Study on the Antimicrobial and Antioxidant Activities of Some Herbal Plants of Sikkim

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SIKKIM UNIVERSITY
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DECLARATION

I declare that the thesis entitled **"Studies on the Antimicrobial and Antioxidant Activities of Some Herbal Plants of Sikkim"** submitted by me for the award of **Master of Philosophy (M.Phil) Degree in Microbiology** of Sikkim University is my original work. The content of this thesis is based on the experiments which I have performed myself. This thesis has not been submitted for any other degree to any other University. The content of this M.Phil. Dissertation has been subjected to the Anti-Plagiarism Software (Ephorus) and it was found satisfactory.

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Date: 25th June 2012.

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Certificate

This is to certify that the thesis entitled **"Study on the Antimicrobial and Antioxidant Activities of Some Herbal Plants of Sikkim"** submitted to the **Sikkim University** for the award of **Master of Philosophy (M.Phil.)** degree **in Microbiology,** embodies the results of *bona fide* research work carried out by **Ms. Pramila Koirala** under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate-ship and fellowship.

All the assistance and help received during the course of the investigation have been acknowledged by her.

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PRAMILA KOIRALA

CONTENTS

LIST OF ABBREVIATIONS USED

1. BSI- Botanical Survey of India
2. DMSO- Dimethyl Sulphoxide
3. DPPH- 2, 2 Diphenyl-1- Picryl Hydrazyl
4. GAE- Gallic Acid Equivalent
5. MIC- Minimum Inhibitory Concentration
6. MRSA- Methicillin Resistant *Staphylococcus aureus*
7. MTCC- Microbial Type Culture Collection Centre
8. NCCLS- National Committee for Clinical Laboratory Standard
9. UTI-Urinary Tract Infection

1. INTRODUCTION

1. INTRODUCTION

"Antimicrobials are the broad classes of substances acting against microorganisms by either killing or inhibiting their growth" (Maartens *et al.*, 2011). Antimicrobial agent disrupts microbial processes or structures of pathogens by hampering cell wall synthesis, disrupting microbial membrane structure and function, inhibiting microbial protein and nucleic acid synthesis or blocking metabolic pathway through inhibition of key enzymes (Goswami and Purohit, 2001; Tenover, 2006).

Microorganisms are ubiquitous in nature and are most adaptable life forms on this planet (Mishra *et. al.*, 2010). They have extremely short generation times and ability to survive in hostile environments. Many microorganisms are beneficial to human beings, for example; they colonize in the guts and provide key enzymes for digestion and produce metabolites that are essential for normal biological function (Bengmark, 1998). But beside their beneficial effects they are responsible for numerous infectious diseases such as tuberculosis, pneumonia, toxic shock syndrome, which are difficult to treat (Baarlen *et. al.*, 2007). Treatments of infectious diseases were dramatically improved when penicillin was discovered by Alexander Fleming in early 1940s; hence many infections which were incurable became curable (Sharma *et al.*, 2010). The discovery and development of antibiotic in 19th century have decreased numerous health hazards resulting from microbial infections. However, microorganisms developed the ability to defend themselves by evolving resistance against many of the antimicrobial drugs (Pieboji *et al.*, 2009; Tenover, 2006). Alexander Fleming predicted the future and possibility of antimicrobial resistance when he said "The time may come when penicillin can be bought by anyone in the shops. Then there is a danger that the ignorant man may easily under dose himself and by exposing his microbes to non lethal quantities of the drug make them resistant"(Sharma *et al.*, 2010).

Development of resistance by microorganisms against many common antimicrobials mainly antibiotics because of their indiscriminate use has created a major challenge for the treatment of infectious diseases (Ahmad *et al.*, 1998; Dash *et al.*, 2005). Antibiotics are also associated with undesirable side effects (Cunha, 2001). All these facts necessitated a search for antimicrobial substances from alternative sources including plants. Plant based antimicrobials are advantageous over antibiotics as they have fewer side effects, are relatively less expensive, have better patient tolerance, have an

acceptance due to long history of use and are renewable in nature (Gur *et al.*, 2006). Since the ancient time the use of plant preparation as a source of medicine have made large contributions to human health and wellbeing (Abukakar *et al.*, 2008). The medicinal value of wide varieties of herbal plants has been documented in ancient Indian literature (Sampathkumar *et al*, 2008). Plant produces biologically active compounds to protect themselves from predators. Naturally occurring compounds found in dietary and medicinal plants, have been shown to posses antimicrobial activities (Kyung *et al.*, 2007). The antimicrobial properties of the medicinal plants are reported from all over the world and are used in treatment of many diseases (Ahmad and Beg, 2001).

All over the world, the scientists are exploring medicinal plants for finding out pharmacologically active compounds. Screening of medicinal plants for their phytochemicals, antimicrobial, antioxidant and anticancer activities is the prime concern (Agbafor *et al.*, 2011; Karmegam *et al*, 2008). Most of these types of works are concerned with the study of aqueous and solvent extracts of plant parts and testing of individual extract for selective pharmacological activities, such as antibacterial activity (Mishra and Mishra, 2011). Recent studies on synergistic effects of plant extract shows that the plant extracts in combination of two or more are exhibiting effective antibacterial activity against a wide range of microorganisms including drug resistant bacteria. However, their activity in combined form is unavailable. (Karmegam *et. al.*, 2012; Mabrouk, 2012).

Apart from antimicrobial property, herbal plants also posses' antioxidant activity. Antioxidants are the substances that fight against oxidant that causes formation of free radicals (Cooper, 1997). They also protect important cell components by being oxidised themselves or repairing the damage caused by oxygen (Cooper, 1997). Antioxidants can be classified as water soluble- acts primarily in the aqueous phase or lipid soluble, which act in the lypophilic region of the cell. The hydrophilic antioxidant includes ascorbic acid and urea. Lipid soluble antioxidant includes ubiquinols, retinoids, carotenoids, tocopherol and flavonoids. These compounds have ability to scavange various free radicles and ROS (Jayaprakasha *et al.*, 2004).

In relation to pathogenesis of many important diseases, Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite

radicals, play an important role in oxidative stress (Shyur *et al.*, 2005; Finkel and Holbrook, 2000). Normally in healthy individuals, the production of free radicals is balanced by the antioxidative defence system. Oxidative stress is generated when equilibrium favours free radical generation as a result of a depletion of antioxidant levels. The oxidation of biological molecules such as lipid, DNA, protein, carbohydrates by toxic ROS may cause DNA mutation and damage target tissues. This often results in cell death or may lead to cancer (Park and Pezzuto, 2002). Knowledge and application of potential antioxidant activities in reducing oxidative stresses has prompted many researchers to search for potent and cost-effective antioxidants from various sources including plants (Park and Pezzuto, 2002; Zi *et al,*. 1997; Hu and Kitts, 2000; Chiang *et al.*, 2004; Wang *et al.*, 2004). It has been reported that the majority of the antioxidant activity in plant extract is due to the presence of phytochemicals such as flavones, isoflavones, flavonoids, anthocyanin, coumarin lignans, catechins and isocatechins (Khalaf *et al.*, 2008).

Flavonoids present in plants act as antioxidants by stabilising the reactive oxygen species. Radicals are made inactive due to high reactivity of the hydroxyl group of the flavonoids (Robert *et al.*, 2001). Phenolic compounds present in the plants have potential health benefits mainly due to their antioxidant properties such as ROS scavenging, electrophile scavenging and metal chelation. Some epidemiological studies support the relation between the consumption of phenolic rich food products and low incidence of coronary heart disease, stroke and certain forms of cancer (Ibrahim and Jaafar, 2012). It has also been reported that the phenolic compounds exhibit certain pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, and antioxidant activity (Randhir *et al.*, 2004).

Medicinal plants have been used since ancient times to treat human diseases as it represents a rich source of antimicrobial agents (Khan *et al.*, 2008). Nearly 80% of the world's population rely mainly on traditional therapies which involve the use of plant extracts or their active substances (WHO, 1993). Plants contain phytochemicals which are responsible for antimicrobial activity (Nascimento *et al.*, 2000). Phytochemicals are wide variety of chemical compounds produced by plants, which includes flavanoids, tannins, saponin, alkaloids and vitamins. It has been reported that many of these

compound possess antimicrobial and antioxidant activity which protect human against many diseases (Mojab *et al.*, 2003; Nascimento *et al.*, 2000).

Sikkim has a unique geographical, topographical, climatic and ecological position that makes it a treasure house of bio-diversity (Singh *et al.*, 2002; Tamang, 2010). The region has vast reserve of medicinal plants and rich culture of folk medicine. More than 400 plants are used by different tribes of Sikkim to cure various diseases and very few of these are used on commercial basis (Singh *et al.*, 2002; Hussain and Hore, 2007). Therefore, it is necessary to establish a scientific rationale for the use of these traditional plants for prevention and cure of various diseases.

For our study we have selected some herbal plants of Sikkim region which are consumed as vegetable in regular diet and also have ethnic medicinal values, as well as herb used only for medicinal purposes. Plants selected for study include *Cyphomandra betacea* (Local name-'*Rookh tamater*'), *Capsicum annuum* var *cerasiforme* (Local name-'*Dallhae khorsani*'), *Dicentra scandens* D. Don (Local name-'*Jogi laharra*', '*Kundaley*') and *Heracleum nepalense* (D.Don) Walpers (Local name-'*Chimphing*'), (Singh *et al.*, 2002; Sharma and Sharma, 2010). *Capsicum annuum* and *Cyphomandra betacea* are part of our regular diet and also possess some medicinal value.

Cyphomandra betaceae belongs to the family *Solanaceae* and is a rich source of vitamin C, B_{12} and Niacin (The Wealth of India, 1950). Fruit of *Cyphomandra betaceae* is used to prepare "*chutney*"or pickle by the people of this region and is part of diet. An invertase inhibitory protein isolated from *Cyphomandra betacea* has broad spectrum antimicrobial activity against plant pathogens (Ordonez *et al.*, 2006). Its antimicrobial activity against human pathogens has not been investigated.

Capsicum annuum belongs to the family *Solanaceae* (Sharma and Sharma, 2010). Fruit of *Capsicum annuum* has carminative properties and is used as an appetizer. It is useful in chronic ulcers and stomach disorders (Sharma and Sharma, 2010). People residing in rural areas of this region believe that the *Capsicum annuum* locally called as '*Dallhae khorsani*' cures gastritis.

Rural people cultivate and sell the *Capsicum annuum* in the local market in good price as compared to other varieties of *Capsicum*. It has become a source of income for many people in rural areas. Fruit of *Capsicum annuum* is usually consumed as a part of

regular diet by the people of this region. The fruit is soaked in salted water and kept in air tight container and are stored for long period of time. During off season, people consume preserved fruits of *Capsicum annuum*.

There are different types of pepper available across the globe such as *Capsicum annuum*, *Capsicum frutescens*, and *Capsicum chinese*. Among the species, there are different varieties of *Capsicum*. Work has been done to evaluate antimicrobial and antioxidant activity of *Capsicum* species. But no work has been reported for the antimicrobial and antioxidant activity of *Capsicum annuum* var. *cerasiforme*.

Heracleum nepalanse is a plant belonging to the family *Umbillifereae* and is extensively used in folk medicine. Fruit of *Heracleum nepalense* is taken against stomach disorder, cough, cold, nausea and diarrhoea. Decoction of root is taken as tonic (Singh *et al.*, 2002). The root of *Heracleum nepalense* is reported to have antimicrobial and antioxidant activity (Dash *et al.*, 2005). Fruit of the plant is used against many diseases but the antimicrobial and antioxidant property of fruit has not been investigated.

Dicentra scandens is a climbing herb with a perennial rootstock and is widely used in traditional medicine. It belongs to the family *Fumariaceae.* Juice extracted from the root of *Dicentra scandens* is believed to cure gastritis by the traditional healers. Leaf paste or juice is applied on cuts and wounds (Singh *et al.*, 2002; Sharma and Sharma, 2010). As per the reference of local people *Dicentra scandens* is locally called as '*Kanchi lahara*'/ '*Kundley*'/ '*Jogi lahara*' but traditional healers never disclose the name of the medicinal herbs. They believe that, by disclosing the name, the medicinal property of herbal plants will be lost. Due to this reason the traditional knowledge are getting lost generation after generation. It is therefore important to preserve the traditional knowledge. *Dicentra scandens* is herb used only for medicinal purpose, but the medicinal property has not been investigated. Therefore scientific investigation is required to validate their medicinal properties.

For our study we have chosen these plants to determine their antimicrobial and antioxidant properties. There are not sufficient scientific studies to support the antimicrobial activity of these plants. The study looks into antimicrobial activity of these plants against some Gram positive and Gram negative bacteria, which are the

common cause of microbial infection and diseases in human beings. Most of the severe infections in human beings are caused by bacteria like different species of *Bacillus, Staphylococcus, Klebsiella, Pseudomonas* and *Escherichia coli* (Shihabudeen *et al.*, 2010).

To evaluate the antimicrobial activity the test microorganisms belonging to both Gram negative and Gram positive bacteria were selected. Gram negative bacteria include *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus vulgaris.* Gram positive bacteria were *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus.* These organisms were selected for the study as they are common pathogens causing different types of human diseases like urinary tract infection, kidney infection and food borne illness (Talaro, 2008).

Rationale and scope of the study

Sikkim being a biodiversity hotspot zone, there are number of indigenous plants having medicinal value and most of the plants are still unexplored. The region has a rich culture of folk medicine too (Singh *et al.*, 2002). Therefore, it is important to scientifically validate this traditional knowledge.

The wide use of antibiotics in treatment of bacterial infections has lead to the emergence and spread of resistant strains (Dash *et al.*, 2005). This has posed a serious problem in the treatment of many infectious diseases. Antibiotics are sometimes associated with many adverse side effects (Cunha, 2001). Plants provide an alternative strategy as a good source of antimicrobial agent. Plant based antimicrobials are advantageous over antibiotics because of easy availability, accessibility; cost effectiveness and posses fewer side effects (Gur *et al.*, 2006).

Free radicals are implicated in several disease processes such as carcinogenesis, ageing, fibrosis, inflammation. The oxidation of biological molecules such as lipid, DNA, protein, carbohydrates by toxic reactive oxygen species may cause DNA mutation and damages target tissues (Shyur *et al.*, 2005). Extracts from plant materials are reported to have antioxidant activities that help in reducing oxidative stresses (Park and Pezzuto, 2002; Zi *et al.*, 1997).

Major source of nutritional requirements in our day to day life is fulfilled by plant sources. Most of the herbal plants have been used in traditional folklore medicines (Singh *et al.*, 2002). Much of the traditional knowledge about medicinal plants has not been scientifically investigated and requires experimental analysis to validate its medicinal properties. This will consequently lead to the usage of the plants as economic and safe alternative to treat various diseases caused by microorganisms.

With this background, the present study aims to evaluate the antimicrobial and antioxidant activities of selected herbal plants against the different test microorganisms.

Objectives of the present study

1. To evaluate the antimicrobial activity of selected herbal plants against different test microorganisms.

2. To evaluate the possible synergistic antimicrobial effect of different combinations of selected plants against different test microorganisms.

3. To examine the phytochemicals present in the crude extract of the selected herbal plants.

4. To evaluate the antioxidant activity of crude extract of the selected plants.

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Antimicrobial therapy has been used to treat infectious diseases since many years. Synthetic antibiotics are used for the treatment of many bacterial infections. However, overuse and misuse of antibiotics has lead to emergence of multi-drug-resistant strains of various microorganisms (Shai *et al.*, 2008). Phytochemicals from plants have been reported to possess antimicrobial properties hence antimicrobial agents derived from medicinal plants are gaining popularity (Fullerton *et al.*, 2011).

Plant based drugs are usually categorised into two types; namely first generation and second generation. First generation include usage of plants in their crude forms that is in their natural state. Some examples include cinchona and opium. The second generation plant based drugs involve the scientific processing of the plant extracts and use of their active constituents. An example includes quinine which is a phytoconstituent derived from cinchona (Iwu *et al.*, 1999).

Most of the phytochemicals are secondary metabolites produced by the plants (Preethi *et al.*, 2010). Phytochemicals such as aldehydes, phenolic compounds, steroids, tannins, resins, alkaloids and flavonoids are the major constituents or active principles of many plant based drugs. The combinations of these secondary products present in the plants are responsible for the medicinal effects of plant materials (Hashim *et al.* 2010). Flavonoids are synthesized by plants in response to microbial infection (Dixon *et al.*, 1983) and are found to be effective *in vitro* as antimicrobial substance against a variety of microorganisms. Tannins are polymeric phenolic substances possessing the astringent and antimicrobial property (Das *et al.*, 2010; Shyamala and Vasantha, 2010). Saponins are reported to have many pharmacological activities such as antibacterial, antifungal, antiviral, hepatoprotective anti-inflammatory and anti-ulcerative (Soetan *et al.*, 2006). Plant steroids are known to have antimicrobial properties and cardiotonic activity (Shyamala and Vasantha, 2010). Alkaloids are of pharmacological importance since it is reported to have antiprotozoal, cytotoxic, anti-inflammatory and antimicrobial properties (Karou *et al.*, 2005).

Antimicrobial activity of flavonoid is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Marjorie, 1999). Flavonoids also exhibit biochemical effects, it inhibits number of enzymes such

as aldose reductase, xanthine oxidase, phosphodiesterase, Ca^{+2}-ATPase, lipoxygenase, cycloxygenase (Narayana *et al.*, 2001). Tannins interfere with the protein synthesis by binding with proline rich proteins (Shimada, 2006). Saponin has been reported to have antimicrobial activity due to its ability to cause leakage of proteins and certain enzymes from the cell (Zablotowicz *et al.*, 1996). Antimicrobial actions of steroids are mainly associated with membrane lipid by causing leakages from liposomes (Epand, 2007).

The effectiveness of bioactive compounds in the treatment of various diseases contributed to their antimicrobial and antioxidant activities (Prasad *et al.*, 2005; Preethi *et al.*, 2010). Antioxidants scavenge free radical and hence provide protection to human against many diseases including cancer (Dash *et al.*, 2005; Polterait, 1997). Reactive Oxygen Species (ROS) such as superoxides, peroxides, hydroxyl radicals contributes to tissue and cell damage in many disease processes. There are some external sources of ROS like tobacco smoke, alcohol, pesticides, certain pollutants and microbial infection that contribute to cell damage (Dash *et al.*, 2005). It has been reported that the antioxidant effects of plant extracts are mainly due to the presence of phytochemicals such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Preethi *et al.*, 2010; Adedapo *et al.*, 2008). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which act as reducing agents. These compounds also possess wide range of medicinal properties, such as antiallergic, anti-inflammatory and antimicrobial (Balasundram *et al.*, 2006).

Several factors influence the concentration of the active phytoconstituent such as phenolic compounds present in the plants. Some of the factors include period of plant collection, geographical origin and climatic conditions. It has been reported that, all these factors sometimes attributes to even absence of active constituents in the same plant collected from different regions and hence absence in activity (Bilia, 2002; Houghton, 1998; Banerjee *et al.*, 2011).

The determination of phytochemicals present in the plants depends largely upon type of solvent used in the extraction procedure. The properties of good solvents used for extraction includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate (Tiwari *et al.*, 2011).

Water is a solvent used in various medicinal preparations. It is also used to extract plant products with antimicrobial activity. Though traditional healers use mostly water extracts, various antimicrobial assays also use water as the solvent to extract plant products. But plant extracts from organic solvents like methanol have been found to give more consistent antimicrobial activity compared to aqueous extract (Tiwari *et al.*, 2011).

Acetone is used as an extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. Phenolic compounds and tannins are better extracted in acetone than in methanol. Alcohol (methanol and ethanol) are good solvent for extraction of phytochemicals. Saponin, flavones, tannins, steroid are extracted in methanol. Most of the phytochemicals are better extracted in methanol, so methanol is used as solvent of extraction in most of the studies. Other solvents like Chloroform, ether are also used (Tiwari *et al.*, 2011).

Himalayan region is rich in vegetation and harbours a huge number of medicinal plants. Some of the plants of the region like *Lantana camara, Ageratum conyzoids and Eupatorium adenopphorum* are commonly found herbs. These plants are widely used in folk medicine as antimicrobial, antiseptic, antipyretic and used in treatment of colic pain, fever and diarrhoea (Padalia *et al.*, 2010). Many phytochemicals including essential oil, terpenoids and other biologically active constituents possessing antimicrobial properties have been reported from these plants (Padalia *et al.*, 2010; Kurade *et al.*, 2010).

Aconitum hetrophyllum is used traditionally to treat dyspepsia and flatulence. A phytoconstituent alkaloid, isolated from *Aconitum hetrophyllum* has been reported to have antimicrobial, antipyretic and enzyme inhibition activity (Ahmad *et al.*, 2008). *Acorus calamus* is a semi aquatic perennial herb and is widely used in traditional medicine. Rhizome powder is taken against fever, pneumonia, cough bronchitis, gastric trouble and sore throat. Paste made out of rhizome is applied on cuts, wounds and skin infections (Sharma and Sharma, 2010). The plant has been reported to have antimicrobial and antioxidant activity (Zahin *et al.*, 2009; Vasinauskiene *et al.*, 2006). *Drymeria cordata* is used traditionally for the treatment of sinusitis and the herb has been reported to have antibacterial property. It was found to be effective against *Staphylococcus aureus* (ATCC 29737), *Escherichia coli* (ATCC 10536), *Bacillus*

subtilis (ATCC 6633) and *Pseudomonas aeruginosa* (ATCC 25619) (Mukherjee *et al.*, 1998).

It has been reported that invertase inhibitory protein isolated from ripe fruit of *Cyphomandra betaceae* inhibited the growth of xylophagous and phytopathogenic fungi. The xylophagous fungi includes *Ganoderma applanatum*, *Schizophyllum commune*, *Lenzites elegans*, *Pycnoporus sanguineous*, *Penicillium notatum*, *Aspergillus niger*, *Phomopsis sojae* and *Fusarium mango* and phytopathogenic bacteria *Xanthomonas campestris* var vesicatoria CECT 792, *Pseudomonas solanacearum* CECT 125, *Pseudomonas corrugata* CECT 124, *Pseudomonas syringae* pv. *syringae* and *Erwinia carotovora* var carotovora (Ordonez *et al.*, 2006).

Extensive work has been done in antimicrobial activities of medicinal plants. Different extract from traditional plants have been investigated. There are many reports suggesting the effectiveness of antimicrobials extracted from plants against a wide range of microorganisms including *Escherichia coli, Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Pseudomonas aureginosa, Klebsiella pneumonia* (Rajput *et al.*, 2011; Mukherjee *et al.*, 1998).

Escherichia coli are predominant species in the intestine of humans. Many strains of *Escherichia coli* are not highly infectious but some strains have developed virulence due to plasmid transfer or as opportunists. It is one of the most frequent causes of many common bacterial infections including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI) and kidney infection. It causes diarrhoea due to the production of enterotoxin by the microorganism. *Escherichia coli* also cause inflammation and ulceration of large intestinal mucosa. *Escherichia coli* are the common cause of infantile diarrhoea. Apart from intestine *Escherichia coli* also invade extraintestinal sites such as urinary tract, kidneys and is responsible for 50%-80% of urinary tract infection (Talaro, 2008).

Klebsiella pneumonia belongs to the family *Enteriobactericiae*. It is a Gram negative, facultatively anaerobic and non motile bacteria. *Klebsiella pneumoniae* causes pneumonia (Talaro, 2008).

Pseudomonas is the most important genus of family *Pseudomonadaceae*. It is Gram negative, curved rods. *Pseudomonas aeruginosa* infect people with low resistance for

example cystic fibrosis patients. It causes urinary tract infection and also invades burn areas (Baarlen *et al.*, 2007; Talaro, 2008).

Proteus vulgaris is a member of family *Enteriobactericeae*. The organism can cause different types of infections. The organism can become deadly when in sinus and respiratory tissues, if left untreated (Sampathkumar *et al.*, 2008).

Bacillus cereus is a facultative anaerobe and is harmful to humans and cause food borne illness. This organism is common airborne contaminants and multiplies very readily in cooked foods such as rice, potatoes and meat. The spores of *Bacillus cereus* survive short periods of cooking and reheating. Hence the spores germinate and releases enterotoxins when food is stored at room temperature. Therefore, the ingestion of such contaminated food causes nausea, vomiting, diarrhoea and abdominal cramps (Talaro, 2008).

Bacillus subtilis cause disease in severely immunocompromised patients and it may contaminate food rarely causing food poisoning (Talaro, 2008). Despite of using various preservation method food poisoning is still a concern for both consumers and the food industry due to the increasing number of illness outbreaks caused by some pathogenic and spoilage microorganisms in foods. The increasing antibiotic resistance of some pathogens that are associated with food borne illness is another concern for food processors, food safety researchers and regulatory agencies (Shan *et al.*, 2007).

Staphylococcus aureus is one of the most important organisms that cause many human diseases. As nosocomial or hospital acquired pathogen, *Staphylococcus aureus* have caused considerable morbidity and mortality in early 1950s and 1960s. Later, emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has become a major problem in treatment of nosocomial infections. One way by which *Staphylococcus aureus* become resistant is through acquisition of *mecA*, a chromosomal gene that encodes an alternate target protein which is not inactivated by methicillin family of β-lactam drug. Many strains of *Staphylococcus aureus* are also resistant to many commonly used antibiotics including latest generation of cephalosporin (Prescott *et al.*, 2005).

Most of the bacterial infections are cured but some of the strains are acquiring resistance to almost all the available antibiotics. Bacteria may be intrinsically resistant

or may acquire resistant gene from other organisms. The resistant genes enable a bacterium to produce enzymes that inactivate the action of antibacterial drug. It prevents the drug from reaching its intracellular target, modifies the target site of drug and bypasses the action of the drug by producing alternative metabolic pathway (Tenover, 2006).

Bacterial resistance is a matter of serious concern because resistant bacteria, particularly *Staphylococci, Enterococci, Klebsiella pneumoniae,* and *Pseudomonas* sp., are associated with the mortality of patient having bloodstream infection. Bacterial resistance to antibiotics often results in treatment failure in critically ill patients (Tenover, 2006).

There is immediate need of alternative source of antimicrobial drugs to overcome the problem of drug resistance. Plant derived potentially useful structures are important source for the development of chemotherapeutic agents (Hidayathulla *et al.*, 2011).

3. MATERIALS AND METHODS

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3.1. Culture media used

Nutrient broth

Composition:	**Grams/litre**
Sodium chloride (Merck, ME9M591006)	5
Peptone (Himedia, RM001)	5
Yeast extract (Himedia, CR027)	2
Beef extract (Himedia, RM002)	1

Nutrient agar

Composition:	**Grams/litre**
Sodium chloride	5
Peptone	5
Yeast extract	2
Beef extract	1
Agar (Himedia, RM666)	15

3.2. Chemicals used

All the chemicals and reagents were obtained from Merck and Himedia.

1. DPPH (2,2-diphenyl-l-picryl hydrazyl)- Himedia, RM5169
2. Ascorbic acid- Himedia, RM1014
3. Folin- Ciocalteu reagent- Merck, AC9A590103
4. Gallic acid- Himedia, RM233
5. Potassium ferricyanide- Himedia, RM1034
6. Trichloroacetic acid- Himedia, RM7570
7. Methanol- SRL, 11528235
8. Acetone- Merck, SF8F580421
9. Ferric chloride- Merck, MK9M592642
10. Sodium hydroxide- Merck, ML0M603751
11. Hydrochloric acid- Merck, HI6H560677
12. Sulphuric acid- Merck, HG8H580592
13. Dimethyl sulfoxide- Merck, SL0S600706
14. Ethanol- Bengal Chemical, Kolkata

3.3. Reagents used

1. Mayer's reagent:

   1. 36 gm of Potassium iodide and 5 gm of Mercuric chloride was added in 100 ml of distilled water.

2. Wagner's reagent:

   2 gm of Iodine and 6 gm of Potassium iodide was dissolve in 100 ml of distilled water.

3. Molisch's reagent:

   15 gm of α-nepthol was dissolved in 100 ml of alcohol.

4. Ninhydrin reagent (Merck, MH8M581782)
5. Benedict's reagent (Merck, AK9AF59455)

3.4. Organisms used

Bacteria		**Obtained from**
Gram negative	*Escherichia coli*	Subashshree Biotech, Kolkata
	Klebsiella pneumonia	MTCC Chandigarh (MTCC- 3384)
	Pseudomonas aeruginosa	MTCC Chandigarh (MTCC 1034)
	Proteus vulgaris	MTCC Chandigarh (MTCC 742)
Gram positive	*Bacillus cereus*	Food Microbiology lab SGC, Gangtok
	Bacillus subtilis	Subashshree Biotech, Kolkata
	Staphylococcus aureus	MTCC Chandigarh (MTCC 7443).

3.5. Collection of plant materials

Some herbal plants of the region were selected for study. Fresh plant parts were then collected from different localities of Sikkim .The plants were identified and authenticated by the Taxonomists at Botanical Survey of India (BSI), located at Zero point, Gangtok, Sikkim. Copies of voucher specimen were deposited at Botanical Survey of India, Gangtok as well as at Department of Microbiology, Sikkim University for future reference.

3.5.1. Plants used for study

Sl.no.	Name of the plants	Parts used for study
1	*Cyphomandra betaceae*	Fruit
2	*Capsicum annuum*	Fruit
3	*Dicentra scandens*	Root
4	*Heracleum nepalense*	Fruit

3.6. Preparation of plant extracts

3.6.1. Preparation of plant extracts from fresh plant parts

Preparation of plant extracts from fresh plant parts was done by method of Bissa and Bohra, 2011 with modification. Fresh plant parts were taken and washed 3 to 4 times with tap water and then with distilled water. The fresh plant materials were grounded in distilled water for aqueous extract. The macerates were squeezed through gauge and then the aliquot was centrifuged at 8000 rpm for 20 min at room temperature using Centrifuge machine (Remi, C30-BL). The supernatant was then filtered through 0.45 micron cellulose acetate membrane filter made up of polycarbonate (Sartorious). The extracts were used for the *in vitro* studies (Bissa and Bohra, 2011).

3.6.2. Preparation of plant extracts from dried plant parts

The selected plants was washed thoroughly and then dried under shade. The dried plant material was grounded into a fine powder using Waring blender (Cole Parmer, RZ-04245-21). The powdered material was extracted in a Soxhlet apparatus for 24 hour

using different solvents (methanol and acetone). The solvent was then evaporated using Rotary evaporator (Bhuchi, Switzerland, R-3) to get crude extract, which was then stored in a refrigerator at 4°C for further use (Bissa and Bohra, 2011).

Prior to antimicrobial assay stock concentration of 200mg/ml extract was prepared in 0.25% DMS0 (Dimethyl sulfoxide), and was filtered through 0.45 micron cellulose acetate membrane filter (Sartorious). With the filtrate, further dilutions were made to get the concentrations of 100 mg/ml, 75 mg/ml, 50 mg/ml, 25 mg/ml, 10 mg/ml, 7.5 mg/ml, 5 mg/ml, 2.5 mg/ml and 1 mg/ml, which were then used for antimicrobial assay.

3.7. Test microorganisms

The test microorganisms for the study include four genera of Gram negative bacteria namely, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and two genera of Gram positive organism, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*. Test microorganisms were maintained at 4°C on nutrient agar slants. Active cultures for experiments were prepared by transferring loopful of culture in a flask containing nutrient broth, and were incubated at 37°C for 24 hours.

3.7.1. Preparation of bacterial suspension

For each isolate, three to five isolated colonies were picked from fresh agar plate and with the help of sterile loop; selected colonies were transferred into a tube containing 3-4 ml of suitable nutrient broth medium. After proper mixing, the broth culture were incubated at 37°C for 24hr. Turbidity was assessed by Spectrophotometer (Eppendrof make Biophotometer) by measuring the absorbance of the suspension. The absorbance should be in the range of 0.08-0.13 OD at 625 nm (McFarland standard 0.5). Turbidity was adjusted by adding sterile distilled water, saline or bróth, when the turbidity was too high, or by adding more bacterial colony when turbidity was too low or by incubating further (Wiegand *et al.*, 2008).

3.8. Screening of antimicrobial activity of selected plants against test organisms (well diffusion method)

The antimicrobial assay was performed by agar well diffusion method: Method established by National Committee for Clinical Laboratory Standard (NCCLS, 1999).

About 20 ml of nutrient agar medium was poured into the Petri plates and was left to solidify. To the solidified medium 100 µl of bacterial suspension was added and was spread uniformly with the glass spreader. Four wells were prepared in the plates with the help of a cup-borer (0.8 cm). Into the two wells, 100 µl of the Plant extract (test compound) was introduced and in one well 100 µl positive control gentamicin (10µg) (Shihabudeen *et al.*, 2010) and in opposite well 100 µl of 0.25% DMSO (negative control) was introduced. The plates were then incubated overnight at 37 °C. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition (Perez *et al,*. 1990). For each bacterial strain, a negative control is maintained where pure solvents are used instead of the extract (Parekh and Chanda, 2006). The experiment was performed four times and the mean values were presented.

To evaluate the synergistic antimicrobial activity of selected plants in combination of two, well diffusion method was followed. Four wells were prepared in the plates. Into two opposite well each extract (50 µl +50 µl) was added and in another opposite wells individual extract was added. After incubation at 37°C for 24 hours the observation were made. The zone of inhibitions formed by combination of extract was compared with the zone formed by individual extract.

3.9. Minimum Inhibitory Concentration (MIC)

The lowest concentration or highest dilution of the plant extract that inhibits the visible growth of test microorganism is known as Minimum inhibitory concentration (Wiegand *et al.*, 2008; Shihabudeen *et al.*, 2010).

The standardization of the bacterial cell number or preparation of bacterial suspension is of critical importance for susceptibility testing and obtaining accurate minimum inhibitory concentration (Wiegand *et al.*, 2008).

The minimum inhibitory concentration assay was performed by using agar dilution method (Wiegand *et al.*, 2008)

3.9.1. Agar dilution method

Different concentrations of plant extract were prepared. Around 20 ml of nutrient agar was prepared in each test tube and after autoclaving at 121°C, 15 lb for 15 minutes, medium was allowed to cool at 45-50°C, then different concentration of plant extract

were added in the respective test tubes to make the final concentration of 0.5-10 mg/ml, which was then poured into the Petri plates after mixing properly. The plates were left to solidify. Standard inoculums of test organisms were prepared. Test microorganisms were spotted in the agar plates with the help of micropipette set at 1µl to deliver the spots (Wiegand *et al.*, 2008). Control experiment were also done to see the effect of antibiotic and solvent alone (without plant extract) on the growth of all the test organisms (Shihabudeen *et al.*, 2010). The Plates were incubated at 37°C for 24hrs. The lowest concentration of the plants extract that inhibits the growth of test microorganism was considered as the MIC of the extract (Wiegand *et al.*, 2008).

3.9.2. Extract dilution chart for agar dilution method

Extract concentration (mg/ml)	Volume of extract stock solution (ml)	Final concentration when adding 20 ml agar (mg/ml)
200	1	10
100	1	5
75	1	3.75
50	1	2.5
25	1	1.25
10	1	0.5

3.10. Phytochemical analysis

To detect the various phytochemicals present, preliminary phytochemical screening was carried out for all four plant extract; *Cyphomandra betaceae*, *Capsicum annum*, *Dicentra scandens* and *Heracleum nepalense*. Qualitative phytochemical tests were performed for methanol, acetone and water extracts, to get the general idea regarding the nature of phytochemicals present.

Plant extract were subjected to standard phytochemical analysis to find the presence of different phytoconstituents such as tannins, phenolic compounds, steroid, saponin, alkaloid, flavanoids, glycosides, carbohydrates, proteins and fats (De *et al.*, 2010; Sofowora, 1993; Tiwari *et al.*, 2011; Tresa and Evans, 2009).

3.10.1. Test for Tannins and Phenolic compounds

3.10.1.1. Ferric chloride test: 0.5 grams of powdered material was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of filtrate, occurrence of blue black or green precipitate indicates the presence of tannins (Sofowora, 1993; Tresa and Evans, 2009).

3.10.1.2. Gelatin test: Plant extracts were treated with 1 % gelatin solution containing 10% sodium Chloride. White precipitates indicate the presence of tannin (De *et al.*, 2010).

3.10.2. Test for Steroids: 2 ml of acetic acid was added in 0.2 grams of plant extract. After allowing the solution to cool in ice concentrated H_2SO_4 was added carefully. Colour development from violet to blue or bluish-green indicate the presence of a steroidal ring (Sofowora, 1993; Tresa and Evans, 2009).

3.10.3. Test for Saponins

3.10.3.1. Froth test: 1gram of plant extract was boiled in 5 ml distilled water, and the contents were filtered. To the filtrate, about 3 ml of distilled water was added and after shaking it vigorously for 5 min frothing develops. Frothing which persists on warming is an indication for presence of saponins (Sofowora, 1993; Tresa and Evans, 2009).

3.10.4. Test for Flavonoids

3.10.4.1. Sodium Hydroxide Test for Flavonoids: 0.5 gm of powdered material is dissolved in water and then filtered. To the 2 ml of filtrate 10% aqueous sodium hydroxide is added to produce yellow colouration. A change in colour from yellow to colourless on addition of dilute Hydrochloric acid is an indication for presence of flavonoids (Sofowora, 1993; Tresa and Evans, 2009).

3.10.5. Test for Alkaloids

3.10.5.1. Mayer's test: To 0.5 gm of plant material, 5 ml of 1% aqueous Hydrochloric acid was added and stirred on a water bath and the water content was filtered. To 1 ml of filtrate Mayer's reagent was added. Appearance of buff coloured precipitate indicates presence of alkaloid (Sofowora, 1993; Tresa and Evans, 2009).

3.10.5.2. Wagner's test: Plant extracts were dissolved in dilute Hydrochloric acid and was filtered. To the filtrate Wagner's reagent was added. Formation of brown or reddish precipitate indicates the presence of alkaloids (Tiwari *et al.*, 2011).

3.10.6. Test for Glycosides

3.10.6.1. Bromine water test: Plant extracts were treated with bromine water. Formation of yellow precipitate indicates the presence of glycosides (De *et al.*, 2010).

3.10.7. Test for Carbohydrates

Plant extracts were dissolved in 5 ml distilled water and the content was filtered. The filtrates were used to test the presence of carbohydrates by following tests.

3.10.7.1. Molisch's test: To the filtrates two drops of alcoholic α-naphthol solution were added in a test tube. Formation of the violet ring at the junction of the filtrate and the solution indicates the presence of Carbohydrates (Tiwari *et al.*, 2011).

3.10.7.2. Benedict's test: Filtrates of plant extract were treated with Benedict's reagent and heated gently. Formation of orange red precipitate indicates the presence of reducing sugars (Tiwari *et al.*, 2011).

3.10.7.3. Fehling's test: Filtrates were hydrolysed with dilute Hydrochloric acid, neutralized with alkali (Sodium hydroxide) and heated with Fehling's solutions A and B. Red precipitate indicates the presence of reducing sugars (Tiwari *et al.*, 2011).

3.10.8. Tests for Proteins and Amino acids

3.10.8.1. Xanthoproteic Test: To the extracts few drops of concentrated Nitric acid was added. Formation of yellow colour indicates the presence of proteins (Tiwari *et al.*, 2011).

3.10.8.2. Ninhydrin Test: To the plant extract, 0.25% w/v ninhydrin reagent was added and was boiled for few minutes. Formation of violet to blue colour indicates the presence of amino acid (De *et al.*, 2010; Tiwari *et al.*, 2011).

3.10.9. Test for fats and fixed oils

3.10.9.1. Stain test: Small quantity of plant extract was pressed between two filter papers. Presence of stain on first filter paper indicates the presence of fixed oils and fats (De *et al.*, 2010).

3.11. Antioxidant activity assay

Determination of antioxidant activity of methanol extract of four plants extract was done by DPPH free radical scavenging activity assay, Reducing power assay and determination of total phenolic compound.

3.11.1. DPPH free radical scavenging activity assay

Methanol extract were prepared at the concentration of 1000 µg/ml. From the stock solution, different concentrations, 1, 10, 20, 30, 40, 50, 60, 80, 100 and 200 µg/ml were prepared and used for antioxidant assay. Ascorbic acid was used as standard for study of DPPH scavenging assay. The stock concentration of 1000 µg/ml was prepared in methanol. From the stock concentration all other concentrations ascorbic acid, 1, 10, 20, 30, 40, 50, 60, 80, 100 and 200 µg/ml were prepared.

The antioxidant activity of the plant extracts was estimated by using the DPPH (2,2-diphenyl-l-picryl hydrazyl) radical scavenging protocol (Macwan and Patel, 2010; Sutharsingh *et. al.*, 2011).

For analysis of antioxidant activity, 0.1 mM DPPH solution was prepared in methanol. In different test tubes 3 ml of extract at various concentration was added, to each test tube 1ml of 0.1 mM DPPH in methanol solution was added. Control was prepared by using deionized water. The reaction mixtures were incubated in dark at room temperature for 30 min and the absorbance of the reaction mixtures were taken at 517 nm by using Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer, L600-00BB). The decrease in optical density of DPPH on addition of test plant extract in relation to control was used to calculate the antioxidant activity as percentage inhibition (%IP) of DPPH radical (Macwan and Patel, 2010).

$$\text{DPPH Scavanged (\%) or \%IP} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{(\text{Absorbance}_{\text{control}})} \times 100$$

IC_{50} values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals (Shyur *et al.*, 2005).

3.11.2. Reducing power assay

The reducing power of methanolic extract of plants was determined by the method of Oyaizu, 1986. Various concentration of plant extract (1-200 µg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5ml Potassium ferricyanide (1%). The reaction mixture was kept in water bath at 50°C for 20 minutes. After cooling, aliquot of 10% trichloroacetic acid (2.5 ml) was added and centrifuge at 3000 rpm for 10 minutes. After centrifugation the upper layer (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml of freshly prepared 0.1% ferric chloride solution. The absorbance of reaction mixture was measured at 700 nm using Lambda 25 UV/Vis spectrophotometer (Perkin Elmer, L600-00BB). Control was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increase in the absorbance of reaction mixture indicates increase in reducing power of extract (Macwan and Patel, 2010).

3.11.3. Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteu and Gallic acid as standard (Slinkard and Singleton, 1977). To estimate the total phenolic compound in plant extract (1000 µg/ml), 0.1 ml of extract was mixed with 1ml Folin-Ciocalteu reagent (10%) after 3 minutes, 3 ml of aqueous sodium carbonate (2%) was added. The reaction mixture was incubated at room temperature for two hours and the absorbance was recorded at 760 nm using Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer, L600-00BB). All the determinations were carried out in triplicate. Gallic acid was used as standard. Different concentration of gallic acid (10-1000 µg/ml) was used to prepare the calibration curve (Patil *et al.*, 2009). Total content of phenolic compound in the plant extract in gallic acid equivalents (GAE) was calculated by: T= C.V/M Where, T= Total content of phenolic compounds, milligram per gram of plant extract, in GAE; C= the concentration of gallic acid established from the calibration curve, milligram per millilitre; V= the volume of extract, millilitre; M= the weight of extract, gram.

4. RESULTS

4. RESULTS

4.1. Identification of selected plants

The plants after being collected from different places of Sikkim were taken to Botanical Survey of India, Gangtok for identification. Selected plants were identified and authenticated by Taxonomists. The details of plants identified are shown below (Table 1).

Table1: Identification of the plants used for study

Serial Number	Plants identified as	Common Name	Family	Place of Collection
1	*Cyphomandra betaceae* (Canniviles) Sendtner	*Rookh tamater*	*Solanaceae*	Temi Tarku, South Sikkim
2	*Capsicum annuum* var. *cerasiforme*	*Dallhae Khorsani*	*Solanaceae*	Lingee Payong, South Sikkim
3	*Heracleum nepalense* D. Don	*Chimphing*	*Umbelliferae*	Damthang, South Sikkim
4	*Dicentra scandens* (D.Don) Walpers	*Jogi laharra/ Kanchi lahara/ Kundaley*	*Fumariaceae*	Damthang, South Sikkim

PHOTOGRAPHS OF THE PLANTS SELECTED FOR STUDY

Photograph 1: ***Cyphomandra betaceae***

Photograph 2: ***Capsicum annuum***

Photograph 3: ***Dicentra scandens***

Photograph 4: ***Heracleum nepalense***

4.2. Phytochemical analysis

Phytoconstituents such as alkaloids, flavonoids, tannins, phenols and steroid and other constituents present in the plants are attributed to antimicrobial activity (Shihabudeen *et al.*, 2010). The present study revealed the presence of various bioactive constituents in selected plant extracts.

4.2.1. Phytochemical analysis of aqueous extract of fresh plant parts

The aqueous extract of *Cyphomandra betaceae, Capsicum annuum, Dicentra scandens* and *Heracleum nepalense* were subjected to phytochemical analysis. The results are presented in table 2.

Table 2: Phytochemical constituents of fresh aqueous extract

PHYTOCHEMICAL TESTS	*Cyphomandra betaceae*	*Capsicum annuum*	*Dicentra scandens*	*Heracleum nepalense*
TANNINS	-	-	-	+
SAPONIN	-	-	+	-
STEROID	+	-	+	-
FLAVONOID	+	+	-	-
ALKALOID	-	-	+	-

'+' indicate present; '-' indicate absent.

The analysis of phytochemicals of *Cyphomandra betaceae* revealed the presence of steroids and flavonoids. Presence of flavonoids was detected in *Capsucum annuum*. Extract of *Dicentra scandens* revealed the presence of saponins, steroids and alkaloids. Similarly the phytochemical analysis of *Heracleum nepalense* showed the presence of tannins.

4.2.2. Phytochemical analysis of methanol extract of plants

The analysis of phytochemical of methanol extract of *Cyphomandra betaceae, Capsicum annuum, Dicentra scandens* and *Heracleum nepalense* revealed the presence of various phytoconstituents. The results are presented in Table 3.

Table 3: Phytochemical constituents of methanol extract

PHYTOCHEMICAL TESTS	*Cyphomandra betaceae*	*Capsicum annuum*	*Dicentra scandens*	*Heracleum nepalense*
TANNINS				
Ferric Chloride Test	-	-	-	+
Gelatin Test	-	-	-	+
PHENOLS	+	+	+	+
SAPONIN				
Froth Test	+	-	+	+
STEROID	-	+	+	-
FLAVONOID				
Sodium Hydroxide Test	+	+	-	-
ALKALOID				
Mayer's Test	-	-	+	-
Wagner's Test	-	-	+	-
Hager's Test	-	-	+	-
CARBOHYDRATE:				
Molisch Test	-	-	-	-
Benedict's Test	-	-	-	-
Fehling's Test	-	-	-	-
PROTEINS AND AMINOACIDS				
Ninhydrin Test	-	-	-	+
Xanthoprotic Test	-	-	-	+
FIXED OILS AND FAT				
Stain test	+	+	+	+

'+' indicate present; '-' indicate absent.

Analysis of phytochemicals of methanol extract of *Cyphomandra betaceae* revealed the presence of phenols, saponins and flavonoids. Phytoconstituents such as phenols, steroid and flavonoids were found to be present in the methanol extract of *Capsicum annuum.* Extract of *Dicentra scandens* revealed the presence of phenols, saponins,

steroids and alkaloids. *Heracleum nepalense* showed the presence of tannins, phenols and saponins in methanol extract. Stain test revealed the presence of fixed oil and fat in all the four plant extracts. Test for presence of protein and amino acids were positive for *Heracleum nepalense*.

4.2.3. Phytochemical analysis of acetone extract of plants

The acetone extract of the plants revealed the presence of various phytoconstituents (Table 4).

Table 4: Phytochemical constituents of acetone extract

PHYTOCHEMICAL TESTS	*Cyphomandra betaceae*	*Capsicum annuum*	*Dicentra scandens*	*Heracleum nepalense*
TANNINS AND PHENOLIC COMPOUNDS				
Ferric Chloride Test	+	-	+	-
Gelatin Test	-	-	-	-
SAPONIN	+	-	-	-
Froth Test				
STEROID	-	-	-	-
FLAVONOID	-	-	-	+
Sodium Hydroxide Test				
ALKALOID				
Mayer's Test	-	-	+	-
Wagner's Test	-	-	+	-
CARBOHYDRATE:	-	-	-	-
Molisch Test	-	-	-	-
Benedict's Test	-	-	-	-
Fehling's Test				
PROTEINS AND AMINOACIDS	-	-	-	-
Ninhydrin Test	-	-	-	-
Xanthoprotic Test				
FIXED OILS AND FAT (Stain test)	+	+	+	+

'+' indicate Present; '-' indicate Absent.

The analysis of phytochemicals of acetone extract of *Cyphomandra betaceae* revealed the presence of phenolic compounds and saponins. *Dicentra scandens* revealed the presence of phenolic compounds and alkaloids. Bioactive constituent flavonoids were detected in the acetone extract of *Heracleum nepalense.* Whereas all the tested phytochemicals were absent in acetone extract of *Capsicum annuum* except for presence of fixed oils and fat.

4.3. Antimicrobial activity

The antimicrobial activity of *Cyphomandra betaceae*, *Capsicum annuum*, *Dicentra scandens* and *Heracleum nepalense* was evaluated according to their zone of inhibition against different test organisms.

4.3.1. Preliminary screening of antimicrobial activity (Aqueous extracts)

Preliminary screening of antimicrobial activity of herbal plants was done against three microorganisms namely *Escherichia coli*, *Bacillus cereus* and *Bacillus subtilis* with aqueous extract prepared out of fresh plant materials. Fresh aqueous extract were evaluated for antimicrobial activity. Fresh aqueous extract of *Cyphomandra betaceae* showed antimicrobial activity by inhibiting the growth of all three test microorganisms. The largest zone of inhibition (19.5 mm) was observed against *Bacillus cereus* followed by *Escherichia coli* (17.5 mm) and *Bacillus subtilis* (17.25 mm).

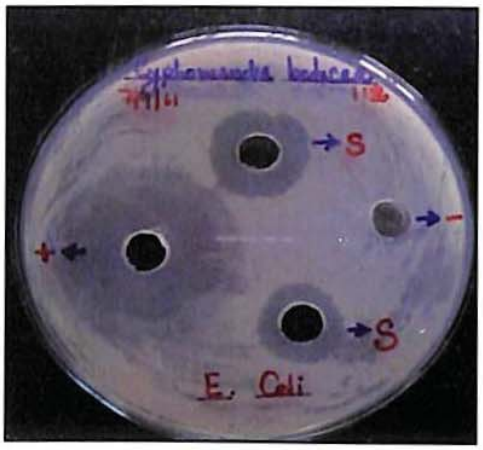
Fresh aqueous extract of *Capsicum annuum* showed antimicrobial activity against *Bacillus cereus* with the zone of inhibition of 21 mm.

The fresh aqueous extract of the root exhibited antimicrobial activity against *Escherichia coli* and the zone of inhibition obtained was 14.7 mm.

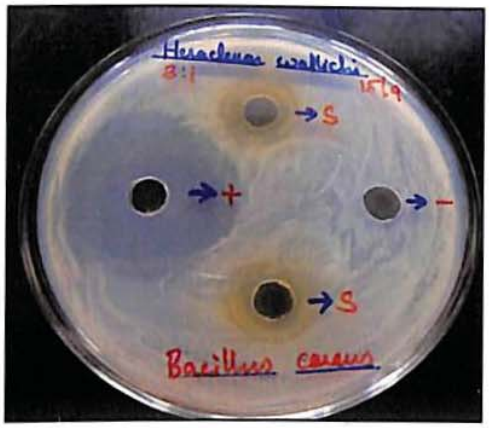
Fresh aqueous extract of *Heracleum nepalense* showed antimicrobial activity against *Bacillus cereus* and the zone of inhibition obtained was 13 mm.

After screening the aqueous extract of selected plants for antimicrobial activity, further analysis of antimicrobial activity was carried out using methanol and acetone extract.

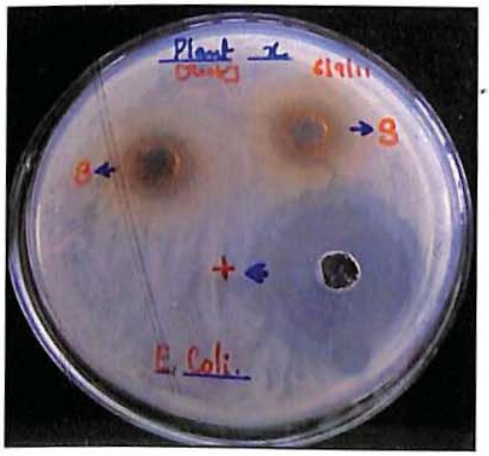
Antimicrobial activity of fresh aqueous extract



Photographic plate 1: Zone of inhibition formed by *C. betaceae* (aqueous extract) against *E. coli*. '+' indicate zone formed by Gentamicin and '_' is distilled water.



Photographic plate 2: Zone of inhibition formed by *H. nepalense* (aqueous extract) against *B. cereus*. '+' indicate zone formed by Gentamicin and '_' is distilled water.



Photographic plate 3: Zone of inhibition formed by *D. scandens* (aqueous extract) against *E. coli*. '+' indicate zone formed by Gentamicin and '_' is distilled

4.3.2. Antimicrobial activity of solvent extracts

Methanol and acetone extract of all the selected plants were tested against some Gram positive and Gram negative organisms. Gram negative bacteria selected for present study were *Escherichia coli*, *Klebsiellae pneumonia*, *Pseudomonas aeruginosa* and *Proteus vulgaris* and Gram positive bacteria were *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*.

4.3.2.1. Antimicrobial activity of methanol extract of *Cyphomandra betaceae*

Methanol extract of *Cyphomandra betaceae* showed antimicrobial activity against all the seven test microorganisms (Table 5, Figure1 and Figure 2).

Table 5: Antimicrobial activity of *Cyphomandra betaceae* (methanol extract) tested against microorganisms by well diffusion method

	Mean diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*P.v*	*B.c*	*B.s*	*S.a*
100	19	12.25	18	20	22.75	22	24.75
75	16	-	15.25	18	18.5	18	21
50	15	-	14	16	18	17.50	20
25	14	-	-	12.5	12.5	12.75	17
10	13.25	-	-	-	-	-	
7.5	12.75	-	-	-	-	-	-
5	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
Positive control	28.25	28.25	26.50	30.25	30.25	31.25	30.50
Negative control	-	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; P.v., Proteus vulgaris; B.c, Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Each value represent mean (n=4). '-': No zone of inhibition, Negative control: DMSO (0.25%), Positive control: Gentamicin (100 µg/ml). Zone of inhibition: including the well diameter of 8 mm

At the concentration of 100 mg/ml the largest zone of inhibition (24.75 mm) was observed for *Staphylococcus aureus* followed by *Bacillus cereus* (22.75) mm, *Bacillus subtilis* (22 mm), *Proteus vulgaris* (20 mm), *Escherichia coli* (19 mm), *Pseudomonas aeruginosa* (18 mm) and *Klebsiella pneumoniae* (12.25 mm). The extract at lower concentrations of 10 mg/ml and 7.5 mg/ml did not inhibit the growth of test organisms except for *Escherichia coli.* At the concentration of 7.5 mg/ml the extract inhibited the growth of *Escherichia coli* with inhibition zone of 12.75 mm. Concentrations below 7.5 mg/ml did not show any zone of inhibition. The zone of inhibition observed for the control antibiotic Gentamicin (100 µg/ml) was 31.25 mm for *Bacillus subtilis*, 30.50 mm for *Staphylococcus aureus*, 30.25 mm for *Bacillus cereus* and *Proteus vulgaris*, 28.25 mm for *Escherichia coli* and *Klebsiella pneumoniae* and 26.50 mm for *Pseudomonas aeruginosa* (Table 5).

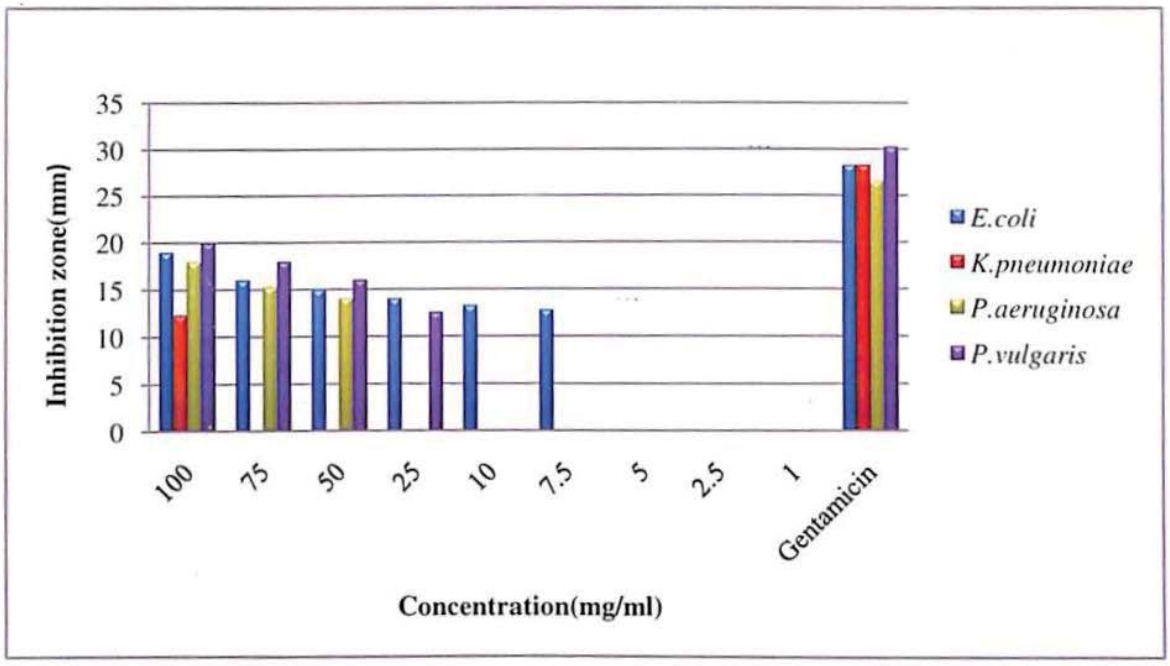


Figure 1: Antimicrobial activity of *Cyphomandra betaceae* (methanol extract) against Gram negative bacteria

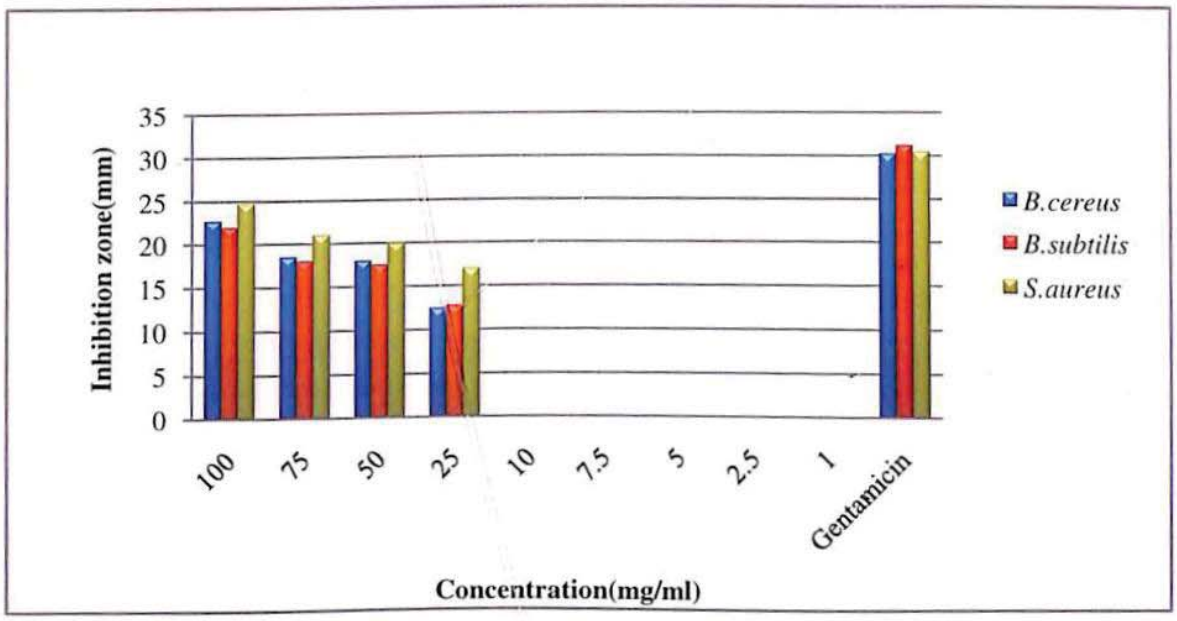
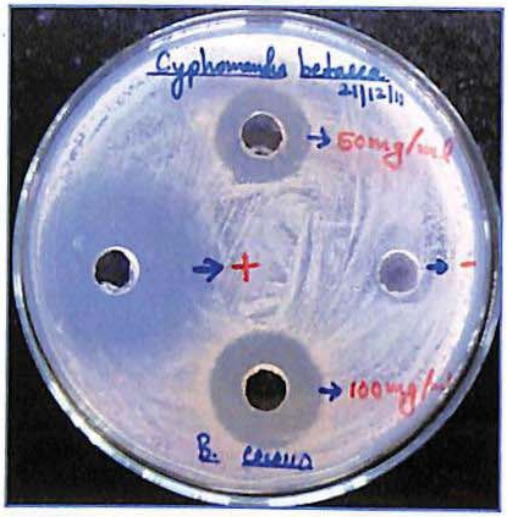


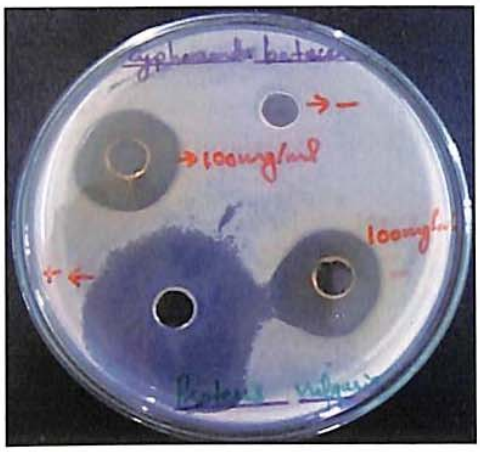
Figure 2: Antimicrobial activity of *Cyphomandra betaceae* (methanol extract) against Gram positive bacteria

Antimicrobial activity of methanol extract of *Cyphomandra betaceae*



Photographic plate 4: Zone of inhibition formed by *C. betaceae* (methanol extract) against *B. cereus* at concentration of 100 mg/ml and 50 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

Photographic plate 5: Zone of inhibition formed by *C. betaceae* (methanol extract) against *B.subtilis* at concentration of 75 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.



Photographic plate 6: Zone of inhibition formed by *C. betaceae* (methanol extract) against *P. vulgaris* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

Photographic plate 7: Zone of inhibition formed by *C. betaceae* (methanol extract) against *P.aeruginosa* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

4.3.2.2. Antimicrobial activity of acetone extract of *Cyphomandra betaceae*

The acetone extract of *Cyphomandra betaceae* showed antimicrobial activity against all the seven test microorganism (Table 6, Figure 3 and Figure 4).

Table 6: Antimicrobial activity of *Cyphomandra betaceae* (acetone extract) tested against microorganisms by well diffusion method

	Mean diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*P.v*	*B.c*	*B.s*	*S.a*
100	23	15.25	17	22	25	25	22.25
75	19	13	15	19.75	22	22	20
50	18.25	11.25	14	17	22	21.25	17
25	15	-	12.25	15.50	19	17	14.75
10	13	-	-	12	14	14.25	12
7.5	11.25	-	-	-	12.5	11.75	11
5	11	-	-	-	-	11	11
2.5	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
Positive control	28.25	28.25	26.50	30.25	30.25	31.25	30.50
Negative control	-	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; P.v., Proteus vulgaris; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Each value represent mean (n=4). '-': No zone of inhibition, Negative control: DMSO (0.25%), Positive control: Gentamicin (100 µg/ml). Zone of inhibition: including the well diameter of 8 mm.

At the concentration of 100 mg/ml the largest zone of inhibition (25 mm) was observed for *Bacillus cereus* and *Bacillus subtilis* followed by *Escherichia coli* (23 mm), *Staphylococcus aureus* (22.25 mm), *Proteus vulgaris* (22 mm), *Pseudomonas aeruginosa* (17 mm) and *Klebsiella pneumoniae* (15.25 mm). As compared to methanol extract, the acetone extract of *Cyphomandra betaceae* showed antimicrobial activity even at lower concentrations. At the concentration of 5 mg/ml the extract inhibited the growth of *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* and the zone of

inhibition observed was 11 mm. Concentrations below 5 mg/ml did not show any zone of inhibition (Table 6).

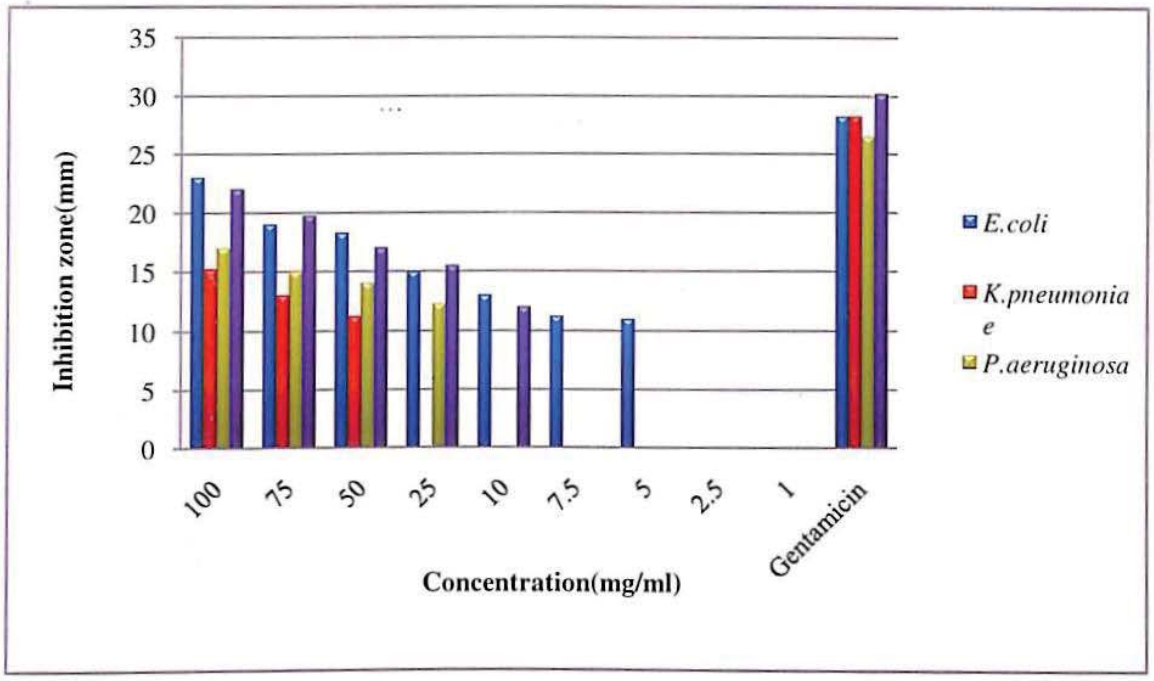


Figure 3: Antimicrobial activity of *Cyphomandra betaceae* (acetone extract) against Gram negative bacteria

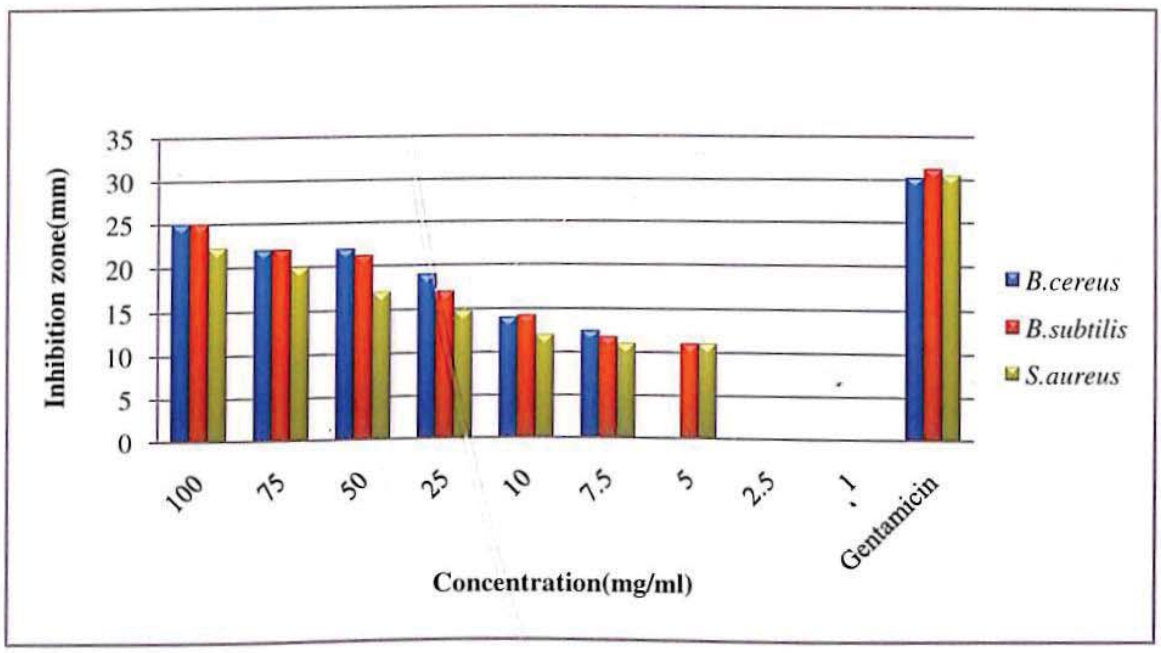
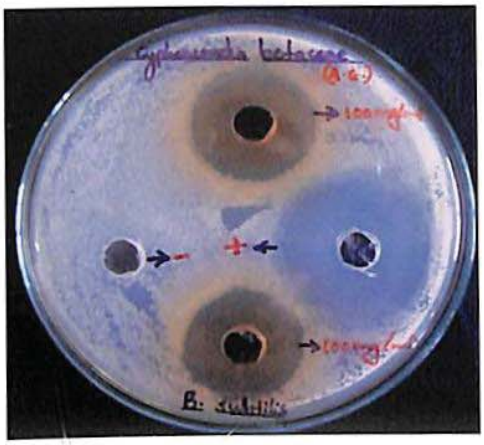


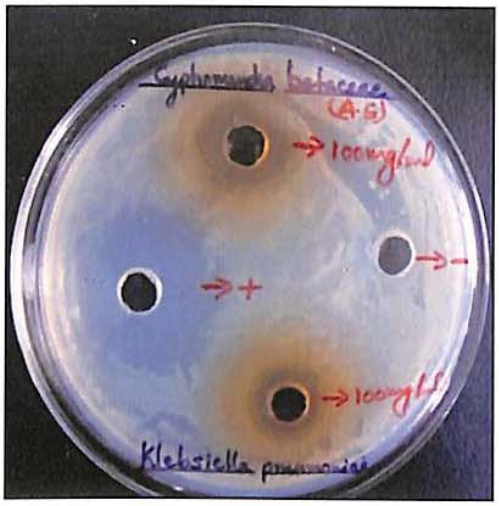
Figure 4: Antimicrobial activity of *Cyphomandra betaceae* (acetone extract) against Gram positive bacteria

Antimicrobial activity of acetone extract of *Cyphomandra betaceae*

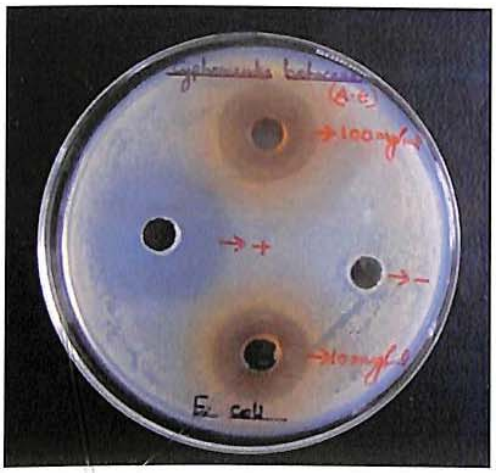
Photographic plate 8: Zone of inhibition formed by *C. betaceae* (acetone extract) against *B.cereus* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.



Photographic plate 9: Zone of inhibition formed by *C. betaceae* (acetone extract) against *B.subtilis* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.



Photographic plate 10: Zone of inhibition formed by *C. betaceae* (acetone extract) against *K. pneumoniae* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.



Photographic plate 11: Zone of inhibition formed by *C. betaceae* (acetone extract) against *E. coli* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

4.3.2.3. Antimicrobial activity of *Capsicum annuum*

The antimicrobial activity of methanol extract of *Capsicum annuum* was done against seven test microorganisms. The extract inhibited the growth of all the three test Gram positive bacteria and only one Gram negative test bacteria *Escherichia coli* (Table 7, Figure 5 and Figure 6).

Table 7: Antimicrobial activity of *Capsicum annuum* (methanol extract) tested against microorganisms by well diffusion method

	Diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*P.v*	*B.c*	*B.s*	*S.a*
200	22.25	-	-	-	11.75	20.75	20.75
100	21.75	-	-	-	11.25	18	18
75	20.25	-	-	-	-	16.75	17
50	18.75	-	-	-	-	16.75	16
25	17.75	-	-	-	-	14.25	14.25
10	11.75	-	-	-	-	-	11.50
7.5	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
Positive control	28.25	28.25	26.50	30.25	30.25	31.25	30.50
Negative control	-	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; P.v., Proteus vulgaris; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Each value represent mean (n=4). '-': No zone of inhibition, Negative control: DMSO (0.25%), Positive control: Gentamicin (100 µg/ml). Zone of inhibition: including the well diameter of 8 mm.

The largest zone of inhibition (22.25 mm) was observed for *Escherichia coli* at the concentration of 200 mg/ml, followed by *Staphylococcus aureus* and *Bacillus subtilis* (20.75 mm) and *Bacillus cereus* (11.75 mm). At the concentration of 100 mg/ml the extract inhibited the growth of *Escherichia coli* with zone of inhibition as 21.75 mm,

Bacillus subtilis and *Staphylococcus aureus* (18 mm) and *Bacillus cereus* (11.25 mm). The extract at lower concentration of 10 mg/ml showed inhibition zone of 11.75 mm for *Escherichia coli* and 11.50 mm for *Staphylococcus aureus*. Concentrations below 10 mg/ml did not show any zone of inhibition. On the other hand the acetone extract of *Capsicum annum* did not show antimicrobial activity against any of the seven test organism.

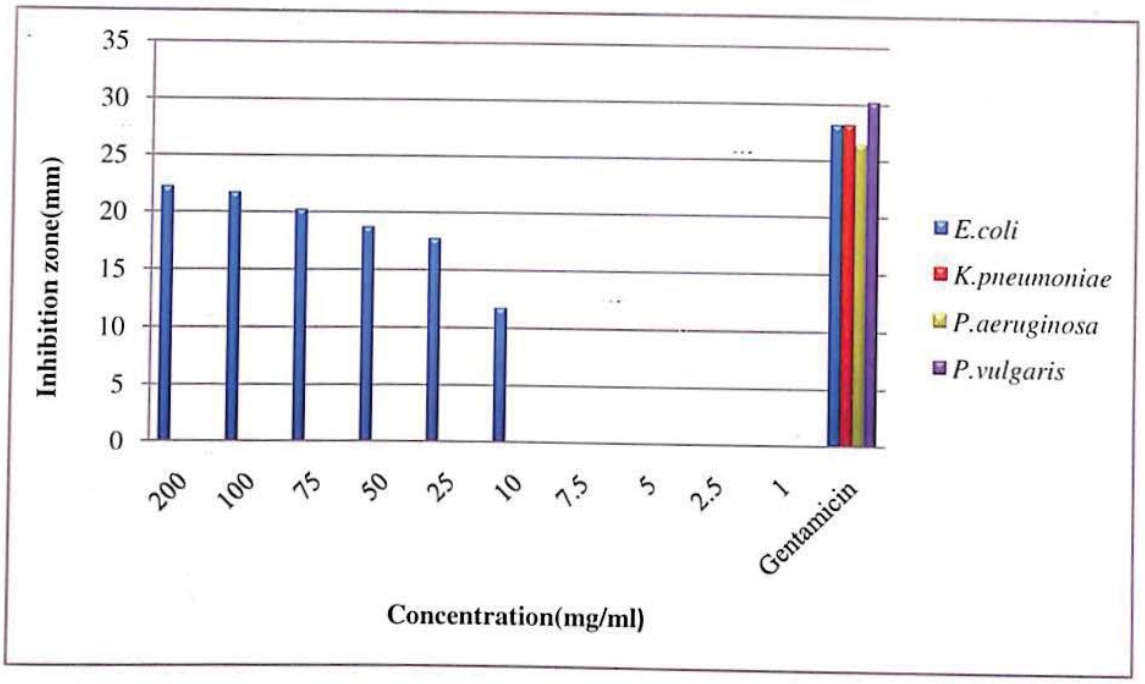


Figure 5: Antimicrobial activity of *Capsicum annuum* (methanol extract) against Gram negative bacteria

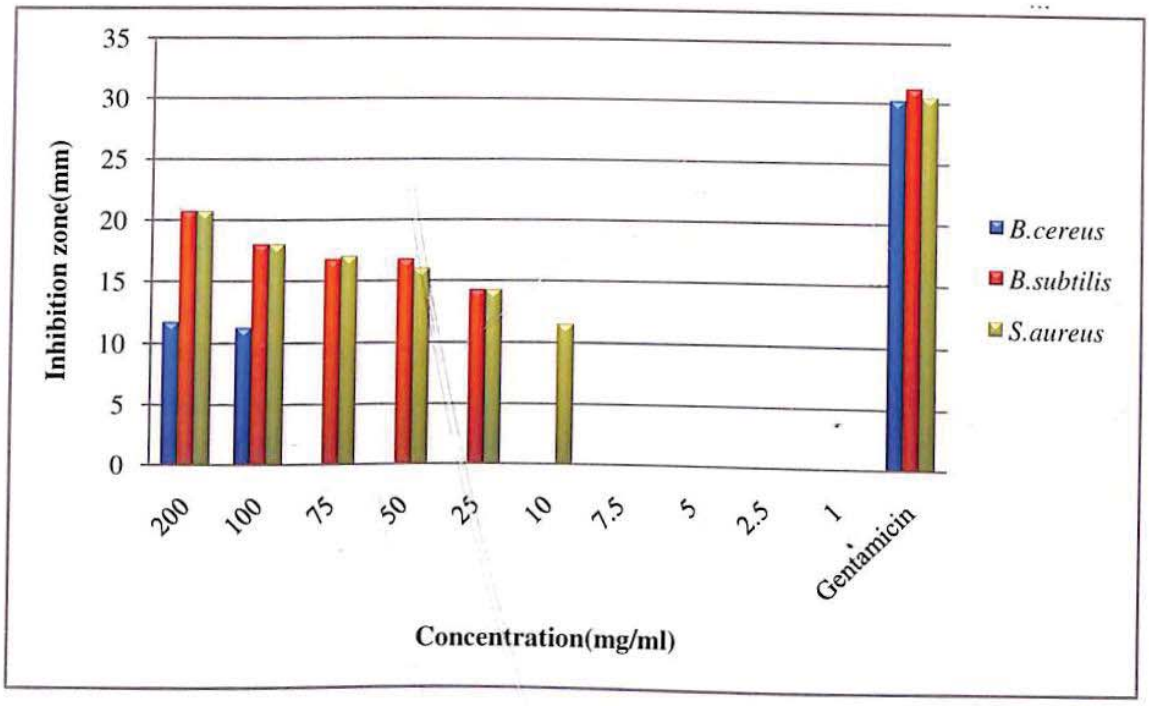
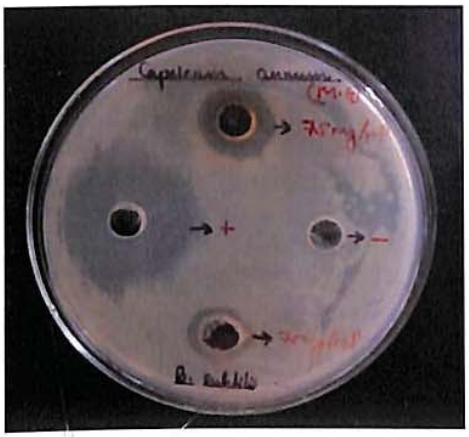


Figure 6: Antimicrobial activity of *Capsicum annuum* (methanol extract) against Gram positive bacteria

Antimicrobial activity of methanol extract of *Capsicum annuum*

Photographic plate 12: Zone of inhibition formed by *C. annuum* (methanol extract) against *S.aureus* at concentration of 50 mg/ml and 25 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.



Photographic plate 13: Zone of inhibition formed by *C. annuum* (methanol extract) against *B. subtilis* at concentration of 75 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

4.3.2.4. Antimicrobial activity of methanol extract of *Dicentra scandens*

The antimicrobial activity of *Dicentra scandens* was tested against microorganisms by well diffusion method. The methanol extract of the herb inhibited the growth of all the seven test organisms (Table 8, Figure 7 and Figure 8).

Table 8: Antimicrobial activity of *Dicentra scandens* (methanol extract) tested against microorganisms by well diffusion method

	Diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*P.v*	*B.c*	*B.s*	*S.a*
200	16	19.75	14	13.75	12	11.75	18.50
100	14.75	15	-	-	11	11	15.50
75	12	-	-	-	10.25	-	13
50	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
7.5	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
Positive control	28.25	28.25	26.50	30.25	30.25	31.25	30.50
Negative control	-	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; P.v., Proteus vulgaris; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Each value represent mean (n=4). '-': No zone of inhibition, Negative control: DMSO (0.25%), Positive control: Gentamicin (100 µg/ml). Zone of inhibition: including the well diameter of 8 mm.

The largest zone of inhibition (19.75 mm) was observed for *Klebsiella pneumoniae* followed by *Staphylococcus aureus* (18.50 mm) *Escherichia coli* (16 mm), *Pseudomonas aeruginosa* (14 mm), *Proteus vulgaris* 13.75 mm, *Bacillus cereus* (12 mm) and *Bacillus subtilis* 11.75 mm) at the concentration of 200mg/ml. At the concentration of 100 mg/ml the largest zone of inhibition (15.50 mm) was observed for

Staphylococcus aureus, followed by *Klebsiella pneumoniae* (15 mm), *Escherichia coli* (14.75 mm), *Bacillus cereus* (11 mm) and *Bacillus subtilis* (11 mm). At this concentration the extract did not inhibit the growth of *Pseudomonas aureginosa* and *Proteus vulgaris*. The extract inhibited the growth only up to the concentration of 75 mg/ml. The zone of inhibition was not observed at a concentration below 75 mg/ml (Figure 7 and Figure 8).

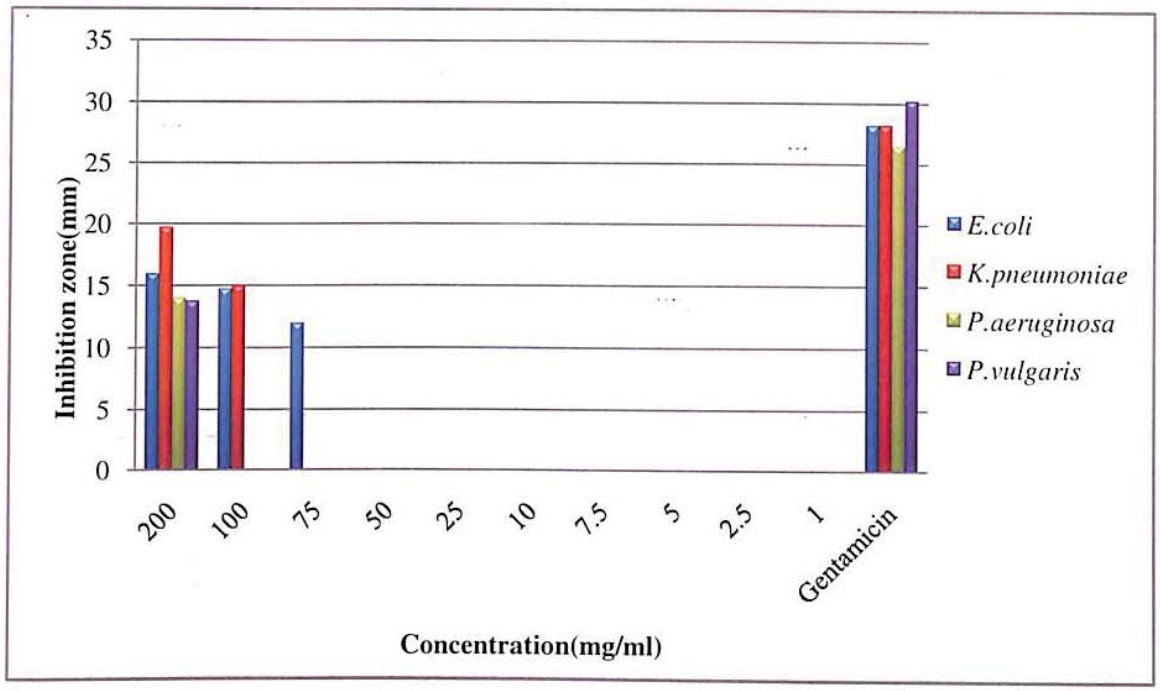


Figure 7: Antimicrobial activity of *Dicentra scandens* (methanol extract) against Gram negative bacteria

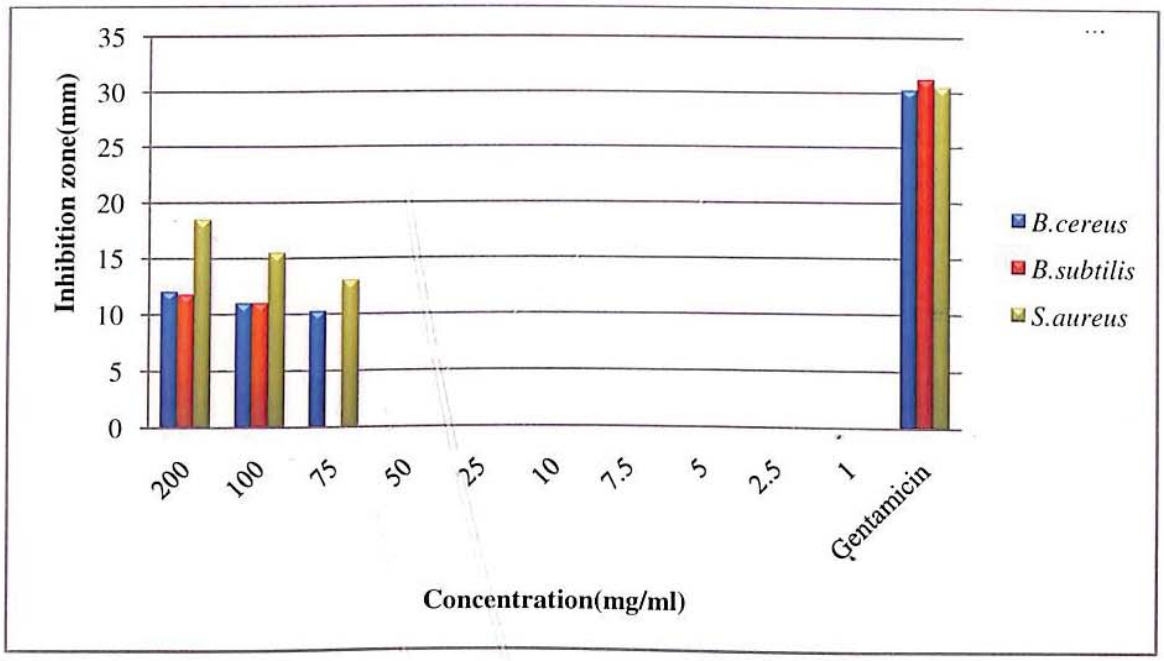


Figure 8: Antimicrobial activity of *Dicentra scandens* (methanol extract) against Gram positive bacteria

Antimicrobial activity of methanol extract of *Dicentra scandens*

Photographic plate 14: Zone of inhibition formed by *D. scandens* (methanol extract) against *K. pneumoniae* at concentration of 200 mg/ml and 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

4.3.2.5. Antimicrobial activity of acetone extract of *Dicentra scandens*

The acetone extract of *Dicentra scandens* was evaluated for antimicrobial activity. The extract inhibited the growth of three Gram negative test microorganism and two Gram positive test microorganisms (Table 9, Figure 9 and Figure 10).

Table 9: Antimicrobial activity of *Dicentra scandens* (acetone extract) tested against microorganisms by well diffusion method

	Mean diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*P.v*	*B.c*	*B.s*	*S.a*
200	23.25	22.50	-	22.50	20.75	19.50	-
100	18	17.25	-	21	17	17	-
75	17	16	-	21	15.75	15	-
50	14.25	14.50	-	16	12	13	-
25	-	-	-	14	-	-	-
10	-	-	-	11	-	-	-
7.5	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
Positive control	28.25	28.25	26.50	30.25	30.25	31.25	30.50
Negative control	-	-	-	-	-	-	-

E.c,. Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; P.v., Proteus vulgaris; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Each value represent mean (n=4). '-': No zone of inhibition, Negative control: DMSO (0.25%), Positive control: Gentamicin (100 µg/ml). Zone of inhibition: including the well diameter of 8 mm.

The acetone extract of *Dicentra scandens* showed promising antimicrobial activity against *Escherichia coli*, *Klebsiella pnumoniae*, *Proteus vulgaris*, *Bacillus cereus* and *Bacillus subtilis* (Table 9). The zone of inhibition observed was 23.25 mm for *Escherichia coli*, 22.50 mm for *Klebsiella pneumoniae* and *Proteus vulgaris* and 20.75

mm and 19.50 mm respectively for *Bacillus cereus* and *Bacillus subtilis* at concentration of 200 mg/ml. As compared to methanol extract, the acetone extract of *Dicentra scandens* showed better antimicrobial activity even at lower concentrations. At the concentration of 50 mg/ml, zone of inhibition observed for *Escherichia coli* and *Klebsiella pneumoniae* was 14.25 mm and 14.50 mm respectively. At the concentration of 10 mg/ml the extract inhibited the growth of *Proteus vulgaris* and the zone of inhibition was 11 mm. Concentration below 10 mg/ml, the zone of inhibition was not observed. The acetone extract of *Dicentra scandens* did not show any zone of inhibition against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

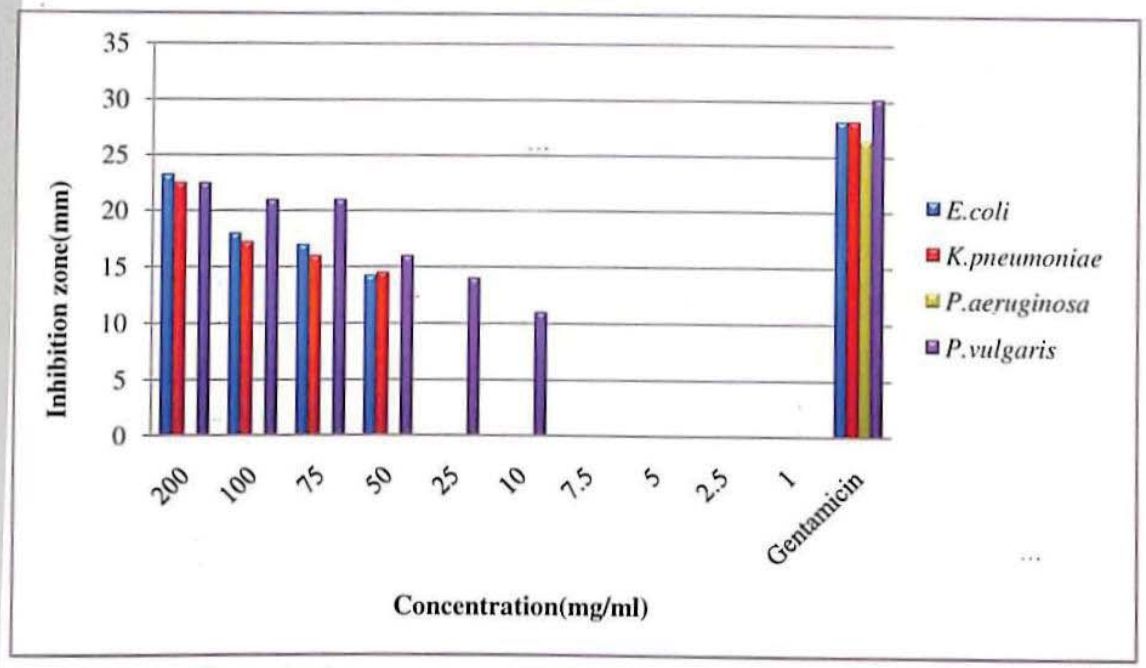


Figure 9: Antimicrobial activity of *Dicentra scandens* (acetone extract) against Gram negative bacteria

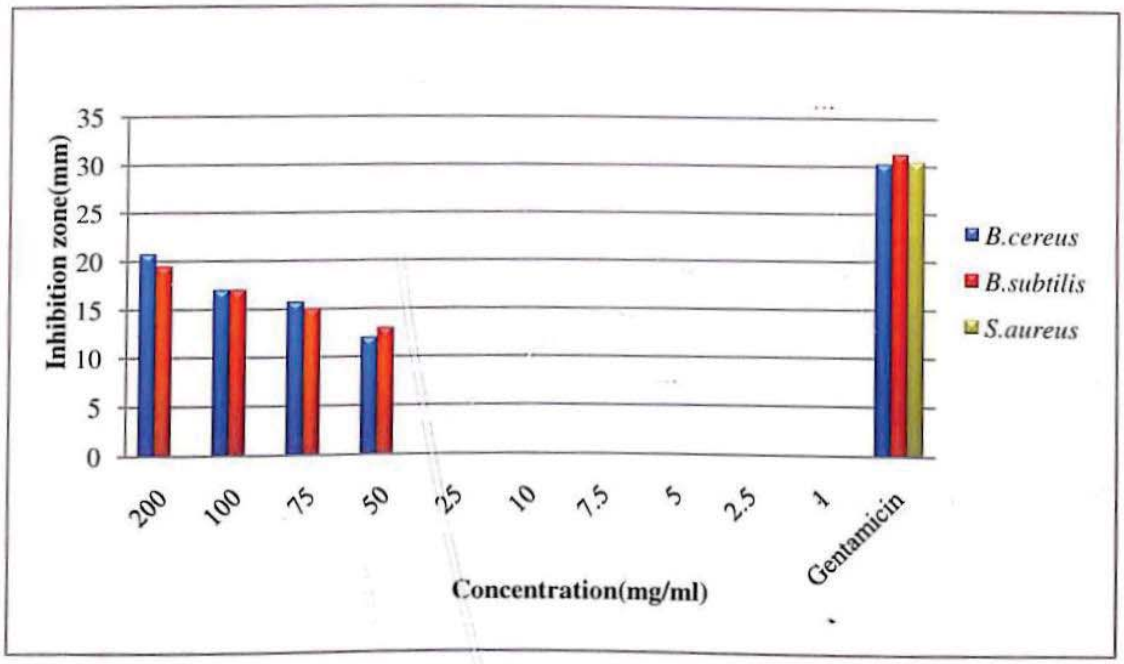


Figure 10: Antimicrobial activity of *Dicentra scandens* (acetone extract) against Gram positive bacteria

Antimicrobial activity of acetone extract of *Dicentra scandens*

Photographic plate 15: Zone of inhibition formed by *D. scandens* (acetone extract) against *P. vulgaris* at concentration of 200 mg/ml and 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

Photographic plate 16: Zone of inhibition formed by *D. scandens* (acetone extract) against *B. subtilis* at concentration of 200 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

Photographic plate 17: Zone of inhibition formed by *D. scandens* (acetone extract) against *K. pneumoniae* at concentration of 200 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

4.3.2.6. Antimicrobial activity of *Heracleum nepalense*

The methanol extract of *Heracleum nepalense* was evaluated for antimicrobial activity. The extract exhibited antimicrobial activity mostly against Gram positive test microorganisms (Table 10, Figure 11).

Table 10: Antimicrobial activity of *Heracleum nepalense* (methanol extract) tested against microorganisms by well diffusion method

	Mean diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*P.v*	*B.c*	*B.s*	*S.a*
200	11.75	-	-	-	13.50	14	14
100	11	-	-	-	13	13	11.25
75	-	-	-	-	11.75	13	-
50	-	-	-	-	12	12	-
25	-	-	-	-	11.50	-	-
10	-	-	-	-	-	-	-
7.5	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
2.5	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-
Positive control	28.25	28.25	26.50	30.25	30.25	31.25	30.50
Negative control	-	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; P.v., Proteus vulgaris; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Each value represent mean (n=4). '-': No zone of inhibition, Negative control: DMSO (0.25%), Positive control: Gentamicin (100 µg/ml). Zone of inhibition: including the well diameter of 8 mm.

At the concentration of 200 mg/ml the largest zone of inhibition (14 mm) was observed for *Bacillus subtilis* and *Staphylococcus aureus*, followed by *Bacillus cereus* (13.50 mm). At the concentration of 100 mg/ml the extract inhibited the growth of *Bacillus cereus* and *Bacillus subtilis* with the zone of inhibition of 13 mm, and *Staphylococcus aureus* (11.25 mm). The extract at lower concentration of 25 mg/ml inhibited the

growth of *Bacillus cereus* and the zone of inhibition observed was 11.50 mm. below the concentration of 25 mg/ml, the zone of inhibitions were not observed. On the other hand the acetone extract of *Heracleum nepalense* did not show antimicrobial activity against all the seven test organisms.

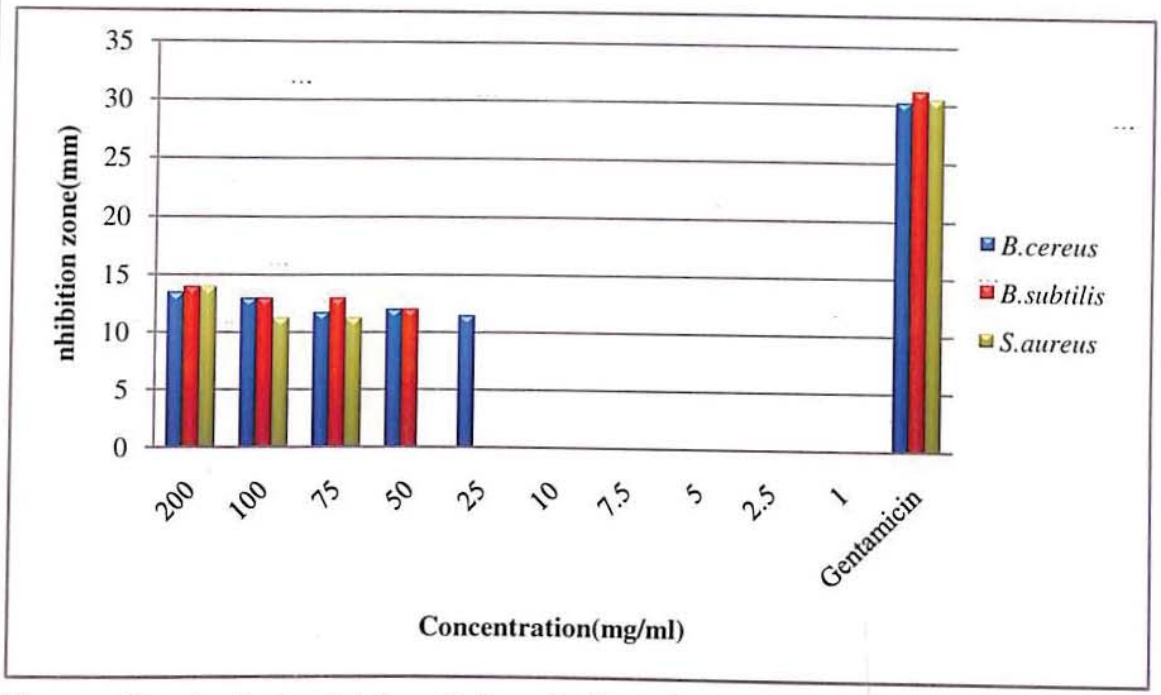
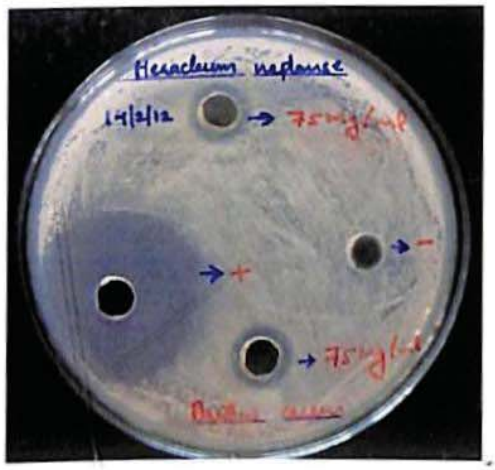


Figure 11: Antimicrobial activity of *Heracleum nepalense* (methanol extract) against Gram positive organisms

Photographic plate 18: Zone of inhibition formed by *H. nepalense* (methanol extract) against *B. subtilis* at concentration of 100 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.



Photographic plate 19: Zone of inhibition formed by *H. nepalense* (methanol extract) against *B. cereus* at concentration of 75 mg/ml. '+' indicate zone formed by Gentamicin and '_' is 0.25% DMSO.

4.3.2.7. Antimicrobial activity of methanol extract of all the four selected plants against *Escherichia coli* and *Staphylococcus aureus*

The methanol extract of the plants selected showed comparatively better activity against *Escherichia coli* among Gram negative bacteria and *Staphylococcus aureus* among Gram positive bacteria (Figure 12 and Figure 13).

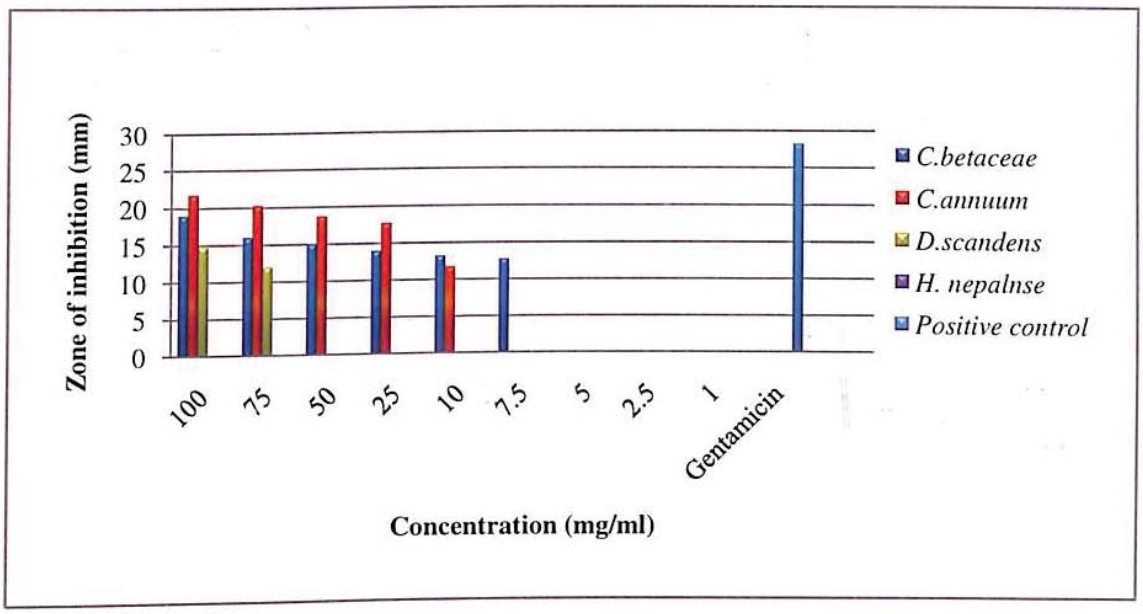


Figure 12: Antimicrobial activity of all four selected plants against *Escherichia coli*

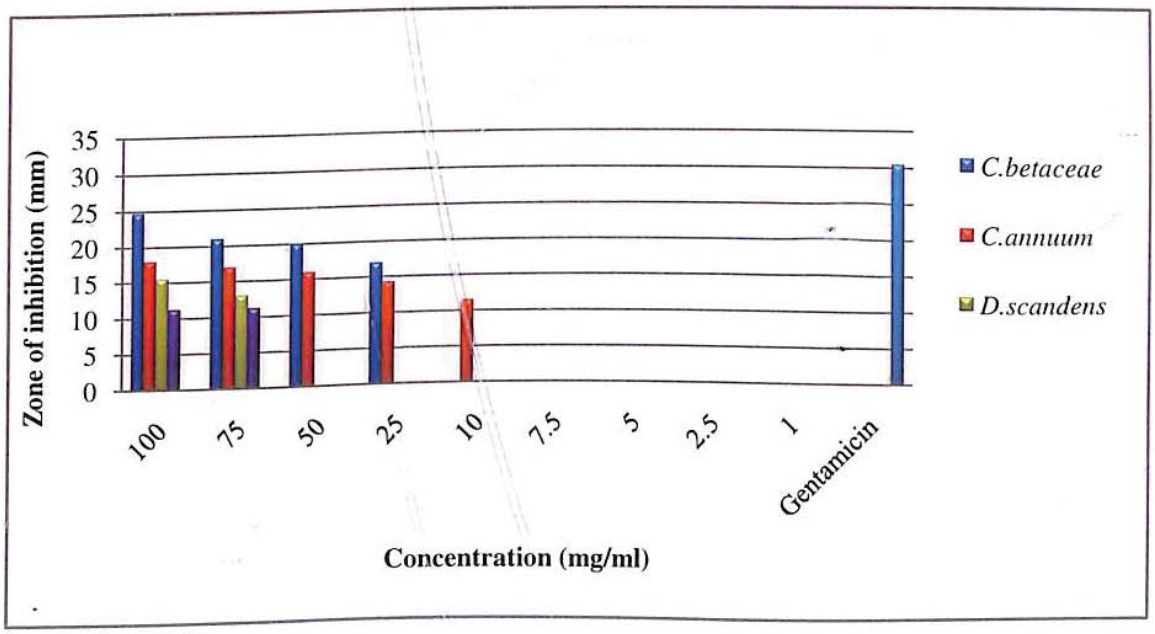


Figure 13: Antimicrobial activity of all four selected plants against *Staphylococcus aureus*

4.4. Minimum inhibitory concentration (MIC) assay of plant extracts on seven test organisms

The minimum inhibitory concentration (MIC) assay was performed with both methanol and acetone extract. The MIC of plant extracts was determined by employing agar dilution method. The results are summarized in table 11 and table 12.

Table 11: Minimum inhibitory concentration (MIC) of methanol extracts of plants on seven test organisms

	Cyphomandra betaceae (mg/ml)	*Capsicum annuum* (mg/ml)	*Dicentra scandens* (mg/ml)	*Heracleum nepalense* (mg/ml)
Escherichia coli	2.5	> 10	> 10	>10
Klebsiella pneumoniae	10	-	> 10	-
Pseudomonas aeruginosa	3.75	-	> 10	-
Proteus vulgaris	3.75	-	> 10	-
Bacillus cereus	3.75	> 10	> 10	> 10
Bacillus subtilis	3.75	> 10	> 10	> 10
Staphylococcus aureus	2.5	> 10	> 10	> 10

MIC assay was performed against organism whose growth was inhibited by extract in well diffusion test. '-' indicate MIC assay not performed.

The MIC value of methanol extract of *Cyphomandra betaceae* was found to be 2.5 mg/ml for *Escherichia coli* and *Staphylococcus aureus*. In case of *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus cereus* and *Bacillus subtilis* the MIC value was 3.75 mg/ml. The MIC value was 10 mg/ml for *Klebsiella pneumoniae.*

The MIC value of methanol extract of *Capsicum annuum, Dicentra scandens* and *Heracleum nepalense* was found to be greater than 10 mg/ml.

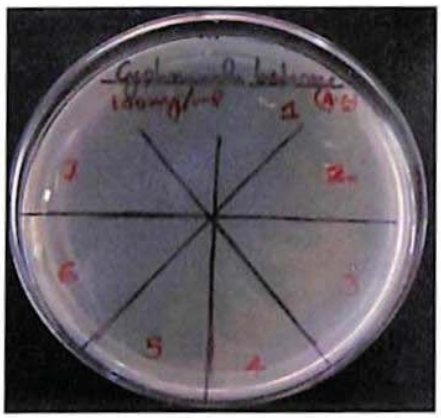
Table12: Minimum inhibitory concentration (MIC) of acetone extracts of plants on seven test organisms

	Cyphomandra betaceae (mg/ml)	*Dicentra scandens* (mg/ml)
Escherichia coli	3.75	> 10
Klebsiella pneumoniae	5	> 10
Pseudomonas aeruginosa	2.5	-
Proteus vulgaris	2.5	> 10
Bacillus cereus	2.5	3.75
Bacillus subtilis	3.75	5
Staphylococcus aureus	2.5	10

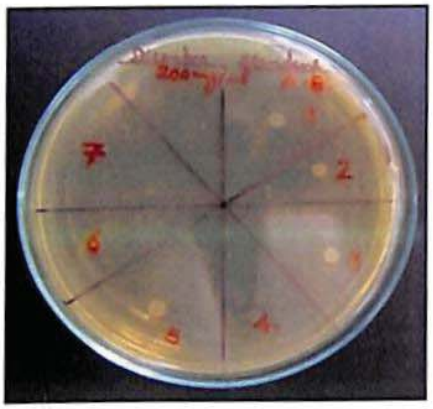
MIC assay was performed against organism whose growth was inhibited by extract in well diffusion test. '-' indicate MIC assay not performed.

Table 12 shows the Minimum inhibitory concentration of acetone extract of plants. The MIC value of acetone extract of *Cyphomandra betaceae* was 3.75 mg/ml for *Escherichia coli* and *Bacillus subtilis*, 5 mg/ml for *Klebsiella pneumoniae* and 2.5 mg/ml in the case of *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus cereus* and *Staphylococcus aureus*. The acetone extract of *Dicentra scandens* showed lower MIC value than methanol extract against *Staphylococcus aureus* (10 mg/ml), *Bacillus subtilis* (5 mg/ml) and *Bacillus cereus* with 3.75 mg/ml. For other test organisms, the MIC value was greater than 10 mg/ml.

Minimum Inhibitory Concentration assay (Agar dilution method)



Photographic plate 20: MIC assay of *Cyphomandra betaceae* (acetone extract). At concentration of 5 mg/ml the extract inhibited growth of all test bacteria. In photographic plate 1 indicates *E. coli*, 2: *P. aureginosa*, 3: *K. Pneumoniae*, 4: *P. vulgaris*, 5: *S. aureus*, 6: *B. cereus*, 7: *B. subtilis*.



Photographic plate 21: MIC assay of *Dicentra scandens* (acetone extract) at concentration of 10 mg/ml. In photographic plate 1 indicates *E. coli*, 2: *P. aureginosa*, 3: *K. Pneumoniae*, 4: *S. aureus*, 5: *P. vulgaris*, 6: *B. cereus*, 7: *B. subtilis*.

Photographic plate 22: Positive control (Gentamicin)

Photographic plate 23: Negative control (solvent)

4.5. Synergistic antimicrobial activity of combination of different plant extracts

To evaluate the possible synergistic antimicrobial activity, the combination of *Cyphomandra betaceae* and *Capsicum annuum* and combination of *Cyphomandra betaceae* and *Heracleum nepalense* were considered for the study.

4.5.1. Combinatorial effect of *Cyphomandra betaceae* and *Capsicum annuum*

Methanol extract of *Cyphomandra betaceae* and *Capsicum annuum* was evaluated for possible synergistic activity. The extracts in combination showed additive effect against some test microorganisms (Table 13).

Table 13: Synergistic antimicrobial activity of combination of *Cyphomandra betaceae* and *Capsicum annuum* (methanol extract)

Mean diameter of growth inhibition zone(mm)						
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*B.c*	*B.s*	*S.a*
Cy100	19	13	17	16	20	23.5
Cp100	20	-	-	11	20.5	22
S	20.5	-	13	16.5	21.5	24
Cy75	16	11	14	15.5	19	19
Cp75	19	-	-	-	19.5	20
S	19.5	-	-	11	23	22.5
Cy50	15	-	13	15	16.5	18.5
Cp50	15	-	-	-	16	16
S	15.5	-	-	-	18	19
Cy25	14	-	-	12.5	13	-
Cp25	-	-	-	-	-	-
S	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Cy; *Cyphomandra betaceae*, Cp; *Capsicum annuum.* Each value represent mean (n=4). Zone of inhibition: including the well diameter of 8 mm. '-': No zone of inhibition.

Synergistic effect of *Cyphomandra betaceae* and *Capsicum annum* was evaluated using four concentrations (25, 50, 75 and 100 mg/ml) (Table 13). At the concentration of 100 mg/ml the individual extract of *Cyphomandra betaceae* showed the zone of inhibition of 19 mm against *Escherichia coli* and *Capsicum annuum* at the same concentration showed 20 mm. The extracts in combination showed the zone of inhibition of 20.5 mm. The extracts (100 mg/ml) in combination inhibited the growth of *Bacillus subtilis* with zone of inhibition of 21.5 mm, the individual extract of *Cyphomandra betaceae* showed zone of inhibition of 20 mm and the extract of *Capsicum annum* showed the zone of inhibition of 20.5 mm when used individually. Combinatorial effects of the extract against *Bacillus cereus* and *Staphylococcus aureus* was comparatively larger than the zone shown by individual extract.

4.5.2. Combinatorial effect of *Cyphomandra betaceae* and *Heracleum nepalense*

The methanol extract of *Cyphomandra betaceae* and *Heracleum nepalense* was evaluated for possible synergistic activity. The extract in combination showed additive effect against test microorganisms (Table 14).

Table14: Synergistic antimicrobial activity of combination of *Cyphomandra betaceae* and *Heracleum nepalense* (methanol extract)

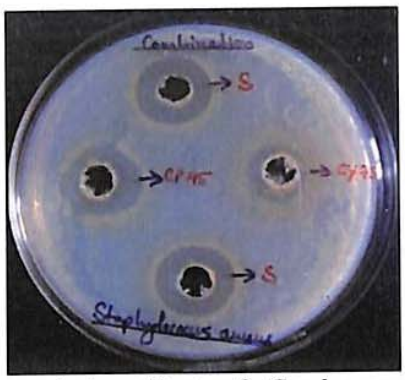
	Mean diameter of growth inhibition zone(mm)					
Concentration (mg/ml)	*E.c*	*K.p*	*P.a*	*B.c*	*B.s*	*S.a*
Cy100	16	14	16	19.5	21	22
H100	11	-	-	12	13	-
S	19.5	11	12	21	21.5	17
Cy75	15	12	14.5	16	18	16
H75	-	-	-	11	-	-
S	-	-	11	17.5	13	13
Cy50	14	-	13	15	16	13
H50	-	-	-	-	-	-
S	-	-	-	-	12	-
Cy25	13	-	-	12	12	-
H25	-	-	-	-	-	-
S	-	-	-	-	-	-

E.c., Escherichia coli; K.p., Klebsiella pneumoniae; P.a., Pseudomonas aeruginosa; B.c., Bacillus cereus; B.s., Bacillus subtilis; S.a., Staphylococcus aureus. Cy; *Cyphomandra betaceae*, H; *Heracleum nepalense*. Zone of inhibition: including the well diameter of 8 mm. '-': No zone of inhibition.

Combinatorial effect of *Cyphomandra betaceae* and *Heracleum nepalense* was evaluated for their synergistic effects against six test organisms (Table 14). The extracts in combination, at the concentration of 100 mg/ml inhibited the growth of *Escherichia coli* with the zone of inhibition of 19.5 mm. The zone of inhibition observed for the individual extract of *Cyphomandra betaceae* and *Heracleum nepalense* was 16 mm and 11 mm respectively. The zone of inhibition observed in case of the combinatorial plant

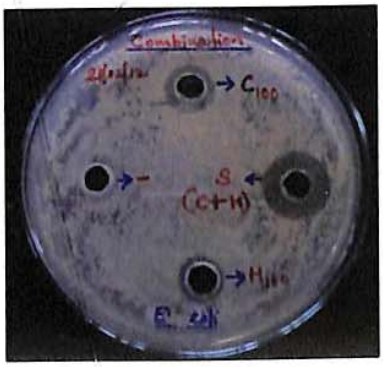
extract for *Bacillis cereus* was 21 mm whereas the individual extract of *Cyphomandra betaceae* showed the zone of inhibition of 19.5 mm and *Heracleum nepalense* was 12 mm. At concentration of 75 mg/ml the extract in combination inhibited the growth of *Bacillis cereus* and the zone of inhibition observed was 17.5 mm, whereas the zone of inhibition observed was 16 mm and 11 mm for individual extract of *Cyphomandra betaceae* and *Heracleum nepalense* respectively. The zone of inhibition observed in case of the combinatorial plant extract for *Bacillis subtilis* was 21.5 mm whereas the individual extract of *Cyphomandra betaceae* showed the zone of inhibition of 21 mm and *Heracleum nepalense* showed inhibition zone of 13 mm.

Synergistic antimicrobial activity



Photographic plate 24: Synergistic effect of *Cyphomandra betaceae* and *Capsicum annuum* at concentration of 75 mg/ml against *S. aureus*. Cy_{75} is the individual extract of *C. betaceae*, Cp_{75} is of *C. annuum* and S in the plate indicates the combination of two extracts.

Photographic plate 25: Synergistic effect of *Cyphomandra betaceae* and *Capsicum annuum* at concentration of 75 mg/ml against *B. subtilis*. Cy_{75} is the individual extract of *C. betaceae*, Cp_{75} is of *C. annuum* and S in the plate indicates the combination of two extracts.



Photographic plate 26: Synergistic effect of *Cyphomandra betaceae* and *Heracleum nepalense* at concentration of 100 mg/ml against *E. coli*. C_{100} is the individual extract of *C. betaceae*, H_{100} is of *H. nepalense* and S in the plate indicates the combination of two extracts.

4.6. Antioxidant activity assay

In the present study, we have evaluated the DPPH free radical scavenging activity, ferric reducing power and total phenolic contents of methanolic extract of *Cyphomandra betaceae*, *Capsicum annuum*, *Dicentra scandens* and *Heracleum nepalense*.

4.6.1. DPPH scavenging assay

The antioxidant activity of the plant extracts was evaluated by DPPH radical scavenging protocol. DPPH free radical scavenging activity of plants extract was compared with ascorbic acid as standard (Table 15).

Table 15: Percentage inhibition of standard (ascorbic acid), *Cyphomandra betaceae*, *Capsicum annuum*, *D*icentra *scandens* and *Heracleum nepalense* at various concentrations (µg/ml) in DPPH scavenging model

Concen-tration (µg/ml)	Standard	*C. betaceae*	*C. annuum*	*D. scandens*	*H.nepalense*
1	33.59±0.19	24.41±0.25	28.83±0.39	29.91±0.54	26.88±0.25
10	43.89±0.38	31.77±0.66	33.37±0.55	33.41±0.41	34.41±0.46
20	54.37±0.27	41.55±0.12	36.24±0.47	41.03±0.34	38.26±0.49
30	61.68±0.38	49.53±0.93	44.92±0.27	55.97±0.59	42.55±0.27
40	70.04±0.27	58.26±0.27	52.55±0.80	60.86±0.27	44.58±0.54
60	81.16±0.46	69.06±0.73	57.49±0.27	67.27±0.34	53.29±0.27
80	88.74±0.19	72.85±0.22	60.93±0.53	75.71±0.72	59.35±0.34
100	95.62±0.39	80.25±0.46	78.74±0.90	78.39±0.27	64.84±0.27

Each value represents mean ± SD (n=3)

At the concentration of 100 µg/ml, the scavenging activity of methanol extract of *Cyphomandra betaceae* was 80.25±0.46 %, followed by 78.74±0.90 % of *Capsicum annuum*, 78.39±0.27 % of *Dicentra scandens* and 64.84±0.27 % of *Heracleum nepalense* while at the same concentration, scavenging activity of ascorbic acid was 95.62±0.39 %. At the concentration of 20 µg/ml, the percentage inhibition of free radicals by ascorbic acid was 54.37±0.27 %, *Cyphomandra betaceae* 41.55±0.12 %,

Capsicum annuum 36.24±0.47 %, *Dicentra scandens* 41.03±0.34 % and *Heracleum nepalense* 38.26±0.49 %. The IC_{50} value was defined as the concentration (in µg/ml) of the extract that scavenges the DPPH redicals by 50 %. The IC_{50} value of standard was 20 µg/ml with percentage inhibition of 54.37±0.27 %. For *Cyphomandra betaceae* and *Dicentra scandens* the IC_{50} value was 30µg/ml with inhibition percentage of 49.53±0.93 % and 55.97±0.59 % respectively. The IC_{50} value of *Capsicum annum* was 40 µg/ml with inhibition percentage of 52.55±0.80 % and *Heracleum nepalense* was 60 µg/ml with scavenging activity of and 53.29±0.27 %. The inhibitory effect of the four test plant extracts on DPPH radicals followed dose-dependent manner (Figure 14, 15, 16 and 17).

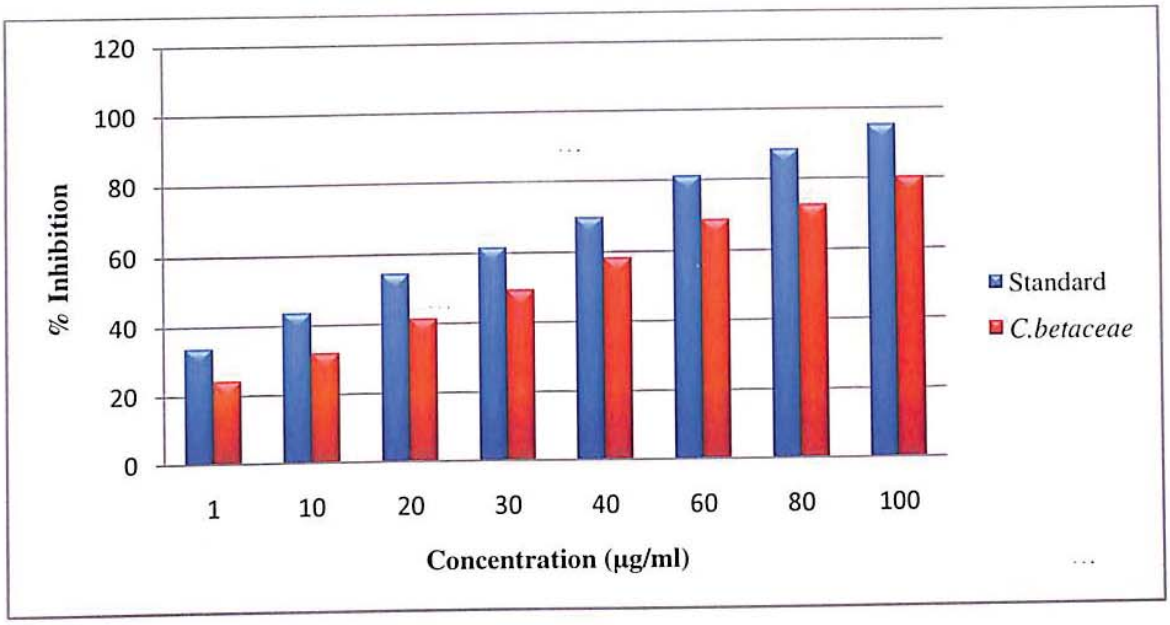


Figure14: DPPH scavenging activity of methanol extract of *Cyphomandra betaceae* and standard ascorbic acid

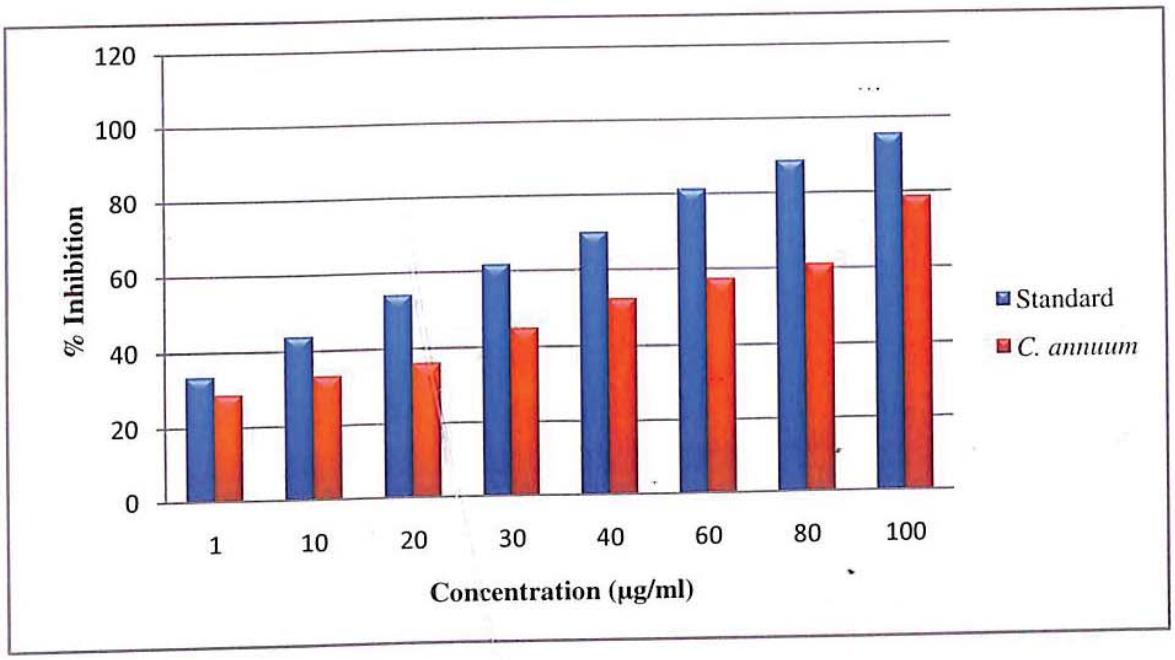
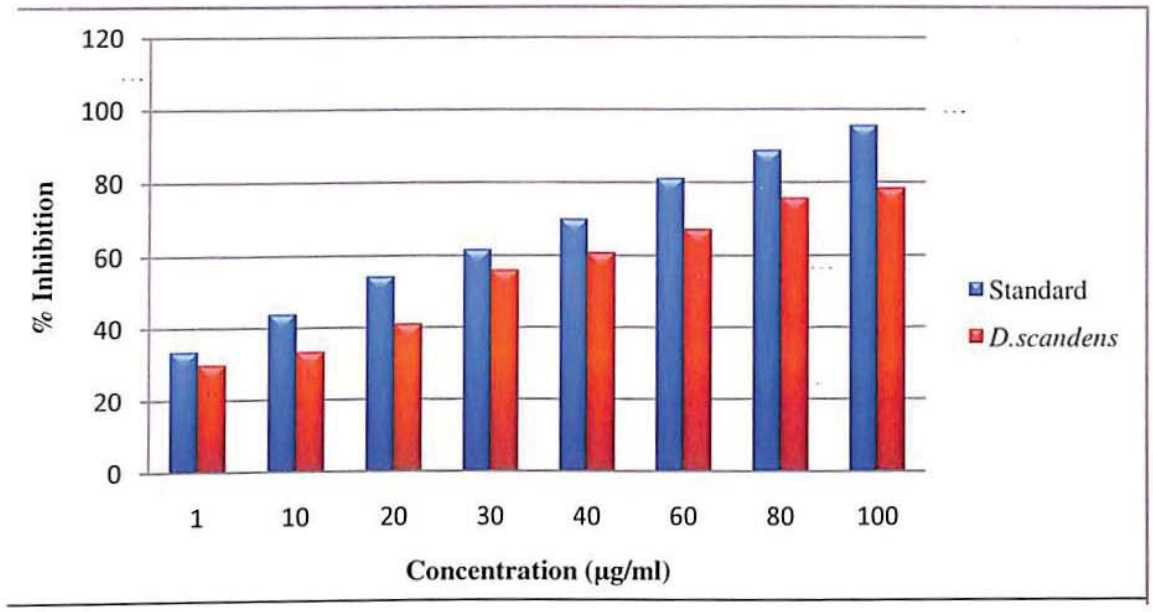
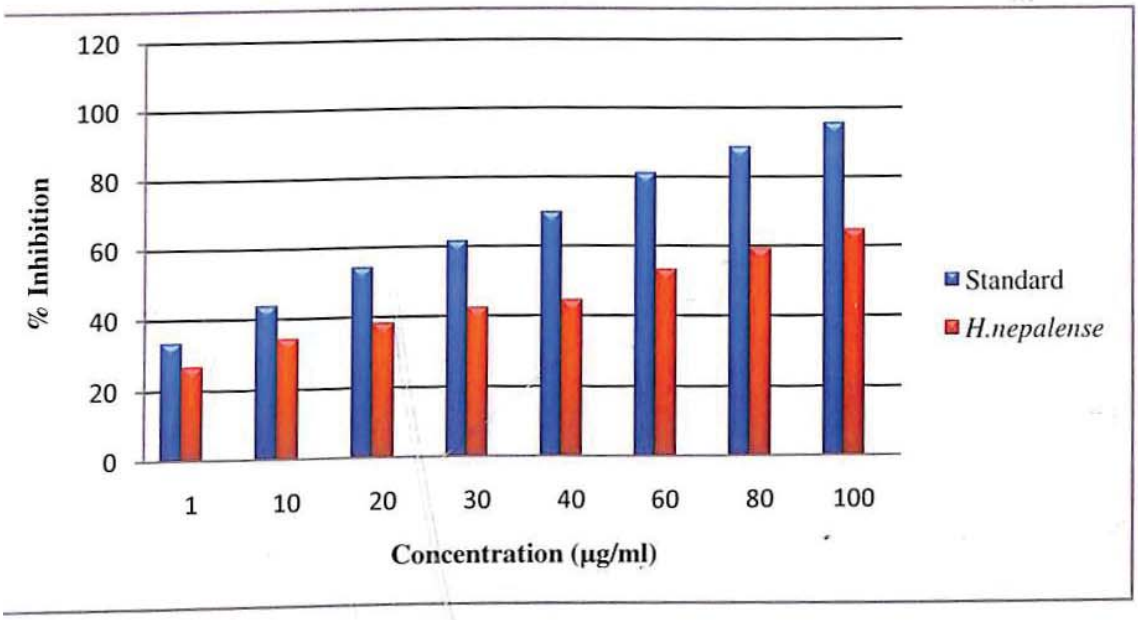


Figure15: DPPH scavenging activity of methanol extract of *Capsicum annuum* and standard ascorbic acid



igure16: DPPH scavenging activity of methanol extract of *Dicentra scandens* and andard ascorbic acid



igure17: DPPH scavenging activity of methanol extract of *Heracleum nepalense* ıd standard ascorbic acid

4.6.2. Reducing power assay

The reducing power of the plant extract was compared to ascorbic acid as standard. The assay is based on the principle that the plant extract which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which further reacts with ferric chloride to form ferric ferrous complex, that has an absorption maxima at 700 nm. The reducing capability of the extract increases with increase in the concentration of extract.

Table 16: Absorbance of Standard (ascorbic acid), *Cyphomandra betaceae*, *Capsicum annuum*, *Dicentra scandens* and *Heracleum nepalense* at various concentrations (µg/ml) in ferric reducing power determination model

Conce-ntration (µg/ml)	Standard	*C. betaceae*	*C. annuum*	*D. scandens*	*H. nepalense*
10	0.036±0.0016	0.032±0.0018	0.034±0.0037	0.026±0.0017	0.030±0.0007
20	0.054±0.0089	0.050±0.0007	0.045±0.0018	0.027±0.0007	0.032±0.0014
40	0.099±0.0000	0.052±0.0003	0.053±0.0039	0.028±0.0002	0.032±0.0018
60	0.134±0.0053	0.061±0.0005	0.058±0.0007	0.036±0.0005	0.032±0.0021
80	0.170±0.0051	0.065±0.0020	0.064±0.0009	0.036±0.0002	0.036±0.0021
100	0.218±0.0048	0.084±0.0020	0.082±0.0018	0.060±0.0090	0.041±0.0072
200	0.338±0.0106	0.122±0.0011	0.100±0.0008	0.082±0.0005	0.043±0.0048

Each value represents mean ± SD (n=3)

In all the four extracts tested, it was found that with the increase in concentration of extract, the absorbance of all four extract increases. The maximum reducing power was observed in *Cyphomandra betaceae,* followed by *Capsicum annuum* ((Table 16, Figure 18, 19) and *Dicentra scandens*. The minimum activity was observed in *Heracleum nepalense*. Though, plant extract had lesser reductive activity than standard of ascorbic acid, it was found that the plant extract could reduce Fe^{3+} ions.

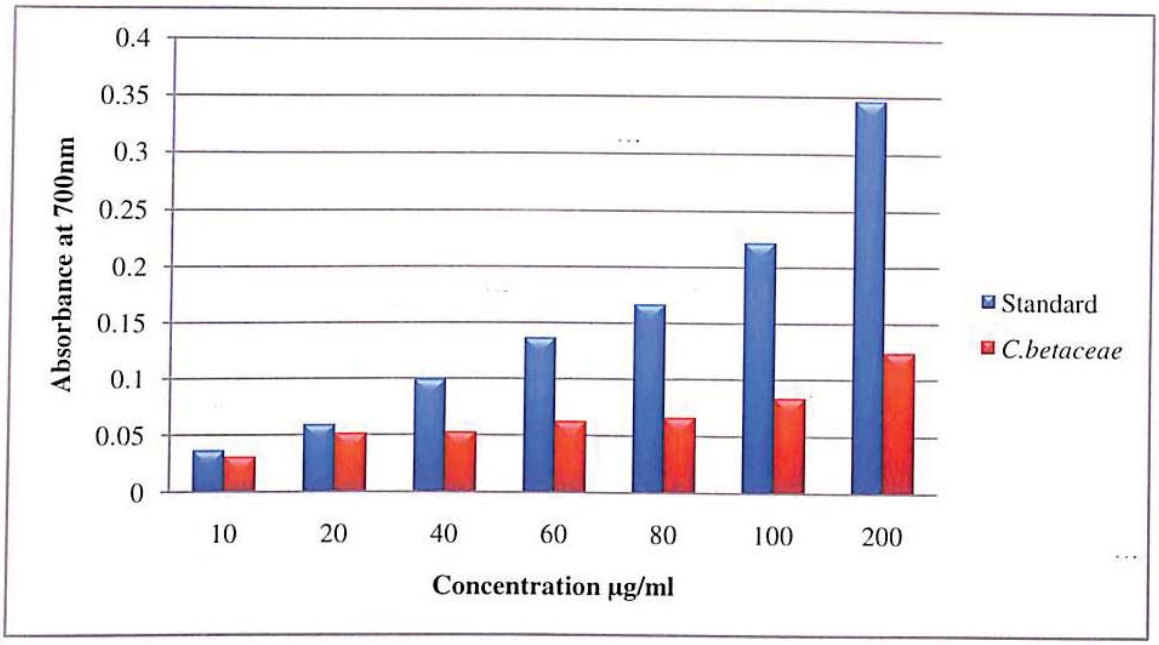


Figure 18: Ferric reducing power determination of methanolic extract of *Cyphomandra betaceae* and standard ascorbic acid

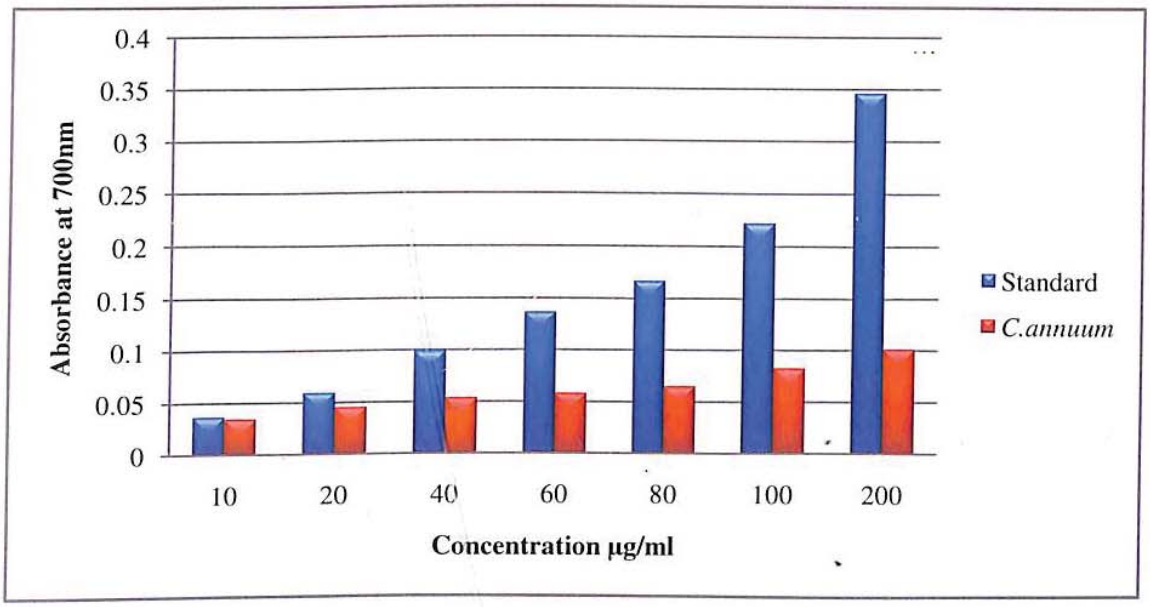


Figure 19: Ferric reducing power determination of methanolic extract of *Capsicum annuum* and standard ascorbic acid

5. DISCUSSION

5. DISCUSSION

5.1. Antimicrobial activity

The present study carried out in plant extract revealed the presence of various phytochemicals. The analysis of phytochemicals had shown the presence of bioactive compounds phenol, saponin, steroid, alkaloids and flavonoid in most of the selected plant extracts which could be responsible for observed antimicrobial activity.

Phytochemical analysis of *Cyphomandra betaceae* aqueous extract revealed the presence of flavonoids and steroid, methanol extract revealed the presence of phenols, saponin and flavonoid and presence of tannin and phenolic compounds in the acetone extract. Among three solvents used acetone extract of *Cyphomandra betaceae* showed more consistent antimicrobial activity than other solvent extract. As phenols and saponins were detected in acetone extract, hence the presence of phenolic compounds in combination with saponin in acetone extract of *Cyphomandra betaceae* could be responsible for observed antimicrobial activity. It has been reported that phenolic compounds are better extracted in acetone than in methanol (Tiwari *et al.*, 2011). The methanol extract of *Cyphomandra betaceae* also showed effective antimicrobial activity against all test microorganisms. It has been reported that flavonoids such as flavonols and anthocyanin and saponins are extracted in methanol (Tiwari *et al.*, 2011). The antimicrobial activity of methanol extract could be due to the presence of these phytochemicals, mainly flavonoids. The aqueous extract also showed different degree of zone of inhibition against *Escherichia coli*, *Bacillus subtilis* and *Bacillus cereus*. Flavonoids like anthocyanin are water soluble, so the antimicrobial activity of aqueous extract is likely due to the presence of flavonoids.

It has been reported that an invertase inhibitory protein isolated from *Cyphomandra betacea* has broad spectrum antimicrobial activity against plant pathogens (Ordonez *et al.*, 2006). The present study had shown that the extract of *Cyphomandra betaceae* is equally active against different microorganisms which are the common cause of various infections and diseases in human beings. A virulent strain of most common bacteria *Escherichia coli* causes gastroenteritis, urinary tract infections, neonatal meningitis; *Klebsiella pneumoniae* is a causative organism of pneumonia; *Pseudomonas aureginosa* infect burns, wounds, urinary tract and respiratory tract (Shihabudeen *et al.*,

2010); *Proteus vulgaris* can become deadly when in sinus and respiratory tissues and if left untreated. Among the Gram positive bacteria taken for study, *Staphylococcus aureus* causes wound infections, toxic shock syndrome and contaminates food products; *Bacillus cereus* causes food borne illness; *Bacillus subtilis* cause disease in severely immunocompromised patients and it may contaminate food rarely causing food poisoning (Talaro, 2008). The extract of *Cyphomandra betaceae* inhibited the growth of all these test microorganisms.

In the present study, the phytochemical analysis of aqueous extract of *Capsicum annuum* revealed the presence of flavonoid, methanol extract revealed the presence of phenols, steroid, flavonoid and alkaloid whereas all the tested phytochemicals were absent in the acetone extract. It was found that aqueous extract of *Capsicum annuum* showed the antimicrobial activity against *Bacillus cereus* with zone of inhibition of 21 mm, interestingly it was observed that the methanol extract showed zone of inhibition of 11.75 mm against *Bacillus cereus*. Since flavonoids are mostly water soluble and we observed that the antimicrobial activity of aqueous extract was comparatively much higher than methanol extract against *Bacillus cereus* and water extract revealed the presence of flavonoid, hence the observed activity could be due to the presence of flavonoids. The methanol extract of *Capsicum annuum* inhibited the growth of *Escherichia coli* but the growth of other test Gram negative bacteria were not inhibited by the concentration of extract employed for study. It is thought that observed differences may result from the doses used in this study, which need to be further evaluated. The dose levels employed may be insufficient to inhibit the growth of other test organisms. Lack of activity can thus only be proven by using large doses (Parekh and Chanda, 2007).

The phytochemicals such as steroid, phenol, flavonoids and alkaloids could be attributed for observed antimicrobial activity of methanol extract of *Capsicum annuum*. Phenols, steroids, and flavonoid were reported to have antimicrobial activity and are known to act by different mechanisms. Flavonoids are the hydroxylated phenolic substances and are effective antimicrobial substances (Dixon *et al.*, 1983). It has been reported that their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Marjorie, 1999). An antibacterial property of steroid is because of its ability to associate with membrane

lipid which exerts its action by causing leakages from liposomes (Epand, 2007; Shihabudeen *et al.*, 2010). Interestingly it was found that the acetone extract of the plant did not inhibit any of the seven test organism which may be related to the fact that no phytochemicals were detected in acetone extract. The phytochemicals responsible for antimicrobial activity of the extract were not extracted in acetone or were not present in sufficient amount as flavonoids are mostly extracted in water and methanol (Tiwari *et al.*, 2011). Hence, it can be speculated that the antimicrobial activity of *Capsicum annuum* is most likely due to the presence of flavonoids.

The rural folk believe that the fruit of *Capsicum annuum* is useful in gastrointestinal ulcers and stomach disorders. Interestingly, it was found that the extract of *Capsicum annuum* inhibited the growth of *Escherichia coli*, is an enteric pathogen causing gastrointestinal disorders. The extract also inhibited the growth of *Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus.* These bacteria are food contaminants and are associated with food poisoning. Hence, the use of *Capsicum annuum* as a part of diet and as an appetizer may be helpful in the preventing food borne illness.

The phytochemical analysis of aqueous extract of *Dicentra scandens* showed the presence of saponin, steroid and alkaloid, methanol extract revealed the presence of phenol, saponin, steroid and alkaloid and presence of phenolic compound and alkaloid in acetone extract. The aqueous extract of *Dicentra scandens* inhibited the growth of *Escherichia coli.* The observed antimicrobial activity could be due to the presence of these phytochemicals mainly saponin as saponins are mostly extracted in water (Tiwari *et al.*, 2011). The antimicrobial activity of acetone extract of *Dicentra scandens* was comparatively better then methanol extract, this is probably because of the fact that phenolic compounds were better extracted in acetone. Both acetone and methanol were found to extract saponins which are known to have antimicrobial property (Tiwari *et al.*, 2011). It has been reported that the antimicrobial activity of saponin is due to its abilty to cause leakage of protein and certain enzymes from the cells (Zablotowicz *et al.*, 1996; Shihabudeen *et al.*, 2010).

Traditionally the root extract prepared from *Dicentra scandens* is used by folk healers to treat various enteric infections like gastritis. In the present study, it was found that all the three solvent extract of *Dicentra scandens* showed antimicrobial activity against *Escherichia coli* which is a common cause of many enteric infections. Hence, our

results provide a scientific basis for the use of *Dicentra scandens* in traditional medicine.

The phytochemical analysis of *Heracleum nepalense* revealed the presence of tannins in aqueous extract, presence of tannin, phenols and saponins in methanol extract whereas most of the tested phytochemicals were absent in acetone extract except flavonoid. The aqueous extract of *Heracleum nepalense* showed antimicrobial activity against *Bacillus cereus* and methanol extract showed activity mostly against Gram positive test bacteria i.e. *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*. The acetone extract did not inhibit the growth of any of the test microorganisms which may be related to the fact that the most of the tested phytochemicals were not detected in the acetone extract. Antimicrobial property of the extract is probably due to presence tannins as tannins are better extracted in water and methanol than in acetone (Das *et al.*, 2010; Tiwari *et al.*, 2011). The mechanism of antimicrobial activity of tannin is probably due to its ability to bind with proline rich proteins and interfere with the protein synthesis (Shimada, 2006). The antibacterial activity of methanol extract of *Heracleum nepalense* was observed mostly in Gram positive bacteria. Researchers have reported that Gram positive bacteria are more susceptible to plant extracts as compared to Gram negative bacteria (Rabe and Van, 1997; Vlietinck *et al.*, 1995). This may be due to the fact that cell wall in Gram positive bacteria is of single layer whereas the Gram negative bacteria have multilayered structure (Sinha, 2012; Yao and Moellering, 1995) and hence the passage of the active compound through the Gram negative cell wall may be inhibited (Parekh and Chanda, 2007). Since, phenols were present in the methanol extract; the antimicrobial activity may be attributed to phenols. It has been reported that the phenols and phenolic comounds causes the injury of membrane function (Davidson and Branen, 1981).We observed differences in antimicrobial activity of plant extracts, which was due to the difference in phytochemical properties.

Traditionally the fruit of *Heracleum nepalense* is taken against stomach disorders, cough, nausea, vomiting and diarrhoea. The methanol extract of the *Heracleum nepalense* showed the antimicrobial activity against *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*. Since, nausea, vomiting and diarrhoea are mainly the symptoms of food poisoning and as the extract exhibited the antimicrobial activity

against organisms associated with food contamination, the results of the study provide justification in traditional use of *Heracleum nepalense*.

Isolation of bioactive compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Parekh and Chanda, 2007). Though the traditional healers use primarily water as the solvent for various medicinal preparations, but in our study we found that the plant extracts prepared by using methanol provided more consistent antimicrobial activity compared to those extracted by acetone and water. This might have resulted from the lack of solubility of the active constituents in aqueous solutions. All the four plant extract showed different degree of antimicrobial activity. Further study using different solvents of various polarities will explore the effects of solvent on extract efficacy (Romero *et al.*, 2005).

5.2. Synergistic antimicrobial activity of different combination of plant extracts

5.2.1. Combinatorial effect of *Cyphomandra betaceae* and *Capsicum annuum*

The methanol extract of *Cyphomandra betaceae* and *Capsicum annuum* were evaluated for possible synergistic activity and it was observed that the extract in combination showed synergistic antimicrobial activity against some of the test organism. There was increase in zone of inhibition in combination than in individual extract. Methanol extract of *Cyphomandra betaceae* contains phenol, saponin and flavonoid whereas *Capsicum annuum* was found to possess phenol, steroid and flavonoid. The additive effect was possibly due to the presence of saponin and steroid in combination of extract along with flavonoid.

5.2.2. Combinatorial effect of *Cyphomandra betaceae* and *Heracleum nepalense*

Combinatorial effect of *Cyphomandra betaceae* and *Heracleum nepalense* was also evaluated against six test organisms. The extracts in combination, at the concentration of 100 mg/ml inhibited *Escherichia coli* with the zone of inhibition of 19.5 mm, which was much higher than the individual extract. The zone of inhibition observed for the individual extract of *Cyphomandra betaceae* and *Heracleum nepalense* was 16 mm and 11 mm respectively. Similarly the extract in combination showed additive effect against *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*. Since, the methanol extract of *Cyphomandra betaceae* revealed the presence of flavonoid and methanol

extract of *Heracleum nepalense* revealed the presence of tannin, hence the additive effect could be due to the presence of both the bioactive compound in combination of extract, which when present together showed additive activity.

5.3. Antioxidant activity

The present study showed that all four plant extracts have free radical scavenging ability, reductive capability and contain good amount of phenolic compounds.

The DPPH scavenging model is a widely used method to evaluate the free radical scavenging ability of various samples (Lee *et al.,* 2003). The antioxidant activity of the extract was also evaluated by ferric reducing power assay. It was found that the free radical scavenging activities and ferric reducing power of all the four methanol extracts increased with increasing concentration. Presence of both flavonoid and phenolic compound in the methanol extract of *Cyphomandra betaceae* and *Capsicum annuum* may have contributed to high antioxidant activity of these plant extracts. Phytochemical analysis of methanol extract of *Dicentra scandens* revealed the presence of phenol. Though the flavonoid was not detected in the extract, on estimation of total phenolic content it was found that the extract contained good amount of phenolic compound. Hence, the antioxidant activity of *Dicentra scandens* is possibly due to the presence of high amount of phenolic compound in the extract. The phytochemical analysis of methanol extract of *Heracleum nepalense* revealed the presence of phenols. The total phenolic compound estimated was comparatively lower than that of *Dicentra scandens* and the flavonoid was absent in the extract. This may possibly relate to the comparatively lower antioxidant activity of *Heracleum nepalense* than the other plant extracts.

Phenols and polyphenolic compounds, such as flavonoids, are widely found in plants, fruits and vegetables and they have been shown to possess significant antioxidant activities (Klimczak *et al.,* 2007). It has been reported that flavonoids present in plants act as antioxidants by stabilising the reactive oxygen species (Robert *et al.,* 2001). The correlation between total phenol contents and antioxidant activity has been widely studied in fruit, vegetables and herbal plants (Klimczak *et al.,* 2007; Kiselova *et al.,* 2006; Jayaprakasha *et al.,* 2008; Kedage *et al.,* 2007). Various reports suggested that

antioxidant activity of phenolic compounds is because of its ability to scavenge free radicals by virtue of hydrogen donating ability (Patil *et al*., 2009; Ghafer *et al*., 2010).

6. SUMMARY

6. SUMMARY

Medicinal plants are used traditionally in treatment of various kinds of diseases since time immemorial. The present study was carried out to evaluate the antimicrobial and antioxidant activities of four herbal plants of Sikkim. Among them *Dicentra scandens* and *Heracleum nepalense* are used traditionally in folklore medicine. *Cyphomandra betaceae* and *Capsicum annuum* are part of diet and also posses' medicinal value. The aqueous, methanol and acetone extracts of the plants were subjected to phytochemical analyses, which revealed the presence of phenol, tannin, flavonoid, steroid, saponin and alkaloid in most of the selected plants.

Methanol and acetone extract of *Cyphomandra betaceae*, *Capsicum annuum*, *Dicentra scandens* and *Heracleum nepalense* were investigated for *in vitro* antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus* by well diffusion method. The aqueous extract of the plants were used for screening of antimicrobial activities of the plants selected, which inhibited the growth of one or other test organism. Methanol and acetone extract of *Cyphomandra betaceae* and *Dicentra scandens* showed promising antimicrobial activity against all test organisms. Methanol extract of *Capsicum annuum* inhibited the growth of all the three test Gram positive bacteria and the growth of *Escherichia coli* among the Gram negative test organism; on the other hand the acetone extract did not show any activity against all the test organisms. Methanol extract of *Heracleum nepalense* was mostly found to inhibit the growth of Gram positive test organisms. The acetone extract did not inhibit the growth of any test organisms.

The Minimum inhibitory concentration (MIC) of the plant extracts were also determined against different test organisms. The MIC value of methanol extract of *Cyphomandra betaceae* extract ranged from 3.75 mg/ml to 10 mg/ml and that of acetone extract ranged from 2.5 mg/ml to 5 mg/ml. For methanol and acetone extract of *Dicentra scandens*, the MIC value ranged from 3.75 mg/ml to greater than 10 mg/ml. For methanol extract of *Capsicum annuum* and *Heracleum nepalense* MIC value was greater than 10 mg/ml. The methanol extract of selected plants were evaluated for possible synergistic antimicrobial activity. The synergistic activity was studied against six microorganisms namely *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas*

aureginosa, *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*. It was found that combination of extracts of *Cyphomandra betaceae* and *Capsicum annum* showed additive effect against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* as compared to the individual plant extracts. Synergistic effect of *Cyphomandra betaceae* and *Heracleum nepalense* was also evaluated. The extracts in combination showed additive effect against *Escherichia coli*, *Bacillis cereus* and *Bacillis subtilis*.

The methanol extract of all the four selected plants were evaluated for antioxidant activity by using free radical scavenging activity assay (DPPH method) and reducing power assay. The results of both the methods were compared with ascorbic acid as standard. The IC_{50} value of standard (ascorbic acid) was found to be 20 µg/ml and for, *Cyphomandra betaceae* and *Dicentra scandens* the IC_{50} value was 30 µg/ml, for *Capsicum annuum* the IC_{50} value was 40 µg/ml and for *Heracleum nepalense* it was found to be 60 µg/ml. The reducing power of the extract was found to increase with increase in concentration. Total phenolic content was determined by using gallic acid as standard. It was found that the methanol extract of *Dicentra scandens* contain relatively high amount of phenolic compound (56 µg/ml).Based on the results obtained it was found that methanolic extract of the plants possess good antioxidant activity.

On the basis of results obtained it can be concluded that plant extracts under study exhibits different level of antimicrobial and antioxidant activity.

7. CONCLUSION

7. CONCLUSION

In the present study, the methanol extract of *Cyphomandra betaceae* and *Dicentra scandens* showed the antimicrobial activity against *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Bacillus cereus, Bacillus subtilis and Staphylococcus aureus.* Methanol extract of *Heracleum nepalense* and *Capsicum annuum* exhibited inhibitory activity mostly against Gram positive test organisms. Acetone extracts of *Cyphomandra betaceae* and *Dicentra scandens* also exhibited effective antimicrobial activity against all the test organisms. Among the four test plants studied, both methanol and acetone extract of *Cyphomandra betaceae* and *Dicentra scandens* showed promising antimicrobial activity against all the test organisms.

The antibacterial action of various extracts of selected plants may indicate their potential as antibacterial herbal remedies. Further work is required for identification, isolation and characterization of the active constituents of plant extracts responsible for the antibacterial activity.

All the selected plants showed promising antioxidant activity. Based on the results obtained it can be concluded that extract of *Cyphomandra betaceae*, *Capsicum annuum*, *Dicentra scandens* and *Heracleum nepalense* had shown different level of antioxidant activity. Further study shall be aimed at isolating and identifying the substances responsible for the antioxidant activity of plant extracts, which may be further exploited in herbal formulations.

Estimation of total phenolic content showed the presence of good amount of phenolic compound in the extracts. Therefore, it is necessary to investigate further and understand the relationship between antibacterial activity and antioxidant activity of phenolic compound in the plant extracts under study. Furthermore, in vivo antioxidant activity of these extracts needs to be assessed.

8. BIBLIOGRAPHY

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