CHAPTER I: INTRODUCTION

1.1 Background

The plant kingdom is a treasure house of potential drugs. In the recent years there has been an increasing awareness about the importance of medicinal plants. Drugs from the plants are easily available, less expensive, safe and efficient and rarely have side effects. Thus Plants have been looked at as a very promising source of new lead compounds for drug discovery and development (Kong et. al., 2003). Many products of medicinal plants prove to be very useful in prolonging longevity and achieving positive health care system (Kaushik et. al., 2002). According to World Health Organization (WHO), medicinal plants would be the best source to obtain variety of drugs. Many modern drugs are derived from natural plant products. Herbal medicines are often used to provide first line basic health service for the people living in remote areas where it is the only affordable remedy (WHO, 1998). About 25% of the drugs prescribed worldwide come from plants. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin. One example of important drug obtained from plant is digoxin from *Digitalis* spp. A significant number are synthetic drugs obtained from natural precursors(Rates, 2001). It is estimated that 60% of anti-tumor and anti-infectious drugs already on the market or under clinical trial are of natural origin (Yue-Zhong Shu, 1998). The vast majority of these cannot yet be synthesized economically and are still obtained from wild or cultivated plants.

Plants are self-generating machines, producing a range of active medicinal compounds. These active constituents are actually plant secondary metabolites and a particular combination of such metabolites may be taxonomically distinct attributing uniqueness of medicinal actions to particular plant species (Ahmad *et al.*, 2011; Parekh *et al.*, 2006, 2007). These natural products can be obtained from any part of the plant i.e., roots, rhizomes, shoots, leaves, bark, flowers, fruits or seeds but it solely depends upon the nature of compounds as presence of some compounds may be restricted to some parts but not others or there may be concentration differences (Cragg and Newman, 2001).

According to World Health Organization consultative group, a plant (tree, herb or shrub, fresh or dried) with at least one of its parts constituting substances which can be employed for therapeutic purposes or which are precursors for the manufacturing of useful drugs is known as medicinal plant (Bernhoft, 2008). The use of medicinal plants as the treatment of several ailments dates back to prehistory and people of all continents have this old tradition (Mungole and Chaturvedi, 2011). About 80 percent population around the world depends on traditional use of plant based pharmaceuticals for primary healthcare needs, especially in the rural areas (Akinmoladun *etal.,* 2007). Their importance can be inferred from the fact that 12 out of the world's 25 popular pharmaceutical agents are derived from natural products (Ahmad et al., 2011). Since ancient days humans have been used plants for medicinal purposes.The number of such plants is very large. It is difficult to ascertain how many of the plant species are used for medical purposes. However more than 35,000 plant species are being used in various human cultures around the world (Lewington 1993).

Nepal is rich in varieties of medicinal plants. Among the 7000 species of medicinal plants recognized all over the world, more than 900 types of precious medicinal plants are said to be found in Nepal (Manandhar, 2000).From the time immemorial many medicinal plants are well known within the mankind. About 1500 plants are systematically used in indigenous system of medicine, like Ayurveda, Unani and Siddha (Joshi et al., 2010). The history of medicine and medicinal plants can be traced back to the Vedic periods when Nepal Himalaya was mentioned as sacred heaven of potent medicinal and aromatic plants. The world is blessed with a rich wealth of medicinal plants. Herbs have always been the principal form of medicine and they are becoming popular throughout the world, as people strive to stay healthy in the face of chronic stress and pollution. Plants have been considered as a source of medicines that work in concert with the body's own defenses (Manandhar, 1993).

Nepal is rich in traditional medical system including Ayurveda, Naturopathy, homeopathy etc. that relies heavily on the plant products. Medicinal property of the plant is due to the active chemical constituents present in different parts of the plants. These chemical substances produce definite physiological action on the human body. The main group of active components are alkaloids, glycosides, saponins, essential oils, mucilage, tannins, bitter principles etc. (Kruger, 1992). Components derived from medicinal plants are non-nutritive phytochemicals which have some disease

preventive properties. They are not required by the human body for life sustenance but they offer protection against pathogens (Kokate et. al., 2006).There are different ways in which phytochemicals can work. Some phytochemicals like polyphenols, carotenoids can act as an antioxidant and protect the body from free radical damage. Terpenes can stimulate certain enzymes, thereby reducing the risk of breast cancer. They may also act as an antimicrobials (http:// www.phytochemicals.info/accessed Nov 20, 2011).

Paris polyphylla Smith, Synonym – *Daiswa polyphylla* (Sm.) Raf. Local name – Satuwaa is an important perennial (herb) medicinal plant growing under the canopy of moist temperate forest. It belongs to family Trililiaceae. In Nepal it has been reported to be found in eastern, mid and western regions between 2000-3000 m elevation ranges. The species *Paris polyphylla* produces spider-like flowers that throw out long, thread-like, yellowish green petals throughout most of the warm summer months and into the autumn. In the fall, the flowers are followed by small, scarlet berries. This flowering plant usually grows up to 90 cm (3 feet) high and spreads out about 30 cm (1 feet) wide. Its leaves grow in a single whorl below a flower growing in two whorls.

While considering the importance of the plant in the field of disease control, whole plant as well as different parts separately have different uses. Rhizome of *Paris polyphylla* is widely used in Nepal as an antihelmintic, antispasmodic, digestive stomachic, expectorant and vermifuge (IUCN 2004; Bhattarai and Ghimire 2006). Powder from rhizome is used for fever and food poisoning. Root paste is applied as an antidote to snake bites and poisonous insect bites and also to alleviate narcotic effects. A decoction of root is used in the treatment of ulcers, diphtheria, epidemic Japanese B encephalitis, appendicitis, lymphadenopathy, tonsillitis, parotitis, mastitis and rheumatism. It causes the subsidence of swelling, alleviates pain and relieves boils, carbuncles, sore throat and traumatic pain. The whole plant is considered to be a febrifuge. Rhizome paste of *Paris polyphylla* can be applied to wounds for rapid healing and also for fever, headache, stomach problems of both man and animals medicine. The rhizomes have high demands in both national and international markets for its valuable rootstock to treat variety of ailments (Bhattarai & Ghimire 2006). It is also an important folk medicinal herb of China. It is found to be used as a primary herb in the treatment of liver, stomach, nose, lung, throat, and breast cancer in traditional Chinese medicine (Vassilopoulos 2009). The major bioactive constituents

that have beenidentified and screened for their in-vivo and in-vitro anticancer activity of *Paris* rhizome are steroidal saponins (Yan et al., 2009).

In the last few decades the rate at which new antibiotics are being produced is slowing (Russel et. al., 2002). Thus the research for novel antimicrobial agents seems to be needed. Plant products have been part of phytomedicines since time immemorial. These can be derived from barks, leaves, flowers, roots, fruits, seeds (Criagg et.al, 2001). Knowledge of the chemical constituents of plants is desirable because such informations are valuable for the synthesis of complex chemical substances (Parekh et. al., 2008). Throughout the development of human culture, the use of natural products has been found to assume magical-religious significance. Different points of view regarding the concepts of health and disease existed within each culture. The importance of natural products is clearly enormous.

In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. This interest in drugs of plant origin is due to several reasons, namely, conventional medicine can be inefficient, abusive use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to pharmacological treatment, and folk medicine and ecological awareness suggest that natural product are harmless. Though a number of scientists and research institutions have been initiated research activities to explore the bioactive constituents and medicinal values of the plant product, potential use of higher plants as a source of new drugs is still poorly explored. High dependency of majority of population in Nepal on plants as a source of medicine proves the importance of medicinal plants in the rural Nepalese society. Therefore the biological resources of Nepalneed to be managed and used properly. The herbs of Nepal are potential natural assets. These could be used for a wide range of medical and cosmetic products and some other uses. The effect of some of the herbal medicine has been well proven for hundreds of years. In recent days people prefer their own choice of medicine and medical treatment than modern medicine. Yet, there are so many herbal plants whose contents and effects are not researched adequately in many parts of the world. Thus initiation of the research activities regarding the isolation and identification of the therapeutic potential of a number of medicinal plants seems to be the present day need.

Generally, these medicinally active plants are first needed to be extracted using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixture of metabolites in liquid or semisolid state or in dry powder form (Tiwari et al., 2011). In the present study rhizome portion of a medicinal plant, *Paris polyphylla* has been selected as a substrate for chemical tests. Methanol extract of each sample was prepared and further tests were performed in different laboratories.

1.2 Rationale and Scope of the Study

Paris polyphylla Sm. (Satuwaa) is one of the medicinal plants listed as vulnerable by the IUCN (Madhu et. al., 2010). Seed viability was found to be low and the seeds did not germinate in laboratory conditions even under different chemical treatments (Madhu et al., 2010). There seems to be a need for raising awareness amongst people who live in environments in which *Paris polyphylla* propagates. Scientists must make known the sustainable use of the rhizome and its cultivation practice for the conservation of this plant. If some part of the rhizome containing the bud is left underground, it is thought that the plant would become more sustainable and would help in conserving its population in the future (Madhu et al., 2010).In a study done in Nepal, it was observed that overharvesting, harvesting of plants before seed maturity, low viable seed production and long dormancy of seeds are the major threats to the plant's propagation (Madhu et al., 2010).

Paris polyphylla is considered to be a traded plant and it might have become less abundant in the past decade and this could be due to deforestation (Harold, 1999).In context of our country Nepal,*Paris polyphylla* Sm. has been used by the local inhabitants since ancient times for fevers, headaches, burns, wound healing and many livestock diseases mainly to neutralize poison. There used to be a large scale collection and trade of the rhizome from different parts of the country. Though the plant is going to be extinct research works related to the plant samples from different parts of Nepal about its chemical composition, biological properties and medicinal importance has not been explored properly yet except some studies related to its socio-ecological status and antibacterial activity against some bacterial strains. Thus present study has been carried out with an aim of exploring antibacterial as well as antioxidant property of *Paris polyphylla* collected from different parts of Nepal.

1.3 Objectives of the Study

The main objective of the study is to assess antibacterial and antioxidant activity of rhizome of a medicinal plant, *Paris polyphylla* Sm. Some specific objectives are as follows:

- To identify the active chemical constituents present in the rhizome extract of *Paris polyphylla* Sm.
- To determine the total polyphenol content in rhizome part of *Paris polyphylla*.
- To determine the total flavonoid content in rhizome part of *Paris polyphylla*.
- To determine the antioxidant activity of *Paris polyphylla* rhizome using DPPH radical.
- To determine the antibacterial activity of *Paris polyphylla* rhizome against Gram positive and Gram negative bacteria.

1.4 Limitations of the study

Nevertheless, this study helps to investigate theantibacterial and antioxidant activity; it doesn't offer complete information towards the exploration of pharmacological application of *Paris polyphylla* Sm. Rare availability of plant material in wild condition, lack of continuous power supply at the time of solvent extraction,time limitation and lack of well equipped laboratory to carry out experimental processes effectively are some limitations associated with this research work.

CHAPTER II: LITERATURE REVIEW

Medicinal plants continue to be a major source of drugs and natural products on the basis of their therapeutic potentials (Anwannil and Atta, 2006). The plants possess potent bioactive compounds capable of preventing and treating most diseases. In developing countries, the use of medicinal plants in the treatment of infectious disease is high because of the high cost of effective drugs. Thus potential indigenous plants exploited for medicinal purposes are needed to undergo basic phytochemical screening and bioassay as first step towards the ultimate development of drugs (Odebiyi and Sofowora, 1998).

The study of natural products of plant is called phytochemistry. Biologically active compounds present in plants used for food and medicine are referred as phytochemicals or phytoconstituents. A great deal of interest has been generated recently in the isolation, characterization and identification of biological activity of these phytochemicals which are responsible for protecting the plant against microbial infections by pests (Doughari *et al*., 2009). A number of biologically active components have been isolated and characterized from fruits such as grapes and apples, vegetables such as broccoli and onion, spices such as turmeric, beverages such as green tea and red wine, as well as many other sources too(Doughari *et al*., 2009).

Medicinal plants are increasingly gaining acceptance even among the literates in urban settlements due to the increasing inefficacy of many modern drugs used for the control of many infections such as typhoid fever, gonorrhea, and tuberculosis. Other reasons include increase in resistance by several bacteria to various antibiotics, increasing cost of modern drugs and for the maintenance of personal health (Smolinski *et al*., 2003). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action. Researchers are increasingly turning their attention to folk medicine to develop better drugs against microbial infections. Screening techniques of biologically active compounds have been conducted on well-known species of plants used in different traditional medicines. (Rabe and Staden, 1997).

Knowledge of the chemical constituents of plant is needed for the identification of new sources of economically important bioactive constituents such as tannins, gums,

oils etc. In addition this type of knowledge becomes fruitful in order to know the actual importance of traditional medicines.Plant-derived antioxidants, especially, the phenolic compounds have gained considerable importance due to their potential health benefits. Epidemiological studies have shown that consumption of plant foods containing antioxidants is beneficial to health because it down-regulates many degenerative processes and can effectively lower the incidence of cancer and cardio-vascular diseases.

Solvent Extraction:

Plants are air dried to a constant weight, powdered and then extracted with suitable solvent. Extraction is the separation of medicinally active components of plant tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, semisolid extracts or powdered decoction. A number of methods have been found to be employed for the extraction of biologically active components in the form of crude extracts examples include hot continuous extraction, Soxhlet extraction, aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction etc. For aromatic plants, hydro distillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation etc. are employed (Handa et al., 2008).

The process of solvent extraction is generally employed either for the isolation of dissolved substances from the solution or for the removal of undesired soluble impurities from solid mixtures (Chatwal and Anand, 1998).

Methanol Extract Preparation

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions 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. The factors affecting the

choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants (Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Das et. al., 2010)

Varieties of different solvents are used to test the biological properties of medicinal plants. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Cowman, 1999). Present study has been carried out using methanol as solvent for the extract preparation.

Methanol is a polar solvent. Nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds. They are most often obtained through initial ethanol or methanol extraction (Cowan, 1999). Alcoholic extracts have higher activity as compared to the aqueous extract has been attributed to the presence of higher amount of polyphenols. Water extract have found to exhibit polyphenol oxidase which degrade the polyphenols making them biologically less active in comparison to methanolic and ethanolic extract. Tiwari et al., 2011 had stated that methanolic extract of the plant is successful for the isolation of almost all the bioactive constituents of plant. Khalaf et al., 2007 also used methanol for crude extract preparation of *Zingiber officinale* Roscoe for phytochemical screening and antioxidant activity determination. Similarly Yaday and Agarawala (20ll) used methanol extract for phytochemical screening and total phenol and flavonoid content determination of *Ricinus communis* (root). Bhattarai (2014)also carried out phytochemical screening, determination of antibacterial activity, total phenol and flavonoid content and antioxidant activity of *Swertia chiriyata* in methanol extract. In the present experiment also methanol was used as solvent for extract preparation.

*Paris polyphylla***Sm.**

Cheetri et al. (2012) studied socio-ecological status, antibacterial activity and ethno-medicinal use of *Paris polyphylla* from Panchase area of Kaski district, Nepal. Rhizome of this plant in the study site was found mainly applied to wounds for rapid healing and antidote to the bite for poisonous insects and snakebite, fever, headache and stomach problem of both human and domestic animals. Different extracts of aerial parts of the plant when subjected to antibacterial test these were found active against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aureginosa, Escherchia coli* and *Salmonella flexinerai* whereas, rhizome extract was active only against *S. aureus*. Petroleum ether extract from the rhizome of *P. polyphylla* was found most effective against *S. aureus* bacteria.

Gewali (1992-1993) and (1997-1998) reported *Paris polyphylla* Sm. one of thetwenty most traded medicinal plants from the Dolpa district of Nepal. He had also reported the plant as a rich source of diverse steroid saponins like diosgenin, paristerone.Madhu et al. (2010) carried out an ecological study on *Paris polyphylla* Sm. in mid-hills of Nepal with an objective of documentation of its ecological information. The plant was found to reproduce mainly by vegetative propagation in the field thus suggested for its sustainable use and conservation.Yan et al. (2009) experimented in-vivo and in-vitro anticancer activity of steroid saponin of *Paris polyphylla* var. Yunnanensis. He isolated eight known steroidal saponins from the rhizome extract. Evaluation of antitumor activity of Diosgenin, the main steroid saponin of *Paris polyphylla*, and its aglycogen portionon LA795 lung adenocarcinoma in T739 inbred mice showed remarkable cytotoxicity and caused typical apoptosis in a dose-dependent manner. Diosgenin and its aglycogene portion significantly inhibited tumor growth, by 29.44% and 33.94%, respectively.Zhao et al. (2010) studied antimicrobial metabolites from the endophytic fungus *Pichia guilliermondii* isolated from *Paris polyphylla* var. yunnanensis. Three steroids and one nordammarane triterpenoid were isolated and identified as ergosta-5, 7, 22-trienol (1), 5α, 8α-epidioxyergosta-6, 22-dien-3β-ol (2), ergosta-7, 22-dien-3β, 5α, 6β-triol (3), and helvolic acid (4). *Agrobacterium tumefaciens, Bacillus subtilus, Escherichia coli, Helicobacter pylori, Pseudononas sp., Sarcina lutea, Staphylococcus aureus, Staphylococcus haemolyticus and Xanthomonas vesicatoria, Aspergillus niger, Candida albicans* and *Magnaporthe* were selected as test organisms. Microdilution-

colorimetric and spore germinationassays were employed to evaluate their antimicrobial activity. Among them Helvolic acid exhibited the strongest antibacterial activity against all the tested bacteria.Zhu et al. (2011) studied in-vitro antitumour activity and antifungal activity of Pennogenin steroidal saponins from *Paris polyphylla* var. yunnanensis. Antitumor properties were determined by using an MTT assay in addition to ethidium bromide and acridine orange staining techniques. Compounds exhibited significant anti-proliferative activity against HepG2 cells. Compounds also exhibited antifungal activities against *Saccharomyces cerevisiae* and *Candida albicans.*

Phytochemical Screening

Anyasor et. al. (2010) examined flavonoids, phenols, anthraquinones, cardiac glycosides, terpenoids, alkaloids and tannins bioactive components in methanolic and aqueous stem extract of *Costus afer* Ker Gawl.Arunkumar and Muthuselvam (2009) analyzed phytochemical constituents in *Aloevera* L.Result showed the presence of tannin, saponin, flavonoids and terpenoids. Twenty six bioactive phytochemical compounds were identified in GC-MS analysis of the ethanolic extract of the plant material based on the peak area, molecular weight and molecular formula of the compounds.Similarly Ayoola et.al. (2008) found the presence of flavonoids, terpenoids, saponins, tannins and reducing sugars in *Carica papaya*(leaves*), Magnifera indica*(stem bark), *Psidium guajava*(leaves) and *Vernonia amygdalina* (leaves). *M. indica* did not contain cardiac glycosides and alkaloids while *P. guajava* also showed the absence of alkaloids and anthraquinones. Anthraquinones was also found absent from *V. amygdalina*.Bhattarai (2014) conducted the primary phytochemical screening of *Swertia chirayita* (whole plant) collected from different localities of Nepal. Methanol extracts of all the samples were observed for the presence of saponin, alkaloid, phenols, glycoside, tannin, terpenoid and phytosterol.A qualitative phytochemical analysis was performed by Chhetri et al. (2008) for the detection of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugar in eight different medicinal plants of Nepal*, Azadiracta indica, Ocimum santrum, Colquhounia coccinea, Rhododendron setosum , Zanthoxylum aromatum, Curcuma longa Ocimum santrum* and *Eucalyptus globulus.Ocimum santrum* contained all the chemicals except flavonoids and reducing sugar however

the*Colquhounia coccinea* lacked alkaloids and reducing sugar. The extract of *Rhododendron setosum*was found most effective against *Escherichia coli* and the extracts of *Azadiracta indica* and *Elsholtzia fructicosa* were found to be most effective against *Klebsiella* species.Edoega et al. (2005) carried out phytochemical analysis of ten Nigerian medicinal plants (*Cleome nutidosperma, Emilia coccinea, Euphorbia heterophylla, Physalis angulata, Richardia bransitensis, Scopania dulcis, Sida acuta, Spigelia anthelmia, Stachytarpheta cayennensis* and *Tridax procumbens*). All the plants were found to contain alkaloids, tannins and flavonoids except for absence of tannins in *S. acuta* and flavonoids in *S. cayennsis* respectively. In the same way a qualitative phytochemical analysis was performed by Joshi et. al. (2010) in aqueous ethanolic extract of four medicinal plants, *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem) confirmed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugars.Peteros and Uy (2010) examined the presence of flavonoids, tannins, triterpenes, steroids, anthrquinones, anthrones, flavonoid glycosides, and coumarin bioactive constituents in crude methanol extract of Philippine medicinal plants *Brucea amarissima, Intsia bijuga, Laportea meyeniana*, and *Pipturus arborescens*.Rachh et al. (2009) examined the presence of flavonoids, phenols, saponins, tannins and triterpenoids inpreliminary screening of alcoholic extract of Gymnema sylvestre.Different bioactive compounds such as alkaloids, coumarins, flavonoids, terpenoids, steroids and glycosides were reported by Sah et al. (2012) in methanol and petroleum ether extracts of *Asparagus racemosus*(tuber),*Catharanthus roseus*(whole part), *Hedychium coronarium*(rhizome), *Mimosa pudica*(whole part) and *Terminalia chebula*(fruits).Phytochemical analysis in five different solvent extract (n-hexane, ethanol, methanol, chloroform, water and ethyl acetate)of *Calotropis gigantean* revealed the presence of cardic glycosides, saponins, flavonoids, steroids and terpenoids in most prominent amount while alkaloids and tannins in less amount. Anthraquinone was absent in all the plant extracts (Seniya et al., 2011). Similarly phytochemical analysis of six folk medicinal plants of India in methanol extract revealed the presence of coumarins, flavonoids, glycosides, phenols, tannins, saponins and steroids whereas no alkaloids were detected in any of the six tested plant extracts (Shihabudeen et al., 2010)

Antibacterial Activity

Arunkumar and Muthuselvam (2009) studied antimicrobial activities of aqueous, ethanol and acetone extracts of *Aloevera* L. against clinical pathogens by agar diffusion method. The maximum antibacterial activity was observed in acetone extract with zone of inhibition values of (20±0.35mm), (20±0.57mm), (12±0.45mm) and (15±0.38mm) against *Streptococcus pyogenes, Pseudomonas aeruginosaStaphylococcus aureus and Escherichia coli* respectively.Bhattarai (2014) tested thirteen different samples of Nepalese *Swertia Chirayita*collected from different localities against two gram positive and four gram negative bacteria using modified agar diffusion method with some modifications. Methanol extracts of all the samples were found to exhibit antibacterial action against *Escherichia coli, Salmonella typhi, Klebsiellapneumoniae, Staphylococcus aureus* and *Enterococcus faecalis* but no activity was recorded against *Pseudomonas aeruginosa*.Ethanol extract of eight medicinal plants of Nepal, *Azadiracta indica, Ocimum santrum, Colquhounia coccinea, Rhododendron setosum, Zanthoxylum aromatum, Curcuma longa, Ocimum santrum* and *Eucalyptus globulus* were experimented for their antimicrobial activity against three species of microorganism *Staphylococcus aureus, Escherichia coli* and *Klebsiella species.* The extract of *Rhododendron setosum* was found most effective against *Escherichia coli* and the extracts of *Azadiracta indica* and *Elsholtziafructicosa* were found to be most effective against *Klebsiella* species (Chhetri et. al., 2008)

Joshi et al. (2010) carried out an experiment for the determination of antimicrobial properties of four medicinal plants, *Ocimum sanctum* (Tulsi), *Eugenia caryophyllata* (Clove), *Achyranthes bidentata* (Datiwan) and *Azadirachta indica* (Neem) against human pathogenic *Escherichia coli, Salmonella typhi, Salmonella paratyphi, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*.Aqueous ethanolic extract of each sample when examined employing cup diffusion methodhighest zone of inhibition (22mm) was obtained in *Eugenia caryophyllata* (Clove) against *Salmonella typhi.* All the plants were ineffective against *E. coli* and *K. pneumonia* whereas *Achyranthes bidentata* was found to be ineffective against all the tested organisms. Kala and Senthikumar (2010) studied antimicrobial activity of methanol, ethanol, chloroform, acetone, ethyl acetate, benzene and hexane extract of

leaf of *Acanthephippium bicolor* Lindley against thirty five gram positive and gram negative bacteri and fungi separately. Among them, mostly Gram positive organisms *Staphylococcus aureus, Streptococcus faecalis, Bacillus cereus, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Shigella dysenteriae, Escherichia coli, Microsporum audouinii, Microsporum fulvum, Candida albicans* and *Trichophyton rubrum* were found more susceptible to methanol extract of plant material than gram negative microorganisms. Similarly Khakurel et al. (2014) in an experiment found 50% ethanolic extract of *Chlorophytum arundinaceum* (perennial) and *Tagetes minuta* moderately active against *Enterococcus faecalis*and *Salmonella typhi* respectively whereas methanolic extract of *Punica granatum* showed encouraging action against*Eshcherichia coli, Shigella dysenteriae, Staphylococcus aureus* and*Enterococcus faecalis.*Prasai (2002) studied the antimicrobial activity of eight different medicinal plants against seven gram negative bacteria. Among the selected medicinal plants four plants (*Alnus nepalensis, Ficus religiosa, Myrica esculenta* and *Rhododendron arboretum*) were found to exhibit antibacterial action against *Escherechia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, Shigella spp*. and *cholera vibrio* whereas all the materials were found ineffective against *Pseudomonas aeruginosa.*Sah et al. (2012) reported the antibacterial potential of methanol extract of *Terminalia chebula*(fruit) against *Staphylococcus aureus*, *Escherichia coli*, *Klebsella* spp., *Salmonella paratyphi*, and *Bacillus subtilis.*When five different solvent extracts (n-hexane, ethanol, methanol, chloroform, water and ethyl acetate)of *Calotropis gigantea* were tested against *Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi* and *Micrococcus luteus* for their antibacterial activity. Ethyl Acetate leaves extract exhibited maximum and aqueous leaves extract the minimum value of diameter of zone of inhibition (Seniya et al., 2011).Shihabudeen et al. (2010) found to have highest antibacterial activity of methanolic extract of *Eugenia jambolana* and *Cassia auriculata* against *Staphylococcus aureus, S. epidermidis, Escherechia coli, Klebsiella pneumonia and Pseudomonas aeruginosa* among the six Indian folk medicinal plants used in the experiments.Seven indigenous plants of Nepal, *Mallotus philippensis, Pogostemon cablin, Colebrookea oppositifolia, Mussaenda macrophylla, Celosia argentea, Pilea symmeria* and *Thysanolaena maxima* were studied by Subba and Basnet (2014).Methanol extracts of all the plant samples were found to exhibit antibacterial

activity against *Staphylococcus aureus, Klebsiella, Proteus vulgaris* and *Escherichia coli.* Similarly methanol extract of *Machilus odoratissima* (kaaulo) was reported for its antibacterial activity against *Staphylococcus aureus* bacteria in dose dependent manner (Subedi et al., 2012).Timsina (2003) evaluated the antimicrobial activity of 20 different medicinal plants of Nepal against *Candida albicans, Bacillus subtilis, Escherechia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 49132), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella paratyphi, Salmonella typhi, Shigella dysenteriae, Staphylococcus aureus* (ATCC 29213) and *Vibrio cholera*. Among the selected medicinal plants, *Rhododendron anthopogan* and *Rhus javanica* were found most active against all the tested microorganisms.

Antioxidant Activity

Anyasor et al. (2010) when carried out preliminary screening of free radical scavenging activity of *Costus afer* Ker Gawl. extract with 2,2-Diphenyl l-1-picrylhydrazyl (DPPH) using thin layer chromatography tested positive. The aqueous extract was observed for higher free radical scavenging activity than methanol extract with IC50 values of 64.42µg/ml and 92.33µg/ml respectively. Likewise the total phenolic content in aqueous extract (0.66±0.02mg gallic acid equivalent/g) was found significantly higher ($p<0.05$) than methanol extract (0.52±0.01mg gallic acid equivalent/g).A research work related to the determination of antioxidant activity of four medicinal plants*Carica papaya*(leaves*)*, *Magnifera indica*(stem bark), *Psidium guajava*(leaves) and *Vernonia amygdalina* (leaves) showed potent inhibition of DPPH radical, *Psidiumguajava* being the most potent with IC50 value 0.04mg/ml (Ayoola et.al., 2008).Bhattarai (2014) analyzed antioxidant activity of Nepalese *Swertia Chirayita* population using DPPH assay. Methanolic extracts of all thirteen different samples (both wild and cultivated samples) were found to exhibit antioxidant property with maximum inhibition of wild sample collected from Makawanpur district (IC50 value 42.07±3.39). Smilarly Borkataky (2013) determined antioxidant activity of a medicinal herb, *Eclipta alba* (L.) Hassk in both aqueous and methanol extracts. In aqueous extract IC50 value of 1145.586µg/ml was obtained where the total phenolic content value was 1.46mgGAE/g and IC50 value of 190.782µg/ml was observed in ethanol extract where total phenol content value of 8.933mgGAE/g was recorded.Gaikwad et al. (2011) reported the acetone extract of leaves, stem and fruits

of *Cassiaauriculata* L. is a potential source of natural antioxidant when four different extracts (acetone, ethanol, methanol and distilled water) of plant material were screened in-vitro, for their possible antioxidant activity by employing 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and nitric oxide (NO) reducing power with ascorbic acid as a standard.In an experiment, Genwali et al. (2013) studied the antioxidant activities and total phenolic contents in different extracts of medicinal herbs *Adhatoda vasica* Nees, *Berginia ciliata, Phyllanthus emblica, Terminalia bellirica, Terminalia chebula*and *Vitex negundo* Linnaeus. He demonstrated a positive correlation between free radical scavenging activity of plant extracts and total phenolic content values. Highest total phenol content value of 357.08mgGAE/g was noted in 70% acetone extract of Bergenia *ciliata as* highest total phenol content value with least IC50 value of 21.11µg/ml among all the selected samples.Khalaf et al. (2008) also evaluated the antioxidant property of some commonly available medicinal plants. The methanolic crude extracts of the plants were screened for their free radical scavenging properties using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and ascorbic acid as standard antioxidant. The overall antioxidant activity of *Camellia sinensis* (green tea) was found strongest, followed in descending order by Camellia sinensis (black tea), *Eugenia caryophyllus, Piper cubeba, Zingiber officinale, Piper nigrum, Trigonella foenum graecum* and *Elettaria cardamomum* With IC50 value ranging between 6.7 – 681.5µg/ml. Similarly Mensor et al. (2001) tested sixteen Brazilian plant species against stable DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free-radical. Extracts were prepared using four different solvents, hexane, dichloromethane, ethyl acetate and n-butanol. Among them plant samples extracted with more polar solvents (ethyl acetate and n-butanol) were found to exhibit higher antioxidant activity.An experiment on determination of free radical scavenging property in crude methanol extract of Philippine medicinal plants namely *Brucea amarissima, Intsia bijuga, Laportea meyeniana,* and *Pipturus arborescens* carried out by Peteros and Uy (2010) reported *Brucea amarissima* (LC50 value 37.7µg/ml) as most potent antioxidant and *Intsia bijuga* (LC50 value 89.5µg/ml) as least potent among the four studied samples.Sharma et al. (2013) studied *Argemone maxicana* Linn. for its highest values of total phenolic content (23.5mgGAE/g), total flavonoid content (34.5mgQE/g) and antioxidant activity in methanol extracts of flower part of the plant in comparison to other body parts (leaves, stems, roots and fruits).Subba and Basnet (2014) evaluated the highest antioxidant property of *Pogostemon cablin* (ethanol extract)with IC50

value of 32µg/ml among the seven indigenous plants (*Mallotus philippensis, Pogostemon cablin, Colebrookea oppositifolia, Mussaenda macrophylla, Celosia argentea, Pilea symmeria* and *Thysanolaena maxima*) of Nepal used for the experiment.*Machilus odoratissima* (kaaulo) was found as a strong antioxidant with IC50 value of 3.37µg/mlwhen Subedi et al. (2012) measured the antioxidant activity using DPPH(1, 1-diphenyl-2-picrylhydrazil) radical using methanol extract.

CHAPTER III: MATERIALS & METHODS

3.1 Laboratory Arrangement

Preliminary phytochemical screening was carried out in Plant - Biochemistry and Biotechnology laboratory of central Department of Botany, spectrophotometric work was conducted in the laboratory of Central Department of Bio-technology and antimicrobial test was carried out in Annapurna Research Center, Maitighar.

Chemicals Used: following chemicals were used for different experiments.

Soxhlet extraction:

i. Methanol (CH_3OH)

Primary Phytochemical Screening

i. Distilled water (H_2O)
ii. Sodium hydroxide (NaOH)
iii. Chloroforrm ($CHCl_3$)
iv. Acetic acid (CH_3COOH)
v. Conc. Sulphuric acid (Conc. H_2SO_4)
vi. Mayer's reagent [Potassium iodide + mercuric iodide]
vii. Ferric chloride ($FeCl_3$)

Total polyphenol content determination

i. Folin- Ciocalteu Phenol reagent
ii. Sodium carbonate (Na_2CO_3)
iii. Gallic acid ($C_7H_6O_5$)
iv. Methanol (CH_3OH)
v. Distilled water (H_2O)

Total Flavonoid Content Determination

i. Aluminium chloride ($AlCl_3$)
ii. Potassium acetate (CH_3COOK)
iii. Methanol (CH_3OH)
iv. Distilled water (H_2O)
v. Quercetin

Antioxidant Activity Test

i. 1,1- Diphenyl-2- Picryhydrazyl (DPPH)
ii. Methanol (CH_3OH)
iii. Ascorbic acid (C_6H_8O)

Antibacterial Test

i. Mueller Hinton Agar (MHA)
ii. Nutrient Agar (NA)
iii. Nutrient Broth (NB)
iv. McFarland standards ($BaCl_2 + H_2SO_4$)
v. Dimethyl sulphoxide (DMSO)
vi. Antibiotic discs (Tetracycline, 30 mg)

Apparatus Used

i. Electric mixture
ii. UV - Visible Spectrophotometer
iii. Soxhlet- apparatus
iv. Hot air oven
v. Incubator
vi. Weighing balance
vii. Refrigerator
viii. Vortex
ix. Autoclave
x. Distillation plant

Glasswares and Others

Test tubes, Stand, Pipette, Micro- pipette, Petriplates, Thimble, Filter paper, Cork borer (6mm diameter), Measuring scale, Inoculating loop, cotton swabs, Aluminium foil, Forcep, Burner, dropper.

3.2 Collection of Plant Material

Samples of *Paris polyphylla* were collected from different localities as presented in the following table:

Table 1: Details of samples collected for the experiment:

S. N.	District	Geographical location	Site of collection	Month of collection	Plant part used
1	Dolakha (Wild)	Central	Mainapokhari	October	Rhizome
2	Dolakha (Cultivated)	Central	Mainapokhari	October	Rhizome
3	Rolpa (Wild)	Mid-western	Thawang	July	Rhizome
4	Parbat (Wild)	western	Banau	October	Rhizome

3.3 Processing of Plant Material

3.3.1 Chopping and Shade Drying

Rhizome part of the plant was taken. It is cleaned and chopped into small pieces. Finely chopped samples were spread under shade separately in blotting paper and left for drying.

3.3.2 Packaging and Storage

Completely dried materials were packed in water proof bags and stored at room temperature away from direct contact of sunlight.

3.3.3 Grinding of Plant Material

Before extraction stored dried material was grinded to fine powder using electric grinder. The fine powder of each sample was collected separately on polythene bag and used for extraction.

3.4 Preparation of Plant Extract

3.4.1 Methanol Extract Preparation

Known weight of powdered material of each sample was subjected to Soxhlet extraction using methanol as a solvent. For extraction, sample and solvent was used in a ratio of 1:10 (w/v).10gm powder material of each sample was loaded separately in clean and dry thimbles and kept into the soxhlet extractor. Extractor was then fitted

with appropriate sized pre-weighed, dried, and properly labeled round bottom flask having capacity of 250ml. About 100ml of methanol was slowly poured from upper mouth of the soxhlet extractor. Upper part was fitted with condenser. Set up was maintained with the help of stand. Constant heat (temperature below the boiling point of solvent (65°C for methanol) was maintained with the help of heating mantle. Constant temperature evaporates the solvent kept in round bottom flask, gets condensed in the form of vapor as the upper part of the extractor is fitted with condenser, then collected in the side tube, drops on the material kept inside the thimble and dissolve soluble compound and recollected in the round bottom flask. The process was continued for about 7 hours. Finally the round bottom flask with extracted compounds dissolved in extracting solvent was collected.

3.4.2 Removal of Solvent

After extraction, round bottom flasks containing extracts wereseparated from the whole set up. Each extract was then poured separately in clean weighed labeled petriplate in order to allow the evaporation of solvent at room temperature. The condensed crude extracts were then collected in sterile tubes fitted with rotary vapor evaporator at reduced pressure. The flask was constantly heated in rotating condition by using water bath below 55°C. Solvent was allowed to evaporate. The condensed crude extracts were then collected in clean weighed labeled sterile vials.

3.4.3 Calculation of Percentage Extract Yield of Plant Sample

Weight of each empty sterile vial was noted before adding crude extract. Again weight of sterile vial was noted when crude extract was kept in it. Then, weight of each empty sterile vial was subtracted from the weight of sterile vial with crude extract and values were noted. This is the value of dry weight of the resulting extract per 10 gm dry weight of plant powder used. Then Percentage yield of crude extract of the respective plant sample was calculated by using following formula.

Percentage yield (%) = (Dry weight of extract / dry weight of plant powder used) * 100%

The resulting dry extract was then sealed and stored at 4℃ until use.

3.5 Preparation of Stock Solution

100mg of crude extract of each sample was weighed and dissolved in 1ml of methanol. Thus prepared 100mg/ml stock solution was used (after dilution to required concentration) for the determination of antioxidant activity, total phenolic and flavonoid content. Similarly 500mg of crude extract of each sample was weighed and dissolved in 1 ml of DMSO. Thus prepared stock solution of 500mg/ml was used for the detection of antibacterial activity of plant extract against gram positive and gram negative bacteria.

3.6 Primary Phytochemical Screening

Methanol crude extract of each sample was used for phytochemical screening by using the protocols following Harborne and Baxter, 1973 and Todkar *et al.*, 2010.

Test for Saponins

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for Alkaloids

Crude extract was mixed with 2ml of 1% HCl and heated gently. Mayer's reagent was then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Test for Phenols and Tannins

Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for Terpenoids

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish color indicated the presence of terpenoids.

Test for Glycosides

Liebermann's test: Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated H_2SO_4 was

added. A color change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

Test for Flavonoids

Alkaline reagent test: Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow color was formed which turned colorless on addition of few drops of diluted acid which indicated the presence of flavonoids

3.7 Quantitative Phytochemical Analysis

3.7.1 Total Polyphenol Content Determination

The total phenol content of each sample was estimated using the Folin-Ciocalteu phenol reagent (Chang *et al.,* 2002; Roy *et al.,* 2010) with slight modification. The reagent was used after dilution with distilled water in a ratio of 1: 10. Stock solution of 100mg/ ml was diluted to 2.5mg/ml and used for this experiment. 0.1ml of each extract was separately mixed with the 1ml of Folin-Ciocalteu phenol reagent (Merck Specialities Pvt. Ltd, India (1:10 dilution with the distilled water) and 0.8ml of aqueous 1M Na_2CO_3 solution. The reaction mixture was allowed to stand for about 15 minutes and the absorbance of the reactants was measured at 765nm using the UV- visible spectrophotometer. The calibration curve was obtained using the solution of gallic acid (Moly Chem, Mumbai, India) as standard in methanol and water (50:50 v/v) using the concentration ranging from 25-250µg/ml. Based on this standard graph, the concentration of the individual samples were calculated. The total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of thedry mass (mgGAE/g). Three replicates were made for each extract and values were noted carefully.

Total polyphenol content was determined using following formula:

Polyphenol content = Concentration determined from the standard curve * Volume of working solution used during assay/ Mass of the extract

Here, volume of working solution used during assay was 0.1ml and mass of extract in 0.1ml working solution of 2.5mg/ml was 0.25mg.

In this way total polyphenol content in different samples were determined.

3.7.2 Total Flavonoid Content Determination

The total flavonoid content in different samples was estimated using the Aluminium chloride ($AlCl_3$) colorimetric method with slight modifications (Begum *et al*., 2011). Stock solution of 100mg/ml of each extract prepared in methanol was diluted to 10mg/ml and used for this experiment. 0.25ml of extract was mixed with the 0.75ml of methanol, 0.05ml of the 10% aluminium chloride, 0.05ml of the 1M potassium acetate (CH_3COOK) and 1.4 ml of the distilled water. Same process was carried out for all the four samples. The reaction mixtures were allowed to stand for about 30 minutes in room temperature. The absorbance of each was measured at 415nm using the UV – visible spectrophotometer. The calibration curve was obtained with the help of the quercetin (Sigma) standard solutions in methanol with the concentration ranging from the 10-100µg /ml. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of dry mass (mgQE/g). For each experiment three replicates were made and results were noted carefully. Then total flavonoid content was determined using following formula:

Flavonoid content = Concentration determined from the standard curve * Volume of working solution used during assay/ Mass of the extract

Here, volume of working solution used during assay was 0.25ml and mass of extract in 0.25ml working solution of 10mg/ml was 2.5mg.

3.8 Determination of Antioxidant Activity of Plant Samples

3.8.1 Preparation of 0.2 mM DPPH solution

1, 1- diphenyl-2 picryhydrazyl (DPPH) is a stable free radial having molecular weight of 394.32 gm/mol. Thus, 100 ml of 0.2mM solution of DPPH was prepared using 7.886mg of DPPH and dissolving it in methanol. Final volume of 100ml was maintained by adding methanol.

3.8.2 Measurement of DPPH Free Radical Scavenging Activity

The antioxidant activity of methanol extract of different samples of *Paris polyphyll* was estimated on the basis of the radical scavenging effect of 1, 1- diphenyl-2 picrylhydrazyl (DPPH) free radical activity following the protocol of Singh *et al*., (2002) with slight modification. Stock solution of 100mg/ml of each sample was diluted to various concentrations and used for the experiment. Each plant extract of concentration (20-100

mg/ml) and ascorbic acid of concentration (10-100µg /ml) was prepared in methanol on the clean labeled test tubes. 1 ml of the sample volume was taken and 1ml of the 0.2mM DPPH solution was added to this solution. The tubes were shaken vigorously for the uniform mixing. These tubes were allowed to stand for half an hour in dark. The control was prepared by mixing 1ml of methanol with 1ml of 0.2mM DPPH solution, without adding plant extract or ascorbic acid. It was also kept in dark for half an hour. Methanol was used for the calibration of spectrophotometer. After half an hour, absorbance was taken on spectrophotometer at 517nm.

Radical scavenging activity is often expressed as percentage inhibition and is often calculated using the formula:

% radical scavenging activity = [($A_{control} - A_{test}$)/ $A_{control}$] * 100%

Where Acontrol is the absorbance of the control (DPPH solution without test sample) and Atest is the absorbance of the test sample (DPPH solution plus extract).

Standard graph was plotted taking the concentration on the X-axis and percentage scavenging activity on the Y-axis. Based on this graph, IC50 value of each sample was calculated using polynomial equation obtained from each graph.Plant sample having the lowest IC50 was considered to have the best antioxidant property among the used samples.

3.9 Antibacterial Activity Test

3.9.1 Preparation of working Suspension

The stock solutions of 500mg/ml prepared in DMSO were used for antibacterial activity test. 0.5ml of stock solutions of each sample were diluted to the concentration of 250mg/ml. Both 500mg/ml and 250mg/ml working solutions of methanol extract were used for the determination of zone of inhibition against six different bacterial strains.

3.9.2 Preparation of Culture Media

Nutrient agar (NA), Nutrient broth (NB) and Muller – Hinton agar (MHA) media were prepared as described below.

3.9.2.1 Nutrient Agar (NA) Media

Twenty eight gram of nutrient agar powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and poured in distilled water in a conical flask. Final volume of 1000ml was maintained. For uniform mixing of agar powder in distilled water it was allowed to boil with constant shaking for few minutes in low heat. This media was sterilized in an autoclave at 15lbs pressure and 121°C for 15 minutes. After this the media was taken out of the autoclave and poured on sterilized and properly labeled petridishes. About 20ml of the media was poured on each petridishes of 9cm diameter. The plates were then left for the solidification. All these activities were carried out under aseptic condition. Thus prepared media was used to subculture microorganisms or stored at 4℃ for future use.

3.9.2.2 Nutrition Broth (NB) Media

Nutrient broth media was prepared as recommended by manufacturer (Hi- Media Laboratories Pvt. Ltd., Mumbai, India). About 13gm of nutrient broth powder was added in distilled water in a conical flask. Final volume of 1000ml was maintained. The mixture was boiled with constant shaking for uniform mixing of powder in distilled water. Appropriate volume (1 or 2ml in each test tube) of media was poured carefully in suitable sized test tubes and sealed properly with cotton plug. Test tubes were thensterilized in an autoclave at 15lbs pressure and 121°C for 15 minutes. Thus preparedmedia were used for the preparation of bacterial suspension during antibacterial assay or stored at 4℃ for future use.

3.9.2.3 Mueller Hinton Agar (MHA) Media

Thirty eight grams of MHA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was weighed and suspended in distilled water. The final volume was maintained 1000ml. The content was heated to boiling for uniform mixing of powder in distilled water. The media was sterilized by autoclaving at 15lbs pressure and 121°C for 15 minutes. It was then cooled to 50℃ and poured on sterilized and properly labeled petridishes. About 20ml of the media was poured on each petridishes of 9cm diameter .The thickness of medium after solidification approximately equals to 4mm. It was then stored at 4℃ .Thus prepared media was used to inoculate working suspension of different samples.

3.9.3 Collections of Test Organisms

Six different types of bacteria (three gram positive and three gram negative bacteria) were selected for the study. Name of the test organisms and their source of collection are as follow:

Table 2: List of test organisms, their types and sources of collection

S. N.	Name of the test organism	Place of collection	Nature of bacteria
1	*Bacillus subtilis*	Manamohan Memorial Hospital, Thamel	Gram positive
2	*Staphylococcus aureus (25923)*	Manamohan Memorial Hospital, Thamel	Gram positive
3	*Enterococcus faecalis*	Annapurna Research Center, Maitighar	Gram positive
4	*Escherichia coli* (25922).	Manamohan Memorial Hospital, Thamel	Gram negative
5	*Klebsiella pneumonia*	Central Department of Biotechnology, TU	Gram negative
6	*Pseudomonas aeruginosa (27853*	Annapurna Research Center, Maitighar	Gram negative

After collecting the microorganisms, each was streaked separately on nutrient agar plates and incubated at 37°C for overnight. They were reconfirmed by gram stain.

3.9.3.1 Preparation of Stock Cultures

Conformed test organisms were subcultured in nutrient agar plates and incubated at 37℃. The organisms were subcultured on every two days and preserved during the experiment. For future use, a loopful bacterial colony of each bacteria was separately and aseptically transferred to nutrient broth and stored at low temperature in refrigerator**.**

3.9.3.2 Preparationof Standard Culture Inoculums

At the time of experiment, test organisms that have been sub-cultured frequently were streaked on the different nutrient agar plates. Those plates were incubated on the incubator at 37℃ for about 24 hours and pure and isolated colonies were obtained. A Small colony of each bacterium was aseptically transferred to separate tubes containing Nutrient broth (NB) for the suspension culture with the help of the sterilized inoculating loop. The bacterial suspensions were incubated on the incubator for overnight. The turbidity of the bacterial suspension was adjusted at the 0.5

McFarland standards by adding more bacterial solution or more sterile nutrient broth for the antibacterial test. These inoculums were then used for the swabbing on MHA plates to test the antimicrobial effects of the plant extracts.

3.9.3.3 Transfer of Bacteria to MHA Plates

The MHA plates were first labeled properly and standard culture inoculums of turbidity maintained at 0.5 McFarland standard was used for swabbing the plates using sterile cotton swabs. Excess inoculums in the swab were removed by pressing and rotating the swab against the upper side wall of the tube above the liquid culture level. The plate was rotated through an angle of 60°C after each swabbing. Finally the swab was passed around the edge of agar plate. One swab was used for one bacterium only. The culture plates were allowed to dry for a few minutes at room temperature with the lid closed.

3.9.4 Determination of Antibacterial Activity

Working suspension of methanol extract (concentration 250mg/ml and 500mg/ml) prepared in DMSO were used for the determination of zone of inhibition against six different bacteria using agar well diffusion technique. Six wells were prepared on the solid MHA media with the help of the sterile cork borer of 6 mm diameter. With the help of the sterile pipette the 50µl of each individual plant extract were poured in the above prepared well. The DMSO was taken as negative control while tetracycline of 30mg per disc was taken as the positive control. The plates were incubated on the microbial incubator overnight at 37°C and the zone of inhibition was observed and noted for individual plant extracts of individual bacteria for two different concentrations.

3.10 Statistical Analysis

All the experiments were performed in triplicate for each sample. Results were expressed as value±SEM using SPSS (16.0), IC50 values were calculated using Microsoft Excel on the basis of a polynomial equation obtained when percentage scavenging activity values were plotted against the concentration of plant extract used. Total phenol and flavonoid content values were determined based on the linear equation obtained using Excel sheet.

CHAPTER IV: RESULT

Plant samples collected from four different regions were subjected to Soxhlet extraction. 10gm of each sample was used for extract preparation along with methanol as solvent. Extract obtained from this process was used in different experiments. Following results were obtained.

4.1. Yield of Plant Extract:

The amount of crude extract obtained from the samples of same plant species collected in different regions showed only little variation in quantity. The values of methanol crude extract obtained in Soxhlet extraction of 10gm finely powdered material of *Paris polyphylla* collected from different regions are presented in the following table 3. The highest percentage yield i.e.16% was obtained in wild plant sample of Dolakha whereas the lowest percentage yield i.e.13% was obtained in wild sample collected from Parbat district. Cultivated sample collected from Dolakha district and wild sample collected from Rolpa district gave 15% and 13.7% yield of crude extract respectively.

Table 3: Percentage yield of crude methanol extracts of *Paris polyphylla* collected from different districts of Nepal

Name of the plant material	**part used**	**Collected districts**	**% yield**
Paris polyphylla	Rhizome	Parbat (wild)	13
		Rolpa (wild)	13.7
		Dolakha (cultivated)	15
		Dolakha (wild)	16

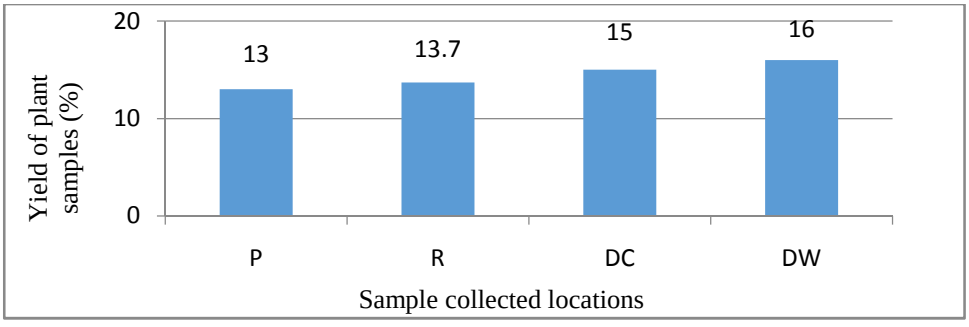

Fig 1: Percentage yields of different samples of *Paris polyphylla*

(P - Parbat, R – Rolpa, DC – Dolakha Cultivated and DW – Dolakha Wild

4.2 Preliminary Phytochemical Screening

Methanol extracts of four different samples were subjected to primary phytochemical screening. Following results were obtained as shown in table (Table 4)

Table 4: Phytochemical screening for the detection of chemically active compounds.

Sample	**Flavonoids**	**Glycosides**	**Terpenoids**	**Saponins**	**Phenols**	**Tannins**	**Alkaloids**
	Alkaline reagent test	**Libermann′s test**	**Salkowski test**	**Froth test**	**Ferric chloride test**	**Ferric chloride test**	**Mayer′s test**
Parbat	++	+	+	++	++	+	+
Dolakha (C)	++	+	+	+++	+	+	+
Rolpa	+	+	+	+	+	+	+
Dolakha (W)	++	+	+	++	+++	+	+

+ = Presence; ++ = moderately presence; +++ = highly presence

Preliminary phytochemical analysis revealed the presence of alkaloids, tannins, phenols, terpenoids, glycosides, saponins and flavonoids in all the four samples. Triplicate of each sample were used in each experiments.

4.3 Total Polyhenol Content Determination

For the determination of total polyphenol content, gallic acid solution ranging concentration between 25µg/ml to 250µg/ml was used as standard solution. A graph was plotted between concentration of standard gallic acid and its respective absorbance at 765nm as shown in fig (fig 2). Thus obtained graph was considered as standard graph. Based on the equation obtained from standard graph, polyphenol content was calculated in different samples. Results obtained were expressed in following figures.

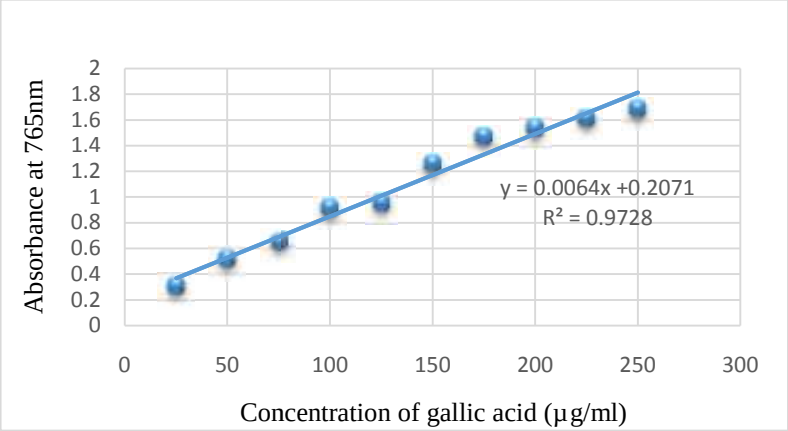

Fig2 Standard graph of gallic acid showing linear equation.

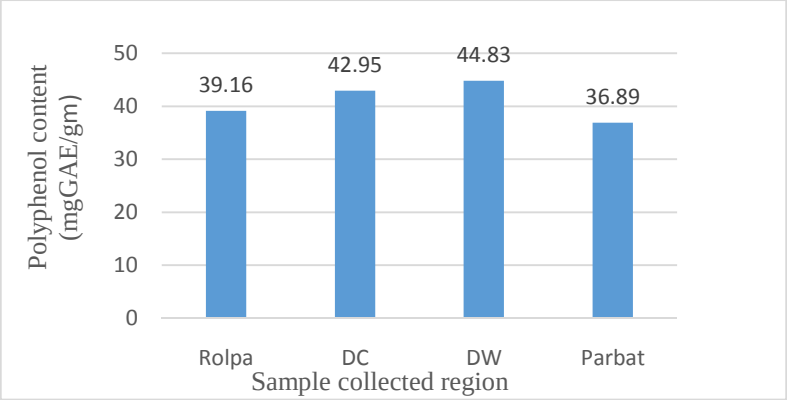

Fig 3 Graphical representation of phenolic content in four different samples of rhizome of *Paris polyphylla* collected from three different localities.

(DC – Dolakha cultivated, DW – Dolakha wild)

Highest phenolic content of 44.83±0.33mgGAE/gm was observed in methanol extract of sample collected in wild state from Dolakha district which was followed by the values of 42.93±0.52mgGAE/gm, 39.16±0.306mgGAE/gm and 36.89±0.074mgGAE/gm in cultivated sample of Dolakha, wild sample of Rolpa and wild sample of Parbat districts respectively.

4.4 Total Flavonoid Content Determination:

Quercetin solution ranging in concentration between 10µg/ml to 100µg/ml was taken as standard solution. When a graph was plotted between concentration of the quercetin solution and their respective absorbance, following calibration curve was obtained (fig 4).

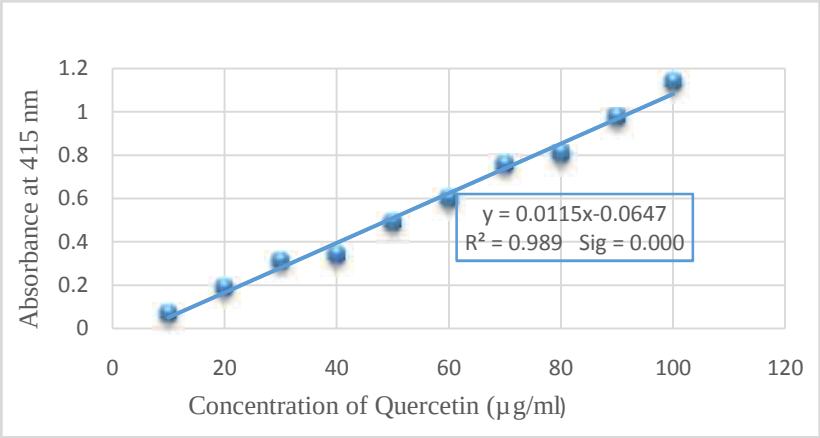

Fig 4: Standard graph of Quercetin solution showing linear equation.

Based on the equation provided by the standard graph of quercetin, total flavonoid content was calculated. Results obtained were presented as shown in the following figure (fig 5).

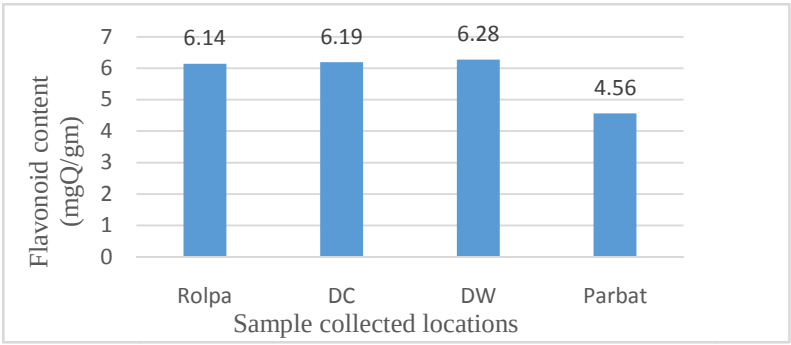

Fig 5: Graphical representation of the total flavonoid content in four different samples of *Paris polyphylla.*

Here, 6.28±0.035mgQ/gm flavonoid content was obtained in wild sample collected from Dolakha district followed by 6.19±0.89mgQ/gm, 6.14±0.35mgQ/gm and 4.56±0.22mgQ/gm flavonoid content in the samples collected from Dolakha in cultivated field, from Rolpa district and from Parbat respectively.

4.5 Determination of the Antioxidant Activity of Different Samples

Antioxidant activity of methanol extracts of *Paris polyphylla* was determined using standard solution of DPPH (0.2mM). Ascorbic acid solution was used as standard solution. The minimum concentration of ascorbic acid solution for 50% inhibition of DPPH free radical i.e. IC50 value for ascorbic acid was found to be 34.65µg/ml. IC50 value was calculated for each plant sample using the polynomial equation obtained

when percentage scavenging activity was plotted against the concentration of plant extract. The value of percentage radical scavenging activity was found gradually increased with increase in concentration of plant sample. This has been graphically expressed in following figures (Fig. 6). The minimum IC50 value of 42.63±1.4389was observed in wild sample ofDolakha district whereas the maximum IC50 value of 47.59±1.2843was observed in sample collected from Parbat district. Similarly IC50 values of 43.11±0.5711and 45.25±0.7736were observed in samples collected from Rolpa and cultivated sample of Dolakha districts. Percentage free radical scavenging activity and IC50 values of different samples has been expressed in following figures (Fig 6 – Fig 10).

1. **Sample collected from Dolakha (wild)**

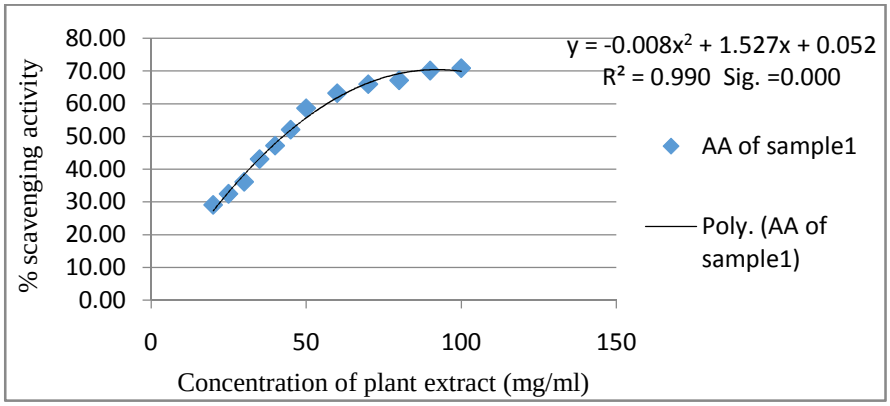

Fig 6 Graphical representation of free radical scavenging activity of Dolakhawild sample plotted against concentration of the extract (mg/ml).

2. **Sample collected from Parbat (wild)**

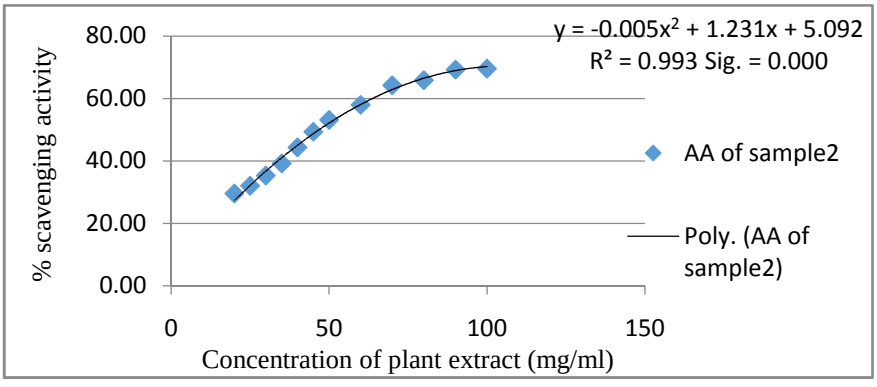

Fig 7 Graphical representation of free radical scavenging activity of Parbatwild sample plotted against concentration of the extract (mg/ml).

5. Sample from Rolpa (wild)

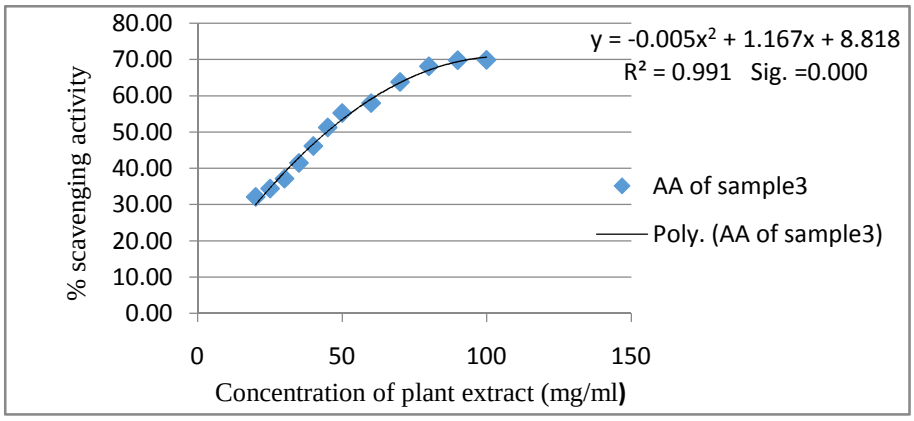

Fig 8 Graphical representation of free radical scavenging activity of Rolpa wild sample plotted against concentration of plant extract (mg/ml).

6. Sample from Dolakha (cultivated)

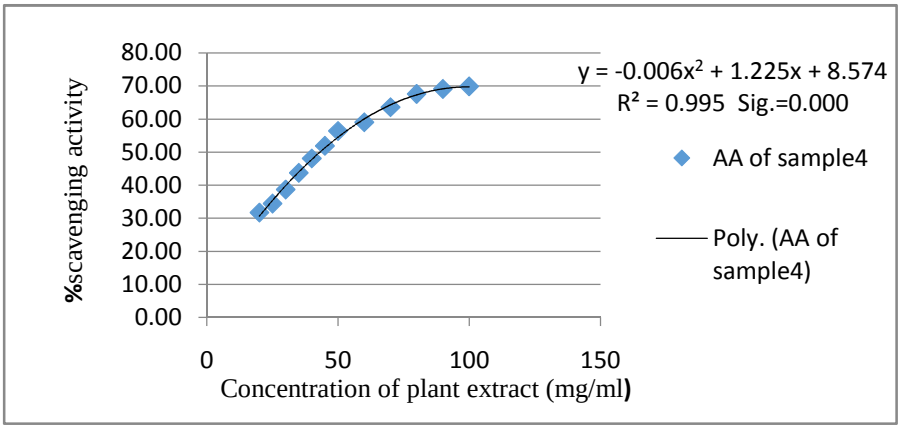

Fig 9 Graphical representation of free radical scavenging activity of Dolakha cultivated sample plotted against concentration of plant extract (mg/ml).

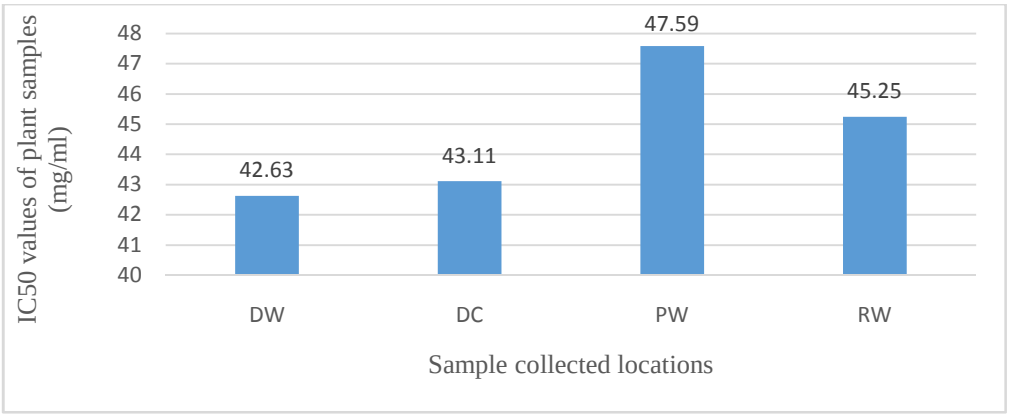

Fig10 IC50 values of different samples.

4.6 Antibacterial Activity of Plant Extract

Antibacterial activity of *Paris polyphylla* was examined in four different samples. Concentration of 250mg/ml and 500mg/ml was used to test the antibacterial potential of different plant extract against both gram positive and gram negative bacteria. Some of the collected bacteria were ATCC culture and two bacteria used were isolated and preserved in Annapurna Neuro Hospital and Research center .Gram positive bacteria were *Staphylococcus aureus (25923), Bacillus subtilis* and *Enterococcus faecalis* and gram negative bacteria were *Klebsiella Pneumoniae (700603), Pseudomonas aeruginosa (27853) and Escherichia coli* (25922).

Among these bacterial strains zone of inhibition was observed only against *Bacillus subtilis* bacteria in all the plant samples. Highest zone of inhibition (14mm including 6mm diameter of well) was obtained in wild sample collected from Dolakha. Concentration below 250mg/ml was not found to show any visible effect. Plant materials of concentrations 500mg/ml and 250mg/ml were used for the determination of zone of inhibition against different bacterial strains.

S.N.	Sample	Nature of the tested microorganism	Name of the tested microorganism	Zone of inhibition (mm) (including diameter of well 6mm)			
				Concentration of extract (mg/ml)		Positive control	Negative control
				500	250		
1.	Parbat	Gram positive	*Staphylococcus aureus*	-	-	24	-
			Enterococcus faecalis	-	-	22	-
			Bacillus subtilis	12	9	24	-
		Gram negative	*Escherichia coli*	-	-	27	-
			Klebsiella pneumonia	-	-	25	-
			Pseudomonas aeruginosa	-	-	22	-
2.	Dolakha (Wild)	Gram positive	*Staphylococcus aureus*	-	-	24	-
			Enterococcus faecalis	-	-	21	-
			Bacillus subtilis	14	11	22	-
		Gram negative	*Escherichia coli*	-	-	23	-
			Klebsiella pneumonia	-	-	21	-
			Pseudomonas aeruginosa	-	-	20	-
3.	Dolakha (cultivated)	Gram positive	*Staphylococcus aureus*	-	-	22	-
			Enterococcus faecalis	-	-	22	-
			Bacillus subtilis	12	11	24	-
		Gram negative	*Escherichia coli*	-	-	24	-
			Klebsiella pneumoniae	-	-	23	-
			Pseudomonas aeruginosa	-	-	21	-
4.	Rolpa wild	Gram positive	*Staphylococcus aureus*	-	-	21	-
			Enterococcus faecalis	-	-	20	-
			Bacillus subtilis	13	10	21	-
		Gram negative	*Escherichia coli*	-	-	22	-
			Klebsiella pneumoniae	-	-	22	-
			Pseudomonas aeruginosa	-	-	20	-

Result obtained from the experiment showed that zone of inhibition is increased with increase in concentration of extract in all the samples. No Zone of inhibition was obtained against *Staphylococcus aureus, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli*. Antibacterial activity exhibited by plant samples were measured in terms of diameter of zone of inhibition exhibited by the plant extract against bacterial solution. Wild sample of Dolakha was observed zone of inhibition value of 14mm at a concentration of 500mg/ml and 11mm at a concentration of 250mg/ml. Similarly zone of inhibition values of 12mm and 10mm were observed in cultivated sample collected fromDolakha, 12mm and 9mm in wild sample collected fromParbat and13mm and 10mm in wild sample collected from Rolpa at concentrations 500mg/ml and 250mg/ml respectively.

CHAPTER VI: DISCUSSION

5.1 Yield of Plant Extract

Plant extract is essential for further separation and characterization of chemical components present in it. It was found that for the same plant, different solvents gave different percentage yields. A same solvent methanol was used for the extract preparation of four different samples of *Paris polyphylla*. Ghimire et al. (2012) had reported 5 to 12% yield in case of Nepalese medicinal plants. In the present experiment percentage yield of dried rhizome powder of each sample was found in a range of 13% to 16%. Maximum yield of 16% was found in the wild sample collected from Dolakha district while minimum yield of 13% was found from the sample of Parbat district. Similarly percentage yield of 15% and 13.7% was found in the sample of Rolpa and cultivated sample of Dolakha districts respectively. Khalaf et al. (2008) had reported 9.2 percentage yield in methanol extract of dried rhizome powder of *Zingiber officinale* Roscoe. Similarly percentage yield of 5.47 was reported by Sah et al. (2012) in methanol extract of *Hedychium coronarium*. In the same manner Timsina (2003) reported percentage yield values of 15, 23.4 and 48.7 in crude methanol extracts of *Amaranthus spinosus* L., *Nardostachys grandiflora* DC. *Nepicrorhiza scrophularii* (Pennell) Hong respectively. These variations in percentage yield of extract in different samples might be due to various factors such as maturation of the material, soil condition of the cultivation area, extent of dryness of the material, differences in the duration of storage period, differences in the genotype and environmental conditions. Generally in the condition where there is incomplete extraction there will be the less yield of the material and when there is incomplete evaporation of solvent used in extraction there will be the chance of higher yield of extract.

5. 2 Preliminary Phytochemical Screening of Plant Material

Qualitative phytochemical screening is carried out to reveal the presence of chemically active components in the selected material. Chemical elements present in the plant as secondary compounds are secondary metabolites. These play an important role in the pharmacological action of the plant material due to their medicinal value. Medicinal property of the plant is usually not attributed to a particular element rather

it is attributed to a group of chemical metabolites present in it. The medicinal action of the plants are unique to particular plant species or groups consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Parekh et al., 2005). Preliminary phytochemical screening of methanol extract of *Paris polyphylla* revealed the presence of flavonoid, alkaloids, saponins, glycosides, terpenoids, tannins and phenols. These compounds are known to exhibit medicinal as well as physiological activities (Dewick, 1996).

Similar experiment when performed by Parekh and Chand (2010) examined the presence of only flavonoids, alkaloid and cardiac glycosides in *Acorus calamus* (rhizome) among six categories of phytochemicals i.e. flavonoids, tannins, steroids, alkaloid, saponins and cardiac glycosides tested. Similarly tannin was also reported together with flavonoids, alkaloid and cardiac glycosides in *Saussurea lappa* Costus (root) whereas steroids and saponins were absent. Saponin, flavonoids and alkaloids were present in *Plumbego zeylanica* (root). Only flavonoids and alkaloids were reported from *Piper nigrum* (root). Tenmozhi *et. al.* (2010) reported flavonoids, saponins, tannins and phenolic compounds from the rhizome of *Asparagus racemosus* whereas tanins, saponins, terpenoids, steroids and flavonoids all were absent in methanol extract of *Hedychium coronarium* (rhizome) only alkaloids, glycosides and reducing sugars were reported from the plant extract (Sah et al., 2012). Yadav and Agrawala (2011) reported the presence of phenols, tannins, steroids, flavonoids, carbohydrates, proteins, saponin and glycosides whereas terpenoids and alkaloids were not detected in the methanol extract of *Ricinus communis* (root). Bagul et al. (2002) also reported the presence of phenols, tannins, flavonoids and sterols in rhizome extract of *Berginia ciliata*.

Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Nobori et. al., 1994). Several workers have reported the antibacterial properties of alkaloids (Stray 1998; Okwu and Okwu 2004). Alkaloids also have pharmacological applications as anesthetics and central nervous system stimulants (Madziga *et al.*, 2010). Likewise glycosides are purely bitter principle. The bitters act on gustatory nerves, which results in increased flow of saliva and gastric juice. Glycosides are known to lower the blood pressure (Nyarko and Addy 1990). Tannins are phenolic compounds of high molecular weight.

These are used as antiseptic due to presence of the phenolic group. Tannin richmedicinal plants are used as healing agents in a number of diseases. In Ayurveda,formulations based on tannin-rich plants have been used for the treatment of diseases likeleucorrhoea, rhinnorhoea and diarrhea (Doughari, 2012). Diterpenes likeresins and taxol are anticancer agents. The triterpenesincluding steroids, sterols, and cardiac glycosides have anti-inflammatory, sedative, insecticidal or cytotoxic activity. Similarly sesquiterpenes have antimicrobial (particularly antiprotozoal) and neurotoxic action.Plant extracts were also revealed to contain saponins which are known to produce inhibitory effect on inflammation. Saponins have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponions include formation of foams in aqueous solutions, cholesterol binding properties and bitterness (okwu, 2004 and Sodipo et al., 2000).

5.3 Total Polyphenol Content Determination

Phenolics or phenols are chemical components that occur ubiquitously as natural colour pigments. Plant phenolics are major group of compounds acting as primary antioxidants or free radical scavengers (Kahkonen et al., 1999). They are able to scavenge reactive oxygen species, due to their electron donating properties (Jothy *et al.,* 2011). Phenolic compounds are commonly found in both edible and inedible plants. These compounds essentially represent a host of natural antioxidants, used as nutraceuticals, and sometimes are anti-inflammatory agents. Several studies have also described the antioxidant properties of medicinal plants which are rich in phenolic compounds (Kings and Berger, 2001). Total polyphenol content determination showed highest phenol content of 44.83±0.33mgGAE/gm in the sample collected in wild state from Dolakha district which was followed by the values of 42.95±0.52mgGAE/gm, 39.16±0.30mgGAE/gm and 36.89±0.074mgGAE/gm from cultivated sample of Dolakha, sample of Rolpa and sample of Parbat districts respectively. Ravipati et al. (2012) had also reported the total phenolic content values of 1.01±3.69 mgGAE/gm and 1.23±1.36 mgGAE/gm in ethanol and aqueous extracts of *Paris polyphylla* respectively. Yadav and Agrawala (2011) determined the total phenolic content value of 29.2mg/gm in aqueous extract of *Ricinus commuis* (root). Similarly Bagul et al. (2002) obtainedtotal phenolic content value of 5.78% (w/w)in methanol extract of *Bergenia ciliate* (Haw.) Sternb. forma*ligulata* Yeo rhizome. Wild

sample of *Paris polyphylla*,collected from Dolakha was found highest amount of phenolic content in comparison to other samples. Polyphenol content in Rolpa sample and Parbat sample was found lower than that of Dolakha wild and cultivated both. There might be some reasons behind that result like variation in the maturity of samples, storage period, climatic and physiographic differences.

5. 4 Total Flavonoid Content Determination

Flavonoids are important group of polyphenols widely distributed among the plant flora. These are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of micro-organisms in-vitro. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Marjorie, 1996). Over four thousand flavonoids are known to exist and some of them are pigments in higher plants and numerous reports support their use as antioxidants or free radical scavengers (Kar, 2007).They are super antioxidants which have anti-inflammatory activity, prevent oxidative cell damage through their water soluble property and also possess strong anti-cancer activity (Gurib-Fakim, 2006; Salah *et al.*, 1995). Materials collected from different places were noted for their different total flavonoid content values. Flavonoid content was found almost equal in three samples i.e. 6.19±0.89mgQ/gm (Dolakha cultivated), 6.14±0.35mgQ/gm (Rolpa wild) and 6.28±0.035mgQ/gm (Dolakha wild) whereas sample of Parbat district showed a little deviation in flavonoid content value (4.56±0.22mgQ/gm). Ravipati et al. (2012) had also reported the flavonoid content values of 2.6±1.96mgQE/gm and 0.72±1.96mgQE/gm in ethanol and aqueous extracts of *Paris polyphylla* respectively.

Yadav and Agrawala (2011) had determined the total flavonoid content value of 6mg/gm in aqueous extract of *Ricinus commuis* (root).These differences in flavonoid content value may be due to different conditions like physiological condition of soil, maturation of plant material, variation in storage period of the materials, nature of the solvent used in extraction.

5.6 Antioxidant Activity of Plant Extracts

Plants contain a wide variety of free radicals scavenging molecules including phenols, flavonoids, terpenoids that are rich in antioxidant activity (Madsen and Bertelsen, 1995, Cai and Sun, 2003). Chemical compounds that have ability to protect the body from damages caused by free radical-induced oxidative stress are antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources .Thus natural compounds with antioxidant properties play an important role as mediators in reactions where reactive oxygen species are being formed. Diminishing harmful potential of these reactive structures, natural antioxidants such as different types of phenol compounds, may prevent development of chronic diseases such as cancer, cardiovascular diseases. (Halliwell, 1996). DPPH is a well-known radical due to the delocalization of the spare electron on whole molecule. Thus DPPH does not dimerize. Delocalization of the electron determines the purple colour with an absorbance band at 517 nm under UV visible spectrophotometer. The scavenging of the stable 1,1-diphenyl-2-picryl-hydrazyl(DPPH) radical is a widely used method to evaluate the free radical scavenging ability of various samples (Chang*et al.*, 2002).When DPPH reacts with the hydrogen donors, the reduced form of DPPH is generated. This is accompanied by the disappearance of the purple color and appearance of paleyellow color or it becomes colorless when neutralized. Antioxidant action of the plant material is determined with reference to percentage radical scavenging activity and IC50 value observed in the experiment. IC50 value indicates the concentration of plant extract required for 50% inhibition of free radical induced damage. Plant material with highest percentage radical scavenging value has lowest IC50 value. The extract with lowest IC50 value is considered as strong antioxidant. In the experiment of measurement of free radical scavenging activity using DPPH solution the IC50 correlates directly with the effectiveness of the extract to scavenge the DPPH radical. In the present experiment, the minimum IC50 value of 43.31±2.33 was observed in wild sample collected from Dolakha district which was found as better antioxidant in comparison to the rest three samples. Similarly IC50 values of 44.94±0.025, 46.59±2.86 and 48.27±2.88 were observed in samples collected from Dolakha (cultivated sample), samples of Rolpa and Parbat districts respectively. In an earlier experiment Ravipati et al. (2012) reported DPPH Scavenging activity (µM

Ascorbate equivalent/g) of *Paris polyphylla.* The values observed were 45.29±1.52 and 19.9±2.12 in ethanol and aqueous extract respectively.

Bagul et al. (2003) had also reported the methanolic extract of *Bergenia ciliata* (Haw.) Sternb. forma*ligulata* Yeo rhizome as a good scavenger of DPPH radical with an EC50 of 36.24µg/ml. Similarly Khalaf et al. (2008) also reported DPPH free radical scavenging activity of rhizome of Zingiber officinale Roscoe with IC50 value 65.1±1.7µg/ml in methanol extract.

According to the findings of some previous experiments higher amount of phenolic content attributes higher free radical scavenging property of the plant materials (Mongkolsilp, 2004; Ghasemzadeh et al., 2010; Ivanov et al., 2011; Bhattarai, 2014). Highest phenolic content was observed in the wild sample collected from Dolakha district and it was also noted for its highest antioxidant value. Thus the result obtained was supported by earlier findings.

5.7 Antibacterial Activity of the Plant Extract

The antibacterial activity has been attributed to the presence of some active constituents in the extracts. Preliminary phytochemical screening revealed the presence of compounds like flavonoids, tannins which are known to have antimicrobial action (Laxmi et al., 2011). Similarly plants phenolics may have important role in plant defense against pathogens thus are applied in the control of human pathogenic infections (Puupponen - Pimia *et al*., 2008).

Chhetri et al. 2012, has examined the antimicrobial activity of both the aerial part and rhizome of *Paris polyphylla* separately against different strains of bacteria .All four different extracts i.e. petrolium ether, chloroform, methanol and water extracts of an aerial part of *Paris polyphylla* was found to be effective against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aureginosa, Escherichia coli* and *Salmonella flexinaria* whereas rhizome extracts was found effective only against *Staphylococcus aureus*. Among the four extracts prepared, petroleum ether extract of rhizome was found to be most active against *staphyloccus aureus* and methanol extract was found less active against this microorganism. In present investigation, methanol extract of rhizome part of *Paris polyphylla* didn't show antibacterial action against *Staphylococcus aureus* whereas the plant extracts were found active only against *Bacillus subtilis* among the six tested microorganisms. Mongalo et al. (2013) also

reported the antibacterial activity of methanol extract of *Jatropha zeyheri* (root extracts) against *Staphylococcus aureus* and *Escherichia coli*. No activity was reported against *Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis* and *Bacillus subtilis*. Similarly Sah et al. (2012) had reported antibacterial action of methanol extract of *Hedychium coronarium* against *Staphylococcus aureus, Bacillus subtilis* and *Klebsiella* spp. and no activity against *Escherichia coli*.

In classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram positive than Gram-negative bacteria (McCutcheon et al., 1992). This finding was also found to be supported by the finding of Timsina, 2003. However the plant extracts were found active only against a single bacterium among the six tested bacteria, the result obtained from the present experiment also supported that finding as the bacteria *Bacillus subtilis* is a Gram positive bacteria.

CHAPTER VII: CONCLUSION

The results obtained from these experiments illustrates the medicinal property of *Paris polyphylla* Sm. Screening of its chemical constituents suggests that the plant material is a rich source of chemically active compounds like saponins, flavonoids, glycosides, tannins, terpenoids, phenols and alkaloids. Many evidences gathered in earlier studies confirmed the identified phytochemicals to be bioactive. Several studies confirmed the presence of these phytochemicals contribute medicinal as well as physiological properties to the plants studied in the treatment of different diseases.

Though the phenolic and flavonoid content values in all samples were not as high as reported in other medicinal plants for example phenolic content in *Swertia chirayita* collected from Makawanpur district was reported the value of 81.64mgGAE/gm dry weight and flavonoid content of 20.57mgQ/gm dry weight (Bhattarai, 2014), highest phenolic content of 44.83±0.33mgGAE/gm was observed in methanol extract of sample collected in wild state from Dolakha district which was followed by the values of 42.93±0.52mg GAE/gm, 39.16±0.306mgGAE/gm and 36.89±0.074mgGAE/gm in cultivated sample of Dolakha, wild sample of Rolpa and wild sample of Parbat districts respectively. Similarly 6.28±0.035mgQ/gm flavonoid content was obtained in wild sample collected from Dolakha district followed by 6.19±0.89mgQ/gm, 6.14±0.35mgQ/gm and 4.56±0.22mgQ/gm flavonoid content in the samples collected from Dolakha in cultivated field, from Rolpa in wild and from Parbat in wild respectively.

Paris polyphylla was reported to have lowest IC50 value of 42.63±1.4389in wild sample of Dolakha district. This sample was considered as best antioxidant among all four tested samples. The maximum IC50 value of 47.59±1.2843was observed in wild sample collected from Parbat district. Similarly IC50 values of 43.11±0.5711and 45.25±0.7736were observed in rhizome extracts of wild sample of Rolpa and cultivated sample of Dolakha districts respectively. Among the four samples collected from different geographical locations, fresh wild sample collected from Rolpa district gave highest yield of 16% which was followed by 15%, 13.7% and 13% values respectively from Dolakha, Rolpa and Parbat samples.

Among all bacteria used in the experiment, only *Bacillus subtilis* was found susceptible to the plant extract whereas *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* and *Enterococcus faecalis* were found resistant to the methanol extract of *Paris polyphylla.* Plant extract exhibited the antibacterial action against gram positive bacteria. Concentration of 500mg/ml was found effective for the determination of antibacterial action of the plant material. Largest zone of inhibition of 14mm was observed in the wild sample collected from Dolakha district

CHAPTER VIII: RECOMMENDATIONS

There are several important works which could be carried out in future in context of medicinal plants of Nepal. Following recommendations are made regarding the scope of *Paris polyphylla* in the field of medicine:

1. Together with rhizome part, aerial part of the plant material should also be studied for its antioxidant action.
2. Identification of different chemical compounds that are responsible for its use as an antidote of poisonous snake bite should be explored.
3. Anticancer activities of *Paris polyphylla* against different cancer cell lines should also be explored.
4. Samples from other different places should be collected and used for different scientific tests considering its medical value.
5. There are various other solvents than methanol that can extract non- alcoholic components from the plant material like petroleum ether extract, chloroform extract, aqueous extract should also be analyzed for the investigation of biological activities of *Paris polyphylla* Sm.
6. Strict rules and regulations should be made and keep in practice regarding the conservation and trade of medicinal plants.
7. People should be motivated for the cultivation of medicinal plants like *Paris polyphylla* considering its economic benefits in future.

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Appendix I

1. Composition of Nutrient agar media

The composition of nutrient agar media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is asfollow.

Components	**gram/L**
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Agar	15.0

PH at 25°C 7.4 ± 0.2

2. Composition of Mueller Hinton Agar (MHA) media

The composition of Mueller Hinton agar (MHA) media (Hi Media Laboratories Pvt. Ltd, Mumbai,India) is as follow.

Components	**gram/L**
Beef infusion form	300
Casein hydrolysate	17.5
Starch	1.56
Agar	17
PH at 25°C	7.4±0.2

3. Composition of Nutrient Broth (NB) media

The composition of Nutrient Broth (NB) media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow:

Components (gram/l)	
Peptone	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
PH at 25°C	7.4±0.2

4. Preparation of 0.5 McFarland Standards

Following components were used for the preparation of 0.5 McFarland Standards (100ml)

Components	**Amount in ml**
Sulfuric acid, 0.18M	99.5
Barium chloride, 0.048M	0.5

Preparation of Reagents

1. Preparation of 1M Na_2CO_3 - 100ml

10.599 gram of the Na_2CO_3 (Merk Specialities Pvt. Ltd, Mumbai, India) was carefully weighedand then dissolved in distilled water, and the volume was adjusted to 100ml at the end.

2. Preparation of Glacial acetic acid (20%) - 200ml

40ml of the commercially supplied glacial acetic acid (Thermo Fisher Scientific, India) was takenand mixed with ethanol. Finally the volume was adjusted to 200ml by the addition of ethanol.

3. Preparation of Aluminium Chloride (10%) -100ml

10 grams of the commercially supplied aluminium chloride (Merk Specialities Pvt. Ltd, Mumbai,India) was weighed and dissolved in water. Finally the volume was maintained to 100ml.

4. Preparation of 1M potassium acetate (CH_3COOK) – 100ml

Weigh 9.814 gram of the potassium acetate (Merk Specialities Pvt. Ltd, Mumbai, India) anddissolved in water. Finally the volume was maintained to 100ml by the addition of water.

5. Preparation of 0.2mM DPPH solution - 100ml

100ml of 0.2mM solution of 1, 1- diphenyl-2 picryhydrazyl (DPPH) was prepared by weighing7.886mg of the DPPH and dissolving it in methanol and finally maintaining the volume to 100mlby addition of methanol.

6. Preparation of the Folin – Ciocalteu phenol reagent (1: 10 dilution)

6ml of the commercially supplied Folin – Ciocalteu phenol reagent (Merk Specialities Pvt. Ltd, Mumbai, India) was taken and mixed it with 54ml of the distilled water to prepare the 60ml of 1:10 dillution of Folin – Ciocalteu phenol reagent.

7. Mayer's reagent

To 1.358gmof mercuric chloride, 60ml of water and 5gm of potassium iodide was addedand dissolved in 10ml water. The two solutions were mixed up and final volume was made 100ml.

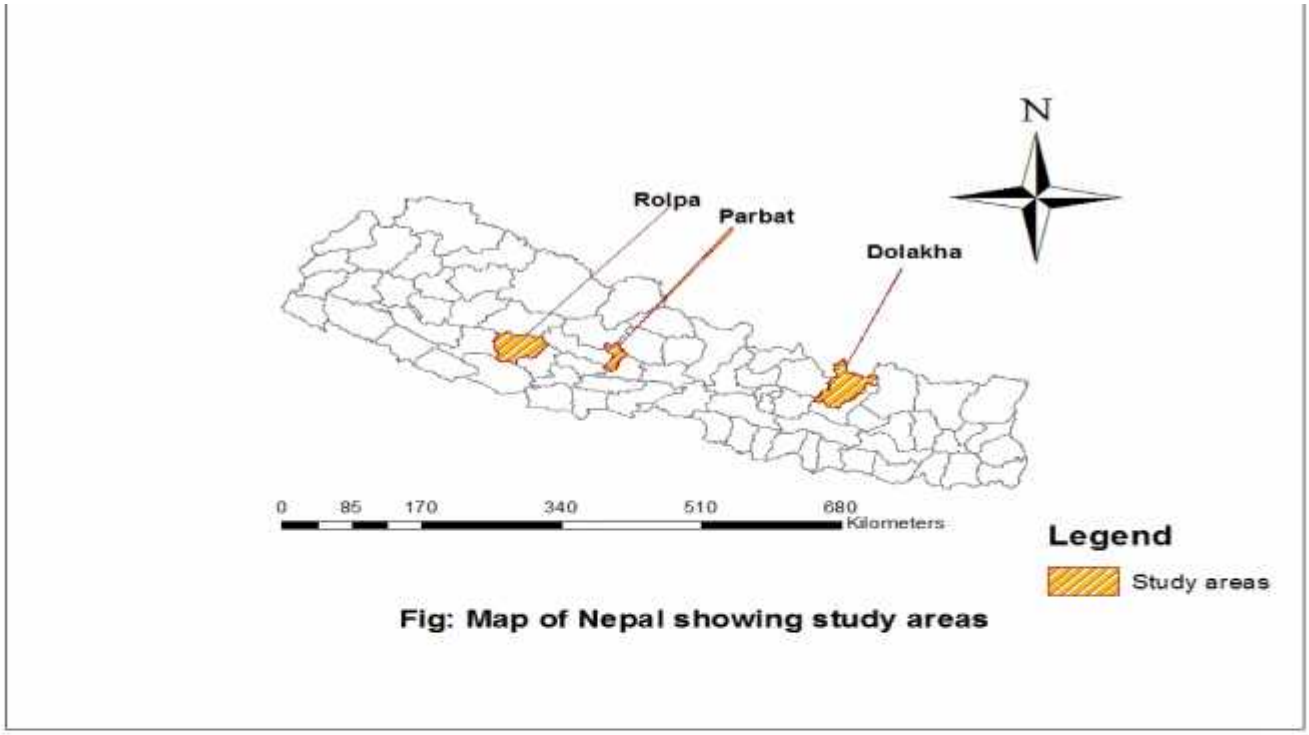

Fig: Map of Nepal showing study areas

a.Paris polyphylla in cultivated field of Rolpa

b. *Paris polyphylla* in wild state (Rolpa)

c. Rhizome of *Paris polyphylla*d. Rhizome in powder form

e. e. e

e.Researcher with Bhim Thami, medicinal plant cultivator of Dolakha district

f. Satuwa in cultivated field of Dolakha

g. g.

g.Zone of inhibition of rhizome extract against *Bacillus subtilis*(at a concentration 500mg/ml)

h.

h.Zone of inhibition of rhizome extract against *Bacillus subtilis*(at a concentration 250mg/ml)

i.Plant extracts of different

j. Phytochemical screening for the detection of presence of

k.Mixture of DPPH and plant extract at the beginning of experiment

l.Mixture of DPPH and plant extract diluted to different concentrations after incubation at dark for 30 minutes