media. They do not ferment lactose, hydrolyse urea rapidly, phenylalaninediaminase positive, ONPG negative, indole positive.

It occasionally causes UTI, they are also recovered from wound infection and abscesses and from cases of otitis media, meningitis, septicemia (Cheesbrough, 2000).

3.7.10 *Pseudomonas aeruginosa*

It is a Gram negative rod that is ubiquitous in nature and an opportunistic pathogen in human. It is non capsulated non sporing, motile usually with a single polar flagellum. It is a strict aerobes and grow well on ordinary media and on most common diagnostic media like nutrient agar.It is oxidase, catalase positive but indole, methyl red, Voges-Proskauer, citrate, H_2S in TSI negative (Collee *et al*., 1996).

It is an etiologic agent of community acquired pneumonia which is rare, it typically affects patients who is compromised by preexisting lower respiratory disease of chronic nature (bronchiectasis, cystic fibrosis), blood dyscrasis or immunosuppressive therapy. This is the commonest cause of hospital acquired pneumonia (Cheesbrough, 2000).

3.7.11 *Salmonella paratyphi* A

It is a Gram negative, non sporing, noncapsulated aerobic or facultative anaerobic bacilli. It grows on ordinary culture media and in MacConkey;s agar and DCA media, it produces small, circular translucent colorless nonlactose fermenting colonies. In Wilson and Blair medium the colonies are jet black with metallic sheen.It is indole. Voges-Proskauer, urease negative and produce H_2S in TSI with acid and gas production.

It causes paratyphoid fever, which is a milder form of febrile illness of shorter duration and incubation period. There is usually diarrhea and vomiting and the entire intestinal tract may be inflamed (Cheesbrough, 2000).

3.7.12. *Salmonella typhi*

It is Gram negative motile rod, with peritrichous flagella, non capsulated and non spore former. It can grow on wide range of media, DCA, BBSA, XLD are the best selective media for this organisms Selenite F broth is probably the best enrichment media for *Salmonella*. It is non lactose fermenter produce gas during fermentation of sugar and can't ferment glucose. It is indole, Voges-Proskauer, urease negative produce H_2 S in TSI, ONPG negative and methyl red positive.

It is responsible for enteric fever, gastrointestinal tract infection nephrotyphoid in these with urinary schistosomisis, oestomyelitis in children with sickle cell disease (Cheesbrough, 2000).

3.7.13. *Salmonella typhimurium*

It is Gram negative rod, motile, non-sporing, non capsulated aerobic and facultative anaerobic bacilli. It grows on ordinary culture media and in MacConkey agar and DCA it produces small, circular translucent colorless nonlactose fermenting colonies. It is indole negative, urease negative, produce H_2S in TSI and gas may be produced.

It causes food poisoning, can also cause bacteraemia, inflammation of the gall bladder etc (Cheesbrough, 2000).

3.7.14 *Shigella dysenteriae*

It is a Gram negative, aerobic, nonmotile, non flagellate and noncapsulated bacilli. It grows optimally at 37º C in ordinary media like nutrient agar, in which colonies are circular, convex, smooth and translucent with 2mm in diameter. In MacCkonkey and DCA, colonies are coloress. It is non lactose fermenting, oxidase positive, and catalase and methyl red positive. It is citrate, indole, urease and H_2S negative. In XLD media it produces red colonies.

It causes bacillary dysentery or shigellosis. In developing countries dysentery) shigellosis) has a high death rate among young children (Chakraborty, 2000, Cheesbrough, 2000)

3.8 Screening and Evaluation of Antimicrobial Activity

Antibacterial susceptibility test measures the ability of an antibacterial agent to inhibit bacterial growth in-vitro. Many compounds either of natural origin or from programmes of chemicals synthesis are examined every year in the search for new structure with activity against microorganisms. In addition many chemical modifications of well established structure are made in attempts to improve their biological activities. There are several stages in determining the likely therapeutic usefulness of a new antibiotic, and it is most important to define what the substance is intended to achieve before embarking on a lengthy evaluation procedure (Hugo and Russell, 1985).

First step of assessment of new antibiotic is screening step and in this step effectiveness of antimicrobial substance is evaluated which is achieved by determination of zone of Inhibition (ZOI), minimum inhibitory concentration (MIC), of the antimicrobial agent (WHO, 1991), and/or minimum bactericidal concentration (MBC), for bacteria and minimum fungicidal concentration (MFC), for fungi (Carpinella *et al.,* 1999).

The lowest concentration that prevents visible growth after over night incubation is known as the minimum inhibitory concentration (MIC), of the antimicrobial agent (WHO, 1991). MBC is the amount of the agent that will prevent growth after subculture of the organisms to antibacterial agent free medium, (Collee *et al.,* 1996).

There are different methods to asses in-vitro antimicrobial activity of a compounds. Some of these include, Poison food technique (PET),Agar disc diffusion(DD) technique. Micro broth two fold serial dilution(TFSD)technique, Micro broth microtitre technique (MT), Agar well diffusion technique also called cup plate technique, etc (Khan, 1997).

3.8.1 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 drugs. In this method the agar is inoculated with the test organisms and the test solution of antimicrobial or formulations are placed in cups(well) cut out of the agar with sterile cork borer. The zones of inhibition are noted after incubation (Hugo and Russell, 1985).This method is suitable diffusion technique for testing aqueous suspension of plant extracts. This method was employed by Peplinjak *et al.*,(1999) for the evaluation of antimicrobial activity of ethanol extract of *Satureja Montana* sps *montana.*

3.8.2 Two- fold serial dilution method

This technique has been recommended by WHO (1991), for quantitative estimation of antibiotic activity, in which graded doses of the test substances are incorporated into the broth and tubes inoculated with the test organisms. The point at which no growth occurs during overnight incubation is taken as the as the minimum inhibitory concentration (MIC), if required the minimum bactericidal concentration (MBC), can be determined by sub culturing the last tube to show a visible growth and all tubes in which there is no growth. The MBC is the lowest concentration of antimicrobial agent required to produce sterile culture (Cheesbrough, 1993).

CHAPTER - IV

4. MATERIALS AND METHODS

4.1 Materials

All the materials used to accomplish this study are given in the Appendix-A

4.2 Method

4.2.1 Collection of Samples

Different parts of selected medicinal plants were collected from different parts of Nepal. The list of medicinal plants, their corresponding parts used in this study ,month of sample collection and location/place of the sample plants are given in Appendix-B

a) Collection of roots and rhizomes:

Roots and rhizomes of medicinal plants were collected by digging the soil with spade and cutting the required part with axe or plant cutter.

b) Collection of bark

Plants part from which the bark is collected was cleaned thoroughly with clean water. Bark was peeled off by using axe.

c) Collection of aerial parts

Aerial parts were collected by cutting small branches of the plants with plant cutter.

d) Collection of fruits:

Fruits were collected by cutting the twigs bearing fruits from the plant.

4.2.2 Identification and Documentation of sample plants

Representative plant samples were collected as voucher specimens following the standard technique (Lawrence,1967; Martin,1995). Medicinal plants were identified according to the descriptions given on different books viz. Flora of Kathmandu valley by HMG/N (1986), Flora of Bhutan (1991), Flora of British India (1992), Medicinal plants of Nepal by HMG/N (1997), Lama *et al.,* (2001), Joshi and Joshi (2001) and other pertinent taxonomic literature. Herbarium were prepared with standard method and their authentic identification was done in collaboration with Central Department of Botany, Tribhuvan University, Kiritipur.

4.2.3 Processing of the samples

a) Washing & Chopping

Bark, root and other underground parts were washed thoroughly to remove soil and extraneous matter such as other parts of the same plant or grasses with herbs or any other unwanted matter in case of rhizome and roots, aerial portion of stem fragments. Stems, roots, twigs and bark were chopped into 3-5 cm into small pieces.

b) Drying of the Samples

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) Packaging and Storage

The completely dried plant matter was packed in water proof bags. Cotton bags were chosen in case of incomplete drying, the air circulation may help to dry and hence prevent rotting during storage.

d) Grinding

The dried samples were cut to small pieces by means of plant cutter and they were subjected to grinding.

4.2.4 Extraction of plant material

4.2.4.1 Soxhlet Extraction with Ethanol

Shade-dried ground powder of various parts of medicinal plants were subjected to continuous extraction with ethanol for 10-15 hours by using soxhlet apparatus to obtain crude ethanol extracts of the respective plants. After complete extraction, the solvent was totally removed by rotary vacuum evaporator. The detail process of extraction is explained below:

Known weight (approx.25 gms) of dried plant powder was loaded in a clean and dried thimble of soxhlet extractor. It was then fitted with appropriate sized pre-dried and properly labeled round bottom flask having a capacity of 250 ml.It was set up with the help of stands and 150 ml of ethanol was slowly poured from upper mouth of the soxhlet extractor. the upper part was fitted with condenser.The flask was constantly heated with heating mantle. The solvent vapours after reaching the condenser through the side tube, dropped on the powder of medicinal plant and dissolved soluble compounds. The solution filters and passed out back into the flask through the siphon

tube (Tewari *et al.*, 1992). The process was allowed to run for 8-15hours or till the coloured solvent appered in the siphon (Shale *et al.*, 1999; Thomas *et al.*, 1999).

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 bath below 55ºC. Solvent was completely removed and was collected in separate round bottom flask of evaporator and it was collected in a sterile bottle. The round bottom flask containing the extract was weighed till constant weight appeared and result was noted. To find out the extract yield, the weight of the pre-weighted round bottom flask was subtracted from the weight of round bottle flask with extract. The crude extract was then transferred in a bottle by sterile spatula and was labeled and stored in a refrigerator.

4.2.4.3 Extraction of essential oil

The essential oil content present in the fresh leaves and stems of the plants *Juniper indica* and *Mentha piperita* was extracted by hydrodistillation method using Clevenger apparatus. 200gm of fresh leaves together with distilled water (5-10times) was taken in a R.B. flask. The content of the flask was heated in heating mantle at 140º C to boiling and boiling was continued moderately briskly for 6hrs. Then the heat was removed, allowed to stand for some time and the stopper of Clevenger apparatus was opened.The water was drawn off slowly until the surface of the oil layer corresponds to the preparation line and allowed to stand for more than one hour at room temperature. The oil was collected in a well -capped tube.Thus obtained oil was labeled and kept in refrigerator.

4.2.5 Preparation of stock/working solution.

100mg/ml of each crude extract was made by taking 1gm of the extract in 9ml of each solvent, i.e. distilled water, DMSO (dimethyl sulfo oxide),methanol and ethanol in clean and capped test tubes. The solution was dissolved by vortexing. After making stock/working solution the test tubes were capped, sealed and stored in refrigerator(2-8º C) until use.

The stock/working solution of essential oil was made in two solvents 2% Tween 80in physiological saline, methanol. 0.5ml of essential oil was dissolved in 0.5ml of each

solvent to make 50% working solution. Then it was vortexed, sealed and kept in refrigerator until use .

4.2.6 Collection of Standard Cultures

Fourteen different types of bacteria were selected for the study. Name of the bacteria and their source of collection are given in Appendix-C

After obtaining the culture, the test organisms were streaked on nutrient agar plates and incubated. From the isolated colony Gram staining was performed. The organism was grown on their selected media and was incubated on biochemical test media. Thus the organisms were tested for their purity and confirmed by their morphological, cultural and biochemical characteristics. The organisms in the study includes:

1. *Staphylococcus aureus*
2. *Streptococcus pneumoniae*
3. *Streptococcus pyogens*
4. *Klebsiella pneumoniae*
5. *Escherichia coli*
6. *Enterobacter aerogens*
7. *Pseudomonas aeruginosa*
8. *Proteus mirabilis*
9. *Proteus vulgaris*
10. *Salmonella typhi*
11. *Salmonella paratyphi*
12. *Salmonella typhimurium*
13. *Shigella dysenteriae*
14. *Bacillus subtilis*

The bacterial culture were maintained in nutrient agar slants in closed vials during the study period. The organisms were subcultured every two weeks.

4.2.7 Preparation of standard culture inoculum

Three-four colonies of similar appearance of the organism to be tested were touched with the inoculating loop aseptically. It was then transferred to a tube containing 5ml of sterile nutrient broth. The tube was then compared with turbidity standard (Mc Farland Nephelometer standard tube 0.5) recommended by WHO (1991) for

antimicrobial susceptibity test and the density of the organism suspension was adjusted either by incubating further or by diluting with sterile nutrient broth.

4.2.8 Preparation of Media

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

4.2.9 Screening and Evaluation of Antimicrobial Activity.

Antimicrobial susceptibility tests measure the ability of an antibiotic or other antimicrobial agent to inhibit growth in vitro (WHO, 1991).

In the present study screening and evaluation of antimicrobial activity was performed by two methods *viz.* agar well diffusion method and two fold broth dilution method. The diameter of zone of inhibition (ZOI) produced by plants extract on particular microorganism was measured for the estimation of the potency 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 antimicrobial activity

The crude extract of medicinal plants were screened for its antibacterial activity. against the tested organisms by agar well diffusion method as given by Dingle *et al.*, (1953).

Sterile Muller-Hinton Agar (MHA) plates of approximately 4mm thickness were prepared. Before using the plates, they were dried under laminar flow or in the incubator at 37° C for 30 minutes to remove excess of moisture from the surface of media. The fresh incoulum comparable with turbidity standard was prepared as in section 4.2.7.

Sterile cottons swabs were dipped into the prepared inoculum and excess of inoculum was removed by pressing and rotating against the upper inside wall of tube above the liquid level and seeded carefully all over the plate.The plate was rotated through an angle of 60° 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).

Then with help of sterile cork borer no 6, wells were made in the inoculated media plates and labeled properly. The diameter of well was 6mm,50 µ l of the working suspension of the plant extract were loaded into the respective wells with the help of micropipette. The solvent itself was tested for its activity as a control at the same time in a separate well. The plates were then left for half and hour with the lid closed so that the extract diffused into media. Then the plates were incubated at 37º C over night.

After proper incubation (18-24 hours) the plates were observed for the zone of inhibition which is suggested by clear area without growth around the well was noted. In case of presence of any zone of inhibition triplicate assays were performed. The zones of inhibition were measured using scale and mean was recorded.

4.2.9.2 Determination of Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) was determined by observing the visible growth of the test microorganisms in two fold serially diluted antimicrobial substances in the broth culture medium. But it couldn't give the actual result of inhibition or death of the microorganisms which suggested the necessity of determination of minimum bactericidal concentration (MBC).

The crude extract of different medicinal plants, which showed antibacterial activity were subjected to two fold serial dilution method of Baron *et al*., (1994) to determine minimum bactericidal concentration (MBC). Media used were nutrient broth prepared as mentioned in Appendix-D.

A set of 12 screw capped test tubes containing 1ml nutrient broth for each bacterium were prepared. The test tubes were labeled as positive growth control, negative growth control, and no. 1to 10. In case of negative growth control the nutrient broth was discarded. Then 1ml of the crude extract from particular medicinal plants was added aseptically to each tube labeled as negative growth control and 1no. labeled test tube.

The 1st tube now contains 1ml of broth and 1ml of extract. After complete homogenization 1ml of its content was transferred aseptically to 2nd tube. Similarly, after complete homogenization of the content in 2nd tube, 1ml of it was transferred to 3rd tube. In the same way , two fold dilution was prepared up to the 10th tube. From

the 10th tube 1ml of the content was discarded hence all the tubes from negative control to no.10 contained equal volume i.e. 1ml with gradually decreasing concentration. No plant extract was added to the tube labeled as positive control. Now with help of micropipette,20μ 1 of inoculum [a 1:100 dilution of a suspension of turbidity equal to a McFarland standard 0.5 supposed to have organism $1.5x10^6$ CFU/ml] was added to all tubes except the one which was labeled by negative control i.e. negative control contains only extract no broth and no organisms; positive control contains broth plus organisms but not medicinal extract while tubes 1to 10 contains all the three medicinal extract broth and organisms. All the tubes were incubated at 37ºC for 24 hours.

After proper incubation of the serially diluted antimicrobial substances in a series of test tubes containing test microorganisms, the results were compared with +ve and -ve growth control tubes. The results were interpreted on the basis of the fact that growth occurs in the +ve control and any other tube in which the concentration of extract is not sufficient to inhibits the growth. The lowest concentration of antimicrobial agent that inhibits the growth of organisms as detected by lack of visible turbidity is the minimum inhibitory concentration (MIC). However, it was difficult to identify whether turbidity was due to the growth of bacteria or due to the turbidity of the plant extract itself. So, all the tubes were sub cultured on nutrient agar plate containing no antimicrobial substance with proper label and incubated at 37º C for 24hrs. Then plates were examined for the growth of bacteria.The tube with minimum concentration of extract in which the growth was completely checked was also clearly notified. Then minimum bactericidal concentration (MBC) value was determined.

Minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial required to kill the bacteria to produce sterile culture.

4.2.10 Bio Assay Guided Fractionation of *Glycyrrhiza glabra* Linn

The antibacterial active ethanolic extract was fractionated using solvent extraction method with solvents of increasing polarity, into four fractions (1,2,3 and 4). This fractionation was necessary in the course of experiment in order to find the active fraction(s) and to trace the active antibacterial chemical constituent(s) for bioassay guided isolation. These fractions were separately assayed for their antibacterial activity.

Shade dried powered (25gm) rhizomes of *Glycyrrhiza glabra* was subjected to continous extraction using soxhlet extractor successively with the solvents of increasing polarity. Four solvents *viz.* hexane, chloroform, n-butanol and distilled water were used. Four fractions (solution) were obtained and each fractions was collected in a separate round bottom flask.

- The hexane extractive solution.
- The chloroform extractive solution.
- The n-butanol extractive solution.
- The aqueous extractive solution.

From each extractive solution, solvent was completely removed by rotary vacuum evaporator separately. The resulting fractions were weighted and percentage yield were calculated.

4.2.11 Determination of Antimicrobial Activity of Each Fraction

Each fraction of *Glycyrrhiza glabra* rhizomes after removal of solvents was subjected to antimicrobial activity tests. This was performed by agar well diffusion method of Dingle *et al.,*(1953) The diameter of the well was 6mm and concentration of each fraction as well as crude extract was 50mg/ml except hexane fraction. The concentration of hexane fraction was 12mg/ml. The sample loaded in each well was 50µl.

After 18-24hrs incubation the plates were observed for the zone of inhibition and was measured and noted.

4.2.12 Antibiotic Susceptibility Test

All the bacterial isolates which were employed in this study were subjected to in-vitro antibiotic susceptibility test by disc diffusion method of Modified Kirby-Bauer method. The antibiotic discs were selected according to the type of bacteria. The antibiotic disc used were Amikacin (30mcg), Amoxycillin (30mcg), Chloramphenicol (30mcg), Chepotaxime (30mcg), Cortimoxazole (25mcg), Ciprofloxacin (5mcg), Erythromycin (15mcg), Gentamicin (10mcg), Nalidixic acid (30mcg), Norfloxacin (10mcg), Tetracycline (30mcg), Vancomycin (30mcg).

For the antibiotic sensitivity test, sterile Muller-Hinton Agar plates were prepared as mentioned in Appendix-D. The inoculum of bacteria was prepared as in section 4.2.7. Cotton swab was dipped into the inoculum and rotated and pressed against the upper

inside wall of the tubes above the liquid level to remove excess inoculum and seeded carefully all over surface of the plates three times rotating the plates through an angle of 60ºC after each application. Finally the swab was passed round the edges of agar surface and was left for 5 to 10 minutes at room temperature.

Antibiotic discs were then taken out from their respective vials with the help of sterile forceps and placed carefully on the plates, at least 15mm away from the edge at the equal distance separated from each other to avoid the overlapping of zones inhibition. The discs were then pressed gently with the help of forceps to make complete contact with the surface of the medium. Then, the plates were incubated at 37ºC for 18-24 hrs. After proper incubation, the diameter of the zone of inhibition in each disc, was measured. According to the size of zone of inhibition (ZOI), the organisms was considered as resistant, intermediate or susceptible to the antibiotic by referring to the standard interpretive chart of antibiotic susceptibility test given in the Appendix-F.

CHAPTER - V

5. RESULT

5.1 Percentage yield of the extract from different plant sample.

The shade dried powder of various parts of medicinal plant *viz*. roots, rhizome, barks, leaves, stems, seeds etc. were subjected to continous extraction for10-15 hours by using soxhlet extractor. The method of extraction, solvent used and percentage yield of the respective extract calculated by the formula (% Yield = Weight of the empty R.B flask / Weight of R.B. flask with extract obtained x 100) is given in the following table.

Table 1 : Percentage yield of Extracts

S.N.	Name of Plants used	Method of extraction	Solvent used for extraction	% yield
1	*A. bidentata*	soxhlet	Ethanol	12.08
2	*A. calamus*	Soxhlet	Ethanol	16.36
3	*A indica*	Soxhlet	Ethanol	12.72
4	*C. cyminum*	Soxhlet	Ethanol	18.68
5	*G. glabra*	Soxhlet	Ethanol	42.44
6	J. humile	soxhlet	Ethanol	39.92
7	J. adhatoda	soxhlet	Ethanol	25.2
8	*M. piperita* (etoH extract)	soxhlet	Ethanol	23.36
9	*M. esculenta*	soxhlet	Ethanol	46.64
10	*O. sanctum*	soxhlet	Ethanol	9.32
11	*P. nigrum*	soxhlet	Ethanol	24.36
12	*S. calava*	soxhlet	Ethanol	29.76
13	*S. aromaticum*	soxhlet	Ethanol	32.44
14	*T. ammi*	soxhlet	Ethanol	18.85
15	*Z. armatum*	soxhlet	Ethanol	28.28
16	*J. indica*	steam distillation	D/W	1.7
17	*M. piperita* (essential oil)	steam distillation	D/W	1.8

5.2 Qualitative screening for antimicrobial activities

The selected medicinal plants for the present study were assayed for their possible antibacterial activity by agar well diffusion method of Dingle *et al.*, (1953).Three readings of the diameter of zone of inhibition were taken from each plant and their mean was recorded as given in table 2. The negative sign in the table indicates that the plant material has no antimicrobial activity against the particular organisms. The positive sign in the table indicates that the particular extract of the respective plants have antibacterial activity and thus zone of inhibition around the well loaded with extract.

Table.2 Qualitative Screening for Antimicrobial Activity

S.N.	Plant Materials	Test organism													
		B. subitilis	*S. aureus*	*S. pneumoniae*	*S. pyogens*	*E. coli*	*E. aerogenes*	*K. pneumoniae*	*P. mirabilis*	*P. vulgaris*	*Ps. aeruginosa*	*S. paratyphis*	*S. typhi*	*S. typhimurium*	*Sh. dysenteriae*
1	***A. bidentata***														
	(D/W suspension	-	-	+	-	-	-	-	-	-	+	-	-	-	-
	DMSO suspension	-	-	+	-	-	-	-	-	-	+	-	-	-	-
	Ethanol suspension	-	+	+	-	-	-	-	-	-	+	-	-	-	-
	Methanol suspension	-	+	+	-	-	-	-	-	-	+	-	-	-	-
2	***A. Calamus***														
	(D/W suspension	-	+	-	+	-	-	-	-	-	-	-	-	-	-
	DMSO suspension	-	+	-	+	-	-	-	-	-	-	-	-	-	-
	Ethanol suspension	-	+	-	+	-	-	-	-	-	-	-	-	-	-
	Methanol suspension	-	+	-	+	-	-	-	-	-	-	-	-	-	-
3	***A indica***														
	(D/W suspension	-	+	-	-	-	-	-	-	-	+	-	-	-	-
	DMSO suspension	+	+	-	-	-	-	-	-	-	+	-	-	-	-
	Ethanol suspension	+	+	-	-	+	-	-	-	-	+	-	-	-	-
	Methanol suspension	+	+	-	-	+	-	-	-	-	+	-	+	-	+
4	***C. cyminum***														
	(D/W suspension	-	-	+	-	+	-	-	+	-	+	-	-	-	-
	DMSO suspension	+	+	+	-	+	-	-	+	-	-	-	-	-	-
	Ethanol suspension	+	+	+	-	+	+	+	+	-	-	-	-	-	+
	Methanol suspension	+	+	+	-	+	+	+	+	-	-	-	+	-	+
5	***G. glabra***														
	(D/W suspension	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	DMSO suspension	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	Ethanol suspension	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	Methanol suspension	+	+	+	+	-	-	-	-	+	-	+	-	+	+
6	***J. humile***														
	(D/W suspension	-	+	-	+	+	+	+	+	-	-	-	-	-	-
	DMSO suspension	+	+	-	+	+	+	+	+	-	-	-	-	-	-
	Ethanol suspension	+	+	-	+	+	+	+	+	+	-	-	-	-	-
	Methanol suspension	+	+	-	+	+	+	+	+	+	-	+	-	-	-
7	***J. indica***														
	Tween 80 suspension	+	+	-	+	-	-	+	-	+	-	-	-	-	+
	Methanol suspension	+	+	-	+	-	-	+	-	+	-	-	-	-	+
8	***J. adhatoda***														
	(D/W suspension	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	DMSO suspension	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	Ethanol suspension	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	Methanol suspension	-	-	-	+	-	-	-	+	-	+	-	-	-	-

Note: + indicates presence of inhibitory activity,
- indicates absence of inhibitory activity

S.N.	Plant Materials	Test organism													
		B. subitilis	***S. aureus***	***S. pneumoniae***	***S. pyogens***	***E. coli***	***E. aerogenes***	***K. pneumoniae***	***P. mirabilis***	***P. vulgaris***	***Ps. aeruginosa***	***S. paratyphis***	***S. typhi***	***S. typhimurium***	***Sh. dysenteriae***
9	***M. piperita***														
	(D/W suspension	-	-	-	-	+	-	+	+	+	+	+	-	-	-
	DMSO suspension	-	-	-	-	+	-	+	+	+	+	+	-	-	-
	Ethanol suspension	-	-	-	-	+	-	+	+	+	+	+	-	-	-
	Methanol suspension	-	-	-	-	+	-	+	+	+	+	+	-	-	-
10	***M. piperita (oil)***														
	Tween 80 suspension	+	-	-	-	+	+	+	+	+	-	+	+	-	-
	Methanol suspension	+	-	-	-	+	+	+	+	+	-	+	+	-	-
11	***M. esculenta***														
	(D/W suspension	+	+	-	+	+	+	-	+	-	+	-	+	+	+
	DMSO suspension	+	+	-	+	+	+	-	+	-	+	+	+	+	+
	Ethanol suspension	+	+	-	+	+	+	-	+	+	+	+	+	+	+
	Methanol suspension	+	+	-	+	+	+	-	+	+	+	+	+	+	+
12	***O. sanctum***														
	(D/W suspension	+	+	+	+	+	-	-	-	-	-	+	-	+	+
	DMSO suspension	+	+	+	+	+	-	-	-	-	-	+	+	+	+
	Ethanol suspension	+	+	+	+	+	-	-	-	-	+	+	+	+	+
	Methanol suspension	+	+	+	+	+	-	-	-	-	+	+	+	+	+
13	***P. nigrum***														
	(D/W suspension	-	-	-	+	-	-	-	-	-	+	-	-	-	-
	DMSO suspension	+	-	-	+	-	-	-	-	-	+	-	-	-	-
	Ethanol suspension	+	+	-	+	-	-	-	-	-	+	-	-	-	-
	Methanol suspension	+	+	-	+	-	-	-	-	-	+	-	-	-	-
14	***S. calava***														
	(D/W suspension	-	-	-	-	-	-	-	-	-	+	+	-	-	-
	DMSO suspension	-	-	-	+	-	-	-	-	-	+	+	-	-	-
	Ethanol suspension	-	-	-	+	-	-	-	-	-	+	+	-	-	-
	Methanol suspension	-	-	-	+	-	-	-	-	-	+	+	-	-	-
15	***S. aromaticum***														
	(D/W suspension	+	+	+	+	+	+	+	+	-	+	+	-	+	+
	DMSO suspension	+	+	+	+	+	+	+	+	-	+	+	+	+	+
	Ethanol suspension	+	+	+	+	+	+	+	+	-	+	+	-	+	+
	Methanol suspension	+	+	+	+	+	+	+	+	+	+	+	-	+	+
16	***T ammi***														
	(D/W suspension	-	+	+	+	-	-	-	+	-	-	-	-	-	-
	DMSO suspension	+	+	+	+	-	-	-	-	-	-	-	-	-	+
	Ethanol suspension	+	+	+	+	+	+	+	+	+	-	-	+	+	+
	Methanol suspension	+	+	+	+	+	+	+	-	+	-	-	+	+	+
17	***Z . armatum***														
	(D/W suspension	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	DMSO suspension	-	-	-	-	+	+	-	+	-	-	-	-	-	-
	Ethanol suspension	+	-	+	-	+	+	-	+	-	-	-	-	-	-
	Methanol suspension	+	+	+	-	+	+	-	+	+	-	-	-	-	-

5.3 Evaluation of antimicrobial activity

Antimicrobial activity of the plant extract under investigation were evaluated by two methods, *viz*. agar well diffusion method and two fold serial dilution method. The diameter of the ZOI given by plant extract against the test bacteria was measured for the estimation of the potency of the particular extract. Similarly, minimum bactericidal concentration (MBC) were determined by using two fold serial dilution method.

The zone of inhibition, and minimum bactericidal concentration (MBC) of the extract of studied plants materials are illustrated in table 3 to table 16.

5.3.1 Antimicrobial activities of the studied plant extracts against *B. subtilis*

The zone of inhibition and MBC for *Bacillus subtilis* due to plant extract are given in table 3. Out of 17 plant extract used it was inhibited by 12extracts. The highest ZOI, was found in the Tween 80 suspension of essential oil of *M. piperita* with diameter of 30mm & lowest ZOI 9mm was seen in DMSO solvent of *P. nigrum*.

Table 3: Antimicrobial activities of studied plant extracts against *B. subtilis*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. indica*	DMSO	13	12.5
		Ethanol	14	6.25
		Methanol	13	6.25
2	*C. cyminum*	DMSO	11	25
		Ethanol	20	12.5
		Methanol	16	12.5
3	*G. glabra*	D/W	12	6.25
		DMSO	16	3.12
		Ethanol	19	1.56
		Methanol	16	1.56
4	*J. humile*	DMSO	12	25
		Ethanol	13	12.5
		Methanol	14	12.5
5	*J. indica*	Tween 80	14	ND
		Methanol	15	ND
6	*M. piperita (oil)*	Tween 80	30	ND
		Methanol	28	ND
7	*M. esculenta*	D/W	16	25
		DMSO	14	25
		Ethanol	13	12.5
		Methanol	17	12.5
8	*O. sanctum*	D/W	10	25
		DMSO	12	12.5
		Ethanol	14	6.25
		Methanol	12	6.25

Table-3(contd..)

9	*P. nigrum*	DMSO	9	12.5
		Ethanol	13	6.25
		Methanol	12	6.25
10	*S. aromaticum*	D/W	13	6.25
		DMSO	15	6.25
		Ethanol	17	6.25
		Methanol	15	1.56
11	*T. ammi*	DMSO	11	6.25
		Ethanol	25	1.56
		Methanol	17	3.12
12	*Z. armatum*	Ethanol	15	12.5
		Methanol	16	12.5

Note: ND indicates not done

5.3.2 Antimicrobial activities of the studied plant extract against *S. aureus*

The ZOI, MBC value of *S. aureus* due to plant extract are given in table 4. Out of 17 plant extracts used it was inhibited by 13 plants extracts. The highest ZOI (27mm) in *T. ammi* and lowest ZOI observed was 10mm in more than one plants.

Table 4 : Antimicrobial activities of the studied plant extracts against *S. aureus*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. bidentata*	Ethanol	11	50
		Methanol	12	12.5
2	*A. calamus*	D/W	10	25
		DMSO	11	12.5
		Ethanol	12	6.25
		Methanol	11	6.25
3	*A indica*	D/W	11	25
		DMSO	12	12.5
		Ethanol	13	6.25
		Methanol	15	6.25
4	*C. cyminum*	DMSO	13	12.5
		Ethanol	16	6.25
		Methanol	15	6.25
5	*G. glabra*	D/W	16	3.12
		DMSO	16	3.12
		Ethanol	23	0.39
		Methanol	20	1.56
6	*J. humile*	D/W	13	25
		DMSO	11	25
		Ethanol	12	6.25
		Methanol	12	6.25
7	*J. indica*	Tween 80	20	N.D
		Methanol	22	N.D

Table-4(contd..)

8	*M. esculenta*	D/W	16	25
		DMSO	14	25
		Ethanol	17	6.25
		Methanol	17	6.25
9	*O. sanctum*	D/W	12	12.5
		DMSO	11	12.5
		Ethanol	15	6.25
		Methanol	16	6.25
10	*P. nigrum*	Ethanol	10	50
		Methanol	12	50
11	*S. aromaticum*	D/W	15	3.12
		DMSO	15	3.12
		Ethanol	23	0.78
		Methanol	22	0.78
12	*T. ammi*	D/W	14	25
		DMSO	17	6.25
		Ethanol	25	3.12
		Methanol	27	1.56
13	*Z. armatum*	Methanol	14	6.25

Note: ND indicates not done

5.3.3 Antimicrobial activities of the studied plant extracts against *S. pneumoniae*

The ZOI and MBC value of different plant extracts in *S. pneumoniae* are given in table 5. Out of 17 plant extracts used only 7 plant extracts had antimicrobial activity against *S. pneumoniae*. The highest activity was observed in DMSO and Ethanol suspension of *A. bidentata* with mean diameter of 21mm and lowest 9mm was seen in D/W suspension of *C. cyminum*.

Table 5: Antimicrobial activities of the studied plant extracts against *Streptococcus pneumoniae*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. bidentata*	D/W	20	6.25
		DMSO	21	6.25
		Ethanol	21	1.56
		Methanol	20	1.56
2	*C. cyminum*	D/W	9	25
		DMSO	12	12.5
		Ethanol	13	12.5
		Methanol	11	12.5
3	*G. glabra*	D/W	12	12.5
		DMSO	12	12.5
		Ethanol	14	6.25
		Methanol	13	6.25

Table-5(contd..)

4	*O. sanctum*	D/W	11	25
		DMSO	10	25
		Ethanol	12	12.5
		Methanol	14	6.25
5	*S. aromaticum*	D/W	10	12.5
		DMSO	14	6.25
		Ethanol	16	3.12
		Methanol	14	3.12
6	*T ammi*	D/W	13	6.25
		DMSO	14	6.25
		Ethanol	14	6.25
		Methanol	16	0.78
7	*Z. armatum*	Ethanol	11	12.5
		Methanol	11	12.5

5.3.4 Antimicrobial activities of the studied plant extracts against *S. pyogens*

The ZOI and MBC value of different plant extract in *S. pyogens* are given in the table 6. Out of 17 different plant extracts used 11 plants extracts showed antimicrobial activity against *S. pyogens*. The greatest ZOI of 22mm in the D/W suspension of *O. sanctum* and lowest in case of DMSO suspension of *S. calava* 9mm.

Table 6 : Antimicrobial activities of the studied plant extracts against *Streptococcus pyogens*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. calamus*	D/W	11	50
		DMSO	14	12.5
		Ethanol	20	6.25
		Methanol	14	6.25
2	*G. glabra*	D/W	17	12.5
		DMSO	12	6.25
		Ethanol	14	3.12
		Methanol	13	3.12
3	*J. humile*	D/W	14	12.5
		DMSO	15	6.25
		Ethanol	16	6.25
		Methanol	16	6.25
4	*J. adhatoda*	D/W	11	50
		DMSO	15	12.5
		Ethanol	15	6.25
		Methanol	15	6.25
5	*J. indica*	Tween 80	17	N.D
		Methanol	16	ND

Table-6 (contd..)

6	*M. esculenta*	D/W	17	12.5
		DMSO	12	6.25
		Ethanol	12	6.25
		Methanol	11	6.25
7	*O. sanctum*	D/W	22	6.25
		DMSO	12	3.12
		Ethanol	13	3.12
		Methanol	12	3.12
8	*P. nigrum*	D/W	12	6.25
		DMSO	14	6.25
		Ethanol	15	3.12
		Methanol	16	3.12
9	*S. calara*	DMSO	9	25
		Ethanol	12	12.5
		Methanol	12	12.5
10	*S. aromaticum*	D/W	10	12.5
		DMSO	17	6.25
		Ethanol	13	6.25
		Methanol	14	1.56
11	*T. ammi*	D/W	12	6.25
		DMSO	20	0.78
		Ethanol	13	1.56
		Methanol	14	1.56

Note: ND indicates not done

5.3.5 Antimicrobial activities of the studied plants extract against *E. coli*

The ZOI and MBC value of the plants extracts are given in the table 7. Among 17 plant extract used, 10 plants extract showed antimicrobial activities against the organism. The largest ZOI was seen in case of *T. ammi* in ethanol suspension (25mm) while least ZOI 9mm was found in D/W suspension of *J. humile*.

Table 7 : Antimicrobial activities of the studied plant extracts against *E. coli*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. indica*	Ethanol	11	6.25
		Methanol	12	6.25
2	*C. cyminum*	D/W	10	12.5
		DMSO	11	12.5
		Ethanol	11	6.25
		Methanol	12	6.25
3	*J. humile*	D/W	9	25
		DMSO	10	12.5
		Ethanol	12	6.25
		Methanol	12	6.25

Table-7(contd..)

4	*M. piperita*	D/W	10	12.5
		DMSO	12	6.25
		Ethanol	12	6.25
		Methanol	13	6.25
5	*M. piperita (oil)*	Tween 80	12	ND
		Methanol	14	ND
6	*M. esculenta*	D/W	11	25
		DMSO	13	12.5
		Ethanol	12	12.5
		Methanol	11	6.25
7	*O. sanctum*	D/W	15	12.5
		DMSO	12	6.25
		Ethanol	13	6.25
		Methanol	12	6.25
8	*S. aromaticum*	D/W	12	6.25
		DMSO	18	3.12
		Ethanol	14	3.12
		Methanol	13	3.12
9	*T. ammi*	Ethanol	25	1.56
		Methanol	18	6.25
10	*Z. armatum*	DMSO	11	12.5
		Ethanol	12	6.25
		Methanol	12	6.25

Note: ND indicates not done

5.3.6 Antimicrobial activities of the studied plants extract against *E. aerogenes*

The ZOI and MBC value of the plants extracts used are given in the table 8. Out of 17 plant extract used only 7 plants showed inhibitory activity with highest ZOI of 19mm in DMSO suspension of *S. aromaticum* and lowest of 10mm in D/W suspension of *J. humile*.

Table 8 : Antimicrobial activities of the studied plant extracts against *Enterobacter aerogenes*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*C. cyminum*	Ethanol	15	6.25
		Methanol	13	6.25
2	*J. humile*	D/W	10	25
		DMSO	12	12.5
		Ethanol	13	6.25
		Methanol	13	6.25
3	*M. piperita (oil)*	Tween 80	14	ND
		Methanol	12	ND
4	*M. esculenta*	D/W	11	50
		DMSO	15	25
		Ethanol	14	25
		Methanol	15	12.5

Table-8 (contd..)

5	*S. aromaticum*	D/W	18	3.12
		DMSO	19	3.12
		Ethanol	17	0.78
		Methanol	18	0.39
6	*T ammi*	Ethanol	18	3.12
		Methanol	18	3.12
7	*Z. armatum*	DMSO	12	6.25
		Ethanol	12	1.56
		Methanol	13	1.56

5.3.7 Antimicrobial activities of the studied plants extract against *K. pneumoniae*

The ZOI and MBC value of the plants extracts are given in the table 9. Among the 17 plant extract used only 7 plant extracts showed antimicrobial activity. The highest ZOI of 26mm in DMSO suspension of *S. aromaticum* while least ZOI 9mm in D/W suspension of *J. humile*.

Table 9 : Antimicrobial activities of the studied plant extracts against *K. pneumoniae*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*C. cyminum*	Ethanol	11	1.56
		Methanol	13	1.56
2	*J. humile*	D/W	9	25
		DMSO	11	12.5
		Ethanol	13	12.5
		Methanol	13	6.25
3	*J. indica*	Tween 80	15	ND
		Methanol	21	ND
4	*M. piperita*	D/W	25	3.12
		DMSO	14	6.25
		Ethanol	12	6.25
		Methanol	12	6.25
5	*M. piperita (oil)*	Tween 80	20	ND
		Methanol	22	ND
6	*S. aromaticum*	D/W	24	6.25
		DMSO	26	0.097
		Ethanol	24	0.19
		Methanol	23	0.19
7	*T ammi*	Ethanol	14	0.78
		Methanol	14	0.78

5.3.8 Antimicrobial activities of the studied plants extract against *P. mirabilis*

The ZOI and MBC value of the plants extracts used are given in the table 10. 9 plant extracts among 17 plant extracts had antimicrobial activity against *P. mirabilis* with largest ZOI of 21mm in DMSO suspension of *C. cyminum* and lowest ZOI of 9mm D/W suspension of *T. ammi*.

Table 10 : Antimicrobial activities of the studied plant extracts against *Proteus mirabilis*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*J. adhatoda*	Methanol	17	6.25
2	*C. cyminum*	D/W	12	12.5
		DMSO	21	3.12
		Ethanol	13	6.25
		Methanol	12	6.25
3	*J. humile*	D/W	12	12.5
		DMSO	15	3.12
		Ethanol	13	3.12
		Methanol	14	3.12
4	*M. piperita*	D/W	10	12.5
		DMSO	12	6.25
		Ethanol	11	6.25
		Methanol	11	6.25
5	*M. piperita (oil)*	Tween 80	15	ND
		Methanol	17	ND
6	*M. esculenta*	D/W	17	6.25
		DMSO	15	6.25
		Ethanol	18	6.25
		Methanol	20	1.56
7	*S. aromaticum*	D/W	12	1.56
		DMSO	12	1.56
		Ethanol	15	0.78
		Methanol	15	0.78
8	*T. ammi*	D/W	9	25
		Ethanol	12	3.12
9	*Z. armatum*	DMSO	20	6.25
		Ethanol	13	3.12
		Methanol	12	3.12

5.3.9 Antimicrobial activities of the studied plants extract against *P. vulgaris*

The ZOI and MBC value of the plants extracts used are given in the table 11. Out of 17 different plants 9 plants extracts showed antimicrobial activity. The largest ZOI was found to be 24mm in the methanol suspension of *S. aromaticum*. While lowest

ZOI 12mm was shown by methanol suspension of *J. indica* D/W suspension of *M. piperita*.

Table 11 : Antimicrobial activities of the studied plant extracts against *Proteus vulgaris*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*G. glabra*	Methanol	15	6.25
2	*J. humlie*	Ethanol	14	6.25
		Methanol	16	6.25
3	*J. indica*	Tween 80	14	ND
		Methanol	12	ND
4	*M. piperita*	D/W	12	25
		DMSO	14	12.5
		Ethanol	15	12.5
		Methanol	15	12.5
5	*M. piperita (oil)*	Tween 80	27	ND
		Methanol	23	ND
6	*M. esculenta*	Ethanol	19	12.5
		Methanol	20	6.25
7	*S. aromaticum*	Methanol	24	0.78
8	*T. ammi*	Ethanol	15	3.12
		Methanol	20	1.56
9	*Z. armatum*	Methanol	14	6.25

Note: ND indicates not done

5.3.10 Antimicrobial activities of the studied plants extract against *P. aeruginosa*

The ZOI and MBC value of the plants extracts are given in the table 12. Out of 17 plants extracts used 10 plants had good antimicrobial activity against it. The largest ZOI was found to be 21mm in D/W, DMSO, ethanol suspension of *S. aromaticum* and lowest 9mm in D/W suspension of *J.adhatoda*.

Table 12 : Antimicrobial activities of the studied plant extracts against *Pseudomonas aeruginosa*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. bidentata*	D/W	11	12.5
		DMSO	11	12.5
		Ethanol	13	6.25
		Methanol	15	6.25
2	*A. indica*	D/W	11	6.25
		DMSO	12	6.25
		Ethanol	15	6.25
		Methanol	13	6.25

Table-12(contd..)

3	*C. cyminum*	D/W	13	6.25
4	*J. adhatoda*	D/W	9	50
		DMSO	10	25
		Ethanol	13	12.5
		Methanol	15	12.5
5	*M. piperita*	D/W	10	12.5
		DMSO	12	6.25
		Ethanol	13	6.25
		Methanol	15	3.12
6	*M. esculenta*	D/W	17	12.5
		DMSO	12	12.5
		Ethanol	19	6.25
		Methanol	13	6.25
7	*O. sanctum*	Ethanol	19	25
		Methanol	13	25
8	*P. nigrum*	D/W	12	25
		DMSO	17	12.5
		Ethanol	12	6.25
		Methanol	14	6.25
9	*S. calava*	D/W	11	12.5
		DMSO	15	6.25
		Ethanol	13	6.25
		Methanol	12	6.25
10	*S. aromaticum*	D/W	21	25
		DMSO	21	12.5
		Ethanol	21	6.25
		Methanol	20	6.25

5.3.11 Antimicrobial activities of the studied plants extract against *S. paratyphi*

The ZOI and MBC value of the plants extracts are given in the table 13. Among the 17 plants extracts used, 7 plant extracts had inhibitory activity against *S. paratyphi*. The highest ZOI 27mm in ethanol suspension of *S. aromaticum* and least activity with ZOI 10mm in DMSO suspension of *M. esculenta*.

Table 13 : Antimicrobial activities of the studied plant extracts against *Salmonella paratyphi*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*G. glabra*	Methanol	13	6.25
2	*J. humile*	Ethanol	12	12.5
		Methanol	11	12.5
3	*M. piperita*	D/W	11	12.5
		DMSO	11	12.5
		Ethanol	12	6.25
		Methanol	11	6.25
4	*M. piperita (oil)*	Tween 80	12	ND
		Methanol	15	ND
5	*M. esculenta*	DMSO	10	50
		Ethanol	14	25
		Methanol	15	12.5
6	*O. sanctum*	D/W	13	12.5
		DMSO	14	6.25
		Ethanol	12	6.25
		Methanol	12	6.25
7	*S. calara*	D/W	15	12.5
		DMSO	13	12.5
		Ethanol	15	6.25
		Methanol	16	1.56
8	*S. aromaticum*	D/W	20	3.12
		DMSO	17	3.12
		Ethanol	27	0.39
		Methanol	18	0.78

Note: ND indicates not done

5.3.12 Antimicrobial activities of the studied plants extract against *S. typhi*

The ZOI and MBC value of the plants extracts are given in the table 14. Out of 17 different plants extracts used, 7 showed activity while the rest doesn't. The largest ZOI of 20mm in methanol suspension of *T. ammi* and least ZOI of 11mm in ethanol suspension of *O. sanctum*.

Table 14 : Antimicrobial activities of the studied plant extracts against *Salmonella typhi*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. indica*	Methanol	12	6.25
2	*C. cyminum*	Methanol	13	12.5
3	*M. piperita (oil)*	Tween 80	17	ND
		Methanol	19	ND
4	*M. sculenta*	D/W	12	25
		DMSO	14	12.5
		Ethanol	12	6.25
		Methanol	13	6.25
5	*O. sanctum*	DMSO	12	12.5
		Ethanol	11	6.25
		Methanol	12	3.12
6	*S. aromaticum*	DMSO	17	1.56
7	*T. ammi*	Ethanol	17	50
		Methanol	20	25

5.3.13 Antimicrobial activities of the studied plants extract against *S. typhimurium*

The ZOI and MBC value of the plants extracts are given in the table 15. Out of 17 plants extracts used, five showed activity. The highest ZOI was found to be 18mm in ethanol suspension of *T. ammi* and least ZOI of 12mm in D/W and ethanol suspension of *O. sanctum*.

Table 15 : Antimicrobial activities of the studied plant extracts against *Salmonella typhimurium*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*G. glabra*	Methanol	14	6.25
2	*M. esculenta*	D/W	14	25
		DMSO	15	12.5
		Ethanol	15	12.5
		Methanol	16	12.5
3	*O. sanctum*	D/W	12	12.5
		DMSO	15	6.25
		Ethanol	12	6.25
		Methanol	15	6.25
4	*S. aromaticum*	D/W	15	12.5
		DMSO	15	6.25
		Ethanol	17	3.12
		Methanol	17	3.12
5	*T. ammi*	Ethanol	18	0.78
		Methanol	18	0.78

5.3.14 Antimicrobial activities of the studied plants extract against *Sh. dysenteriae*

The ZOI and MBC value for *Sh. dysenteriae* are given in table 16. The lowest ZOI was found in 10mm in suspension DMSO of *T. ammi* and D/W suspension of *S. aromaticum* while highest zone of inhibition of 20mm in ethanol suspension of *T.ammi*.

Table 16 : Antimicrobial activities of the studied plant extracts against *Sh. dysenteriae*

S.N.	Name of plant	Suspension	Antimicrobial activity	
			ZOI mm	M BC (mg/ml)
1	*A. indica*	Methanol	12	12.5
2	*C. cyminum*	Ethanol	12	12.5
		Methanol	13	12.5
3	*G. glabra*	Methanol	13	6.25
4	*J. indica*	Tween 80	14	ND
		Methanol	16	ND
5	*M. esculenta*	D/W	13	25
		DMSO	15	12.5
		Ethanol	12	12.5
		Methanol	15	12.5
6	*O. sanctum*	D/W	14	12.5
		DMSO	13	6.25
		Ethanol	13	6.25
		Methanol	12	3.12
7	*S. aroncatium*	D/W	10	25
		DMSO	15	12.5
		Ethanol	15	3.12
		Methanol	18	1.56
8	*T. ammi*	DMSO	10	6.25
		Ethanol	20	3.12
		Methanol	17	3.12

Note: ND indicates not done

5.4 Antimicrobial susceptibility test

Antibiotic susceptibility test was done for bacteria involved in this study by Kirby-Bauer disc diffusion assay. The antibiotic sensitivity test showed that *Pseudomonas aeruginosa* was most resistant since it was not inhibited by the drugs used for the test. The results of all the bacteria tested for antibiotic sensitivity is given in table 17.

Table 17 : Antibiotic susceptibility pattern of the bacteria used

Organism	Antibiotic used												
	Amoxycillin	Amikacin	Chloramphenical	Ciprofloxacin	Cefixime	Cephotaxime	Cotrimoxazole	Erythromycin	Gentamicin	Nalidixic acid	Norfloxacin	Tetracycline	Vancomycin
B. subtilis	I	-	S	-	-	-	R	-	S	-	-	S	-
S. aureus	-	S	R	-	-	-	S	-	-	-	-	I	S
S. pneumoniae	S	-	-	S	-	S	S	S	-	-	-	-	-
S. pyogens	S	-	-	S	-	S	S	S	-	-	-	-	-
E. coli	S	-	S	-	-	-	S	-	S	-	S	-	-
En. aerogens	R	-	-	S	S	-	-	-	-	S	S	-	-
K. pneumoniae	R	I	-	S	-	-	-	-	S	-	-	R	-
P. mirabilis	-	S	-	-	-	-	S	-	-	S	S	S	-
P. vulgaris	-	S	-	-	-	-	S	-	S	S	S	-	-
Ps. aeruginosa	-	R	-	R	-	-	R	-	R	-	-	R	-
S. paratyphiA	S	-	-	-	-	-	S	-	S	R	S	-	-
S. typhi	-	S	-	S	-	S	-	-	S	R	-	-	-
S. typhimurium	I	-	I	-	R	-	-	-	-	S	S	-	-
Sh. dysentriae	-	S	-	-	-	-	-	-	S	R	S	R	-

Note : R = resistance

S = sensitive

I = intermediate

5.5 Bioassay guided fractionation of *Glycyrrhiza glabra* linn (Jethi madhu)

Percentage yield of *G. glabra* in four different fractions are given in the table 18. It was revealed that highest yield was obtained with D/W (32.64%) followed by Chloroform (23.76%), n-butanol (20.12%) and the lowest yield obtained with hexane (16%).

Table 18 : Percentage yield of different fractions of *G. glabra*

S.N.	Name of fractions	yield (%)
1	Hexane	16
2	Chloroform	23.76
3	n-butanol	20.12
4	Aqueous	32.64

5.6 Screening for Antimicrobial activity of different solvent fraction of *Glycyrrhiza glabra*

Table 19 shows the analysis of antimicrobial activity of different fractions of *G. glabra*. It was revealed from the table that n-butanol fractions showed ZOI with seven microorganisms, while hexane fractions showed ZOI with four microorganisms, Cholorform showed activity against two microorganisms but aqueous fraction was inactive against any of the test organisms.

Table 19 : Antimicrobial activity of different solvent fraction of *Glycyrrhiza glabra*

S.N.	Test organisms	Different fractions of *G. glabra*			
		hexane	Chloroform	n-butanol	Aqueous
1	*B. subtilis*	+	+	+	-
2	*S. aureus*	-	-	+	-
3	*S. pneumoniae*	-	-	+	-
4	*S. pyogens*	+	+	+	-
5	*E. coli*	-	-	+	-
6	*P. vulgaris*	+	-	+	-
7	*Sh. dysenteriae*	+	-	+	-

The positive (+) sign indicates the presence of ZOI.

The negative(-) sign indicates the absence of ZOI.

5.7 Comparison of zone of inhibition of n-butanol fraction & crude extract of *G. glabra*

n-butanol fraction of *G. glabra* and its crude extracts were assayed for antimicrobial activity by measuring zone of inhibition produced by n-butanol fraction and crude ethanol extract. The diameter of well (6mm) has been included in the value of ZOI given in table 20. The concentration of n-butanol fraction was 50 mg/ml.

Table 20 : ZOI produced by n-butanol & crude extract of *G. glabra*

S.N.	Test organisms	Zone of inhibition (mm)	
		n-butanol fraction concentration (50mg/ml)	crude extract concentration (100mg/ml)
1	*B. subtilis*	15	12
2	*S. aureus*	14	16
3	*S. pneumoniae*	10	12
4	*S. pyogens*	13	17
5	*E. coli*	15	-
6	*P. vulgaris*	19	15
7	*Sh. dysenteriae*	14	13

CHAPTER VI

6. DISCUSSION

Plants are valuable for modern medicine in four ways: (i) they are used as sources of direct therapeutic agents; (ii) they serve as raw materials base for the elaboration of more complex semi-synthetic chemical compounds; (iii) the chemical structure derived from the plant substances can be used as model for new synthetic compounds; and finally (iv) plants can be used as taxonomic markers for the discovery of new compounds (Akerele, 1993).

Nepal is a developing country, where more than 90% of the population relies directly or indirectly on agriculture and other natural resources for existence including medicinal plants for treatment of various diseases. The majority of people in Nepal have no access to modern therapy and the situation is worse in the hills and mountains due to inaccessibility of transport. Besides this, most people could not afford it, and have to depend on herbal medicine.

Since, Nepal is rich in biodiversity, among 7000 species belonging to vascular plants and 4500 species of non-vascular plants are reported. Among them 700 species medicinal plants have been recorded (HMG/N, 1970).

Herbal medicines are often used to provide first line and basic health services for people living in remote areas, where it is the only afforable remedy (WHO, 1998). Recently, herbal drugs from Nepal are receiving an increased attention in the country and abroad. Hence the wealth of medicinal plants in Nepal may be considered as one of the important natural resources for the promotion of socio-economic condition of this Himalayan Kingdom (HMG/Nepal, 1970).

From the above discussion, it is realized that there is urgent need to explore the medicinal value of the flora of Nepal. This study forwards one step towards this long way.

For this study, those plants were selected which were believed to be useful as cited in literature and floklore for diseases like cough cold, bronchitis, sore throat, diarrhoea and dysentry etc. Altogether 16 plants were selected taking care of geographical as well as application diversity. Herbariums were prepared at spot during sample collection and identification was done in the Central Department of Botany, T.U.,

Kirtipur and by reffering to various literature. The sample plants were shade dried properly as sunlight may cause loss of some active compounds. The dried plant samples were then subjected to grinding to facilitate complete and easy extraction of the active compound.

Extraction of medicinal plants

There is no any specific rule for the selection of the solvent used for extraction. Thus, varieties of different extracts are used to test the antimicrobial properties of medicinal plants. However, in most of the cases methanolic and other alcoholic extracts were found to be most effective (Shale *et al.*, 1999; Thomas *et al.*, 1999). Rabe and Van (1997) found that the majority of antibacterial activity was present in alcoholic rather than aqueous extracts. Besides alcohol other solvents like acetone, chloroform, petroleum ether etc. were also found to be used. Although water, the universal solvent, is found to be used by the traditional healers and herbalists to extract the active compounds, it is generally not used in the antimicrobial tests as it is less effective for nonpolar organic compounds.

It was found that for the same plant, different solvents gave different percentage yields, so a single suitable solvent i.e. ethanol was used throughout the study. Continuous extraction with the help of soxhlet extractor was performed. In this study, green leaves took longer time for complete extraction while the fruits and roots took shortest duration.

In the present study, the highest yield was observed from *Myrica esculenta* (46.64%) followed by *Glycyrrhiza glabra* (42.44%), *Jasminum humile* (39.94%), *Syzygium aromaticum* (32.44%), *Spilanthes calava* (29.76%), *Zanthoxylum armatum* (28.28%), *Justicia adhatoda* (25.2%), *Piper nigrum* (24.36%), *Mentha piperita* (23.36%), *Trachysperum ammi* (18.85%), *Cuminium cyminum* (18.68%), *Acorus calamus* (16.36%), *Azadirachta indica* (12.72%), *Achyranthes bidentata* (12.08%) and least yield with *O. sanctum* (9.32%). The yield of essential oil from *Juniper indica* and *Mentha piperita* was 1.7% and 1.8% respectively.

There is a distinct difference in the percentage yield of extracts from different plant sample. The differences in yield might be due to various factors such as time of extraction, type and part of plant materials, fineness of powder, and extent of dryness,

etc. The old and dried plant material yield lesser than the young ones. Similarly, incomplete extraction results in lesser yield.

The organism used for this study were selected on the basis of traditional uses of the plants. Most of the plants and herbs species under study were found to be used in respiratory compliants (bronchitis sore throat, cough cold) and in common intestinal disorders in traditional medicines. Thus the organisms included in this study covers the common pathogenic organisms like *B. subtilis, S. aureus, S. pneumoniae, S. pyogens, E. coli, E. aerogenes, K. pneumoniae, P. mirabilis, P. vulgaris, Ps. aeruginosa, S. paratyphi A, S. typhi, S. typhimurium* and *Sh. dysenteriae*. The microbial species employed here are also used for general screening of new antimicrobial compound and also recommended by Cleeland and Squires (1991) for in-vitro evaluation of new antimicrobials.

Qualitative screening for Antimicrobial Activity

In this study extracts from 16 different plant species were studied for their antimicrobial property. The extracts from selected plants, *A. bedentata, A. calamus, A indica, C. cyninum, G. glabra, J. humile, J. adhatoda, M. piperita, M. esculenta, O. sanctum, P. nigrum, S. calava, S. aromaticum, T.. ammi and Z. armatum* dissolved in 4 different solvent viz. D/W, DMSO, ethanol and methanol, and in case of essential oil from *J. indica* and *M. piperita* 2% Tween 80 in normal saline was used.

Almost all the tested medicinal plants, showed some degree of inhibitory effect against the tested microorganisms. The least effective plant was *A. calamus, A. bidentata and J. adhatoda, P. nigrum and S. calava.* Which showed antimicrobial activity against less than 5 microorganisms while most effective was *S. aromaticum* which showed inhibitory effect against all 14 test organisms i.e. 100% effective. Here out of 16 medicinal plants tested 11 plants showed antimicrobial activity against 5 or more than 5 microorganisms.

The plant materials in different suspension, which contained antimicrobial substances are able to inhibit the growth of microorganisms produced ZOI around the agar well/cup in the petridish. In this study a ZOI of diameter more than 8mm has been considered as positive and that of less than 8mm as negative.

In this study few samples did not showed inhibitory activity against the tested bacteria, in the water suspension the activity was less this can be due to the fact that

water couldn't dissolve the active components of alcoholic extracts of the plants, while in most of the cases, plant extract were found active in solvents like DMSO, ethanol and methanol against test organisms. From this it is clear that active components are more soluble in these solvent, this is in accordance with Sasidharan (1997).

During the study, it was found that *A. bidentata* was able to show ZOI with *S. aureus, S. pneumomiae* and *Ps. aeruginosa* only. It was in effective in all other organisms included in this study. Ethanol and methanol suspension show more inhibitory activity in these organism. Our result do not tally with Parasi (2002) who found no inhibitory effect of the plant extract in the tested seven bacterial strain.

A. calamus inhibited only two microorganisms *S. aureus* and *S. pyogens.* This is in accordance with Zaiba *et al*., (1999) where the ethanolic extract of *A. calamus* demonstrated only moderate antibacterial activity. Similar antibacterial activity was observed by Gautam (2002) with *A. calamus*. This results also supports our results.

Similarly, *A. indica* produced ZOI against six test organisms *viz. B. subtilis*, *S. aureus, E. coli, Ps. aeruginosa, S. typhi* and *Sh. dysenteriae*. The ethanol and methanol suspension were found potent to Gram-positive and Gram-negative bacteria.

In case of *C. cyminum*, the ethanol and methanol suspensions inhibited 10 out of 14 bacterial strains where as *S. pyogens, P. vulgaris, S. paratyphi* and *S. typhimurium* were found resistant to it at the concentration tested. Its activity is significantly higher in *B. subtilis* and *P. mirabilis*. The ZOI against all other organisms were moderate, ethanol and methanol suspension were more potent. According to Sayed (1986). Cumin showed remarkable activity against *S. aureus*, *E. coli, S. typhi, Sh. dysenteriae* and *V. cholerae*, at quite low concentration which is also comparable with our study. Similarly our results tally with Shetty *et al*., (1994) who found *E. coli* most sensitive to Cumin, Sharma (2000) also found similar resultsin her study of antimicrobial effect of essential oil.

Mean while *G. glabra* produced ZOI against all Gram -positive bacteria and only moderate activity against Gram-negative bacteria like *P. vulgaris, S. paratyphi, S. typhimurium* and *Sh. dysentriae* in its methanol suspension. This result is quite similar to Devkota *et al*., (1999) who reported the crude ethanol extract of *G. glabra* showed best antibacterial activity against all tested bacterial strains. Pokhrel (2000) in his

study also found that *G. glabra* more active towards Gram-positive than Gram-negative bacteria.

Jasminum humile showed antibacterial activity against nine bacteria out of 14 tested. Both Gram-positive and Gram-negative. In all the cases ethanol and methanol suspension gave good inhibition than D/W and DMSO solvent.

Activity of the essential oil extracted from *Juniper indica* showed activity against *B. subtilis, S. aureus, S. pyogens, K. pneumoniae, P. vulgaris* and *Sh. dysenteriae.* It also showed more pronounced effect on Gram-positive bacteria than Gram-negative bacteria.

In case of *Adhatoda vasica*, only 3 out of 14 test organism was found positive *viz. S. pyogens, P. mirabilis* and *Ps. aeruginosa*. In *P. aeruginosa* all the 4 suspension gave good inhibitory effect against it. Brantner and Chakraborty (1998) also found significant activity of alkaloid fraction of *A. vasica* leaves against *P. aeruginosa*. Similar, study done by Baidya (2001) showed similar results with *Ps. aeruginosa* and *P. mirabilis* while in our case *S. pyogens* was also additional and was positive which supported this results.

M. piperiata (ethanolic extract) inhibited only Gram-negative bacteria and had no activity on Gram-positive bacteria. The highest activity was seen in *K. pneumoniae* in D/W solvent. But in case of essential oil of *M. piperita* dissolved in Tween 80 and methanol, the antibacterial effect was more pronounced against Gram-positive and Gram-negative bacteria like *B. subtilis, E. coli, E. aerogenes, K. pneumoniae, P. mirabilis, P. vulgaris, S. paratyphi, S.typhi*. Sampurna and Nigam (1980) observed essential oil of *M. piperita* posses potent antibacterial efficacy against *S. typhi.* Similarly Imai *et al*., (2001) also observed essential oil of the plant was active against *Salmonella* sps and differents strains of *E. coli.*

M. esculenta showed good inhibition zone even in the D/W suspension of most of the test organisms. So, all the 4 suspension showed inhibitory activity against 12 test bacteria and had broad antimicrobial activity. In a similar study done by Prasai (2002), *M. esculenta* gave similar antibacterial activity against the test organisms *viz. E. coli, P. vulgaris. Ps. aeruginosa, S. typhi, Sh. dysenteriae* except in this study *K. pneumoniae* showed some minimal effect but in our study it was not inhibited at all.

*O. sanctum a*lso proved to have broad antibacterial activity being effective against 10 out of 14 test bacteria concluding *B. subtilis, S. aureus, S. pneumoniae, S. pyogens, E. coli, Ps. aeruginosa, S. paratyphi, S. typhi, S. typhimurium* and *Sh. dysenteriae*. Aqil *et al*., (2005) found leaves extracts of the *O. sanctum* exhibited better activity against three MRSA strains. Rajendhran *et al*., (1998) reported that antibacterial effect of extract *O. sanctum* was most effective against *S. aureus, E. coli, Ps. aeruginosa* and *Klebsiella*. But in our study *K. pneumoniae* was found negative.

P. nigrum was found to be very less inhibitory against all the test bacteria. Three Gram-positive and only one Gram-negative bacteria were inhibited, *B. subtilis, S. aureus, S. pyogens* and *Ps. aeruginosa*. In similar study done by Baratta *et al*., (1998) 50% alcoholic solution of black pepper has been studied for antibacterial and antifungal activity by department of forestry and plant research against *B. subtilis, S. dysenteriae, E. coli*, *Candida albicans* and *Sacchromyces cerevisae* and found all the organisms tested were resistant to it (HMG/Nepal, 1983,84). Sharma (2000) found that essential oil of black pepper inhibited only *S. aureus* and *B. subtilis* which is comparable to our results.

The *S. calava* extract inhibited growth of *S. pyogens*, *Ps.aeruginosa* and *S. paratyphi* hence have least activity. In experiment done by Gautam (2002), *S. calava* showed similar result which is can be compared with ours.

Syzygium aromaticum showed relatively broad spectrum of activity. All the four suspension of its extract showed activity against all 14 test bacteria. There was equal inhibition effect in both Gram-positive and Gram-negative bacteria. This results is similar to the results obtained by Deans *et al.,*. (1995), who found the antimycotic properties of clove oil significantly more pronounced than the antibacterial activity. However results differ in case of bacteria as they found *Pseudomonas* the least affected where as in our case *S. typhi* and *P. vulgaris* were least affected. Zaiba *et al.,* (1999), reported that antimicrobial activity of *S. aromaticum* (bud) had significantly broad spectrum activity against Gram-positive and Gram-negative bacteria.

T. ammi, also had broad spectrum antimicrobial activity against test organisms but its ethanol and methanol suspension gave more inhibitory effect than D/W and DMSO suspension. It inhibited all Gram-positive bacteria but was unable to inhibit all Gram-

negative bacteria. Gautam (2002) also observed significant antibacterial activity of *T. ammi*. This finding also complies with our result.

The ethanol and methanol suspension of *Z. armatum* were found more effective against test organisms. It had only moderate antibacterial activity; only three Gram-positive bacteria and four Gram-negative bacteria were inhibited with least ZOI.

In overall it was found that most of the plant material tested were active against Gram-positive and less active against Gram-negative bacteria. This finding is well supported by Sindambiwe *et al*., (1999). They found 80% of aqueous ethanol extract were directly active against Gram-positive bacteria while found to be ineffective to Gram-negative. Caceres *et al*., (1990), while screening 84 medicinal plants for in-vitro antibacterial activity found that 40.8% plant inhibited one or more enterobacteria and Gram-positive bacteria found to be more sensitive towards the ethanol extract of medicinal plants than Gram-negative bacteria. The ZOI obtained in some of the plants in D/W suspension were negligible or not at all present . It might be due to presence of oil in extract that was not fairly soluble in D/W used to prepare working solution, hence oil caused obstruction in diffusion of the extract as well as interference to dissolve antimicrobial compounds present in the plant.

Evaluation of Antibacterial Activity

Evaluation of antimicrobial activity was aspects of our study as compound having broad spectrum of activity may have little potency and such compounds have little value in development of new drugs. The evaluation of antimicrobial substances also becomes the essential step during new drug research from natural product. For this two methods were employed here *viz.* Agar well diffusion method and two fold serial dilution method. In agar well method (first introduced by Dingle *et al.* ,(1953) method, the material under test diffuses from agar well into the surrounding agar in a concentric circle and inhibits or kill the microorganisms that are susceptible. This effect, manifested by a clear zone around the well is measured as zone of inhibition (ZOI).

It was revealed from the result that each medicinal plant shows different degree of inhibition against different microorganisms. Among the plants selected 11 plants

showed antibacterial activity (ZOI) against five or more than five microorganisms and five plants showed antimicrobial activity against less than five microorganisms.

Susceptibility of microorganisms can be defined both in terms of number of medicinal plants which affected them i.e. the plant producing ZOI and in terms of MBC observed.

In this study, for *B. subtilis*, 37 test suspension out of 64 produced zone of inhibition(ZOI)and12 outof 17 plants extracts were effective. Largest ZOI was observed with Tween 80 suspension of *M. piperita* (essential oil) with ZOI 30mm. The smallest ZOI of 9mm was observed with DMSO solvent of *P. nigrum*. The ZOI value for other plant extracts was in between 28-10mm.

Against *S. aureus*, 42 test suspension out of 64 produced ZOI,13 out of 17 plants extracts were found to be active against the organisms. Largest ZOI was found to be exhibited by methanolic suspension of *T. ammi* (27mm). The smallest ZOI value of 10mm was found in one or more cases.

S. pneumoniae was inhibited by seven plants extracts out of the 17 plants extracts. 26 suspensions out of 64 suspension produced zone of inhibition. The largest ZOI (21mm) was observed with DMSO and ethanol solvent of *A. bidentata* and least ZOI was seen in D/W suspension of *C. cyminum*.

Against *S. pyogens*, 11 plants extracts out 17 showed inhibitory activity and produced zone of inhibition. The highest ZOI 22mm was seen in D/W suspension of *O. sanctum* and lowest 9mm in DMSO suspension of *S. calava*. The organism was totally resistant to suspension of *A. bidentata, A. indica, C. cyminum, M. piperita* (etoH. extract) as well as its essential oil and to *Z. armatum*.

In case of *E. coli*, 10 out of 17 plantsextract showed activity;most potent,extract with highest ZOI was found to be ethanolic suspension of *T. ammi* and least effective was D/W suspension of *J. humile*. While the organisms which was found completely resistant to extracts were *A. bidentata, A. calamus, G. glabra, J. indica, J. adhatoda, P. nigrum* and *S. calava*.

E. aerogenes had moderate antibacterial effect. 7 out of 17 plant extracts was found to have inhibitory effect, and produced ZOI. The maximum ZOI of 19mm in DMSO solvent of *S. aromaticum* and minimum 10mm in D/W suspension of *J. humile* was seen. All other plant had ZOI in range of (18-11mm).

K. pneumoniae had moderate inhibitory effect and 7 plant extracts showed activity against it. The highest 26mm in DMSO solvent of *S. aromaticum* and lowest 9mm in D/W suspension of *J. humile* was observed. While *A. bidentata, A. calamus, A. indica, G. glabra, J. adhatoda, M. esculenta, O. sanctum, P. nigrum* and *S. calava* did not have any effect at all.

Against *P. mirabilis* 9 plant extracts showed antibacterial effect, the most potent extract among them with maximum zone 21mm in DMSO solvent of *C. cyminum* and least effective with ZOI 9mm was D/W suspension of *T. ammi*. The range of ZOI of all other plant material was between (20-10mm).

Against *P. vulgaris*, 17 suspensions were positive and showed zone of inhibition, among them, methanolic suspension of *S. aromaticum* with 24mm ZOI was the highest and 12mm was least, in one or more cases.

Ten plant extracts showed positive results against the opportunistic pathogen *P. aeruginosa*. The largest ZOI of 21mm was recorded with Ethanolic suspension of *S. aromaticum* and lowest ZOI 9mm in D/Wsuspension of *J. adhatoda.*The range of ZOI (minimum 10mm and maximum 20mm) was found for other plant material.

In *S. paratyphi*, seven plant extracts under test exhibited the inhibitory effect and produced ZOI. The ethanol suspension of *S. aromaticum* gave largest ZOI (27mm) and least ZOI in case of DMSO solvent of *M. esculenta*.

S. typhi similarly was inhibited by seven plant extracts under study. *T. ammi* was most potent with 20mm ZOI in its methanolic suspension and minimum potency given by ethanolic suspension of *O. sanctum*.

S. typhimurium was the most resistant bacterium inhibited by only 5 plant extracts. The most largest ZOI (18mm) given by ethanol and methanol suspension of *T. ammi* and minimum effect was seen in D/W suspension of *O. sanctum* (12mm). The remaining 12 plants extracts proved futile as they failed to inhibit the growth.

Finally, *Sh. dysenteriae* was inhibited by 8 plant extract in their different suspension. But ethanolic suspension of *T. ammi* gave the highest ZOI 20mm and least 10mm in D/W suspension of *S. aromaticum*.

In overall evaluation, extracts from all the plant material under study were active against both Gram-positive and negative bacteria . The largest ZOI was 30mm given

by Tween 80 suspension of *M. piperita* (oil) and smallest ZOI was 9mm which was found in more than one species.

The comparative evaluation of essential oil from *J. indica* and *M. piperita* showed that *J. indica* had more activity towards Gram-positive organisms while *M. piperita* was more active towards killing Gram-negative bacteria and produced relatively greater zone of inhibition.

The diameter of zone of inhibition largely depends upon the diffusibility of the antimicrobial substances. Hence sometimes the ZOI demonstrated the antimicrobial substance doesn't commensurate the efficacy of the substance. This necessitates the determination of MIC (minimum inhibitory concentration) and minimum bactericidal concentration (MBC) to give a correct picture of antimicrobial potency of the compound. This was done here by two fold serial dilution.

The MBC values obtained in this experimental study, for *B. subtilis*, lowest MBC was found to be 1.56 mg/ml in the ethanolic and methanolic suspension of *G. glabra*, methanolic suspension of *S. aromaticum* and ethanol suspension of *T. ammi* and the highest MBC value was 25mg/ml found in the D/W and DMSO suspension of *M. esculenta*.

In *S. aureus*, lowest MBC value of (0.39 mg/ml) in ethanol suspension of *G. glabra* i.e. the organisms was actually inhibited at this values, and the highest MBC (50mg/ml) was found in ethanol suspension of *A. bidentata*.

For *S. pneumoniae*, *A. bidentata* was the most lethal requiring as low concentration 1.56mg/ml in its ethanolic suspension. While highest MBC value was found to be 25mg/ml against *C. cyminum*, *O. sanctum* in D/W and DMSO suspension.

S. pyogens, had lowest MBC value of 0.78mg/ml in DMSO solvent of *T. ammi* and highest MBC value 50 mg/ml in D/W suspension of *A. calamus* as well as in D/W suspension of *J. adhatoda.*

Against *E. coli*, most lethal plant extracts was found to be ethanol suspension of *T. ammi* with MBC value of 1.56 mg/ml and highest MBC value of 25mg/ml was observed in D/W suspension of *J. humile* and *M. esculenta*.

E. aerogens, only seven plant extracts inhibited it with least MBC value of 0.39mg/ml in methanol suspension of *S. aromaticum* while the highest MBC value of 50mg/ml was seen in D/W suspension of *M. esculenta*.

In *K. pneumoniae* least MBC value was 0.097mg/ml against the organism in DMSO suspension of *S. aromaticum* and maximum MBC value of 25mg/ml in case of D/W suspension of *J. humile*

Against *P. mirabilis*, *S.aromaticum* had lowest MBC of 0.78mg/ml, while *T.ammi* had this potential only at 25mg/ml in D/W suspension and had least cidal activity

In case of *P.s vulgaris* lowest MBC value of 0.78mg/ml was found in *S.aromaticum* and highest MBC value to kill the bacteria was 25mg/ml in D/W suspension of *M.piperita*.

Against *Ps. aeruginosa*, lowest MBC value to kill to organism was 3.12mg/ml in methanol suspension of *M. piperita* and the highest value 50 mg/ml in case of D/Wsuspension of *J. adhatoda* was cidal for the bacteria.

S. paratyphi was inhibited by seven plant extracts and most lethal and least MBC value of 0.39mg/ml by *S.aromaticum* extract and least effective with high MBC value of 50mg/ml in case of DMSO suspension of *M. esculenta*.

For *S. typhi* least MBC value was 3.12mg/ml given by methanol suspension of *O. sanctum* and the highest being 50mg/ml by ethanol suspension *T.ammi.*

S. typhimurium, was the most resistant and only five plant extracts showed activity among them 0.78mg/ml MBC value given by ethanolic and methanolic suspension of *T. ammi* while lowest MBC value to kill the organisms was 25 mg/ml by *M. esculenta* in D/W suspension.

Finally, *Sh. dysenteriae*, the highest value for killing the organisms was found to be : 25mg/ml in D/W suspension by *M. esculenta* and *S. aromaticum* and least value for cidal effect was found to be 1.56mg/ml in methanol suspension of *S. aromaticum*.

In overall analysis, *S. aromaticum* was found to be most effective against both Gram-positive and Gram-negative organism but Gram-positive were more effected, with highest ZOI (26mm) and lowest MBC value (0.097mg/ml) in *K. pneumoniae*. It was also exhibited that, lowest MBC value among Gram-positive was 0.39mg/ml in *S. aureus* by *G. glabra*.

Analysis of different suspension from same extract of plant shows that the D/W suspension was relatively inefficient than DMSO, ethanol and methanol of suspension which gave greater antimicrobial activity.

The diameter of ZOI produced by plant extracts depends both on several factors both intrinsic and extrinsic. The extrinsic factors such as p^H of the medium, period and temperatures of incubation, volume of well, concentration of plant extract and size of inoculum can be fixed and standardized during experiment, hence no errors results due to extrinsic factors. The intrinsic parameters such as nature of medicinal plants including its compounds, solubility and diffusibility are predetermined and hence affect the zone of inhibition. Similarly, the MBC value is also affected by both extrinsic and intrinsic parameters.

The ZOI and MBC value are two different attributes. The compound having large ZOI could have large MBC value as well and vice-versa. 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 test organism and are also able to diffuse through agar medium. 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. the D/W suspension of *M. esculenta* having ZOI of 16mm and MBC value of 25mg/ml against *S. aureus*. Similarly ethanol suspension of *M.esculenta* having ZOI of 19mm and MBC value of 12.5mg/ml against *P. vulgaris*.

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 propects. The ethanolic suspension of *G. glabra* demonstrated a ZOI of 23mm and MBC of 0.39mg/ml against *S.aureus*. On the other hand methanolic suspension of *T. ammi* had ZOI of 27mm and MBC of 1.56mg/ml for the same bacteria.

Small ZOI and small MBC values are given by plants which have good cidal effect but diffuses poorly. In this experiment the D/W suspension of *G.glabra* gave a ZOI of 12mm and it's MBC was 6.25mg/ml against *B.subtilis* while the DMSO suspension of *T.ammi* gave ZOI of 11mm with MBC value of 6.25mg/ml against same bacteria.

Finally, small ZOI and large MBC showed that the plant extract has poor diffusibility coupled with poor antimicrobial property. These categories have very poor prospects of being useful. For eg: against *S.pneumoniae* D/W suspension of *C.cyminum* gave a ZOI of 9mm and MBC of 25mg/ml. Similarly, D/W suspension of *J.humile* gave ZOI of 9mm and MBC of 25mg/ml against *E.coli*.

Antibiotic sensitivity patterns of Bacteria

All the bacteria employed in the research were also subjected to antibiotic sensitivity test on MHA plate by disc diffusion method in order to observe their response against the antibiotics during the study period. The antibiotic discs were chosen according to their clinical uses against the bacteria (Collee *et al*.,1996) and their sensitivity pattern.

During, antibiotic sensitivity test, *B. subtilis* was sensitive to four antibiotics and was resistant to cotrimoxazole. It was most sensitive to Tetracycline and was intermediate with Amoxycillin.

S. aureus was sensitive to three antibiotics and most sensitive was Amikacin while it was resistant to chloramphenicol and intermediate to Tetra cycline. These two Gram positive bacteria were most susceptible in antibacterial screening and was inhibited by most of the plant extracts

S. pneumoniae and *S. pyogens* were found sensitive to all the antibiotics used for it. Both was most sensitive to cephotaxime. They were also inhibited by most plants used for respiratory complaints.

E. coli was found sensitive to all antibiotic used. The organism was inhibited by ten plant extracts during the antimicrobial screening in this study.

E. aerogenes was sensitive four antibiotics and resistant to Amoxycillin. Similarly it was inhibited by seven plant extracts though zone of inhibition were moderate.

K. pneumoniae was sensitive to ciprofloxacin and gentamicin while resistant to Amoxycillin and Tetra cycline and intermediate to Amikacin. While antimicrobial

screening showed that ZOI value quite comparable with the ZOI of the antibiotics used, hence the plants had good inhibitory effect to this organisms.

P. mirabilis was sensitive to all the five antibiotics used. In the antimicrobial screening it was inhibited by 9 plants while most potent was *S. aromaticum* and the plant could be a potential antimicrobial if further analysis is done.

P. vulgaris, sensitive to all of the antibiotic and most effective was norfloxacin. In case of antibacterial screening *M. piperita* oil was most effective against it, hence further analysis can be done.

Ps. aeurginosa was most resistant to all antibiotic used while, in case of the antimicrobial screening process it was inhibited by five or more plants extracts.

S. paratyphi, was sensitive to Cotrimoxazole, Gentamicin and Norfloxacin while resistant to Nalidixic acid. In antibacterial screening it was fairly susceptible and inhibited by seven plant materials.

S. typhi when tested for antibiotics was found to be Nalidixic acid resistant and in screening for antibacterial activity showed that it was inhibited by seven plant extract hence plants are quite effective.

S. typhimurium was resistant to Cefixime and in case of antibacterial screening it was the most resistant bacteria. So, antibiotic therapy could be better option.

Sh. dysenteriae found to be sensitive to three antibiotic. In the screening process from plant extracts most of the plants had good antimicrobial property against it.

Thus, from the result obtained, we can say that the extracts from the medicinal plants had fairly good antibacterial activity and some extracts had inhibited the most resistant organism like *Sh. dysenteriae*, *E. coli* and *S. typhimurium*.

Bioassay guided fractionation of *Glycyrrhiza glabra* Linn.

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 we can identify the active compounds.

In this study, four different fractions were separated using hexane, chloroform, n-butanol and water as solvents of increasing polarity. Different fractions were screened

for their antibacterial property against all test organisms, n-butanol fraction showed activity against most of the test microorganisms and aqueous fraction was found to be ineffective while chloroform was active against two out of 14 test organisms, while hexane gave four positive results against the test microorganisms. This result indicated that the chemical constituents responsible for medicinal property were more soluble in n-butanol than in ethanol or other solvent or the butanolic extract was more diffusible than ethanolic. Thus, *E. coli* which was not inhibited in it's crude extract was inhibited in the n-butanol fraction of the same plant material. This result also indicated that the antibacterial chemical constituent(s) become more specific against Gram-negative *E.coli*, hence related antibacterial constituent(s) have gone in this fraction. With n-butanol, largest ZOI of 19mm was observed in *P. vulgaris* followed by 15mm in *B. subtilis* and *E.coli*. The smallest 10mm was found against *S. pneumoniae*(10mm) while it's ethnolic extract gave 12mm ZOI. By comprasion with activity of ethanolic extract it was found that the potency against different bacteria was independently altered. ZOI observed for *S. aureus*, *S.pneumoniae*, *S.pyogens* showed greater zone in ethanolic extract or possibly the components dissolved in ethanol were more potent.

From overall analysis it could be concluded that *Glycyrrhiza glabra* and its butanolic fraction, contained more than one compounds that are different in their solubilities and spectrum of antibacterial action, as n-butanol is less polar than ethanol, the more polar component got dissolved in the ethanol while the less polar one dissolved in n-butanol.

From this study, it can be concluded that the traditional folklore of using the selected plant materials under study were able to inhibit the causative agent of respiratory diseases and intestinal disorder as well. Hence if studies in medicinal plants are focused to evaluate antimicrobial potency with respect to their traditional uses, medicinal plants could serve as potential source of new drugs that could be efficient in managing the drugs resistant organisms.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

In this experimental study, 16 different medicinal plants were screened and evaluated for their antibacterial activity. Different parts of these plants were taken for extraction with 95% ethanol to assay their antimicrobial property using Soxhlet extractor. The essential oil content of *Juniper indica* and *Mentha piperita* was extracted by hydrodistillation process with cleavenger apparatus.

Highest yield was obtained from *Myrica esculenta*(46.64%), followed by *Glycyrrhiza glabra* (42.44%), *Jasminium humile* (39.92%), *Syzygium aromaticum* (32.44%), *Spilanthes calava* (29.76%), *Zanthoxylum armatum* (28.28%), *Justicia adhatoda* (25.2%), *Piper nigrum* (24.36%), *Mentha piperita* (etoH extract, 23.36%), *Trachysperum ammi* (18.85%), *Cuminium cyminum* (18.68%) *Acorus calamus (16*.36%), *Azadirachta indica* (12.72%), *Achyranthes bidentata* (12.08%), and the least yield with *Ocimum sanctum* (9.32%) . The yield of essential oil was 1.7% and 1.8% for *Juniper indica* and *Mentha piperita* respectively.

The crude extract were then dissolved in four fraction *viz.* distilled water, DMSO, ethanol and methanol. The essential oil was dissolved in two different solvent *viz.* 2% Tween 80, and methanol. These suspension were tested for antimicrobial activity against 14 different bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogens*, *E.coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella typhimurium*, *Shigella dysenteriae*). Agar well diffusion method, and two fold serial dilution method deterimined the zone of inhibition (ZOI), and minimum bactericidial concentration(MBC). Only those extracts, which showed zone of inhibition (ZOI), were assayed for MBC. The water suspension of most of the extract show least inhibitory activity against the tested microorganisms.

Syzygium aromaticum was found to have broadest antimicrobial activity. It inhibited all of the 14 test bacteria. On the other hand *Acorus calamus* had lowest antimicrobial

activity as it was active against only two test oraganisms. Similarly, *Trachyspremum ammi, Myrica esculenta*, *Ocimum sanctum* and *Cuminum cyminum* also showed good antibacterial activity. While *Glycyrrhiza glabra* was most effective towards Gram-positive bacteria, than Gram-negative. During screening process, largest ZOI was 30mm in *B.subtilis* produced by tween 80 suspesion *M. piperita* and smallest ZOI was 9mm which occurred with more than one species.

Among all the test bacteria, *Staphylococcus aureus* was the most suspcetible bacteria being sensitive to 13 medicinal plants extracts. Similarly, 12 plants inhibited *B.subtilis.* Among the Gram- negative, *E.coli* and *Ps.aeruginosa* were inhibited by 10 plants extracts while most resistant was found to be *Salmonella typhimurium*.

In over all analysis of all the antibacterial properties of all the plants used, *Syzygium aromaticum* was found to be most effective against both Gram-positive and Gram-negative bacteria as well with highest ZOI (26mm) and lowest MBC value (0.097mg/ml) found in *K.pneumoniae*.

Antibiotic susceptibility test were also performed by disc diffusion method. *Pseudomonas aeruginosa* emerged as the most resistant species. Comparision of antibiotic sensitivity with that of activities of various extracts under study had fairly good antimicrobial activity. Since, *Ps.aeruginosa*, which was resistance to all the five tested drugs, was sensitive to 10 different plant extracts in different suspension. In most cases alcoholic suspension had more pronounced activity than aqueous suspension.

In bio-assay guided fraction of *G.glabra* n-butanol fraction was found to have extended activity. It inhibited seven test bacteria including *E.coli* which did not show any response to ethanolic extract. *P.vulgaris* showed highest ZOI(19mm) while *S.pneumoniae* with minimum 10mm. The comparative study of the activity of the two extracts and fractions showed that *P.vulgaris*, *B.subtilis*, and *Sh.dysenteriae* showed larger ZOI with n-butanol and the rest produced larger ZOI with with ethanolic extract.

7.2 Recommendations

Based on the study following recommendations are made.

1. Only 16 medicinal plants have been assayed due to constraints of time. There are more than 700 medicinal plant species in Nepal. Antimicrobial activities of other should be studied.
2. Only ethanolic extracts of plants have been used in this study. There are various non alcohol extractable active compounds in the plants which have antimicrobial activity, so other solvents should also be tried for extraction.
3. As, there are many parameters in our body that interfere with antimicrobial agents, *in-vivo* test should be performed for use in human beings.
4. In this study, antibacterial activities of medicinal plants have been studied, Antiviral, Antifungal, activities of medicinal plants should also be studied.
5. Plants, which showed higher antimicrobial activity during evaluation, should be analyzed in detail to identify the active antimicrobial constituents and also their mode of action.
6. Medicinal plants should also be assayed against microorganisms that are not included here, specially those that are of public concern eg., *Mycobacterium tuberculosis*, HIV viruses, Hepatitis viruses, Fungi as well as for anticancer agents.
7. Modern era antimicrobials are becoming less effective due toincrease in emergence of antibiotic resistant microorganisms day by day. Medicinal plants may however from a viable alternative in this case. So, further research in them should be conducted in future.

CHAPTER-VIII

8. REFERENCES

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APPENDIX-A

List of materials used for the study

Soxhlet extraction:

- Condenser
- Grinder
- Heating mantle
- Rotatory Vacuum evaporater
- Round bottom flask
- Sieve
- Soxhlet extractor(Ogawa Seiki Japan)
- Stands

Steam distillation:

- Round bottom distilling flask
- Cleavenger type oil trap
- Condenser
- Heating mantle
- Stands

Media for culture

- Eosin methylene blue(EMB) agar.
- Mannitol salt agar(MSA)
- MacConkey's Agar
- Muller Hinton Agar(MHA)
- Nutrient Agar(NA)
- Nutrient broth(NB)
- Xylose lysine decarboxylase(XLD)agar
- Triple sugar iron agar(TSI)
- Sulphide indole motolity(SIM)
- MRVP broth (Glucose, Phosphate, Peptone broth)
- Simmon's citrate medium.
- Urease medium

Chemical Reagents:

Absoulte ethanol, Tween 80, DMSO, Crystal violet, Gram's iodine, Kovac's reagent, Methyl red, -napthol solution, Barium chloride, Conc. H_2SO_4, Potassium hydroxide, Physiological saline,Blood plasma, n-butanol.Ethanol, Chloroform, Hexane, Saffranin.

Antibiotic Discs: Antibiotic discs from Hi-Media Laboratory

Apparatus and Equipments:

- Aluminium foils
- Autoclave
- Compound Microscope
- Cork borer no5&6
- Cotton roles
- Electric balance
- Filter paper
- Forceps
- Hair drier
- Incubator
- Inoculating loop
- Laminar flow hood
- Micropipette
- Measuring scale
- Plant cutter
- Refrigerator
- Sticker
- Water distillation plant
- Vortex shaker.

Glassware:

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

Preparation of 2% Tween 80 Diluent.

0.85gm of Nacl is dissolved in 100ml of water in volumetric flask and is sterilized by autoclaving. Then 2ml Tween 80 is added it and mixed well, Stoppered and stored in refrigerator.

APPENDIX-B

Lists of Medicinal Plants used in the Evaluation of Antimicrobial Activities

Botanical Name	Family	Parts used	Place of collection	Month of collection	Local name
Achyranthes bidentata Blume.	Amaranthaceae	leaves	Kiritpur, Kathmandu	August	Datiwan
Acorus calamus L.	Aracae	Rhizome	Kiritipur, Kathmandu	November	Bojho
Azadirachta indica A.Juss.	Meliaceae	Leaves	Bhoteodar, Lamjung	September	Neem
Glycyrrhiza glabra Linn.	Leguminosae	Rhizome	Purchased from Ayuervedic shop	-----------	Jethimadhu
Cuminum cyminum Linn.	Umbelliferae	Seeds	Purchased from Ayuervedic shop	-----------	Jeera
Jasminum humile Linn.	Oleaceae	Leaves	Kiritipur, Kathmandu	September	Jai
Juniper indica Bertol.	Cupressaceae	Leaves	Kiritipur, Kathmandu	October	Dhupi
Justicia adhatoda L.	Acanthaceae	Leaves	Bhoteodar, Lamjung	March	Asuro
Mentha piperita Linn.	Labiatae	Leaves	Sanothimi, Bhaktapur	June	Pudina
Myrica esculenta Buch-Ham.ex D.Don	Myricaceae	Bark	Bhoteodar, Lamjung	March	Kaphal
Ocimum tenuiflorum L.	Labiatae	Leaves			Tulsi
Piper nigrum Linn.	Piperaceae	Seeds	Purchased from Ayuervedic shop	------------	Marich
Spilanthes acmella (L)Murr.	Asteraceae	Flower buds	Koteshwor, Kathmandu	August	Marahaththi
Syzygium aromaticum Linn.	Myrtaceae	Flower buds	Purchased from Ayurvedic shop	------------	Lawang
Trachyspermum ammi (L).Sprague.	Umberlliferae	Seeds	Purchased from Ayuervedic shop	-------------	Jwano
Zanthoxylum armatum DC.	Rutaceae	Seeds	Pulchoki, Lalitpur	September	Timur

*Samples of these plants were collected from different people.

APPENDIX-C

Lists of Test Organisms and their Sources

S.N.	Name of Test Organisms	Sources
1.	*Bacillus subtilis*	Central Department of Microbiology, Kirtipur
2.	*Staphylococcus aureus*	Kathmandu Medicial College, Hospital, Sinamingal
3.	*Streptococcus pneumoniae*	T.U. Teaching Hospital, Maharajgunj
4.	*Stertococcus pyogens*	National Public Health Laboratory, Teku
5.	*Escherichia coli*	Central Department of Microbiology, Kirtipur
6.	*Enterobacter aerogenes*	National School of Sciences, Lainchour
7.	*Klebsiella pneumoniae*	T.U. Teaching Hospital, Maharajgunj
8.	*Proteus mirabilis*	Kathmandu Model Hospital, Pradarshani Marg
9.	*Proteus vulgaris*	Kathmandu Medical college, Hospital, Sinagmingal
10.	*Pseduomonas aeruginosa*	T.U. Teaching Hospital, Maharajung
11.	*Salmonella para typhi* A	Kathmandu Model Hospital, Pradarshani Marg
12.	*Salmonella typhi*	Kathmandu Model Hospital, Pradarshani Marg
13.	*Salmonella typhimurium*	National School of Sciences, Lainchour
14.	*Shigella dysenteriae*	Central Department of Microbiology, Kirtipur

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°C) 7.4+/-0.2

Procedure: 28gms of media was dissolved in 100ml of distilled water and heated to dissolve the media. The media was autoclaved at 15lbs pressure at 121°C 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°C) 7.4+/-0.2

Sterilized by autoclaving at 15lbs pressure(121°C) for 15 minutes.

3. Muller Hinton Agar (MHA)

Ingredients gm/litre

Beef Infusion Broth 300.0

Casein Acid Hydrolysate 17.0

Strach 1.0

Agar 17.0

Final pH 7.0+/-0.2

Procedure: 3.8gms of media was suspended in 100ml distilled water, boiled to dissolve and sterilized by autoclaving at 121°C for 15minutes. It was poured while at 45-55°C in sterile 9cm diameter plates in 25ml quantities. 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 0.6ml of 1% (10g/litre) solution of barium chloride dihydrats into a 100ml graduated cylinder, and filling to 100ml with 1% (10ml/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 fold serial dilution:

Code 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
8 th	0.390625	390.6250
9th	0.1953125	195.3125
10th	.09765625	97.6565
+ve control	0.00	0.0000

APPENDIX - F

ZONE SIZE INTERPRETATIVE CHART FOR ANTIBIOTIC SENSITIVITY TEST

Antibiotic or chemotherapeutic agent	Strength	Diameter of zone of inhibition		
		Resistant	Intermediate	Sensitive
Amikacin	30mcg	14	15-16	17
Amoxycillin	30mcg	19	-	20
Ciprofloxacin	30mcg	12	13-17	18
Cephotaxime	30mcg	14	15-22	23
Chloramphenicol	25mcg	12	13-17	18
Cortimoxazole	25mcg	10	11-15	16
Erythromycin	15mcg	13	14-22	23
Gentamicin	10mcg	12	13-14	15
Nalidixic acid	30mcg	13	14-18	19
Norfloxacin	10mcg	12	13-16	17
Tetracycline	30mcg	14	15-18	19
Vancomycin	30mcg	-	-	15

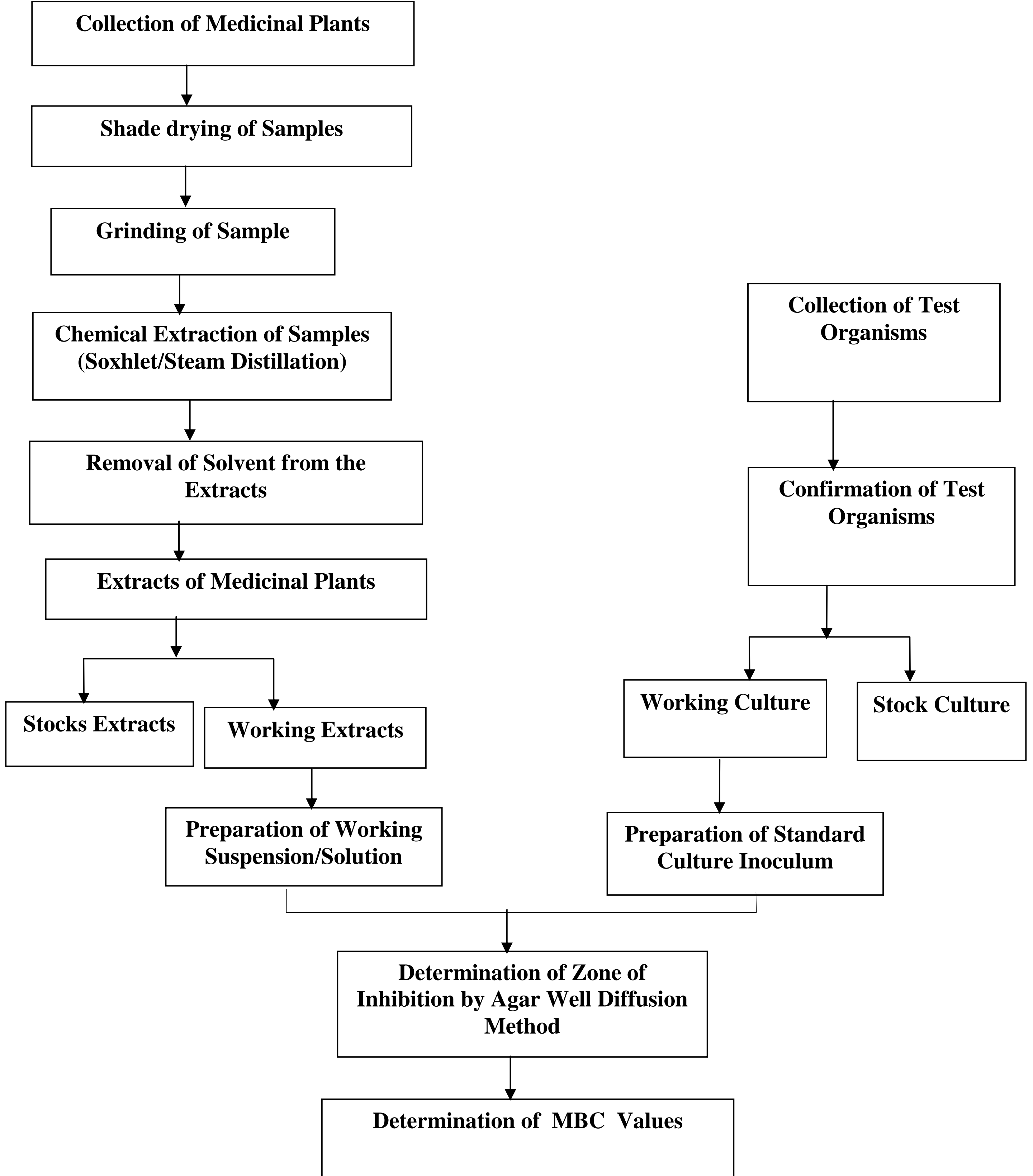
Note: mcg = micro-gram

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

METHODS

Flow Chart of The Methods

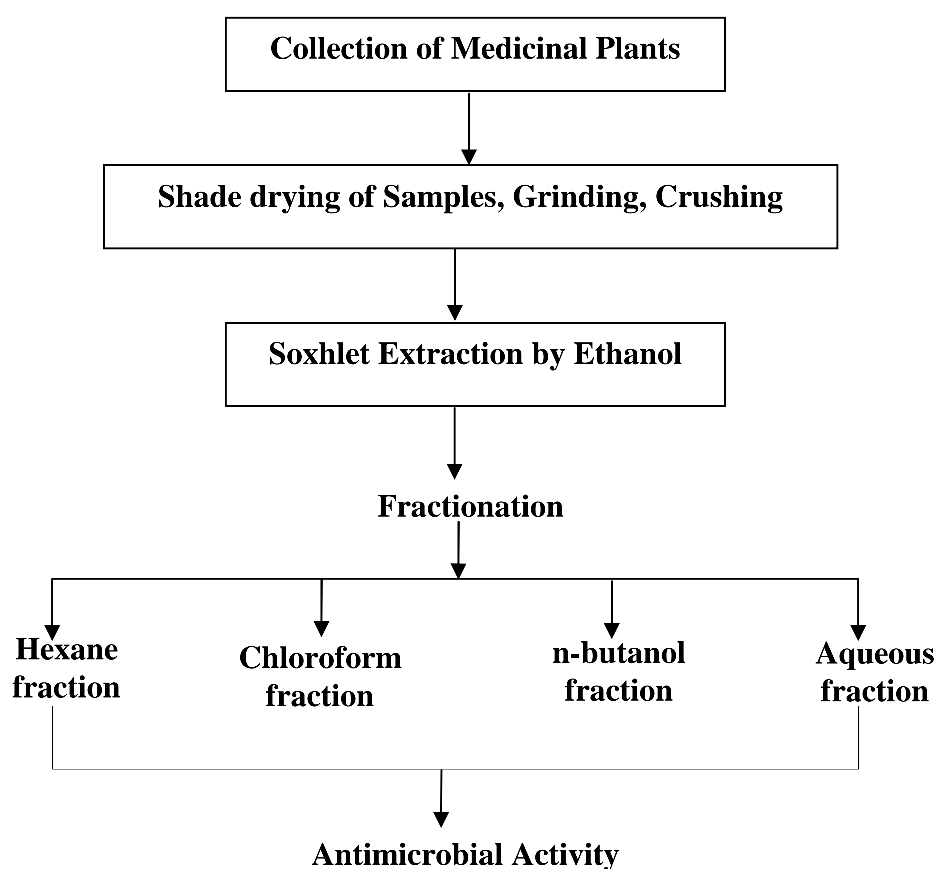
1. Screening and Evaluation of Antimicrobial Activity of Different Medicinal Plants



Bioassay Guided Fractionation of *Glycyrrhiza glabra* Linn

Methodology

(Bioactive Compound)



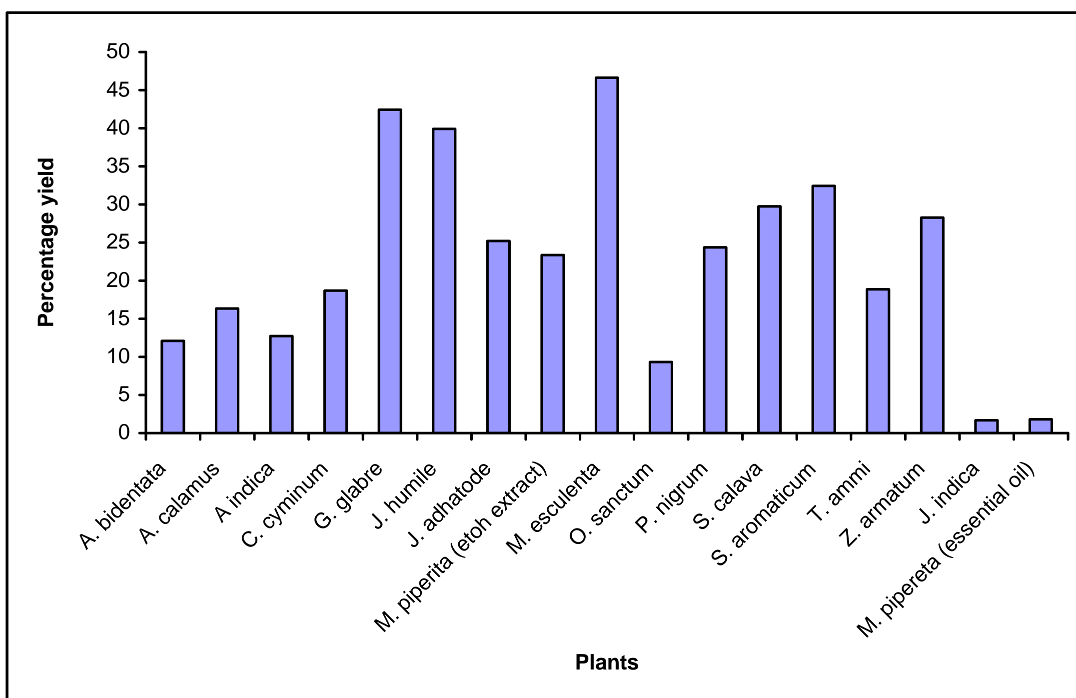


Figure 1: Percentage yield of different extracts from different plant species

The figure 1 shows maximum percentage yeild of *M. esculenta* followed by *G. glabra* respectively. The lowest yield was obtained by essential oil of *J. india* and *M. piperita*.

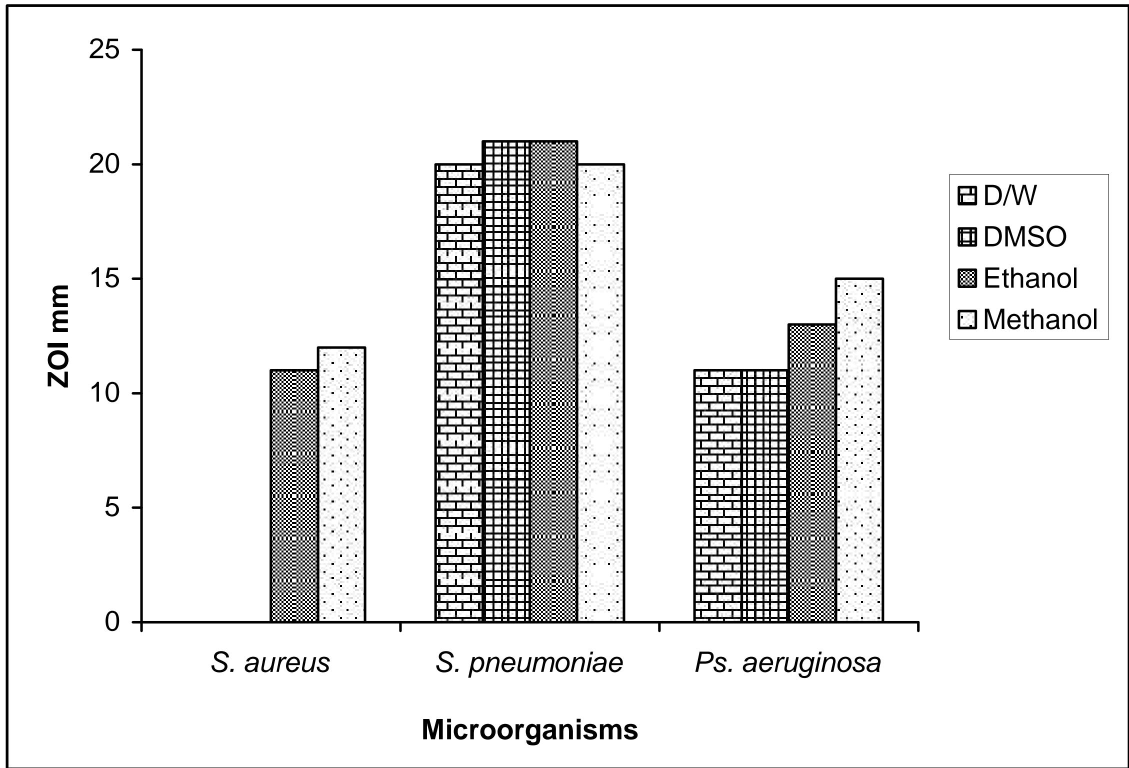


Figure 2: ZOI of *A. bidentata* against test bacteria

The figure 2 shows maximum zone of inhibition of *A. bidentata* against *S. pneumoniae.*

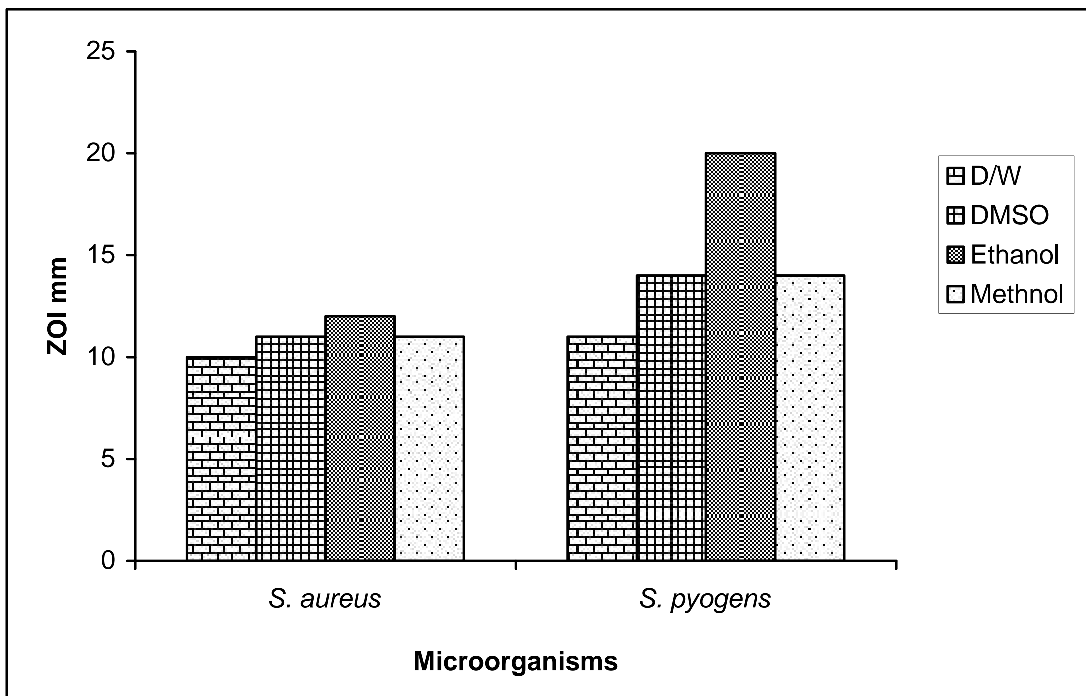


Figure 3: ZOI of *A. calamus* against test bacteria

The figure 3 shows maximum zone of inhibition of *A. calamus* against *S. pyogens*.

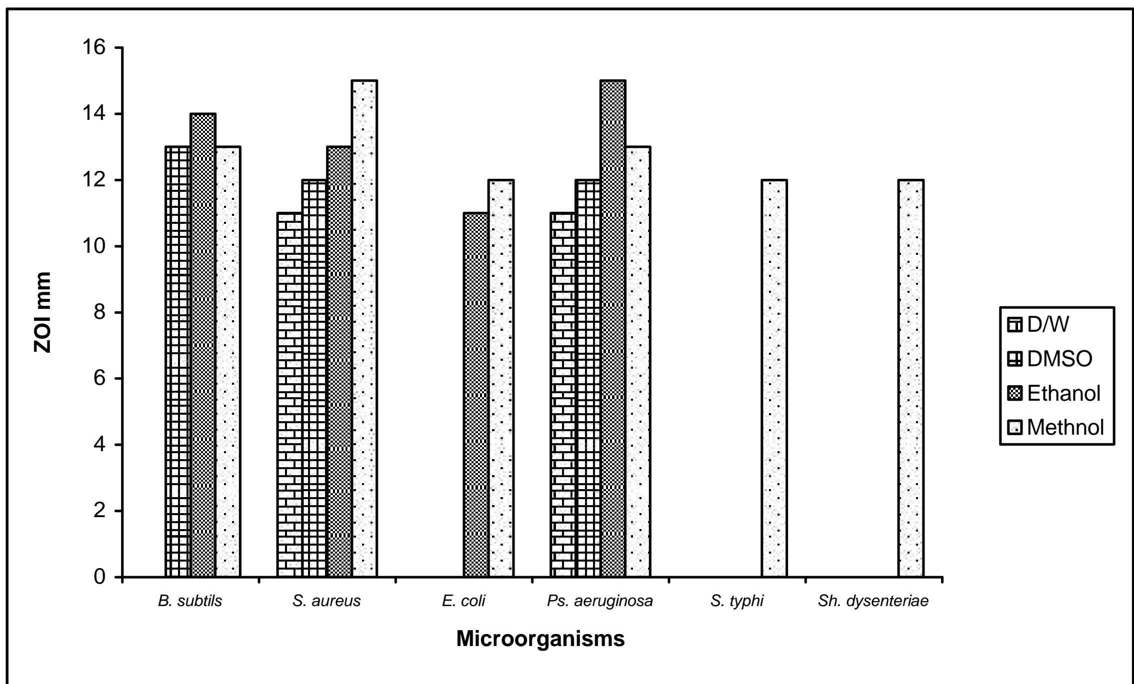


Figure 4: ZOI of *A. indica* against test bacteria

The figure 4 shows maximum zone of inhibition of *A. indica* against *S. aureus* and *Ps. aeruginosa.*

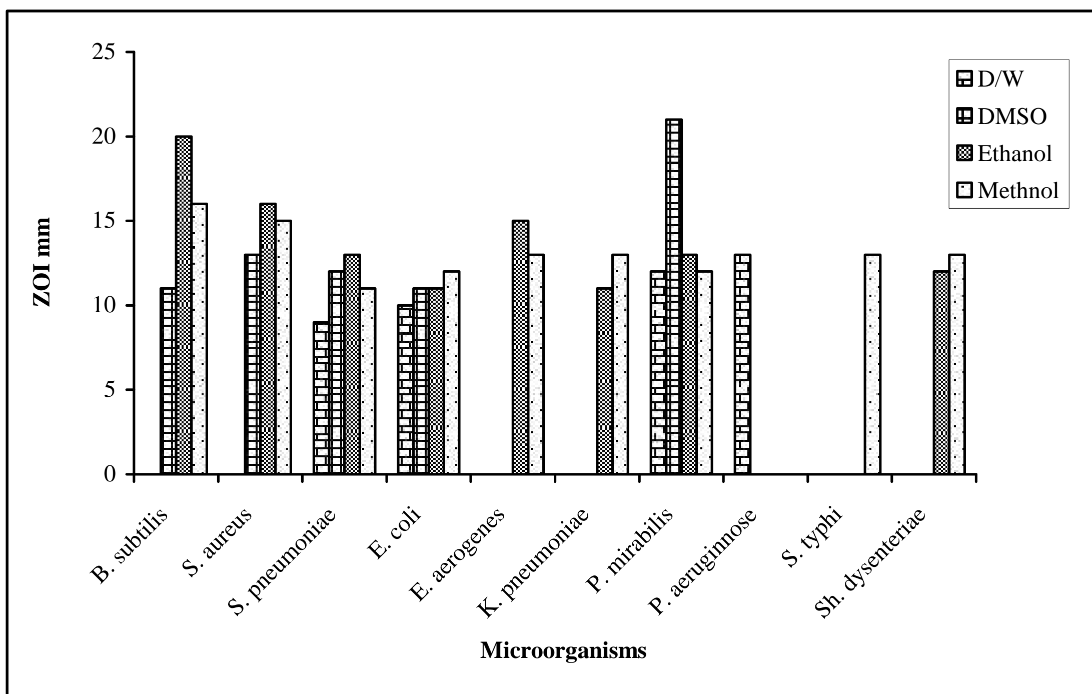


Figure 5: ZOI of *C. cyminum* against test bacteria

The figure 5 shows maximum zone of inhibition of *C. cyminium* against *B. subtilis* and *P. mirabilis*.

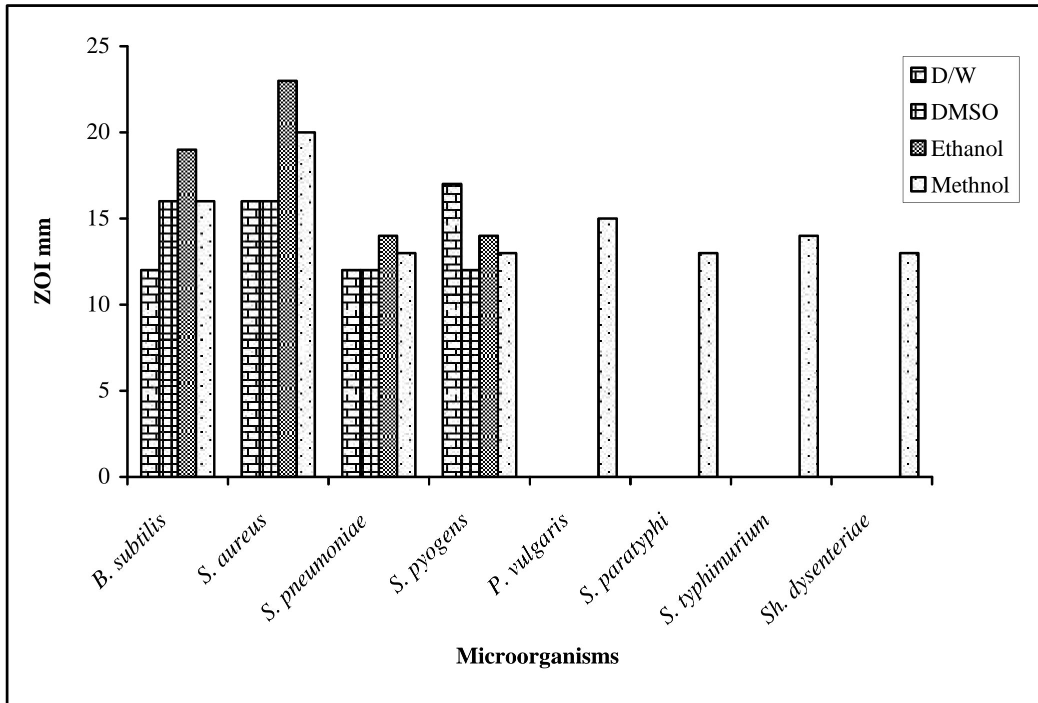


Figure 6: ZOI of *G. glabra* against test bacteria

The figure 6 shows maximum zone of inhibition of *G. glabra* against *B. subtilis* and *S. aureus*.

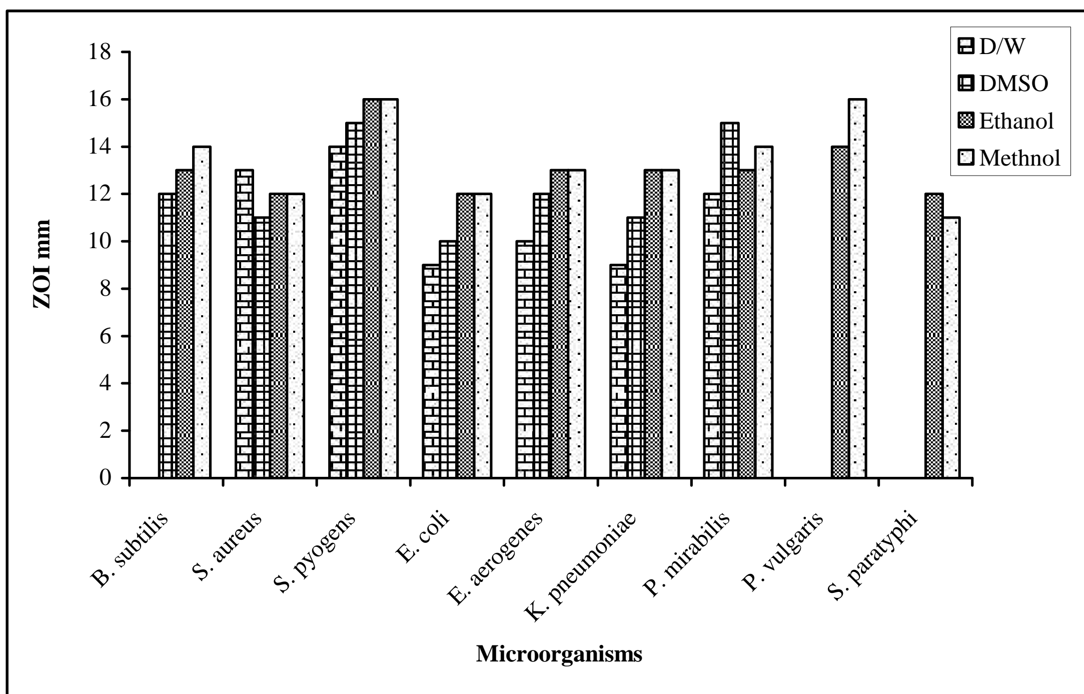


Figure 7: ZOI of *J. humile* against test bacteria

The figure 7 shows maximum zone of inhibition of *J. humile* against *S. pyogens* and *P. vulgaris*.

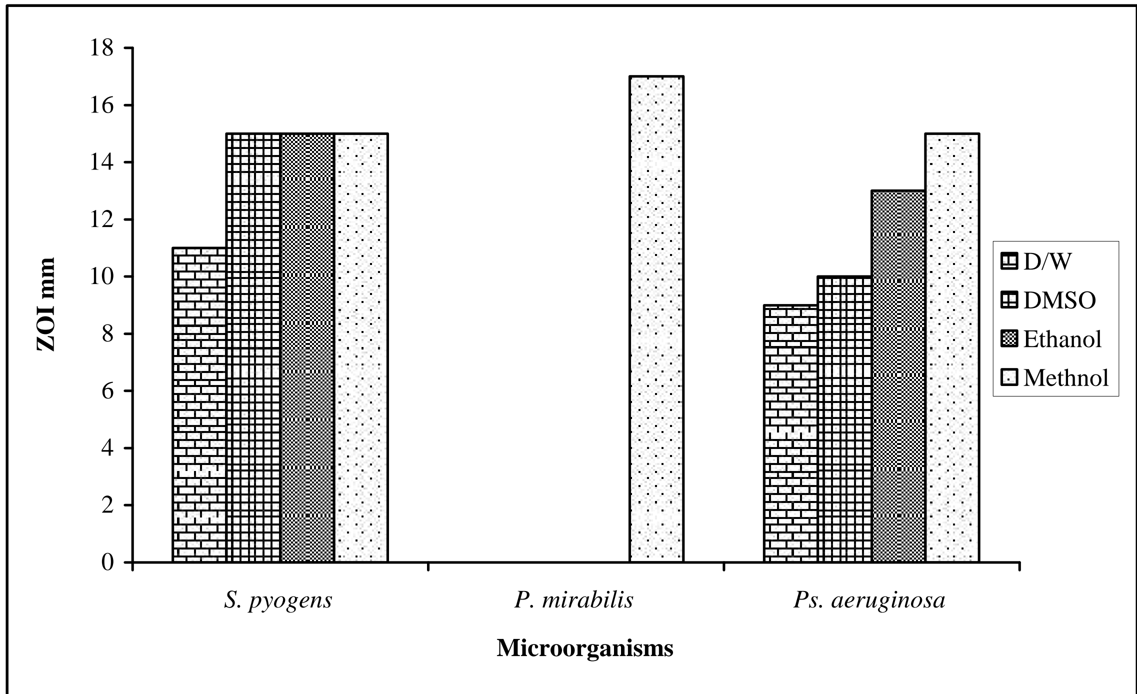


Figure 8: ZOI of *J. adhatoda* against test bacteria

The figure 8 shows maximum zone of inhibition of *J. adhatoda* against *P. mirabilis*.

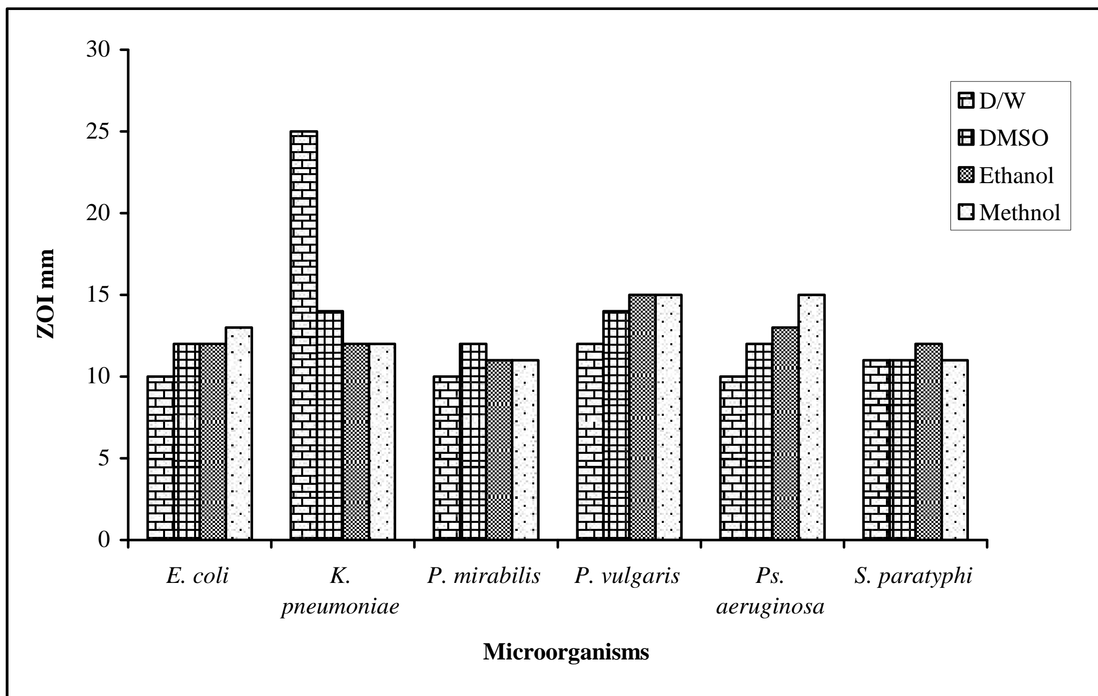


Figure 9: ZOI of *M. pipereta* (etoHextract) against test bacteria

The figure 9 shows *M. piperita* (etoH) extract most active inhibitor against *K. pneumoniae*.

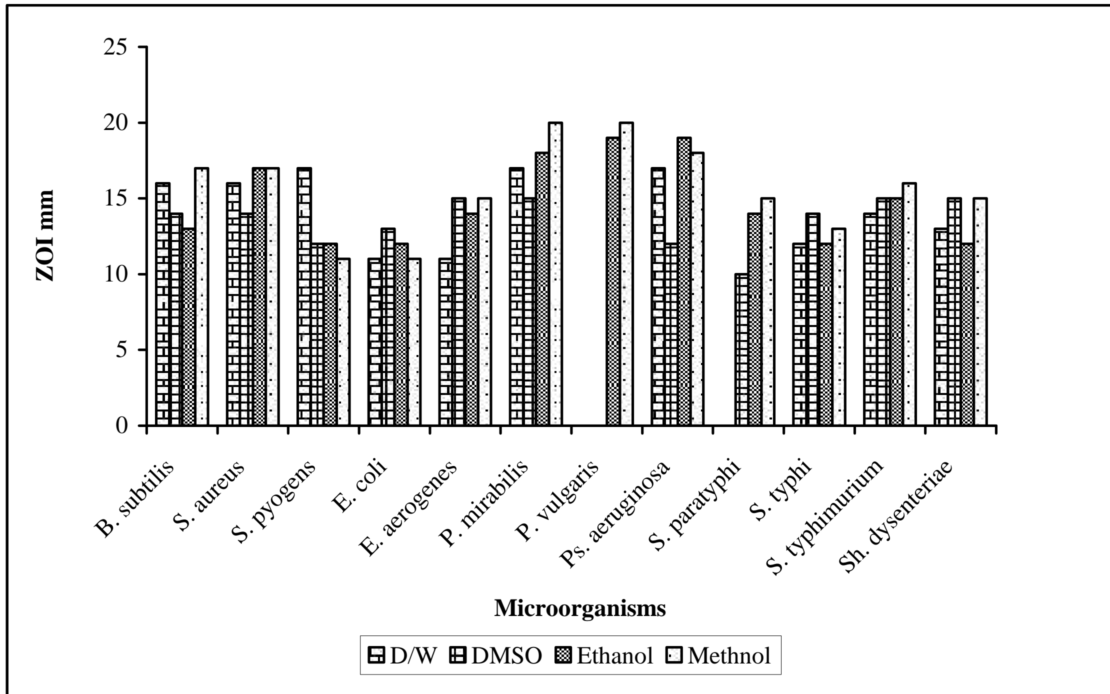


Figure 10: ZOI of *M. esculenta* against test bacteria

Figure 10 shows moderate zone of inhibition of *M. esculenta* against most of the beacteria texted.

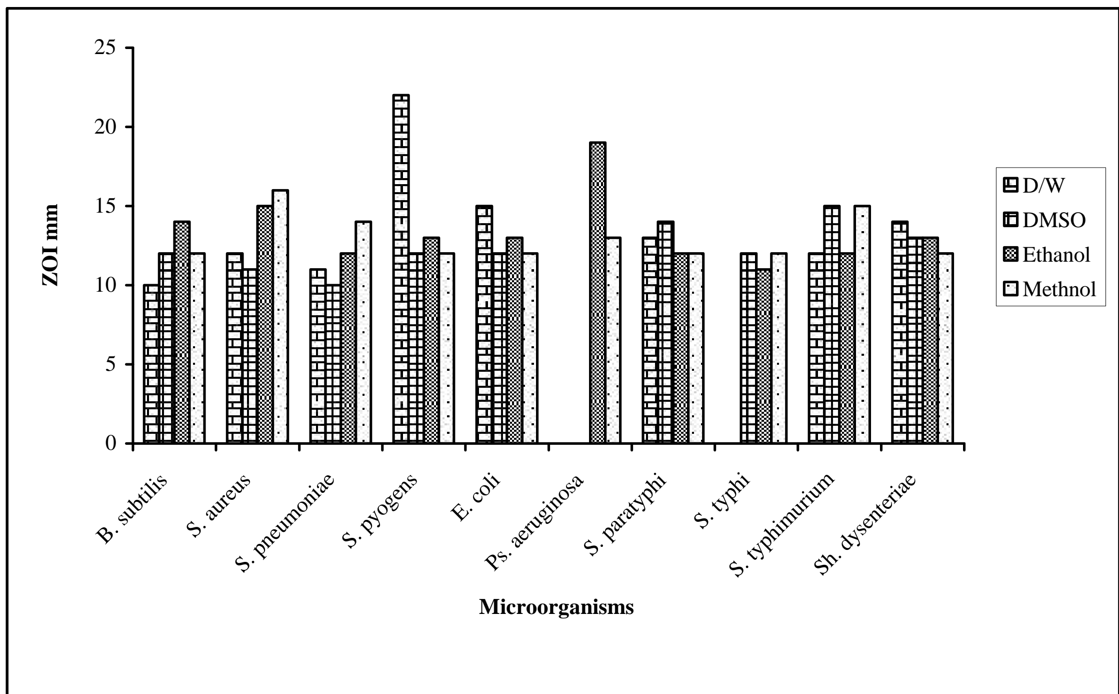


Figure 11: ZOI of *O. sanctum* against test bacteria

The figure 11 indicates maximum zone of inhibition of *O. sanctuan* against *S. pyogens* and *Ps. aeruginosa*.

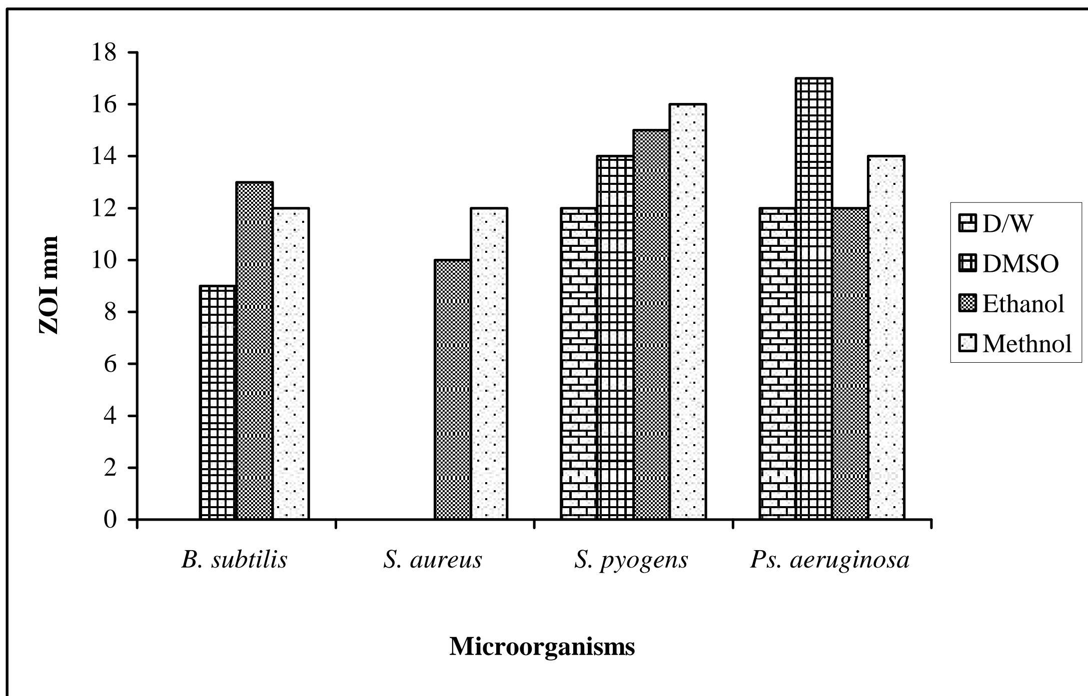


Figure 12: ZOI of *P. nigrum* against test bacteria

The figure 12 indicates maximum zone of inhibition of *P. nigrum* against *Ps. aeruginosa* and *S. pyogens*.

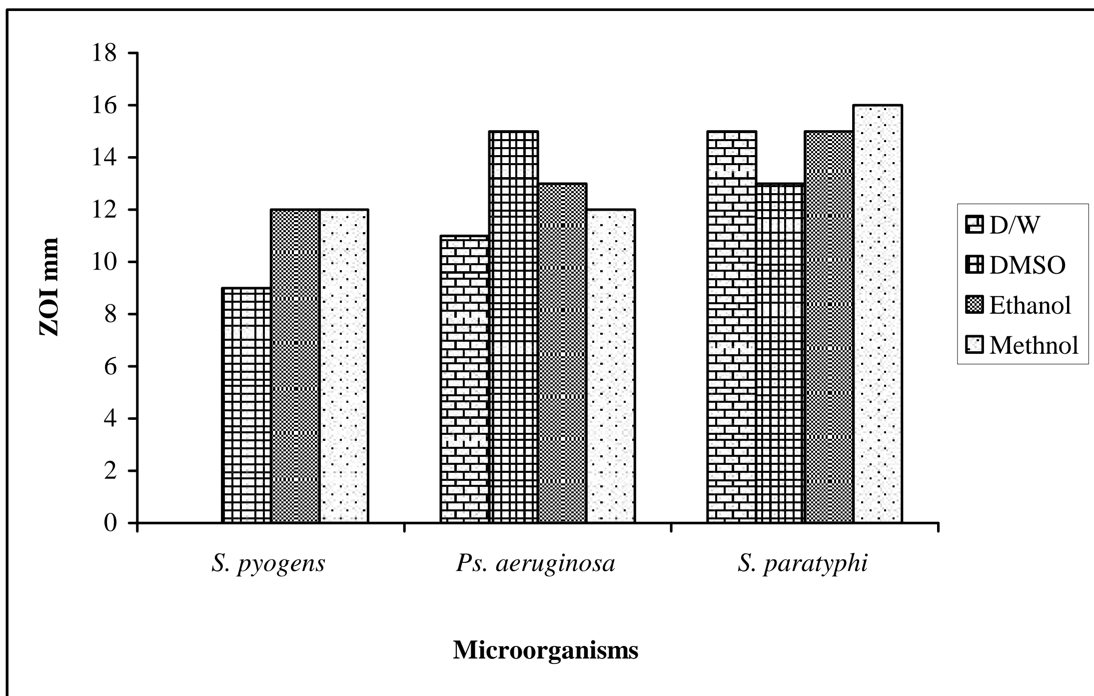


Figure 13: ZOI of *S. calava* against test bacteria

The figure 13 indicates maximum zone of inhibition of *S. calava* against *S. paratyphi* and *Ps. aeruginosa*.

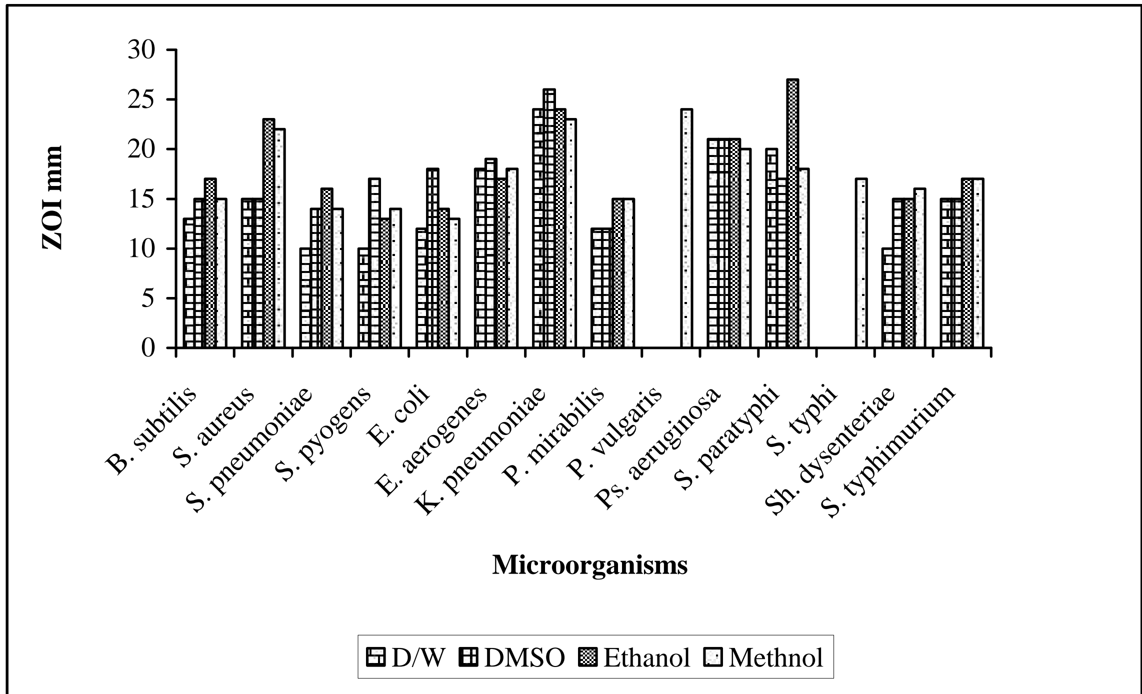


Figure 14: ZOI of *S. aromaticum* against test bacteria

The figure 14 indicates that the zone of inhibition of *S. aromaticum* is moderate against most of the test bacteria.

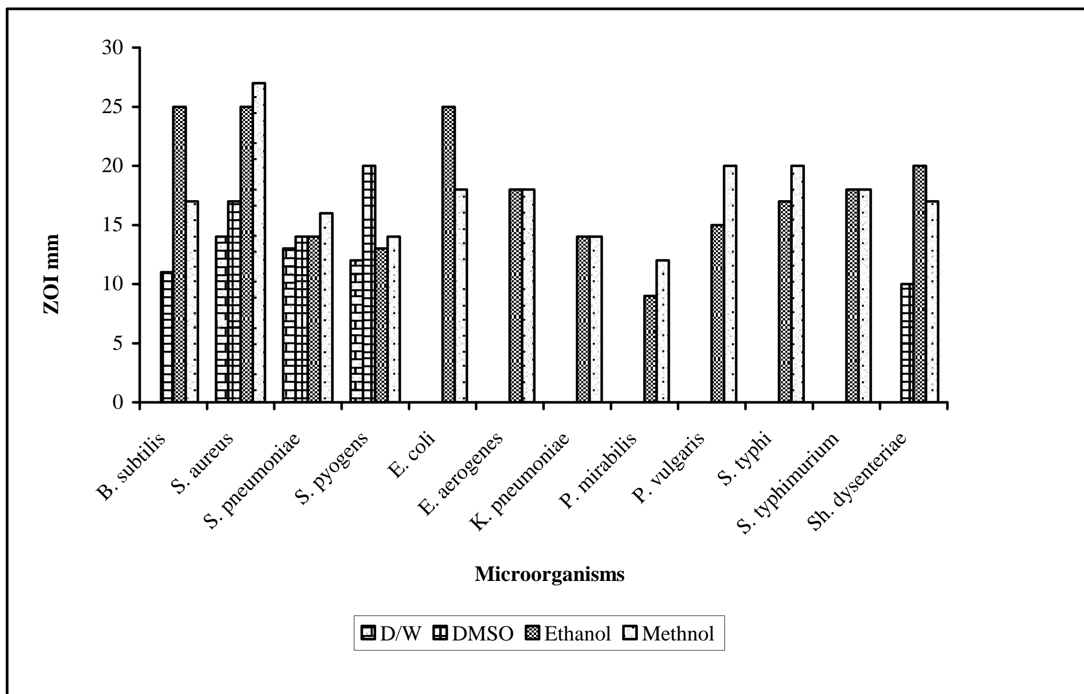


Figure 15: ZOI of *T. ammi* against test bacteria

The figure 15 indicates the maximum zone of inhibition of *T. ammi* against *S. aureus, B. subtilis* and *E. coli.*

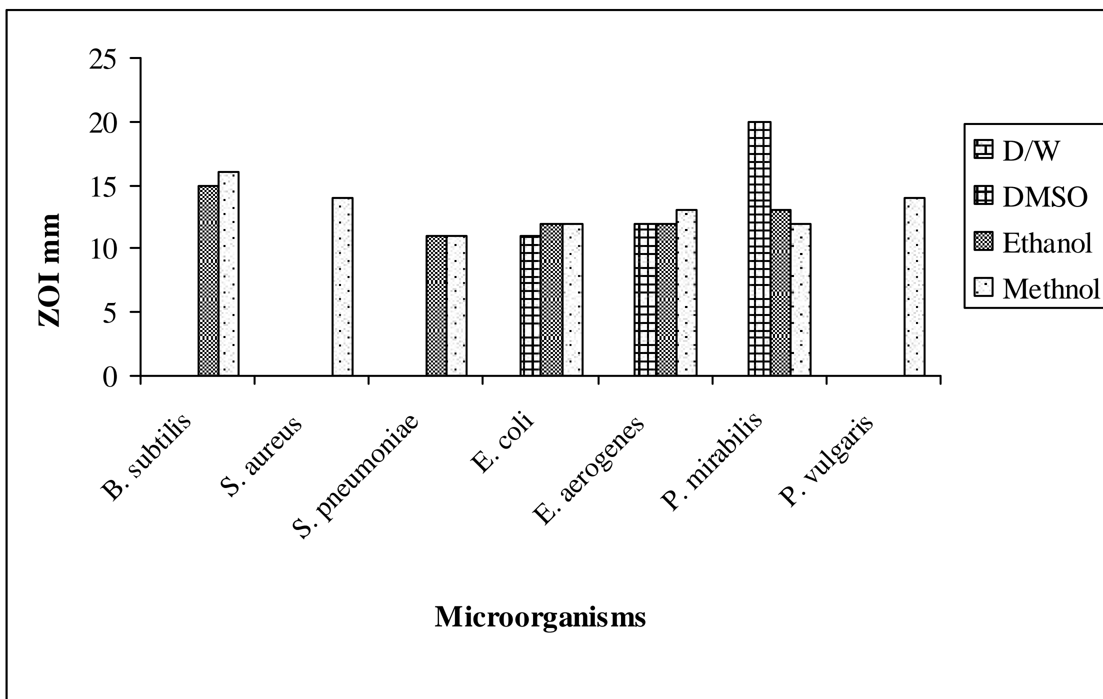


Figure 16: ZOI of *Z. armatum* against test bacteria

The figure 16 indicates maximum zone of inhibition of *Z. armatum* against *P. mirabilis*.

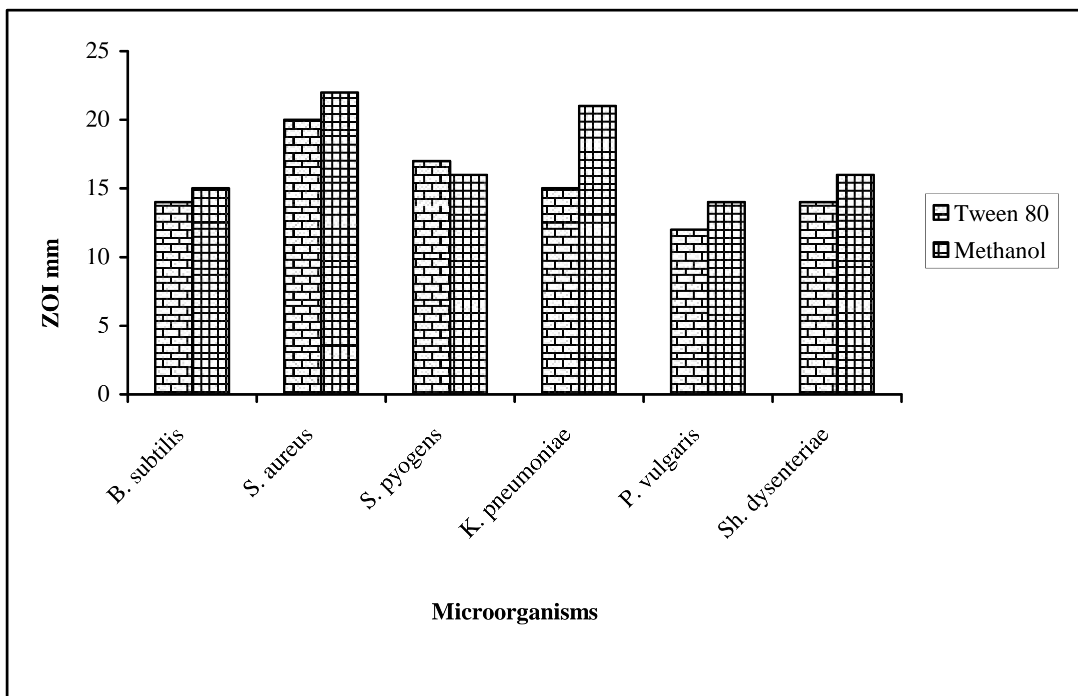


Figure 17: ZOI of essential oil of *Juniper indica* against test bacteria

The figure 17 indicates highest zone of inhibition of *J. indica* against *S. aureus.*

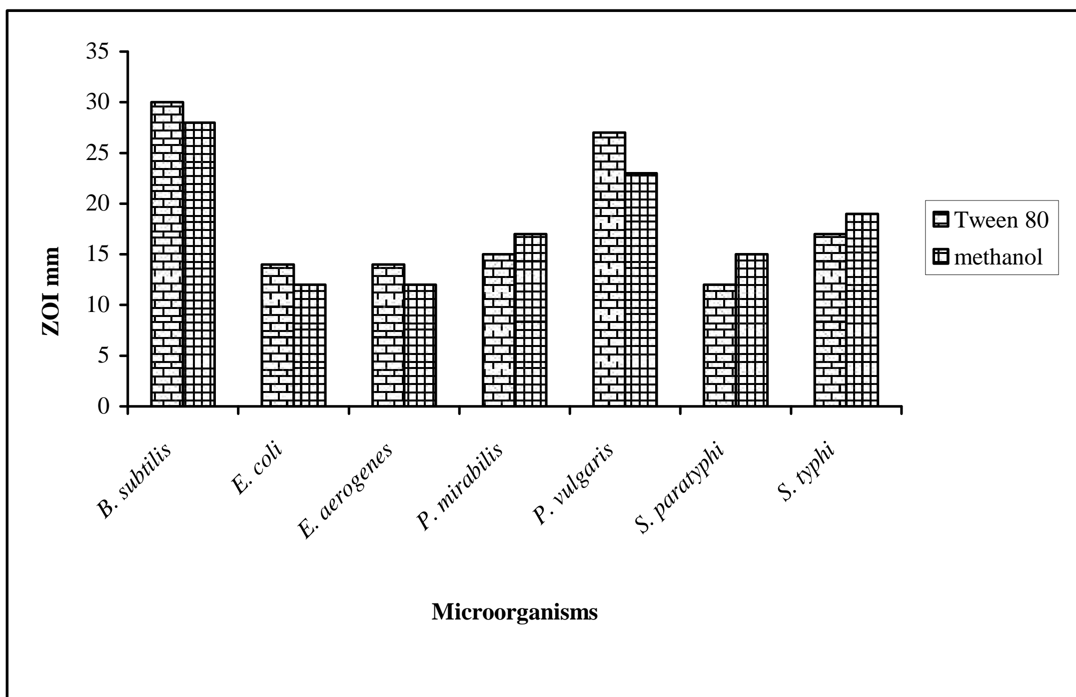


Figure 18: ZOI of essential oil of *M. piperita* against test bacteria

The figure 18 shows maximum zone of inhibition of *M. piperita* (oil) against *B. subtilis* and *P. vulgaris*.

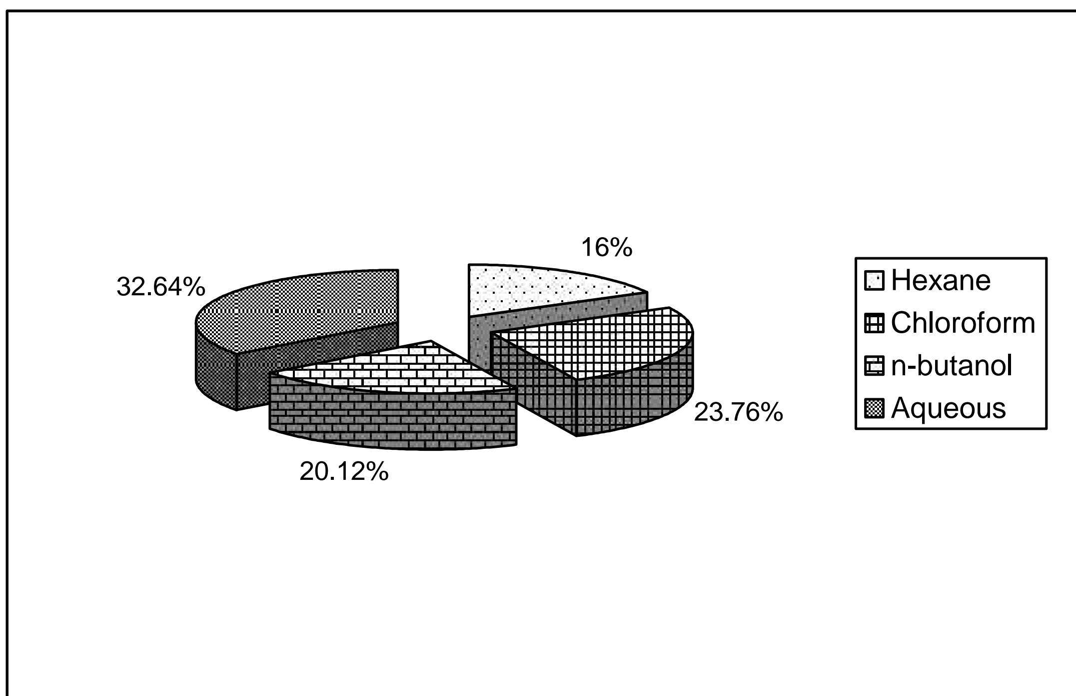


Figure 19: Percentage Yields of Different Fractions of *Glycyrrhiza glabra*

The pie chart indicates maximum and minimum percentage yield of different solvent fractions of *Glycyrrhiza glabra*.