EVALUATION OF ANTIOXIDANT, ANTIBACTERIAL AND ANTIFUNGAL PROPERTIES OF SOME MEDICINAL PLANTS OF NEPAL

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RECOMMENDATION

This is to certify that the research work entitled **"Evaluation of antioxidant, antibacterial and antifungal properties of some medicinal plants of Nepal"** has been carried out by **Ms. Kalpana Bakey** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. All results presented in this thesis are original findings and has not been published in any form till the date. I, hereby, recommend this thesis for final evaluation.

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Kalpana Bakey

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Glossary Acronyms
(List of abbreviation words)

µg	=	Microgram
µl	=	Microlitre
mg	=	Milligram
ml	=	Milliliter
DMSO	=	Dimethyl Sulfoxide
g	=	Gram
L	=	Liter
^0C	=	Degree Celsius
OD_{760}	=	Optical Density at 760nm
NA	=	Nutrient Agar
MHA	=	Mueller Hilton Agar
LB	=	Luria Bertani
PDA	=	Potato dextrose Agar
µM	=	Micro Molar
M	=	Molar
mM	=	Milli Molar
nM	=	Nanometer
RT	=	Room Temperature
UV	=	Ultra Violet
DPPH	=	1, 1 diphenyl 2 picryhydrazyl
SD	=	Standard Deviation
Wt	=	Weight
DW	=	Dry weight
d/w	=	Distilled water
spp.	=	Species
TPC	=	Total polyphenol content
TFC	=	Total flavonoid content
RSA	=	Radical scavenging activity
MeOH	=	Methano

CHAPTER I

INTRODUCTION

1.1 Background

The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal Plants" by World Health Organization. Medicinal plant can be defined as any plant which, in one or more of its organ, contain substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs (Sofowora, 1982). Medicinal plants are the richest bio-resource of drugs of traditional medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al*., 2008).

Medicinal plants are in use since the pre-historic time. Traditional and Folk medicine, Ethno-pharmacology and Ethno-medicine can be used synonymously for they all are derived from traditionally available natural plant products consisting adequate therapeutic value. Traditional medicines system is the heterogeneous term referring to a broad range of ancient health care practices, generally based on medicinal herbs and their extracts (Engebretson, 2002; Schmidt *et al*., 2008) whereas Ethno-pharmacology refers to the scientific study of ethnic groups and their uses as drugs which has played a vital role in our community for maintaining valuable information and exchange of medicinal plant resources (Devkota, 2006; Gaire and Subedi, 2011). Traditional medicine used in various regions of the world has been the effective, safe, accessible and affordable basic healthcare to the local communities (Mainen *et al*., 2010). For these reasons WHO encourages, recommends and promotes traditional/herbal remedies in national health care programs. In fact, traditional medicine is believed to be the foundation of ethno-medicine systems which includes the Sumerians and Akkaidians,Ayurveda and Siddha of India, Ying and Yan principles of Chinese herbal medicines, Jamu of Indonesia, Unani system of Middle and Far East Asia and many others (Natesh, 2000; Srinivasa *et al*., 2013). Whereas the modern medicine has their origin in ethno-pharmacology, the knowledge of entho-pharmacology has eventually led to the innovative and powerful discoveries of Modern age of medicine (Newman *et al*., 2003)

1.2 Plants as source of Drugs

Plants are considered as state-of-art chemical laboratories capable of biosynthesizing numberof biomolecules of different chemical classes.Many of these are proved to be precursors for development of other drugs. The phytochemical drug extraction and manufacturation is of commercial interest in the present world (Smetanska, 2008; Perumal and Gopalakrishnakone, 2010). According to WHOreport, 80% of people from the rural areas in the world depend on the traditional medicinal systems for their primary health care needs. At the subsistence level about 85% of Nepalese rural population is dependent on the various traditional medical practices, 60% in Uganda and Tanzania, 70% in Rawanda and India, 80% in Benin and 90% in Ethopia (WHO, 2002). The folk and traditional medicinal plants are the main sources of 25 - 50 % of modern medicines being manufactured (WHO, 2003). In the present context, the pharmacognostic investigations of plants are extensively being carried out for the discovery of novel drugs or templates for the development of new therapeutic agents (Beringer, 1999). Today, WHO has listed 11% of 252 basic drugs produced from flowering plants and one fourth of all prescribed drugs are directly or indirectly derived from plant sources (Karuppusamy, 2009).The first compound derived from herbal remedies to enter the international market was ephedrine, an amphetamine like stimulant from *Ephedra sinica* Stapf later was artimisinin, a potent antimalarial drug from Artemisia annua L. (Patwardhan *et al*., 2005). Some of them are tabulated below.

Table 1.1: Plant derived drugs with their source plant

Plant based Drugs	**Plant Sources**	**Medical Uses**
Atropine	Atropa belladonna	Anthicholinergic
Cocaine	Erythroxylum coca	Local anaesthetic
Digitoxin	Digitalis purpurea	Cardiotonic
Erythromycin	Tropical fungi spp	Antibiotic
Menthol	Menthe spp.	Rubefacien
Quinine	Cinchona ledgeriana	Antimalarial, Antipyretic
Pilocarpine Glaucoma	Jaborandi spp.	Galucoma
Tubocurarine	Chondodendron tomentosum	Muscle relaxant
Resserpine	Rawolfia serpentia	Antihypertensive
Taxol	Taxus spp	Anticancer
Vinblastine	Catharanthus roseus	For Hodgkins's disease
Vincristine	Catharanthus roseus	For Paediatric leukaemia

The earth is the common niche for 350,000 higher plant species among which 80,000 plant species are reported to have at least some medicinal value and around 5000 species are

known to have specific therapeutic values (Schippmann *et al*., 2006; Tabassum *et al*., 2012). In Asia-pacific only more than 8,000 sps are known for their medical values of which about 10% are in regular uses for treating various ailments and disorders. 20,000 to 35,000 species of these plants are being used in folk medicines, pharmaceuticals, cosmetics and neutraceuticals by different local and indigenous people around the world (Trivedi, 2006). Interestingly, within them some 100 species are involved in 25% of all the drugs prescribed in modern medicine.

In the present time focus on plant research has increased all over the globe enormously.The NCI (National Cancer Institute, USA) has tested more than 50,000 plant samples for anti-HIV activity and 33,000 samples for anti-tumour activity (Seidl, 2002). The latest trend shows the demand and research for therapeutically effective new drugs such as anticancer drugs (Dewick, 1996), antimicrobial drugs (Phillipson and Wright, 1996) antihepatotoxic compounds and multi-drug resistance pathogens (Sieradzki *et al*., 1999) eventually, leading to the era of medicinal plants which has no side effects. It is estimated that 60% of anti-tumor and anti-infectious drugs already on the market or under 3 clinical trials are of natural origin (Shu, 1998). In addition, major pharmaceutical companies, such as Glaxo, CIBA, Boehringer and Merck have established their specific departments dedicated to the study of new drugs from natural sources.

1.3 Plants as a source of secondary metabolites

Each medicinal plant has its own elemental composition and having pharmacologically important phytoconstituents (Hoffman 1998; Dingman, 2002). The phytoconstituents are the non-nutrient plant chemical compounds, making the phytochemical diversity associated with the taxonomic diversity. The distribution of phytoconstituents can be used as taxonomic marker in the utility in standardization of drugs, in detection of adulteration of drugs particularly in trafficking and in formulating quarantine measures.

Plants are limitless bioreactor of bioactive compounds which are responsible for plant defense against microbial infections or infestations by pests (Nweze *et al*., 2004; Doughari *et al*., 2009). These secondary metabolites are numerous and only 12,000 have been isolated, accounting less than 10% of the total (Schultes, 1978). The active bimolecules are broadly categorized into alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols, flavonoids etc. The phytoconstituents are circulated throughout the whole plant or can be deposited into the specific parts of the plants bark, fruit, flower, inflorescence, leaf, root, rhizome, stem, seed, and wood. The underground parts are most frequently attributed to presence of bioactive compounds (Moore, 1994). Concentrations of active phytochemical constituents of some Himalayan medicinal plant species have been reported to be high in populations growing at higher altitude as compared to the populations growing at the lower

altitude (Mikage *et al*., 1987; Yang *et al*., 2005). The factors such as soil type, temperature, precipitation, and abundance of microbial populations also have greater effect on the synthesis and turnover of secondary compounds in MPs (Mikage and Mouri 2000; Yang *et al*., 2005).

1.4 Phytoconstituents and Pharmacological Specificities

It is estimated that 25% of prescription drugs contain active principles derived from higher plants. The medical plants abundantly consist of heterogenous active biomolecules; can be used for various ailments. Phytoconstituents are known to possess antioxidant (Wong *et al*., 2009), antibacterial (Nair *et al*., 2005), antifungal (Khan and Wassilew, 1987; Agarwal *et al*., 2000), antiviral (Taylor *et al*., 1996a, b) antidiabetic, anti-inflammatory, antiarthritic (Mentreddy, 2007; Kumar *et al*., 2008),radio-protective activity (Jagetia *et al*., 2005) etc.

Alkalloids are the largest group of phytoconstituents predominantly found in seed and root. More than 12,000-alkaloids are known to exist in about 20% of plant species and only few havebeen widely exploited for medicinal purposes as anesthetics and CNS stimulants, narcotics, and poisons (Madziga *et al*., 2010). The analgesics morphine and codeine, the muscle relaxant tubocurarine, the anticancer agent vinblastine are the well known alkaloids.

Flavonoids are important polyphenol group having antioxidant properties (Kar, 2007). Over 4000 flavonoids are known to exist and some of them arepigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoidspresent in nearly 70% of plants.Flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, calchones, catechin etc are other flavonoids.

Phenols are the ubiquitously occurring as natural pigments of plant fruits. Caffeic acid is the most common phenol followed by chlorogenic acid (Kar, 2007). These are the natural antioxidants found in apples, green-tea, red wine, known to fight against cancer, inflammation and prevention of heart ailments. Saponins exhibit hypolipidemic and anticancer activity. The sex hormonesprogesterone, cortisone and hydrocortisone in clinical use are commercially produce from steroidal sapogenin (hydrolysed saponin) are diosgenin and hecogenin respectively (Sarker and Nahar, 2007). Tannins are antiseptic so tannin-rich medicinal plants are used as healing agents in a number of diseases like leucorrhoea, rhinnorhoea and diarrhea. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Scalbert, 1991). Plant steroids for its therapeutic application as cardiac drug are also known as cardiaic glycosides. Itwas reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1977). Phytochemicals castanospermine, calanolides, prostratin are potentially useful in the treatment of human autoimmune

deficiency syndrome (AIDS). The triterpenoid betulinic acid is just one of several terpenoids have been shown to inhibit HIV.

1.5 Plants as source of Antioxidants

Natural antioxidants play a key role in health maintenance and prevention ofthe chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNAdamage and ageing (Uddin *et al*., 2008).Phenols, flavonoids, vitamins, terpenoids, β-carotene, ascorbic acid of plants, citrus fruits and leafy vegetables posses the ability to scavenge the free radicals present in the human body. Eventually, prevent the crucial damage of biomolecules like lipids, proteins including those present in all membranes, mitochondria and, the DNA resulting in abnormalities leading to disease conditions (Uddin *et al.*, 2008).Natural antioxidants mangiferin, emblicanins, curcumin, polyphenols are the excellent rejenuvatos (Scartezzini and Speroni, 2000; Govindarajan *et al*. 2005). Natural antioxidants have been reported to prevent thepropagation of chain of free radical reaction and retard lipid oxidative rancidity (Lobo *et al*., 2010). In contrast, the synthetic antioxidants like Butylated hydroxytoluene (BHT), Butylated hydroxyl anisole (BHA), Gallic acid esters etc used as food additives, are capable of inhibiting oxidation via only single mechanism i.e. free radical scavenging and found to have drawbacks like toxicity and carcinogenicity (Hou *et al*., 2003; Almey *et al*., 2010). Thus, world is driving its attention towards herbal medicine.

1.6 Plants as source of Antimicrobial agent

Phytochemical and anti-microbial analysis of various medicinal plants have shown thatthe phenolic, alkaloids, steroids, glycosides, saponins, flavinoids, essential oils and other resins presents in these plants contains effective bioactive compounds against multiple pathogens. Plant inhibits microorganisms, interfere with some metabolic processes or may modulate gene expression and signal transduction pathways (Kris-Etherton *et al*., 2002; Manson 2003; Surh 2003). In general, the mechanism of action is due to inhibition of efflux pump, and quorum sensing and formation of biofilms (Savoia, 2012). phenolic acids prevents the -tract infections (UTI) and the usual dental caries by helping in the reduction ofparticular adherence of organisms to the cells lining the bladder, and the teeth. The bark of the Cinchona tree has been used as historical drug for treatment against malaria (Mates *et al*., 2007). Alkaloid 'quinine' is principally responsiblefor this anti-malarial activity. The data published in 2008 suggest that of the total 109 new antibacterial drugs approved during 1981-2006, 69% were originated from natural products (Newman, 2008). The publication showed the recent trend of antimicrobial drug discovery with their origin in "phytopharmocology".

1.7 Plants as source of Anticarcinogenic agent

Globally, caner has become the life threatening disease. The total number of cancer-related cases ispredicted to increase by 73% in the developing world and by 29% in the developed world (Parkin, 2001). Phytochemicals may either be used as chemotherapeutic or chemo preventive agents with chemoprevention referring to the use of agents to inhibit, reverse, or retard tumorigenesis (D'Incalci *et al*., 2005). More than 3000 plant species have been actively used to treat variety ofcancer including leukemia, melanoma, prostrate, and other form of cancers (Lucas *et al*., 2010). Discovery of alkaloids, vinblastine, vincristine and cytotoxicpodophyllotoxins derived the natural anti-cancer agent (Cragg and Newman, 2005). Phenol acids are found to minimize the formation of the specific cancer-promoting nitrosamines from the dietary nitrites andnitrates. Glucosinolates from various vegetable sources as broccoli, cabbage, cauliflower and Brussel sprouts exert a substantial protective support against the colon cancer. Phytosterols block the development of tumors (neoplasms) in colon, breast, and prostate glands. The taxanes and camptotliecins are found to exhibit anti-carcinogenic in the research conducted by the United States Nation Canser Institute (Gresham *et al*., 2008; Mishra *et al*., 2013). The saponins of Paris polyphylla are discovered to have anti-carcinogenic activities against wide range of cancer. Indeed, the greatest advancement in cancer therapeutics in the last quarter of a century has been the incorporation of compounds and analogs isolated from plants sources and their semi synthetic derivatives.

1.8 Economy sustained by Medical plants

In the last few years there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries. Globally, traditional medicine has a rapidly growing economic importance. The global market for herbal medicines based on traditional knowledge was estimated at US $60,000 million in the late 1990s. The world market for herbal remedies increased to US $19.4 billion in 1999 (Laird and Pierce 2002) while made around US $60 billion in 2000 (Tilbert and Kaptchuk, 2008). The herbal product in international market has played significant role generating US$ 14 billion for China in 2005 and US$ 160 million for Brazil in 2007. It was estimated to reach US $90 billion in 2015 (Global Industry Analysts Inc, 2012).

1.9 Medicinal Plants Prospective in Nepal

Nepal is a globally unique country of topography ranging from lowlands to the Greater Himalayan Highlands, comprising virtually all climate zones within an area of 147,181 sq. km with the latitudinal range from 26°22' to 30°27' N and longitudinal ranges from 80°14' to 88°12' E. The altitudinal variation with all six different climatic conditions of the world

houses globally significant and biologically diverse ecosystems of a wide range of unique and valuable medicinal plants (Pyakurel and Baniya, 2011).

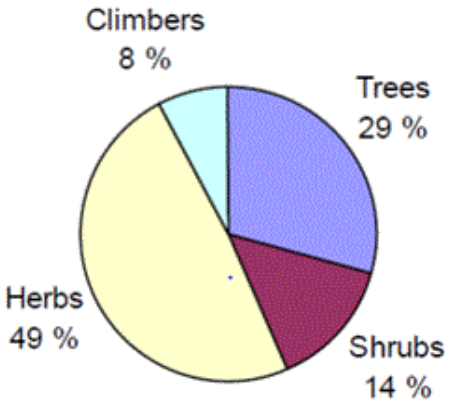
Nepal comprising only 0.3% of the global land area, of 0.09 % of land area on a global scale, it boasts high biodiversity at genetic, species and ecosystem level. Nepal represents 2.2% of the global plants, ranking 25th in biodiversity richness globally (HMGN, 2002; MoFSC, 2009) and ninth-richest flowering plant diversity in Asia according to The Biodiversity Profiles Project 1995.

Nepal is the repository of more than 7000 higher plants, 5% of which are endemic and 10% have medicinal and aromatic values (Joshi and Joshi, 1991). It has been estimated that the traditional use of 1000 species of medicinal plants by various communities in Nepal. There are about 700 species of medicinal plants in Nepal, about 250 of which are endemic to the country (MoFSC, 2009).The cultural and biological diversity of Nepal offers immense opportunities for ethno-botanical studies (Shrestha and Dhillion, 2003; Baral and Kurmi, 2006; Rokaya *et al*., 2010). The traditional medicinal plants and their valuable knowledge are believed to be well-preserved among different ethnic groups either in written or unwritten form that is passed onto generations through different practices like homegrown herbal medicine formula, folk lore, Amchi, and Ayurveda system (Shrestha *et al*., 2004; Kunwar *et al*., 2010). Nepal's floral diversity is a reflection of its unique geographic position, undulating landscape, as well as its altitudinal and climatic variations.

1.10 Medicinal Aromatic Plants (MAPs) of Nepal

Medicinal plants are one of the non timber forest products (NTFPs) or the alternative and secondary products which primarily focus on the commodifiability for rural income, expression of traditional knowledge, component of sustainable forest management and conservation strategies (Glossary of Forestry Terms in British Columbia, Ministry of Forests and Range, Canada, 2008). Medicinal and Aromatic Plants (MAPs) include plants used to produce pharmaceuticals, dietary supplement products and natural health products, beauty aids, cosmetics, and personal care products, as well as some products marketed in the culinary/food sector.

About 3,000 MAP species are in international trade (Lange and Schippmann 1997), while an even larger number of MAP species are traded locally, nationally, and regionally illegally or illegally. The rampant export of NFTPs from many illicit points is an obstacle for collecting actual data of trade of MAPs within or from Nepal. A total of 161 based NTFPs are harvested for commercial purposes of which 50% are medicinally important (Subedi, 2006). According to the medicinal and aromatic



plant data base of Nepal (MAPDON), the number of the medicinal and aromatic plants in the wild, cultivated, imported and naturalized found in Nepal as 1624 spp. (Shrestha *et al*.,2000) which has been recently increased to 1792 spp.(Baral and Kurmi, 2006). However, only 143 species has been considered as commercial MAPs which includes the plant under study *Paris polyphylla* (Satuwa) and *Rubia cordifolia* (manjista) (Bhattarai and Ghimire, 2006). About 100 MAPs are traded annually from Nepal among them 23 are found to be traded in high volume (Amatya, 2000) among which manjista have annual demand over 1000 kg in Kathmandu valley (Tiwari *et al*., 2004). Government of Nepal has identified 30 commercial MAPs for further research and development and for development of agro-technology; among them also 12 plants are given main priority (Sharma *et al*., 2004).The genetic resources deposition defines the most therapeutic preparation of medicinal plants which vary according to juice, decoction, paste, infusion and powder.The commercial MAPs belong to different life forms tree, shrubs, climbers and herbs.

1.11 MAPs and Nepal Economy

In Nepal, MAPs have very high socio-cultural, symbolic and economic values and are recognized as source of increasing revenue, strength the economy, conservation and employment to millions of people living in the region (Olsen 2005; Pyakurel and Baniya, 2011). Revenue collection on NFTPs based trade is above 10% of the total revenue generated from the forest based products (MoFSC, 2009). It has been reported that commercial cultivation of NTFPs has been a source to provide 50% of the family income and found that 10-100% of the households at mountain regions are involved in such trade system (Edwards 1996; Olsen and Larsen 2003).Some estimate that the contribution of NTFPs to Nepal's gross domestic products (GDP) stands at 5%. Department of Foresthad collected royalities about NPR 24.6 million (USD 0.34 million) on the 20 most-traded MAPs in 2006/07, increasing to NPR 29.2 million (USD 0.4 million) in 2007/08 (TEPC, 2009).

Nepal exported about US$3 million worth of MAPs in the world in 2008, in 2009 were about US$9.8 million, which fell to about US$6 million in 2010 (GIZ, 2011). While the global export of essential oils in 2009 was about US$846 million, Nepal exported only worth US$208,000 and was ranked 64th among 159 exporting countries. Nepal had exported 3,400 tons of Maps through legal channels in 1989/90 (Karki *et al*., 2003). The department of Forests estimated the export to be 33,000 tons of MAPs in 2005/06. According to the International Centre for Integrated Mountain development (ICIMOD), global trade in existing Medicinal and Aromatic Plants (MAPs) is valued at around US$ 60 billion in 2000 that is expected to grow to US$ 5 trillion by 2050 with an annual growth rate of 10-12% (Pyakurel and Baniya, 2011).

1.12 Research Plan

1.12.1 Hypothesis

Medicinal plants are in use since the pre-historic periods for the treatment of various ailments and disorders generally without any drawbacks. Hence, the bioactive molecules with pharmacological value should be detectable. The claimed health benefits of these plants must be based on the phytoconstituents deposited on various parts of the plants.

1.12.2 Experimental Design

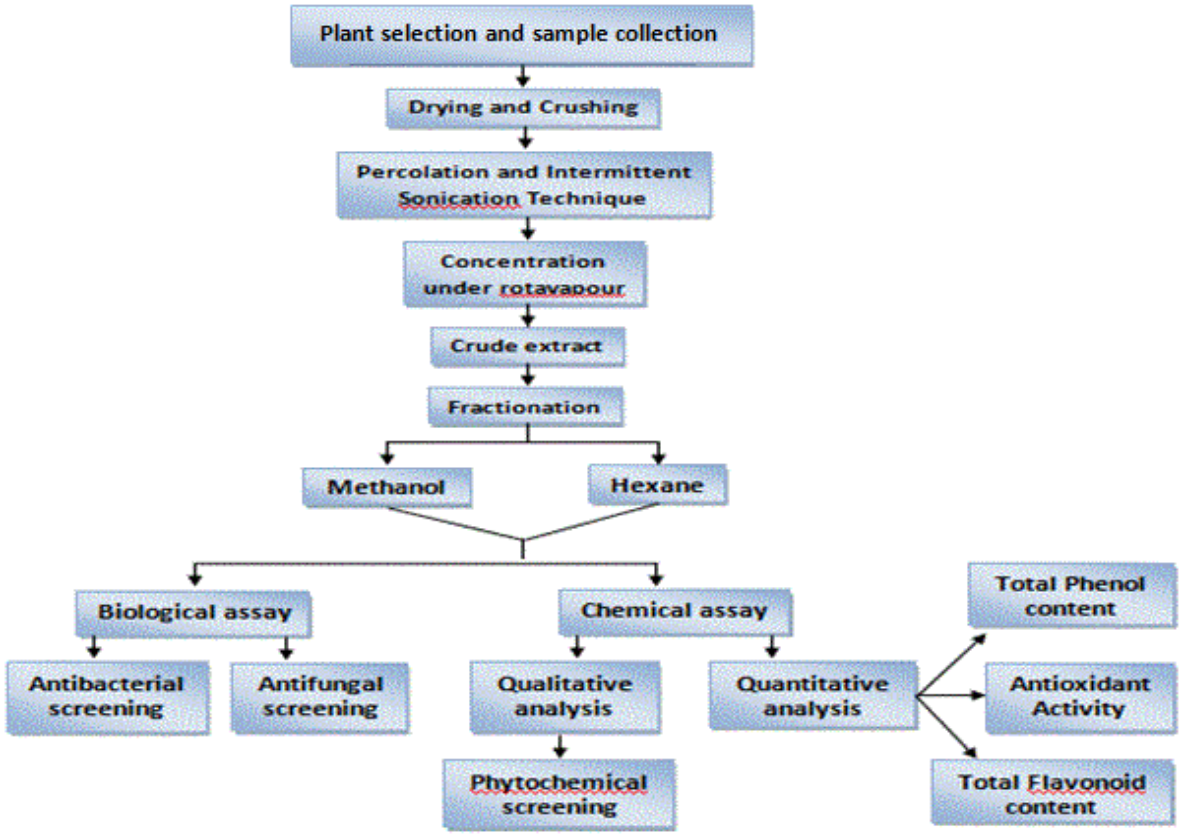


Figure 1.2: Experimantal Design of research work plan

1.13 Objectives

1.13.1 General Objectives

Evaluation of anitoxidnat, antibacterial, antifungal property of the medicinal plants of Nepal, *Paris polyphylla*, *Rubia cordifolia* (commercial MAPs) and *Phylllanthus niruri* having reported significant therapeutic values.

1.13.2 Specific Objectives

- Methanol and Hexane extraction of selected plants.
- Preliminary phytochemical screening of the plants
- Quantification of phytochemicals like alkaloid, flavonoid and phenolic contents.
- Determination of antioxidant activity of the plants under study.
- Evaluation of antibacterial and antifungal activity of the selected plants.

1.14 Rationale

Nepal is considered as one of the richest country in term of biodiversity. The altitudinal variation has been the main factor for the abundant availability of medicinal plants. Moreover, the cultural and ethnic diversity with more than 123 ethnic groups has been the sole key of the ethno-pharmacology. Traditional medicine system has been in use since time immemorial. Presently, the use of traditional medicine is being increased. The traditional medicine system is passed on generation to generation but the scientific records of MAPs with their phytoconstituents, potency and safety is still not sufficient. The scientific validation of their consistency and efficacy is the most. MAPs are not only the primary healthcare system but also sustaining the livelihood of large number of people. Scientific studies directed towards findings the phytoconstituents and active biochemicals for exploring their potential pharmaceutical use is essential step towards utilizing plant-based resource for drug development and uplifting the living standard of the local people. The assessment of phytochemicals of the plant and and the isolation of specific biomolecules lay the foundation of novel drug. Hence, this study will be a preliminary study towards the isolation and characterization bioactive compounds for potential drug development conserving the indigenous knowledge of the people.

1.15 Scope

The preliminary assessment will support thepharmacological studies as antibacterial and antifungal activities, quantification of each phtyoconstituents and evaluation of toxicity of the medicinal plants. Further, the study will promote the study of medicinal plants of Nepal. The exploration and conservation of these plants is important for the initiation of set up of pharmaceuticalcompanies.

CHAPTER II

LITERATURE REVIEW

Medicinal plants have always been the primary focus among the traditional healers, researchers, pharmacologist, and pharmaceutical companies. Medicinal plants have been in use for treatment of various ailments from simple injuries to the fatal diseases even cancer. Plant derived drugs are cheap and easily acceptable by the people. Natural medicines are believed to be with no toxic side effects or with very negligible drawback. Hence, it is believed that natural products based medicines are more compatible than their synthetic counterparts and more readily acceptable by the medical community. At present, the researcher are highly exploring and exploiting the traditional medicine system for the development of novel natural drugs. Thus, laboratories around the world are significantly focused on finding new plant-derived phytochemicals that might have huge pharmaceutical potential.

The plants under study with the taxonomical information, phytoconstituents and their pharmacological aspects have been given below.

2.1 *Paris Polyphylla*

Kingdom	Plantae
Order	Liliales
Family	Melanthiaceae
Genus	Paris
Species	*P. polyphylla*
Common name	Love apple
	Satuwa (Nepali)
Synomym	*Daiswa polyphylla* (Sm.)
Phenology	May -August

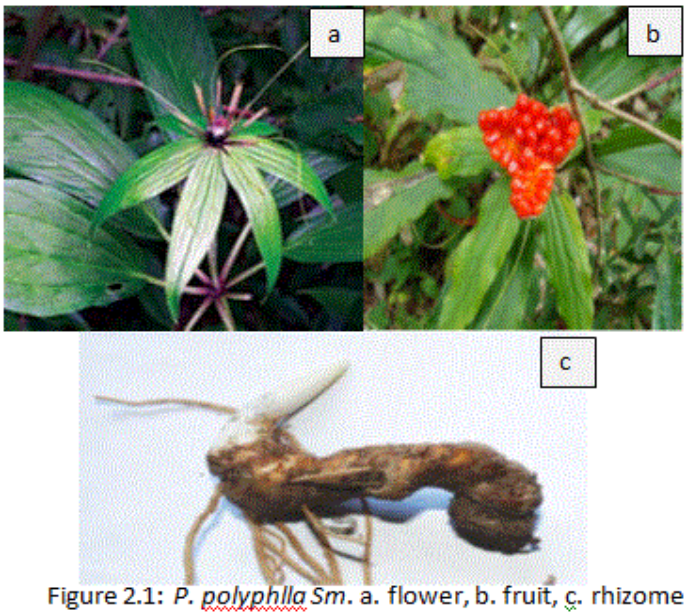


Figure 2.1: *P. polyphlla Sm*. a. flower, b. fruit, c. rhizome

The name “Paris” comes from “Par,” meaning equal, and it refers to the symmetry of the flower. It is a very variable species of the Liliaceae (Trilliaceae) family and has a large number of subspecies. The genus Paris comprises 24 species. This is an important medicinal perennial 10-100 cm tall creeping rhizomatous herb. The stem is smooth, glabrous and erect. The dark green colored leaves may be elliptic, oblong or lanceolate, has glaborous surface which present in 4-9 in number, and arranged in a whorl at the top of the stems. Rhizomes

of female plants are bigger in size than that of male plants. The non-self fertile plant have hermaphrodic flowers (have both male and female organs). The flowers are solitary, terminal, greenish at the apex, subtended by 4-9 lanceolate long-pointed leaf-like bracts. The globular fruit capsules contain numerous scarlet seed which on maturation turn red. (Garbyal, 2005; K.C *et al*., 2010; http://en.wikipedia.org/wiki/Paris_polyphylla).

2.1.1 Habitat and Distribution

P. polyphylla is found in Europe and especially in the south eastern hemisphere of the Asiatic countries like North-West India, Nepal, Bhutan, Southern Tibet, Myanmar, Laos, Thailand, Vietnam and mainland China at the elevation range of 100 to 3500 meter. It prefers phosphorus rich moist and swampy habitat and grows in dense forests, bamboo forests, thickets, grassy or rocky slopes, and in humus-rich moist soil (Manandhar 2002).

The plant is distributed throughout Nepal but is more common in Central and Eastern Nepal within an altitudinal range of 1800-3300m. The highest population of the plant was observed between 2300m to 2700m in Nepal. The plant has studied from Syaprubesi-Khangjung (2000m), between Ghatte Khola and Deorali (2400m), Ramche VDC, Birdim VDC, and Helambu VDC inside Langtang National Park, Ghandruk VDC, Rolpa, Dolpa, Makwanpur and Buffer Zone Area (IUCN 2004; K.C*et al.*, 2010).

2.1.2 Phytochemistry

The constituents of the plant are variable depending on habitats and the state of the plant parts (Wang *et al*., 1990; Deng *et al*., 2007; Kang *et al*., 2012). The bioactive molecules that are predominant in rhizomes are glucosides (α-paridin and α-paristyphnin), glycosides, saponins, phytoecdysone, diosgenin etc (Singh *et al*., 1966; Nohara *et al.*, 1982). *P. polyphylla* is a rich source of diverse steroid saponins which can be of different kinds. Spirostanol saponins, furostanol steroidal saponins, a new cholestane saponin are derived from rhizome (Wu *et al*., 2004). Pariphyllin A-B , Polyphylln A-H , dioscin, Gracillin, Paris I, II, V, VI, VII, other pennogenin and diosgenin derivatives have been isolated from rhizome gained importance in therapeutic properties of the plant (Singh and Thakur, 1980, 1982; Kang *et al*., 2005, Liu *et al*., 2006; Yan *et al*.,2009 a, b; Li *et al*., 2012).

The abundant types of rhizomal saponins of *P.polyhylla* (PRS) are saponins with diosgenin, pennogenin, or prosapogenin and their congeners as the aglycones. About 50 different

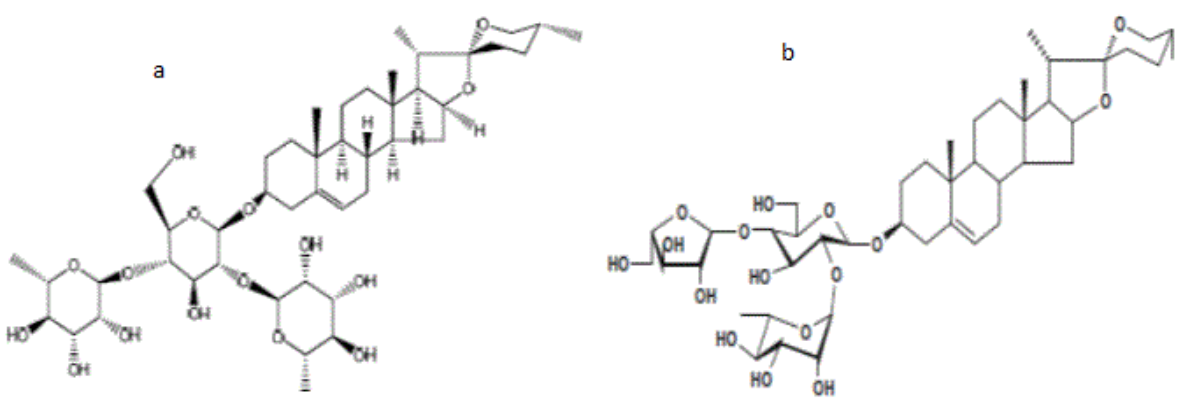


Figure 2.2: a. Dioscin, b. Polyhyllin D

compounds have derived from prototype saponin with the structural diversity in the glycoforms (Hostettmann and Marston, 1995; Man *et al.*, 2010). Trillin is the simplest rhizomal saponins. Tillin is diosgenin 3-O-β-D-glucopyranoside formed by addition of β-D-glucopyranosyl unit at the 3-OH of the steroid aglycones. The disaccharide saponin ophiopogonin C' is derived by substitution of α-rhamnopyranosyl residue at the 2-OH of the first glucose residue (Hostettmann and Marston, 1995; Wang *et al.*, 2007). One sapogenin and 24 steroidal saponins with antimicrobical activity have been isolated from the stems andleaves of *P. polyphylla* var. yunnanensis (Qin *et al*., 2012).

2.1.3Traditional and Ethnomedical Applications

The plant has been in use since the time innumerable for its broad range of significant uses. Each and every part of the plant has medicinal properties. *Paris polyphylla* has been used for the prevention and treatment of cancers in China for thousands of years. The whole plant can be used as febrifuge while the root and rhizome has broad range of uses. The rhizomes are used for injuries from falls, fractures, convulsions and strains (Liang, 2000). The roots are analgesic, antiphlogistic, antipyretic, antispasmodic, antitussive, depurative, febrifuge and narcotic. The decoction of root is used in the treatment of ulcers, diphtheria, epidemic Japanese B encephalitis, appendicitis, lymphadenopathy, tonsillitis, boils, parotitis, mastitis and rheumatism (http://www.pfaf.org). The roots have antibacterial action against *Bacillus dysenteriae, B. typhi, B. paratyphi, E. coli, Staphylococcus aureus*, haemolytic streptococci, Meningococci etc. The herb is primarily used for the treatment of liver, stomach, nose, lung, throat and breast cancer in traditional Chinese medicine. *P. polyphylla* was also found to be potent sedative (Wang *et al*., 1990), spermicidal in rat and human spermand immunostimulating (Zhang *et al*., 2007).

In Nepal, satuwa is locally used for curing fever, headache, wounds and burns. It is also used as a tonic. Traditionally, rhizome is widely used as an antileshmanic, antihelmintic, antispasmodic, digestive stomachic, expectorant and vermifuge (IUCN 2004; Devkota *et al*., 2007). The root paste works as an antidote to snake bites and poisonous insect bites and also to alleviate narcotic effects. It produces vasoconstriction in kidney, vasodilation in spleen and limbs and stimulates the isolated intestine (Baral and Kurmi 2006; Dutta, 2007). The juice of the root or the powder is taken as anthelmintic and in cases of fever and food poisoning (Shrestha *et al*., 1995; Manandhar, 2002). Presently, the rhizome is used as an alternative to the drug Diosgenin (ANSAB, 2012).

2.1.4Pharmacological aspects of *Paris polyphylla*

P. polyphylla is an important member of this genus which can be truly called "jack of all trades" for its very broad properties of curing a number of human and animal aliments from

diarrhea to cancer. The herb has been used to treat liver cancer in China for many years and has been reported as a potent anticancer agent that can overcome drug resistance. It also played an important role in the medicine development for antitumor, immunity adjustment, analgesia, and anti-inflammation (Yan *et al*., 2009a). Saponins have haemolytic activity and exert anti-inflammatory, antifungal and antimicrobial effects (Justyna *et al*., 2011).

2.1.4.1 Anti cancer/anti-tumor activity

The many different phytochemical studies have reported that steroidal saponins exhibit inhibition of tumor growth. Saponins have a potential cytotoxicity against various tumor cells, such as CCRF leukemia cells, ECA109 esophageal cancer cells, CaEs-17 cells, human promyelocytic leukemia HL-60 cells, human liver carcinoma HepG-2 cells, human gastric cancer BGC-823 cells, human colon adenocarcinoma LoVo cells and SW-116 cells (Sun *et al*., 2007; Kang *et al*., 2012; Li *et al*., 2012). The PRS steroidal saponins inhibiting tumor growth has been identified as Paris I, II, V, VI, VII, and H, Dioscin, Gracillin, disogenin, penonogenin, polyphyllin I and polyphyllin D, (Yan *et al*., 2009a; Man *et al*., 2011).

It is reported that PRS exhibited anti-tumor activity via mitochondrial mediated capase activation pathway of apoptosis, attenuation of the inflammation response (Li *et al*., 2012). Diosgenin could suppress proliferation, inhibit invasion, and suppress osteoclastogenesis through inhibiting the expression of NF-κB-regulated gene (Shishodia and Aggarwal, 2006). Polyphyllin I, D exhibited apoptosis via mitochondrial dysfunction caused by enodoplasmic reticulum mediated stress (Cheung *et al*., 2005; Ong *et al*., 2008). Polyphyllin D was also found to inhibit human breast cancer cells and can serve as a candidate for breast cancer treatment (Lee *et al*., 2005).

2.1.4.2 Antimicrobial activity

The isolation of one sapogenin and 24 steroidal saponins from the stem and leaves of the plant have antimicrobical activity. The antibacterial activity of different extracts of aerial parts and rhizome of the plant against *Bacillus subtilis*, *Staphylococcus aureus*, P*seudomonas aureginosa*, *Escherchia coli* and *Salmonella flexinerai* was reported (Chhetri *et al*., 2012). The antibacterial activities of ethanol extract of the plant against fungi *Aspergillus fumigates*, yeast *Candida albicans*, gram negative bacteria (*Acinetobacter baumannii* and *Pseusomonas aeruginosa*) was reported (Zhang *et al.*, 2013).The antitumor and antifungal activity by β-ecdysterone and three pennogenin steroidal saponins isolated from ethanol extract of *P. polyphylla var. yunnanensis* has been studied (Zhu *et al*., 2011). The compounds chonglouoside SL-7, chonglouoside SL-8 and dumoside were the first report of C22-steroidal lactone glycosides isolated from the Paris genus evaluated for their antimicrobial activity against *P. acnes* (Qin *et al*., 2012; Qin *et al*., 2013). The anti-fungal activity of PRS against *Cladosporium cladosporioides, Candida* species and other species has been reported (Wu *et*

al., 2004). Similary, polyphyllin D showed to exhibit antifungal activity even at 2ug/ml against *C*. *albican* (Shi *et al.,* 2011).

2.1.4.3 Hemostatic activity

The steroidal saponins exhibits uterine contractile activity (Zhou, 1991) and can be used for uterine hemorrgage of various etiology (Tian *et al*.., 1986). On basis of the uses of steroidal saponins, drug named as Gaonxuening Capsule has been manufactured for the treatment of abnormal uterine bleeding (Zhao and Shi, 2005). The hemostatic activity of pennogenin saponin with three glycones was stronger than that of diosgenyl saponin.

2.1.4.4 Other pharmocological acitivity

The rhizome of the plant contains sugars (7.9%). Two glucosides, viz. α-paridin and α-paristyphnin produce a tingling sensation on the tongue. α-Paristyphnin is pharmacologically more active and has a depressant action on carotid pressure, myocardium and respiratory movements. A series of steroidal saponins, especially various diosgenin glycosides and pennogenyl saponins, such as polyphyllins VII and polyphyllins II strongly inhibit gastric lesions induced by ethanol and indomethacin (Matsuda *et al*., 2003). The four diosgenin type saponins present in rhizomes were found to be tyrosinase inhibitors as well as antileishmanial agents (Devkota *et al*., 2007)

2.1.5 Trade of *Paris polyphylla*

P. polyphylla Sm. is one of the important medicinal plant listed under vulnerable category (V) (CAMP 2001; IUCN 2004). It is the high value NFTPs mostly traded medicinal plants of Dolpa and Makwanpur district possessing the potential to boost the economy in future (Tiwari *et al*., 2004; Gewali, 2008; KC *et al*., 2010).

The average market price of *P. polyphylla* is Rs. 260-350 per kg in local market whereas cost more than Rs 4000/kg in foreign market. The rhizomes have high demands in both national and international markets for its valuable rootstock to treat variety of ailments (Bhattarai and Ghimire, 2006). In the year 2009/10 only 19,882 kg of the plant has been exported from Nepalgunj to India (Sharma and Shrestha, 2011).

Because of its high trade value, people from mountain region are now very much attracted to *P. polyphylla*'s collection.Forest act 1993 of Nepal has permitted the licensed trade of its rhizomes but the local population is declining continuously at an alarming rate by human interference such as unsustainable harvesting (over- and premature collection), unscientific use, illegal/cross-border trade of rhizomes, habitat destruction, overgrazing, forest-fire, and

soil-erosion, lower number of viable seed production and long dormancy of seeds or very poor seed germination seems to be the major threats to the plant regeneration (K.C. *et al*., 2010). At present, over-exploitation for its root may make this species a threatened one in future with the insurgency for conservation of this medicinally very significant plant.

2.2 *Rubia cordifolia*

Kingdom	Plantae
Order	Gentianales
Family	Rubiaceae
Tribe	Rubieae
Genus	Rubia
Species	*R. cordifolia*
Common name	Manjistha
	Madder
	Manjitho (Nepali)
Phenology	June - October

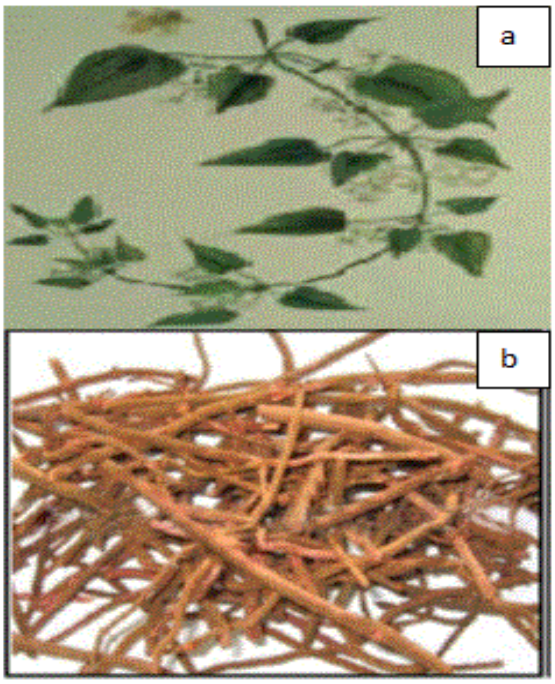


Figure 2.3: a. Leaves, b. Root

Rubia cordifolia Linn.sensu Hook. f., is a perennial herbaceous prickly creeper or climber of 1.5 m with red rhizomatous base and roots height (Wealth of India; 2002). The stems are brittle in prickly. Stems are quadrate in shape. The leaves are long-petioled, ovate, and acute with glabrous or cordate base. The evergreen leaves are 5–10 cm long and 2–3 cm broad, produced in whorls of 4-7 starlike around the central stem. It climbs with tiny hooks at the leaves and stems. The flowers are small (3–5 mm across), with five pale yellow petals. The pinkish or redish or dark purplish flowers are borne in terminal panicled cymes. Fruits are globular, or slightly 2-lobed dark-purplish or black matured into two small seeds. The 5 mm long fruits are fleshy with red juice. It has long cylindrical and typically red colored roots. Roots are long, wide, externally longitudinally striated, and yellowish red. The roots can be over 1 m long, up to 12 mm thick (Adwankar *et al*., 1980). The herb is used as food plants for the larvae of some Lepidoptera species including Hummingbird hawk moth.

2.2.1 Habitat and Distribution

It prefers loamy soils with a constant level of moisture though can grow well in light (sandy), medium (loamy) and heavy (clay) soils. The plant is distributed widely around the world, such as Western Europe, Northern Europe, Mediterranean Coast, Temperate Asia, Africa, Himalayas (More*et al.*, 2007). In Nepal, the plant is distributed in Himalayan region of Nepal

at and around 2400m of elevation. It is prominent at the elevation of 1200 - 2700 m from east to central Nepal (http://www.biosysnepal.com.np/product/herb/madder.php).

R. cordifolia and *P. polyphylla* are the herbs exported from Nepal under the JABAN (Jadibuti assosciation of Nepal). These two plants are the conservation priority plant species of Kanchenjungha-Singalila Ridge, eastern Nepal under ethnobotanial society of Nepal (ESON). *R. cordifolia* is of the herbs under the list of priority species for cultivation of Ashok medicinal and aromatic plants centre run by Dabur Nepal pvt. ltd. It is one of the 143 species assessed as commercial MAPs in Nepal (Bhattarai and Ghimire, 2006). It is the one of the dominant associated shrub species in Khimti hydropower project area and Tinjure-Milke-Jaljale area (Sigdel *et al*., 2013)

2.2.2 Phytochemistry

Rubia cordifolia has been reported for the presence of glycosides, saponins, anthraquinones, tannins, hexapeptides, quinones, triterpenoids (Antarkar *et al*., 1983). *R. cordifolia* is basically known for its anthraquinones and naphthohydroquinones constituents (Itokawa *et al.,* 1989). The major phytoconstituents of *R. cordifolia* reported to include Rubiadin (Rao *et al.,*2006), Rubicordone A (Li *et al.,* 2009) Rubiasins AC (Chang *et al,*. 2000), triterpenoid-Rubiatriol (Arisawa *et al.,*1986), 6-methoxygeniposidic acid an iridoid glycoside (Wu *et al.,*1991) and two pentacyclic triterpenoid- Rubicoumaric acid and Rubifolic acid (Talapatra *et al.,*1981). Mollugin, furomollugin, dehydro-alpha-lapchone are isolated from chloroform fraction of the root (Gupta *et al.,* 1999). α-Fernane derivative rubiatriol, β-sitosterol, scopoletol and oleanolic acid acetate are also isolated from root of the plant, two macrocyclic neoplasm inhibitors – TPC-A and TPC-B, tectoquinone have been isolated. Different forms of quinine, anthroquinone, iridoid glycoside, two naphthohydroquinone dimmers (I and II), and four naphthohydroquinones have been isolated from the plant (Hua *et al.,* 1992). All cultures produced only two major AQs, with munjistin and purpurin representing 90% of the total AQ yield (Bulgakov *et al*., 2002).

The primary chromophores present in *R. cordifolia* are alizarin, purpurin, pseudopurpurin, xanthopurpurin, munjistin, rubiadin with the base 9,10-anthraquinone structure but with different functional groups at carbons 1-4 (Thomas *et al.,* 2010). Preliminary analysis has revealed that *R. cordifolia* has significant amount of reduced glutathione(GSH), Vitamin C, other important antioxidants and polyphenols and important trace elements like Zn, Cu, Vd, Se and Mo. (Rawal *et al*., 2004).

2.2.3 Traditional and ethnomedical applications

R. cordifolia is one of the earliest plant resources possessed important commercial and medicinal values. *R. cordifolia* is an important medicinal plant which is used for treatment of

various ailments in Ayurvedic system of medicine (Adwankar *et al*., 1980; Sertoli *et al*., 1994; Srideviet *al*., 2011).

It is good lymphatic tonic, used to enhance lymphatic drainage in patient with fungal dysbiosis and/or mercury toxicity. The leaves of this plant also studied for its antiviral and in-vitro free radical scavenging activity (Prajapati and Parmar, 2011). The plant posses' antimicrobial, anti convulsant activity together being anxiolytic as it increases dopamine level in body (Tripathi *et al*., 1993; Kasture *et al.*, 2000; Singh *et al.*, 2005). The roots are astringent, digestive, expectorant and hypnotic and used in leprosy, urinary complaints (Sapkota and Adhikari 2001; Senapati *et al.*, 2001; Kunwar *et al.*, 2009). The plant is astringent, antidysenteric and antiseptic in properties (Rajbhandari *et al*., 1995). A root extract is useful for disintegration and elimination of urinary stones (Mischenko *et al*., 1999).

Rubia denotes 'red' as their internal use imparts red color to breast milk and urine (McIntyre, 2005). *R. cordifolia* is popular all over the world for its external as well as internal medicinal uses in various skin related diseases eczema, major burns, itches, psoriasis, herpes, scabies, ulcers , various ailments as chronic pyrexia, puerperal fever, dysentery, tuberculosis, cancer etc. The stem can be used for snake bite and scorpion sting as well as on non diabetic foot ulcer (Ojha *et al*., 1994). The cooling effect of it is extensively applied in Tibetan medicine for treatment of blood disorders; removes excess heat in blood and in the lungs, kidneys, and intestines; reduce swelling. It is a popular remedy for the relief of heat and itching in and also reported successful in treatment of vitiligo when given with honey (Gogte, 2000).

2.2.4 Pharmocological aspects and other significances

R. cordifolia show potent antioxidant activity against lead nitrate and radiation induced toxicity (Lodia and Kansala, 2012; Tripathi and Singh, 2007). Rubiadine, a dihydroxy anthraquinone present abundantly in root and GSH, Vit-C and important trace elements like Zn, Cu, Vd, Se and Mo account of the antioxidant activity ((Rawal *et al*., 2004; Lodia and Kansala, 2012). Terpenes and cyclopeptideas are good antioxidant along with antitumoric agent. Rubiadin is a potent antioxidant, inhibits lipid peroxidation (Tripathi and Sharma, 1998) and immunomodulatory (Jokharapukar *et al*., 2003). The methanolic extract had significant anti-plasmodial activity against *P. knowlesi* whereas had moderate parasitaemia (Nyambati *et al*., 2013). Two triterpenes, maslinic acid and ursolic acid were reported to possess inhibition on the human immunodeficiency virus (HIV-1) protease (Xu *et al*., 1996). The hepatoprotective activity of an aqueous methanol extract of *R. corodifolia* was investigated against acetaminophen and CCl_4- induced damage (Gilani *et al.,* 1994; Rao *et al*., 2006).

2.2.2.4.1 Anti-Carcinogenic/ anti-tumor activity

This plant is in clinical use for the management of several disorders including cancer (Son *et al*., 2008; Patel *et al*., 2011a, b). The eight different types of Hexapeptides exhibited potent anticancer activity against P-388 lymphocytic leukaemia (Parag *et al*., 2010). Rubinocordifolin showed significant cytotoxic activity both in vitro and in vivo, inhibiting the growth of sarcoma ascites in mice at low concentrations (Itokawa *et.al*., 1991). The plant was reported as effective chemotherapeutic agent against N-nitrosodiethylamine induced hepatocellular carcinoma in rat. The methanolic extract of Rubia cordifolia induced typical apoptosis in HEp-2 (Human laryngeal carcinoma) cell line through the elevation of reactive oxygen species generation (Shilpa *et al*., 2012a, b).

The compounds Mollugin, Alizarin and Lucidin primversoside are the potential chemopreventive/chemotheurapetic agents in breast, prostate, bladder, liver, pancreas, skin, lung, colon and brain as these compounds selectively inhibit Cyclooxygenase (COX-2) which suppress malignant cells proliferation and invasion (Ristimaki *et al.*, 2002; Kaur *et al*., 2010; Fotia *et al*., 2012). Anthraquinones III, IV, V and VII had significant antitumor activity against Sarcoma 180 ascites and P388 leukemia in mice (Hasuda *et al*., 2011). Ethyl acetate extract of root inhibits cell growth and promotes terminal differentiation in cultured human keratinocytes strongly suggesting its antipsoriatic activity (Tse *et al*., 2006; Zhou *et al*., 2012) against psoriasis, skin disorder characterized by hyperproliferation and aberrant differentiation of epidermal keratinocytes.

2.2.4.2 Antimicrobial activity

R. cordifolia extracts have antibacterial activity for *E. coli*, *B. cereus*, *B. subtilis*, *S. aureus, S. intermedius, K. pneumonia, P.vulgaris, P. aeruginoasa* (Basu *et al*., 2005, Singh *et al*., 2005). Ethanol extract was found inhibitiroty to all *E.coli* strain isolated from urine samples (Sawhney and Kumar, 2011). Methanol extract was reported to have had highest antioxidant, antibacterial, antifungal activity against *K.pneumoniae*, *S. aureus* and *C. ablican* (Prajapati and Parmar, 2011). The antimicrobial activity is attributed to the known anthraquinones present in the plant (Mishchenko *et al*., 2007; Li *et al*., 2009). A gel formulation containing 0.1 % of anthraquinone rich fraction exhibited optimum anti-acne activity against *P.acne, S.epidermidis, Malassezia furfur* (Joshan, 2010; Khan *et al*., 2012).

2.2.4.3 Anti-Inflammatory Activity

The anti- inflammatory properties has been severally reported (Kasture *et al*., 2001; Pande and Flora, 2002). *R. cordifilia* is very effective for wide range of inflammation caused by musculoskeletal problems, digestive problems such as Peptic Ulcer, Crohn's Disease and Ulcerative colitis. Ranitidine exhibited antiulcer activity. The anti-inflammatory, analgesic, anti-pyretic activity and gastroproctective properties depend on the triterpenoids (Kasture

et al., 2001; Deoda *et al*., 2011). It is used for inflammatory skin conditions like acne, vitiligo, eczema and oozing (Hazra *et al.*, 2005; Zu *et al*., 2010).

2.2.4.4 Wound healing activity

Rubia cordifolia has been evaluated for its wound healing activity (Karodi *et al*. 2009). Pentacyclic triterpenoids, tannins and anthraquinones are the major responsible phytoconstituent for this activity (Rashed *et al*., 2003; Singh and Geetanjali, 2004). The ethanolic extract and its gel formulation of the roots of the plant found to be effective in the functional recovery of the healing of wounds and also in histopathological alterations (Karodi *et al*., 2009).

2.2.4.5 Natural dye and colorant

Rubia cordifolia is very economically important plant in many regions of Asia, Europe and Africa as a source of red pigments. The traditional usage of Majitho as natural food colorants and natural dyes has been commercialized (Gao*et al*., 2000). It has been used since ancient times as vegetable red dye for leather, wool, and cotton, silk and medicinal oils. The purpurin and munjistin are major coloring pigments with small proportion of xanthopurpurin and pseudopurpurin present in the roots (Gupta and Glurajani, 1992). The root and stem extract is extensively used in yarn and fabric industry for its characteristic beautiful colors ranging from orange gold to deep red and light pink or scarlet shades (Barakoti and Shrestha, 2008; Yusuf *et al.,* 2012). The very good UV protection can be achieved by dyeing jute fabric with natural dyes extracted from *R. cordifolia.*

2.2.5 Trade of *R. cordifolia*

It is one of the 43 MAPs being exported in large amount from mostly to India. The annual demand of this herb in Kathmandu valley is 1000 kg. According to Forest Range Office, about 10,000 kgs of Majitho is traded from Basantapur in the year 2001-2002. In year 2005-2006-2007, total 1550 dry kg was traded from Rasuwa district whereas 4,721 kg has been legally exported nationwide in the year 2009/10 only (Humagain and Shrestha, 2009; Sharma and Niraj, 2011).

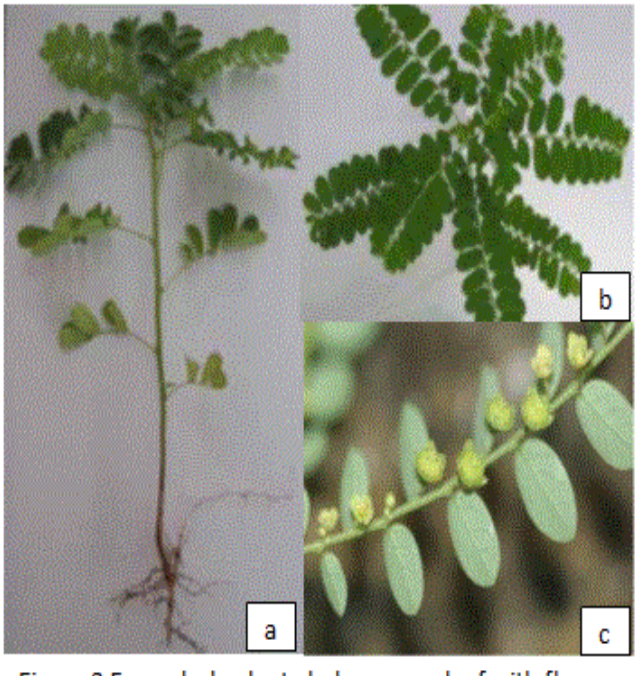


Figure 2.5: a. whole plant, b. leaves, c. leaf with flower

2.3 *Phyllanthus niruri*

Kingdom	Plantae
Division	Magnoliophyta

Class	Magnoliopsida
Order	Euphorbiales
Family	Euphorbiaceae
Genus	*Phyllanthus*
Species	*P. niruri*
Common name	Stone breaker Bhuinamala (Nepali)
Phenology	June - August

The term phyllanthus means "leaf and flower" and the genus got its name for all the plants belonging into this genus have the flowers, fruits are alike leaf (Cabieses, 1993). It is the medicinally potent member of the Euphorbiaceae family which groups over 6500 species in 300 genera of upright or prostrate herbs or shrubs, often with milky acrid juice.

Phyllanthus niruri is an annual herb of 30-60 cm high, quite glabrous, stem often branched at the base. The plants are monoecious or homogamous; leaves are small and appear oblong with very short or absent petiole. The leaves are small and numerous which are arranged like leaflets alternately on lateral branches with single female flower or a group of 1-3 unisexual male flowers at the base of leaves. The minute and numerous, greenish colored flowers are cluster in cup-shaped structures. The fruit is a three-lobed capsule extending from the cup and commonly the long stalk pendant. The capsules are found to be 2.5 mm in diameter, oblate and rounded, depressed globose and smooth scarely lobed. The seeds 0.9 mm long are dark brown and warty (Lewis, 1977; Kirtikar and Basu, 1994).

2.3.1 Habitat and distribution

Plants in the genus *Phyllanthus* are found proliferating throughout tropical and subtropical regions of both hemisphere Asia and America which comprises 600-700 species with minor distinguishing features among them (Unander, 1995). It is wide spread throughout the tropics and subtropics in sandy regions as a weed in cultivated and waste land (Ross, 1999). *Phyllanthus niruri* may be found in profusely branched condition along with crops of gram, wheat, pea, etc. In the wild it is found growing along road sides, in street corners, and dumps of building materials. In Nepal it is found at the altitude of 470-900m altitude, from east to west.

2.3.2 Phytochemistry

The wide variety of phytochemicals and their pharmacological properties of *P. niruri* has been the subject of studies since the mid 1960s. More than 50 different classes of organic compounds with various medical interest have been reported, the major being the lignans, tannins, polyphenols, alkaloids, flavonoids, terpenoids and steroids (Calixto *et al*., 1998; Bagalkotkar *et al*., 2006).

The active phytochemicals, lignans (Murugayiah and Chan, 2009), polyphenols (De Souza *et al*., 2002), flavonoids (Shakil *et al*., 2008), xylans (Mellinger *et al*., 2005), terpenoids and in

particular, tannins (Thakur *et al*., 2000) alkaloids, coumarins and saponins have been identified from various parts of *P. niruri*. The bioactive molecules and chemical agents including phyllanthin, hypophyllanthin, phyltetralin, niranthin, nirtetralin, hinokinin and isolintetralin are the lignans that have been isolated (Calixto *et al*., 1998; Murugayiah and Chan, 2009). Furthermore, various detail work has isolated Rutin (flavonol glycoside), Quercetin, Quercitrin, Astragalin, Gallocatechin, Nirurin Limonene, Ellagic acid, Gallic acid, Repandusinic acid, Norsecurinine, Saponins etc from whole from the hot aqueous extracts of the whole plant (Ishimaru *et al*., 1992; Matsuura *et al.*, 2005; Bagalkotkar *et al*., 2006; Elfahmi *et al*., 2006; Mellinger *et al*., 2008; Wei *et al*., 2012).

2.3.3 Traditional and Ethnomedical Applications

P. niruri is the medicinally important plant with a long history of use as an herbal edible source for the treatment of a broad spectrum of diseases in Brazil, India, Nepal and other countries. It is use in folk remedies around the world in treatment of liver, kidney and bladder problems, diabetes and intestinal parasites (Foo, 1993). It has been a favorite choice of the rural people because of its immense medicinal properties like antidote, against liver diseases, antiviral properties**,** antioxidant, heptoprotective, anti-inflammatory and strong inhibitory effect against neurogenic (Thyagarajan *et al.,* 1998; Kiemer *et al*., 2003; Bhattacharjee and Sil, 2006; Chattopadhyay *et al.,* 2006). It is consumed as a tea in Brazilian folk medicine as an effective remedy to eliminate gallstones, kidney stones, and other genitourinary and liver disorders (Freitas *et al*., 2002).

P. niruri is ethnomedically valuable plant having wide used in the preparation of various ayurvedic formulations (Barros *et al*., 2006; Mahesh and Satish, 2008). Whole plant, fresh leaves, roots and fruits are used to treat various ailments. An aqueous infusion of the whole plant is a typical preparation traditionally being used for fever including malaria and as antispasmodic (Weninger and Haag-Berrurier, 1982), laxative (Halberstein and Saunders, 1978),dysentery and diarrhea (Singh *et al.,* 1986; Holdsworth and Wamoi,1992), analgesic (Santos,1994). and vaginitis, hepatitis B, gonorrhoea, syphilis, tuberculosis (Unander, 1998; Adhikari *et al*., 2007), jaundice (Singh *et al.,* 1986), diuretic, diabetes, apitizer, asthma ulcer,, scapies, ringworm, genitourinary disorders, new borns baby bath, cough (Paithankar *et al.*, 2011, Narendra *et al.*, 2012).

2.3.4 Pharmacological aspects

P. niruri has found to have therapeutic effects in many clinical studies. Phytochemicals exhibit different structural characteristics with various pharmacological actions. The lignans have excellent hepatoprotective (Chang *et al*., 2003; Yan *et al*., 2009b) whereas terpenes exhibit anti-microbial activities (Popova *et al*., 2009). Flavonoids from have been shown to

have antioxidant (Hayashi *et al*., 2012), antileishmanial (Muzitano *et al*., 2006) and anti-inflammatory properties.

The in-vitroand in-vivo anti-plasmodial activities of different extracts, as well as the toxicity of the lyophilized aqueous extract, from *P. niruri*were also reported (Tona *et al*., 2001; Cimanga *et al*., 2004; Luyindula *et al*., 2004; Mustofa *et al*., 2007). The whole plant's extract reduced parasitaemia by 73% showing its effectiveness against malaria (Neraliya and Gaur, 2004).

It has been reported that feeding of *P. niruri* at a dose of 100 mg/kg lowered the elevated level of low-density lipoprotein lipids in hyperlipidemic animals fed with triton and cholesterol (Chandra, 2000). Lignans and phyllanthin reported to exhibit a uricosuric activity in hyperuricemic rats (Murugaiyah and Chan, 2006). The tannin ellagic acid is thought to application in managing diabetic complication such as cataract development (Shimizu, 1989). The herb was found to reduce the blood sugar 18.7 percent at concentration of 1000mg/kg by weight (Okoli *et al*., 2010). *P. niruri* is sold commercially in France as Pilosuryl, an aqueous-alcohol extract for diuretic puroposes (Robineau, 1991).

2.3.4.1 Anticarcinogenic activity

The active ingredients in *P. niruri* that exert anticancer effects may include polyphenols, such as gallic acid ((Ohno *et al*., 1999; Kawada *et al*., 2001), flavanoids or tannins (Markom *et al*., 2007) which are abundant in the herb. The anti-proliferative and selective cytotoxicity due to gallic acid was observed on two cancer cell lines HT29 and HepG2 cells (Pinmai *et al*., 2008). A spray-dried extract from *P. niruri* (SDEPN) induced an increase in cell death of HT29 and HepG2 cells when used in combination with cisplatin (Araujo *et al*., 2012).

2.3.4.2 Antimicrobial activity

The antimicrobial activity has been studied very much. The ethanolic extracts of had inhibited the growth of microorganisms (Crisanto *et al*., 2003). Both aqueous and ethanol extracts were found inhibitory to *E. coli*, *S. aureus* and *Salmonella typhi* (Ekwenye and Njoku, 2006). *P. niruri* DMSO leaves extract was reported very effective in inhibiting the growth of all the selected strains of *S. typhi* (4 strains) and *S. aureus* (3 strains) whereas was non-inhibitory on *E. coli, K. pneumoniae* and *S. paratyphi* even at 400 µg/ml (Sumathi and Parvathi, 2010). The antibacterial test was conducted with water and methanol extract among 14 different test gram positive and gram negative bacteria (Poh-Hwa *et al*., 2011). It was revealed that the methanol extracts of various parts of *P. niruri* have antibacterial activity against five bacterial strains - *E. cloacae, S. aureus*, *P. aeruginosa*, *E. coli* and *S. viridians* and two fungal strains - *A. niger* and *T. viridae (*Mathur*et al*., 2012).

2.3.4.3 Hepatoprotectant / anti-hepatotoxic activity

The plant extract is one of the components of a multiherbal preparation for treating liver ailments (Kapur *et al.*, 1994). The plant is effective against liver damages induced by several different drugs and environmental hepatotoxicants as CCl_4, nimesulide and paracetamol (Chatterjee and Sil, 2006; Harish and Shivanandappa, 2006; Sabir and Rocha, 2008). The hepatoprotective activity along with its antioxidant activity has been proved by inhibiting the progression of thioacetamide (TAA) induced liver cirrhosis by normalizing ROSs. The 4-O-caffeoylquinic acid and quercetin 3-O-rhamnoside in the extract are thought to regulate TGFβ, Collα1, MMP2, and TIMP1 genes expression for preserving and maintaining normal liver function, shape, and appearance (Amin *et al*., 2012, 2013). The anti-hepatotoxic activity of *P. niruri* has been attributed to two novel lignans, phyllanthin and hypophyllanthin against CCl_4 cytotoxicity whereas triacontanal showed it protective activity against galactosamine toxicity (Syamasundar *et al*., 1985).

2.3.4.5 Antiviral activity

It had been postulated that *Phyllanthus niruri* might inhibit proliferation of the virus by inhibiting replication of the genetic material of the virus (Thyagarajan *et al.,* 1988). The antiviral activities of the plant too has been reported ((Venkateswaran *et al*., 1987; Wang *et al*., 1994) together with hypolipidaemic properties of the plant (Khanna *et al*., 2002). The quantitative determination of the anti viral effect against jaundice, hepatitis B and other viral infection has been well-defined *in vitro* systems (Meixa and Yanjin, 1995).*P.* The extract of dried fruit and leaves showed the chromosomal aberration inhibition acitivity (Holdsworth and Wamoi*,* 1992). Even a simple aqueous extract of the plant inhibited HIV-1 reverse transcriptase. The alkaloid extracts of the plant exhibit the sensitive inhibitory reponse on cytopathic effects induced by both the strains of HIV on human MT-4 cells (Ogata *et al*., 1992; Naik and Juvekar, 2003). The regular administration of *P. niruri* along with four other herbs found to improve the clinincal condition of HIV patients (Natarraj, 2000).

2.3.4.6 Antiurolithiasis

The *P.niruri* extract at 0.25 mg/ml concentration reported to inhibit of calcium oxalate crystallization. Triterpenes lupeol and butulin in plant extract are effective against the urolithiasis by interfering with growth and aggregation of calcium oxalate crystals either on rat model and human (Fretas *et al*., 2002). The alkaloids (phyllanthoside) extract exhibit an antispasmodic activity leading to smooth muscle relaxation, mostly evidenced in the urinary tract that eventually facilitate the elimination of stone (Calixto *et al*., 1998).

CHAPTER III
MATERIALS AND METHODS

3.1 Setting of laboratory

This thesis work was conducted on the laboratory of the central department of biotechnology, Tribhuwan University.

3.2 Sample materials preparation

3.2.1 Selection of plant materials

The plants samples were selected on the basis of their reported uses in ethno medicine in Nepal. The two plants are under the commercial MAPs in which *P. polyphylla* is the endangered MAPs. The other one under study is the kidney stone breaker, *P. niruri*. The plants were not at their flowering stages at the time of collection and so they were collected from Ayurveda health home, Dhapasi, Kathmandu, Nepal. The different parts of plant were used as rhizome of *P. polyphylla,* root of *R. cordifolia* and whole plant of *P. niruri*

3.2.2 Phytochemical Extraction

The selected plant materials were cleaned, crushed and grinded by an electric grinder. The fine powder filtered through wire sieve was collected on the polyethylene bag for further use. The percolation method of extraction with intermittent sonication was followed. For methanol fractionation, about 40 grams of fine powder of each plant sample was taken separately and dissolved in 400 ml of 100 % methanol and left for overnight percolation at room temperature. Similarly, 40 g each sample were dissolved in hexane at the ratio of 1:10 (w/v). Next day, the samples transferred to falcon tubes were subjected to intermittent sonication for two hours at 30 kHz. The samples were centrifuged and filtered through Whatman no.1 filter paper (Whatman Ltd, Kent, UK). The filtrates were then subjected to evaporation in a rotary vacuum evaporator under the vacuum at room temperature. The solid mass obtained was weighed carefully to express the percentage yield of the crude extract. Obtained solvent free extracts were stored at 4°c until use. The percentage yield of the extract was calculated by using following formula:

$$\text{Percentage yield (\%)} = \frac{\text{Dry wt.of Extract}}{\text{Dry wt.of plant material}} \times 100$$

3.2.3 Extract dilution

100mg of each solvent free plant extract was carefully weighed and dissolved in 1 ml methanol. Thus, prepared 100mg/ml stock of each plant extract was used for qualitative preliminary phytochemical screening and quantification of the total phenol and total flavonoid. Similary, 100mg of crude extract dissolved in DMSO i.e 100mg/ml DMSO stock of each fractionation of plants were used for the evaluation of antimicrobial and antifungal activity.

3.3 Qualitative phytochemical analysis

The crude methanol and hexane extract of the test plants were subjected to preliminary phytochemical screening to detect the major phytoconstituents following the established methods described by (Tiwari *et al.*, 2011; Parmar *et al.,* 2012).Observations were carried out for colour change, precipitation or formation of an emulsion.

3.3.1 Detection of alkaloids

Crude extract was mixed with 2ml of 1% HCl and heated gently. In Mayer's test, few drops of Mayer's reagent (Potassium Mercuric Iodide) was added form the side of the wall. Formation of white or offwhite colored precipitate was regarded as evidence for the presence of alkaloids. In Wanger's test, formation of brown/reddish precipitate upon the addition of equal amount of Wagner's reagent (Iodine in Potassium Iodide) was regarded as the presence of alkaloids.

3.3.2 Detection of flavonoids

Alkaline reagent test: 5 ml of crude extract was mixed with 2ml of 2% solution of NaOH which instantly give an intense yellow color. Addition of few drops of diluted acid turns the solution colorless indicating the presence of flavonoids.

3.3.3 Detection of phenols

Ferric chloride test: 4 drops of 2% solution of $FeCl_3$ was added to crude extract (1ml). A bluish black coloration or dark green color indicates the presence of phenols.

3.3.4 Detection of tannins

Gelatin test: 0.5ml of 1% gelatin solution with sodium chloride was treated to crude extract (1ml). Formation of white precipitation means the positive result.

3.3.5 Detection of saponins

Frothing test: Crude extract (0.5ml) was mixed with 5ml of D/W in a test tube and it was shaken vigorously. The formation of stable persistent foam was regarded as evidence for the presence of saponins.

3.3.6 Detection of carbohydrates and glycosides

Molisch's test: 0.2g dry extracts were dissolved in 10ml distilled water and filtered. 5ml of the filtrate was treated with 1ml of 1% alcoholic α-napthol solution. 1ml of concentrated Sulphuric acid was added along the sides of the tubes. Appearance of violet colored ring at the junction of two liquid shows the presence of carbohydrates

3.3.7 Detection terpenoids

Copper acetate test: 2 drops of copper acetate solution was added to crude extracts and formation of bright green colour was observed for positive result.

3.3.8 Detection of phytosterols

Salkowski test: 50mg of the extracts were treated with 2ml of Chloroform with 2ml of conc. Sulphuric acid. The mixture was shaken well and allowed standing for some time. Appearance of golden yellow colour represents positive results.

3.4 Quantitative phytochemical analysis

3.4.1 Total polyphenol content determination

The total polyphenol content of the test plants was determined using the Folin–Ciocalteu phenol reagent (Roy *et al*., 2010) with slight modification. For this 0.1 ml of each extract (2.5 mg/ml) was separately mixed with the 1 ml of Folin–Ciocalteu phenol reagent (Merck Ltd, India) (1:10 dilution with the distilled water) and 0.8 ml of aqueous 1 M Na_2CO_3 solution. The reaction mixture was allowed to stand for about 15 minutes and then absorbance was measured at 765 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). A calibration curve was obtained using gallic acid (Moly Chem, Mumbai) in methanol using the concentration ranging from 25-250µg/ml as standard. Based on this standard graph, the concentration of the individual samples was calculated. Total polyphenol content was expressed in terms of the milligrams of the Gallic acid equivalent per gram of the dry mass (mg GAE g^{-1}). Each test was triplicated for the reproducibility of results.

3.4.2 Total flavonoid content determination

The total flavonoid content in the plant extract was estimated using the Aluminium chloride ($AlCl_3$) colorimetric method (Chang et al., 2002) with slight modifications. 0.25 ml of extract (10 mg/ml) was separately mixed with the 0.75 ml of ethanol, 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium acetate (CH_3COOK) and 1.4 ml of the distilled water. The reaction mixture was allowed to stand for about 30 minutes in room temperature. The absorbance of the mixture was measured at 415 nm using the UV – visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained with the help of the quercetin (Sigma) standard solutions in ethanol 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 the dry mass (mg QE/g). For each experiment the tree replication were used for the accuracy and reproducibility of results.

3.4.3 Antioxidant activity assay

The antioxidant activity of the plants in both fractionations was determined through the DPPH free radical scavenging activity described by Singh et al. 2002. The stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to assay the antioxidant activity

The different concentration of plant extract (30-150µg/ml) and ascorbic acid (10-100µg /ml) were prepared in methanol on the clean and clear test tubes. Equal volume of 0.2mM DPPH solution was added to 0.5ml of sample volume. The tubes were shaken vigorously for the uniform mixing. These tubes were allowed to stand for half an hour in dark. Gallic acid was used as positive control and sample blank and control solutions were the solvent and DPPH solution respectively. The absorbance was taken on spectrophotometer at 517 nm (Thermo Fisher Scientific, Genesystem-10-5).

The free radical scavenging activity (RSA) of the plant samples were calculated in percentage by the formula.

$$\%\ \text{Radical Scavanging Activity (RSA)} = \frac{(\text{Abs.Control} - \text{Abs.Sample})}{\text{Abs.Control}} \times 100$$

The standard graph was plotted taking the concentration on the X-axis and percentage scavenging activity on the Y-axis. Based on this graph, IC_{50} value of each sample was calculated based on below formula developed by Dr. Louis and Dr. Paul (Louis and Paul, 2010). The IC_{50} value of the different species was compared. The species having the lowest IC_{50} is considered to have the best antioxidant property.

$$IC_{50} = EXP\left[LN(conc > 50\%) - \left(\frac{signal > 50\% - 50}{signal > 50\% - signal < 50\%}\right) \times LN\left(\frac{conc > 50\%}{conc < 50\%}\right)\right]$$

EXP: exponential function, LN: is natural log function both used in Microsoft Excel 2007 software. Signal >50%: PI value just above 50%, signal <50%: PI just below 50%. Conc >50%: concentration of signal >50% and conc <50%: concentration of signal <50%.

3.4.4 Antibacterial activity

The antibacterial activity of crude extracts of the plants was studied bymodified agar well diffusion method as per the recommendation made by the Lindequist *et al*., 2005 with slight modification. This is the qualitative method of testing anti-bacterial efficacy measuring the observed halozone.

3.4.4.1 Bacterial Strains

ATCC cultures of gram negative bacteria *Escherichia coli, Salmonella typhi, Enterococcus fecalis, Pseudomonas aeruginosa* and *Klebseilla pneumonia* and a single standard gram positive bacteria *Staphylococcus aureus* were used for anti-bacterial assay.

3.3.4.2 Preparation of necessary culture media

3.3.4.2.1 Nutrient Agar Plate and Nutrient Slant

Nutrient agar plate and slant are necessary for antibacterial tests. About 28 gram of the powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and poured in distilled water. The contents were dissolved in water completely and the final volume was maintained to 1000 ml followed by boiling for uniform mixing. This media was sterilized in an autoclave at 15lbs pressure and 121°C for 15 minutes. After cooling the autoclaved media to about 45-50°C and about 20ml was poured on sterilized and properly labeled petridish of of 9 cm diameter. The plates were then left for the solidification. All processes after autoclaving were carried out on the sterile cabinet. For the preparation of the slant media screw tight bottles were filled with the media followed by autoclaving in the condition as mentioned above. The bottles were then placed in an inclined position and left for solidification of the medium.

3.3.4.2.2 Luria Bertani Miller (LB) broth and Mueller Hinton Agar (MHA) plate

The Luria Bertani broth, (LB) Miller medium is necessary for culture and subculture of microorganisms prior to antimicrobial tests. About 25 gram of LB powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and transferred to a conical flask. The content was dissolved in distilled water and final volume was maintained to 1000 ml. This media was transferred to the screw bottles and sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes. Later, the media on cooling dispensed in sterile and dry culture tubes under laminar airflow.

38 grams of MHA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was weighed and suspended in distilled water. The final volume was maintained 1000 ml. The content was heated to boiling to dissolve the medium completely. The media was sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes. The media was mixed carefully

before pouring. The media was poured on sterile petridishes under aseptic conditions for further proposes.

3.3.4.3 Preparation of standard culture Inoculums

The individual pure ATCC culture of bacteria *Escherichia coli*, *Salmonella typhii*, *Staphylococcus aureus, Enterococcus faecalis and Klebsiella pneumoniae* were streaked on the different nutrient agar plates. Those plates were incubated on the incubator at 37˚C for about 24 hours and pure and isolated colonies were obtained. Each distant colony was aseptically transferred to the Luria Bertani (LB broth) for the suspension culture with the help of the sterilized inoculating loop. The inoculated bottles were kept on the shaking incubator at 37°C and 120 rpm for overnight. The turbidity of the bacterial suspension was adjusted at the 0.5 McFarland standards for the antibacterial test. These inoculums were used for the swapping of the MHA plates to test the antimicrobial effects of the plant extracts.

3.3.4.4 Transfer of bacteria on the petriplates

The test plates for the antimicrobial activity were first labeled with date, name of bacteria, and name of the plant sample and the concentration of the plant extract to be added. The MHA plates were inoculated with the appropriate bacterial culture by a sterile cotton swab. One swab was used for one bacterium. The culture plates were allowed to dry for about 30 minutes under aseptic condition.

3.3.4.5 Antibacterial Screening

Five wells were prepared on the previously prepared solid MHA media with the help of the sterile cork borer of 0.4 mm diameter. Three different concentrations (100 mg/ml, 200 mg /ml and 300 mg /ml) of the plant sample were prepared on DMSO. With the help of the sterile pipette the 30 µl of the each individual plant extract were poured in the above prepared well. The DMSO was taken as negative control while the Streptomycin at the concentration of the 50, 25, 12.5 and 6.25 mg/mL 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 for individual plant extract for individual bacteria at different concentrations for further analysis.

3.4.5Antifungal Activity

3.4.5.1 Fungal Strains: *Candida albicans*, *Sacharomyces cerivaseae* and *Pichia pastoris*

3.4.5.2 Preparation of Potato Dextrose Agar (PDA) and Potato Dextrose Broth

About 25 gram of PDA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed, dissolved in distilled water and final volume was maintained to 1000 ml. This

media was autoclaved at 15 lbs pressure and 121°C for 15 minutes, was cooled in laminar airflow and dispensed in sterile petriplates. Likewise, Potato dextrose broth was prepared in culture tubes excluding agar.

3.4.5.3 Preparation of the standard fungal cultures

The individual pure and characterized cultured of *Sachharomyces cerevesiae*, *Pichia pastoris* and *Candida albicans* was obtained from CDBT, TU and were subcultured in PD broth with the help of the sterilized inoculating loop and kept on the shaking incubator at 37°C and 120 rpm for overnight. The turbidity of the subcultured fungal suspension was adjusted to 0.5 McFarland standards. These bacterial inoculums were used for the swabbing on the MHA plates to test the antimicrobial effects of the plant extracts.

3. 4.5.4 Antifungal Screening

The antifungal test performed is described by by modified agar well diffusion method.Properly labeled PDA pertiplates were taken and test cultures were inoculated as per Lindequist et al. (2006). The petriplates were allowed to dry for about 15 to 20 minutes. On the prepared PDA plates, six wells were prepared with the help of the sterile cork borer (3 mm diameter). Five different concentrations (100 mg/ml, 50 mg /ml, 25 mg /ml, 12.5 mg /ml and 6.25 mg /ml) of the plant extracts were prepared in DMSO. With the help of the sterile pipette, 30 µl of the each individual plant extract was poured in the above prepared wells. The DMSO was taken as negative control. Streptomycin at the concentration of the 50, 25, 12.5 and 6.25 mg/mL 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 for individual plant extract for individual fungi at different concentrations.

3.3.7 Statistical Analysis

All the experiments were performed in triplicates for each sample and the values were reported as mean ± SD. The obtained data were also subjected to the analysis of variance and mean values were compared. Differences at $P<0.05$ were considered to be significant. All the statistical analysis was done using Excel software (Microsoft Office 10).

CHAPTER IV
RESULTS

4.1 Phytochemical extraction

The three plants samples were subjected to hexane and methanol extraction. Different parts of plants have been used, their texture, and consistency of the resulting extract showed some variations given in table 4.1. All the extracts were turned into dark color after in vacuo concentration in the evaporator. The hexane extract of the plants was found to be more greasy and sticky in nature. The percentage yield was varied from 0.72% to 8.7% *P. niruri* had highest yield in both extractswhereas *P. polyphylla* had the lowest yield in both extract. The methanol extracts of the plants have higher yield than the hexane extraction. The percentage yield of the plants has been depicted in figure 4.1.

Table 4.1: Physical characterization of methanol and hexane extracts with parts used

Plant	Parts used	Solvent	Characteristics Of Extracts	
			Color	Consistency
P. polyphylla	Rhizome	Methanol	Yellowish	Semisolid
		Hexane	Light red	Semisolid, greasy
R. cordifolia	Root	Methanol	Reddish brown	Semi solid
		Hexane	Dark reddish	Semi solid; sticky
P. niruri	Whole plant	Methanol	Greenish black	Semisolid
		Hexane	Dark greenish black	Semisolid; sticky

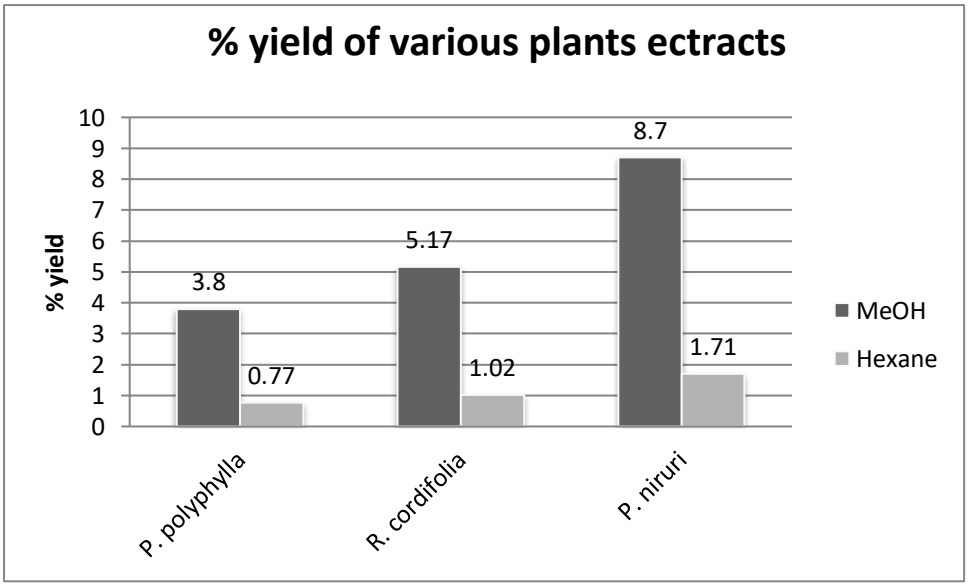


Figure 4.1: Yield of various extracts of the plants (%)

4.2 Preliminary phytochemical screening

Themethanol and hexane extract of the plants were subjected to the preliminary phytochemical tests. The test result revealed thepresence of different kind of chemical groups that are summarized in table 4.2 and 4.3.

Table 4.2: Preliminary phytochemical screening of the plants in methanol extract

Plant Extracts	Alkaloids Mayer's Test	Saponins Forthing Test	Phenols Ferric Chloride Test	Flavonoids Alkaline Reagent Test	Glycosides Molisch's Test	Terpenes Copper Acetate Test	Tannins Gelatin Test	Phytosterol Salkowski's Test
P. polyphylla	-	+	+	+	+	-	+	+
R. cordifolia	-	+	+	-	+	-	+	-
P. niruri	+	+	+	+	+	+	+	+

+ Positive; -Negative or Not Detectable

Table 4.3: Preliminary phytochemical screening of the plants in hexane extract

Plant Extracts	Alkaloids Mayer's Test	Saponins Forthing Test	Phenols Ferric Chloride Test	Flavonoids Alkaline Reagent Test	Glycosides Modified Brontrager's Test	Diterpenes Copper Acetate Test	Tannins Gelatin Test	Phytosterol Salkowski's Test
P. polyphylla	-	+	+	+	-	-	+	-
R. cordifolia	-	+	-	-	-	-	+	-
P. niruri	+	-	-	-	-	+	+	+

+ Positive; -Negative or Not Detectable

4.3 Determination of total phenolic content

The total phenol content of the crude aqueous and methanol extracts were determined in terms of Gallic acid equivalent (mg of GAE/gm dry weight of extract) by using the calibration curve of gallic acid (25-250µg/ml, y = 0.0071).

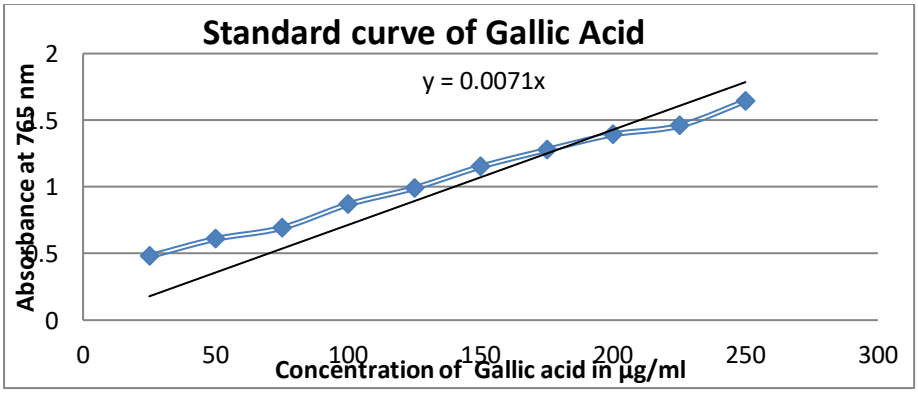


Figure 4.2: Standard graph of Gallic acid

The highest total polyphenol was found in methanol extract of *P. niruri* i.e. 88.34 ± 22.78 mg GAE/g dry wt. *P. polyphylla* hexane extract had the lowest phenol contenti.e. 10.87 ± 10.04

mg GAE/g dry wt. *R. cordifolia* showed the intermediate phenol content in either extracts. The polyphenol content of the methanol and hexane extracts of different plants is as below.

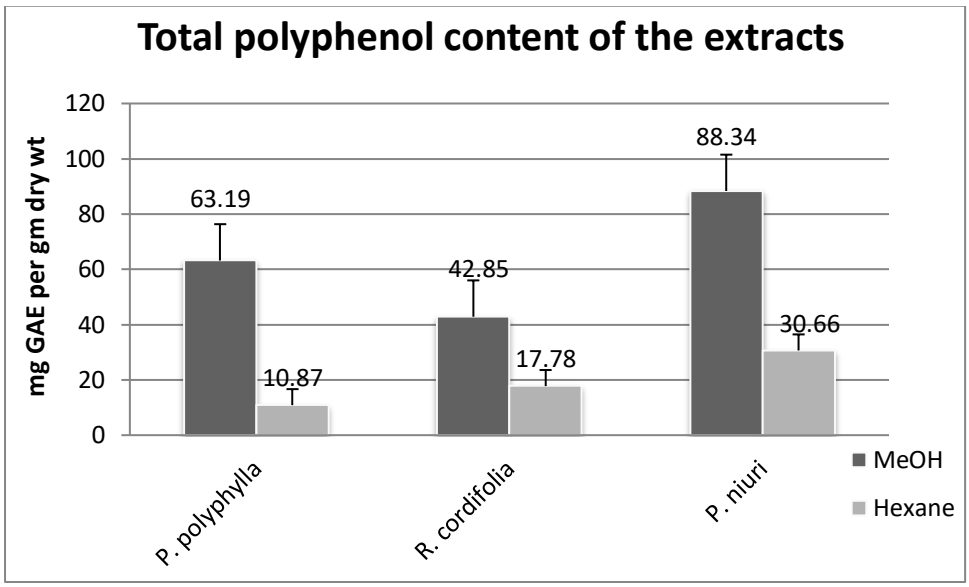


Figure 4.3: Total polyphenol content of various extracts of the plants

4.4 Determination of total flavonoid content

The total flavonoid content of the crude methanol and hexane extracts were determined in terms Quercetin equivalent (mg Quercetin /gm dry mass) by using the calibration curve of Quercetin (0-100µg/ml) with an equation of y = 0.0081X.

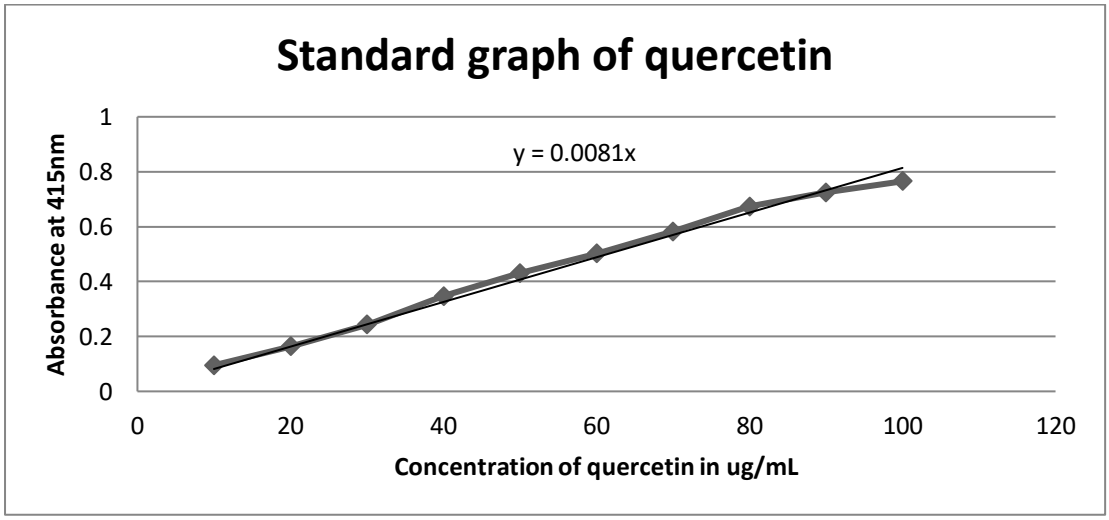


Figure 4.4: Standard graph of Quercetin

Based on this equation, total amount of the flavonoid present in the plant samples was determined. The results were expressed in mg QE /g and shown in figure 4.3. TFC ragnes from 4.86 ± 3.25 to 22.3 ± 1.73mgQE/gm dry mass. *P. niruri* was found to contain highest

amount of total flavonoid both in methanol and heaxane extraxt while *R. cordifolia* showed the moderate flavonoid content in both extract whereas *P. polyphylla* hexane extract had the lowest flavonoid content found to be 4.86 ± 3.25mgQE/gm dry mass.

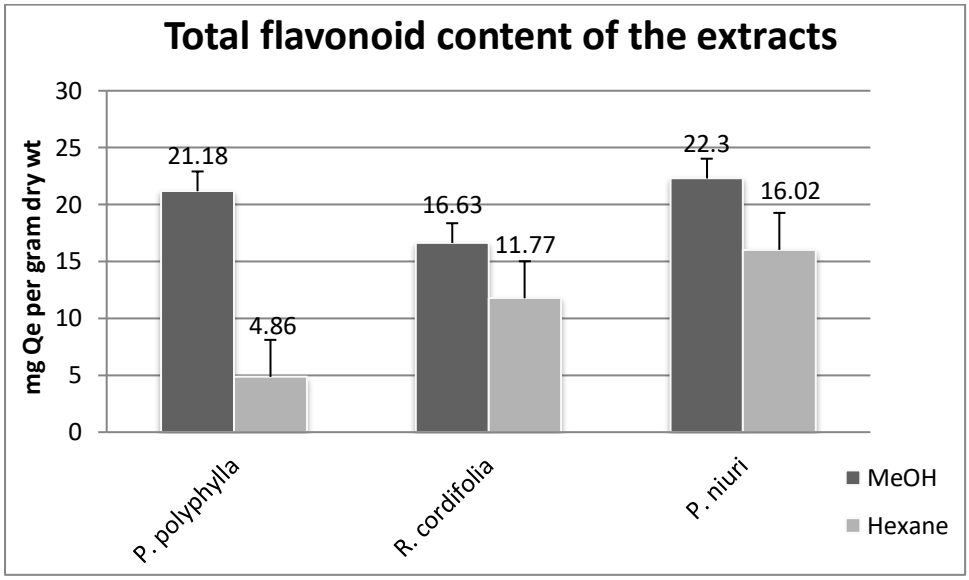


Figure 4.5: Total flavonoid content of the methanol and hexane extracts of the plants

4.5 Antioxidant activity – DPPH assay

The antioxidant activity of the plants was determined using the solution of DPPH (0.2mM) free Radical Scavenging Assay, taking Ascorbic acid as the pure antioxidant reference compound. IC_{50} value was calculated for each sample taking the concentration vs. % radical scavenging activity as described by Louis and Paul (2010). The RSA value of each sample was estimated by comparing with the calculated RSA value of the standard Ascorbic acid.

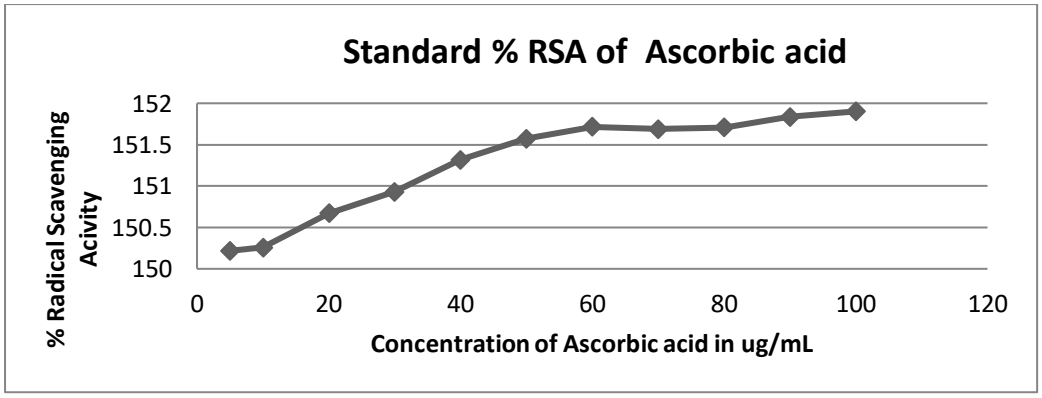


Figure 4.6: Percentage Radical Scavenging Activity of Ascorbic acid

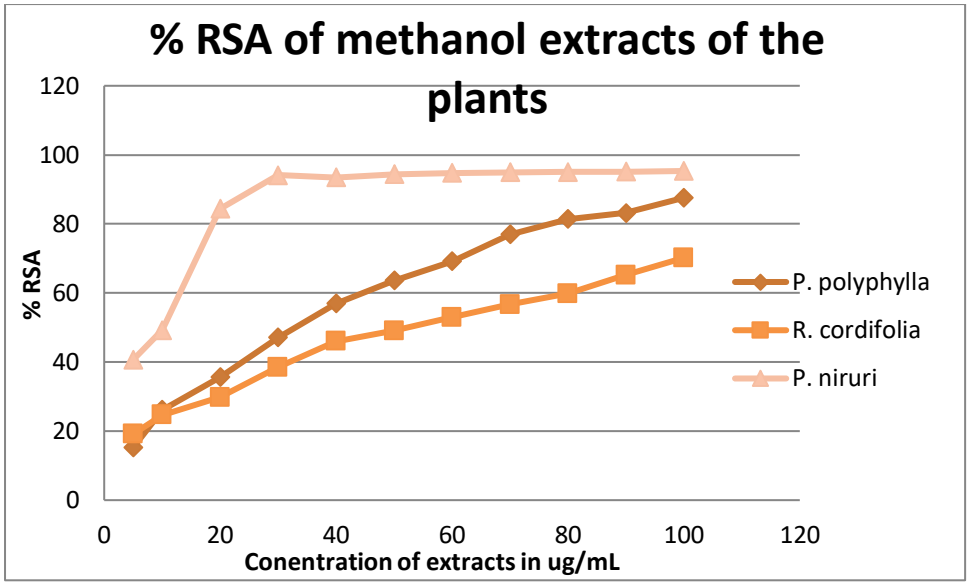
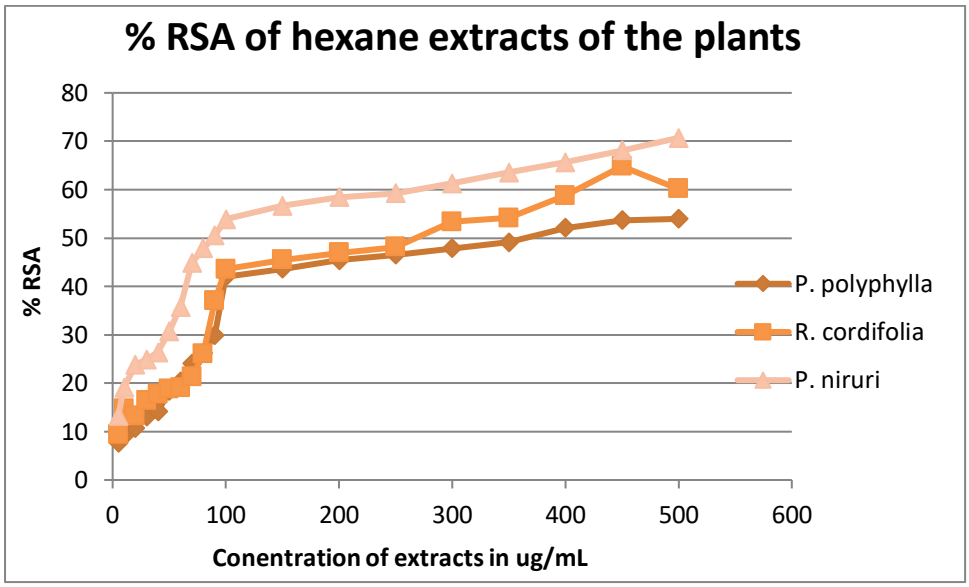


Figure 4.7 a.: % of Radical scavenging activity of extracts of the plants



b

Figure 4.7 b: % of Radical scavenging activity of hexane extracts of the plants

The highest free radical scavenging activity was shown by *P. niruri* in the methanol extract. The IC_{50} value for Ascorbic acid was found to be 26.73 ±2.13 µg/ml. Being based on this, the IC_{50} value was found to belowest in the methanol extract of *P. niruri* (15.37±21.68µg/ml) while highest in hexane extract of *P. polyphylla* (361.18 ± 132.79 µg/ml). The hexane extract exhibited very lower antioxidant activity. The observed result suggest that *P. niruri* have best antioxidant activity among the studied plants. *R. cordifolia* found to posses the lowest antioxidant property.

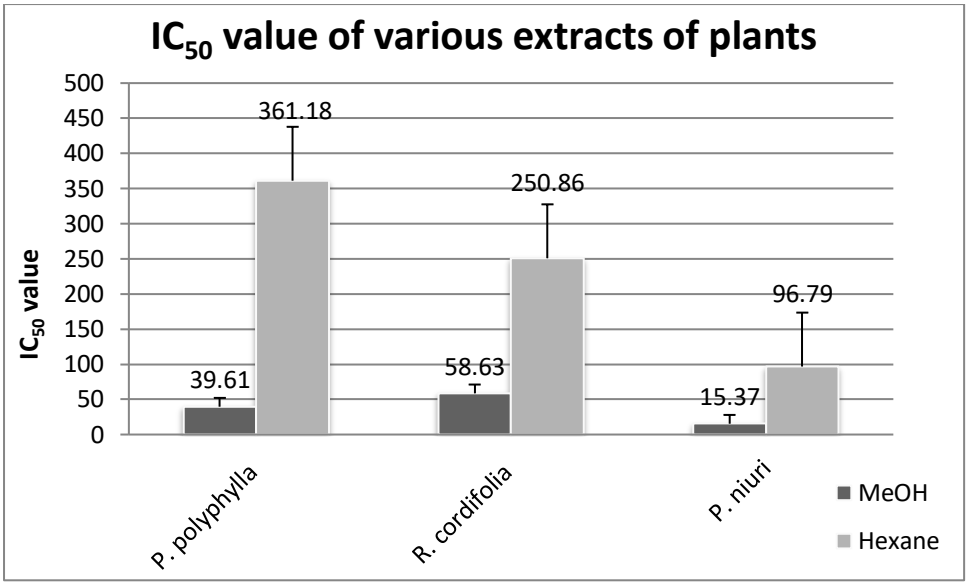


Figure 4.8: IC_{50} values of various extracts of different plants

4.6 Antibacterial activity of the plants

The antibacterial activity of both methanolic and hexane extracts of the plants under study were tested against ATCC cultures of *Staphylococcus aureus, Escherichia coli, Salmonella typhii, Klebsiella pneumonia* and *Enterococcus feacalis.* Antibiotic drug Streptomyin was taken as a positive control and DMSO (the solvent of the plant extract) was taken as a negative control. Different concentrations of plant extract (300mg/ml, 250mg/ml, 200mg/ml, 100 mg/ml, 50mg/ml, 25mg/ml, 12.5 mg/ml and 6.25 mg/ml) were tested against six bacterial strains and compare with the standard Streptomyin. The zone of inhibition was expressed in mm as given on the following tables.

Table 4.4: Zone of Inhibition (in mm) of different bacterial strains in Streptomycin

Bacterial Cultures	Zone of Inhibition (in mm) in Streptomycin		
	50 mg/ml	25mg/ml	10 mg/ml
Escherichia coli	25	24	21
Staphyloccous aureus	29	21	18
Salmonella typhi	30	26	24
Klebsiella pneumonia	41	38	37
Enterococcus feacalis	28	25	23

Table 4.5: Zone of inhibition of methanol extracts of various plants

Plants	Bacterial Cultures	Zone of Inhibition (in mm)								
		300 mg/ml	250 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	-ve control
P. polyphylla	*E. coli*	12	10	9	7	6	-	-	-	-
	S. typhii	8	7	6	5	3	-	-	-	-
	K. pneumoniae	8	7	7	5	4	-	-	-	-
	S. aureus	8	7	6	5	3	-	-	-	-
	E. faecalis	8	7	7	6	4	2	-	-	-
R. cordifolia	*E. coli*	16	17	16	12	9	-	-	-	-
	S. typhii	12	10	8	5	3	-	-	-	-
	K. pneumoniae	11	10	8	7	5	4	-	-	-
	S. aureus	13	12	9	6	5	-	-	-	-
	E. faecalis	12	10	8	6	5	-	-	-	-
P. niruri	*E. coli*	10	9	7	-	-	-	-	-	-
	S. typhii	-	-	-	-	-	-	-	-	-
	K. pneumoniae	9	8	8	4	4	-	-	-	-
	S. aureus	12	10	9	7	4	-	-	-	-
	E. faecalis	8	7	7	6	5	-	-	-	-

Table 4.6: Zone of inhibition of hexane extracts of various plants

Plants	Bacterial Cultures	Zone of Inhibition (in mm)					
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 g/ml	-ve control
P. polyphylla	*E. coli*	7	6	2	-	-	-
	S. typhii	-	-	-	-	-	-
	K. pneumoniae	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-
	E. faecalis	-	-	-	-	-	-
R. cordifolia	*E. coli*	-	-	-	-	-	-
	S. typhii	-	-	-	-	-	-
	K. pneumoniae	4	4	-	-	-	-
	S. aureus	6	-	-	-	-	-
	E. faecalis	3	-	-	-	-	-
P. niruri	*E. coli*	-	-	-	-	-	-
	S. typhii	-	-	-	-	-	-
	K. pneumoniae	-	-	-	-	-	-
	S. aureus	-	-	-	-	-	-
	E. faecalis	-	-	-	-	-	-

All methanolic extracts of all the plants found to have antibacterial activity mostly against *E.coli* and *K. pneumoniae. K. pneumoniae* and *E. faecalis* were observed to be sensitive against 25mg/ml concentration of methanol extract of *R. cordifolia* and *P. polyphylla*

respectively. The methanol extract of *R. cordifolia* seemed to exhibit broad range of antibacterial activity against all bacterial strains than *P. polyphylla* whereas *P. niruri* was determined to be less effective. The hexane extract of *R. cordifolia* exhibited zone of inhibition at concentration of 50mg/ml. Besides, most of the bacterial strains proved to be insensitive to hexane extracts of the plants.

4.7 Antifungal activity of the plants

Both the methanolic and aqueous extracts were tested for their antifungal activity. Three characterized fungal strains were tested against different concentrations of plant extracts ranging from 6.25 mg/ml to either 100 mg/ml or 300mg/ml depending on different fraction. DMSO was taken as negative control. The antifungal activity of the plants was determined being based on zone of inhibition. Among all of the plant extracts methanolic of *R. cordifolia* had shown potent antifungal property against all of the tested species. The antifungal activity of methanol and hexane extract is tabulated in table 4.6 and 4.7 as below.

Table 4.7: Zone of inhibition of plants methanol extract against various fungi

Plants	Bacterial Cultures	Zone of Inhibition (in mm)								
		300 mg/ml	250 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	-ve control
P. polyphylla	*P. pastoris*	16	13	11	11	8	6	-	-	-
	C. albicans	17	16	14	12	9	6	4	-	-
	S. cerevisiae	7	6	6	6	4		-	-	-
R. cordifolia	*P. pastoris*	23	21	19	17	13	10	6	-	-
	C. albicans	24	20	20	16	12	9	6	-	-
	S. cerevisiae	12	8	7	6	5	-	-	-	-
P. niruri	*P. pastoris*	28	25	24	21	17	15	13	-	-
	C. albicans	21	19	18	16	14	11	9	-	-
	S. cerevisiae	11	9	9	7	4	-	-	-	-

Table 4.8: Zone of inhibition of hexane extracts against various fungi

Plants	Bacterial Cultures	Zone of inhibition (mm)					
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	-ve control
	Pichia pastoris	-	-	-	-	-	-

P. polyphylla	*Candida albicans*	4	3	2	-	-	-
	S. cerevisiae	-	-	-	-	-	-
R. cordifolia	*Pichia pastoris*	-	-	-	-	-	-
	Candida albicans	6	5	4	-	-	-
	S. cerevisiae	4	-	-	-	-	-
P. niruri	*Pichia pastoris*	6	5	5	3	-	-
	Candida albicans	5	4	4	3	-	-
	S. cerevisiae	-	-	-	-	-	-

The hexane extract of *P. niruri* was comparatively concluded to have high antifungal activity than other hexane extracts. The methanol extract had shown the potent antifungal activity than the hexane extract. *C. albican* was mostly inhibited by all plants whereas *S. cerevisiae* was less inhibited. No significant difference in zone of inhibition was seen with respect to the concentration of the plant extract used.

CHAPTER V
DICUSSION

5.1 Yield of extracts

The very important factor defining the yield of plant is the extraction process, the polarity of solvent system and the parts of plant used. The extraction technique and solvent used always depends the chemical composition and content of the plant phytochemicals that are present within the plant species. The extraction of the bioactive plant constituents has always been a challenging task for the researchers (Ncube *et al*., 2008).

The percentage yield for medicinal plants in different solvent extractions found in the range of 3.2 to 10 (Tiwari *et al*., 2011) and the results obtained in this study is consistent with aforementioned report. The % yield of *R. cordifolia* in methanol extract is consistent with the % yield reported by Patil *et al.* (2011) in which it was reported to be 5.2%. The lowest % yield in hexane extract in *R. cordifolia* as reported in Kaur*et al.* (2008). The highest % yield was found in *P. niruri* which is consistent to the findings of Bhuju (2013). There was higher percentage yield in methanol extract than hexane extract in all plants as reported by Klejdus *et al.* (2005) and Lay *et al.* (2014). The superiority of methanol over hexane as an extraction medium is possibly due to its high polarity (Rahman *et al*., 2011).

5.2 Qualitative Phytochemical Analysis

The secondary metabolites existing in the plant extract play a key role in the pharmacological actions of any plant or plant parts. It has been documented that different solvents have diverse solubility capacities for different phytochemical constituents (Marjorie, 1996). Saponins, phenols, glycosides and tannins were present in methanolic extracts of all plants. The preliminary test data of *P. polyphylla* is similar to the data of Pfoze *et al*. (2013) where they reported the positive results for flavonoids and saponins; alkaloids were reported to be negative. Similarly, preliminary test results of both methanolic and hexane extracts of *R. cordifolia* showed positive test for saponins and glycosides; negative test for alkaloids confirming earlier reports (Siddiqui *et al*., 2008; Kaur *et al*., 2008) The presence of alkaloids, saponins, flavonoids, glycosides, phytosterols and tannins in the methanolic extracts of *P. niruri* in present investigation are consistent with that of Udoh *et al.* (2010) and Bhuju (2013).

5.3 Total Polyphenol Content

Phenols are the largest group of secondary metabolites found in plant kingdom. Phenolic compounds are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. Phenol is a potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenols is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al*., 1995). Phenol contain hydroxyl substituent next to their aromatic structures and have ability to chelate metals, inhibit lipoxygenase and prevent the adverse effects of reactive oxygen and nitrogen species (ROS/RNS), on normal physiological functions in humans (Gutteridge and Halliwell, 2000; Huang *et al*., 2005).

The total phenol content in methanolic extracts of *P. polyphylla* was found to be higher than that reported earlier by Paonom and Sharma (2014). Simlarly, TPC in methanolic extracts of *R. cordifolia* in present investigation was a bit higher than that reported by Jain *et al*., (2011). TPC in methanolic extracts of *P. niruri* in present investigation are consistent with that of Poh-Hwa *et al.* (2011) and Nimmi *et al.* (2012).

Methanol is more efficient in extracting wider range of phenolic compounds (from polar to semipolar) found in *Phyllanthus* (Nimmi *et al*., 2012; Harish and Shavanandappa, 2006; Ravipati *et al*., 2013). Methanol is able to extract more phenolic compounds than water and hexane (Paonom and Sharma, 2014). The variation in phenol content in the present study and previous repots may be due to various reasons. The recovery of polyphenols from plant materials is influenced by their solubility in the extraction solvent, the type of solvent, the degree of polymerization of phenols, the interaction of phenols with other plant constituents and the formation of insoluble complexes (Galvez *et al*., 2005). The phenol content of a plant depends on a number of intrinsic (genetic, extracting solvent) and extrinsic (environmental, handling and development stage) factors (Fratianni *et al*., 2007). Some or all of these factors might have contributed to the discrepancies in the value of TPC even in the extracts from the same species.

5.3 Total Flavonoid Content

Flavonoids are hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substances against wide array of microorganisms 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). Flavonoids can easily scavenge aqueous free radicals because of their amphipatic characteristics (Riou *et al*., 2002; Paonam *et al*., 2013).

The TFC in methanolic extracts of *R. cordifolia* was reported to be less than 10mgQE/gm dry mass by Mital *et al.* (2012) which is a bit lower than the present study. Nimmi *et al.* (2012)

had reported TFC of *P.niruri* to be 1.396±1.26 mgQE/gm dry which is lower than the calculated result in this study.

It was observed that methanol extracts contain more phenolic and flavonoid compounds than hexane extract, because methanol can release the cell wall bound polyphenols from the cells and also it can neutralize the activity of polyphenol oxidase which degrades the polyphenols in plants (Lapomik *et al*., 2005). The total phenol, flavonoid, tannins and other most of biomolecules are lowest in hexane extract. The total phenolic, flavonoid content and total antioxidant activity of methanol extract was found be in between ethanol and water extract (Paramaguru *et al*., 2012; Medini *et al*., 2014).

5.4 Antioxidant Activity - DPPH Assay

Many methods have been used to determine the antioxidant activity of natural products (Antolovich *et al*., 2002). Free radicals (super oxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypochloric acid and proxynitrite) produced during aerobic metabolism in the body, can cause oxidative damage of amino acids, lipids, proteins and DNA and has been linked to majority of the systemic diseases including cancer, cardiovascular diseases, and type 2 diabetes (Phoboo *et al*., 2013). DPPH is stable and commercially available organic nitrogen free radical. When DPPH is added to the plant extracts containing antioxidant compounds, diphenylpicrylhydrazl is reduced to diphenylpicrylhydrazine and the color changes from purple to yellow proportionally decreasing the absorbance at 517nm. The decrease in absorbance is a measurement of the radical scavenging. The absorbance reduction depends linearly on the antioxidant concentration (Thaipong *et al.*, 2006). Antioxidant property can be inferred on the basis of % radical scavenging activity (RSA) and IC_{50} value. Antioxidant activity DPPH inhibition of the plant extract is expressed as % inhibition of stable radical or inhibition concentration fifty (IC_{50}) in reference to a standard compound. The plant with higher %RSA and corresponding lowest IC_{50} value is considered having better antioxidant properties. The antioxidant activity was found to be increased gradually for all the extracts with the increase of concentrations. A significant difference on IC_{50} value of the studied plant species has been found.

Methanolic extracts of *P. niruri* exhibited the highest antioxidant activity while hexane extracts of *P. polyphylla* showed negligible antioxidant activity. Methanolic extract of *P. niruri* revealed very high potency as antioxidant supporting the fact that the free radical quenching properties with the IC_{50} values at 10–30 mg/ml exhibit high potential antioxidant property (Harish and Shivanandappa, 2004, 2006). The IC_{50} value of methanolic extracts of *P. polyphylla* was reported to be 14.09± 0.08 mg/ml by Paonom and Sharma (2014). Ravipati *et al.* (2013) has reported that IC_{50} value 45.29 ± 1.52 and 19.9 ± 2.12 µg/ml for ethanol and

water extracts respectively. The present result of *P. polyphylla* is consistent as the antioxidant activity of MeOH extract is in between ethanol and water extract.

The present study too observed a positive correlation between the antioxidant capacity and amount of polyphenol and flavonoid present in the plant as previous studies (Castelluccio *et al*., 1995; Kalt *et al*., 1999; Haman *et al*., 2008; Mital *et al.*, 2012). Generally, the total phenolic content increased proportional with the antioxidant activity as found in this study. The total phenolic content is not a specific test for polyphenol compounds and the antioxidant activity may due to more than 1 phenolic group to react. Moreover, other groups of secondary metabolites such as flavanoid may contribute to antioxidant activity as well beside phenolic compounds (Teissedre and Landrault, 2000; Poh-Hwa *et al*., 2011). Polyhydroxyl substituted anthraqunions as rubiadine in *R. cordifolia* are free radical scavengers (Kaur *et al*., 2008; Lodia and Kansala, 2012; Upadhyaya *et al.*, 2013). The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes in *Phyllanthus* sps (Shahidi *et al*., 1992; Tan, 2009).

5.5 Antimicrobial activity

Secondary metabolites and all other active principles of plants have been shown to be responsible for the antimicrobial activities (Nweze *et al*., 2004). Flavonoides and glycosides are a special class of phytochemical which have antimicrobial characteristics. Plants with high flavonoid content have been established to posses antimicrobial activity (Cushnie and Lamb, 2005; Zhang *et al*., 2011, 2013). Saponins of *P. polyphyllla* are also reported to show antimicrobial effects (Justyna *et al*., 2011). The triterpenes of *Rubia* species found to be strong antioxidant (Xu *et al.*, 2012) as well as have antimicrobial and antifungal effect (Fan *et al.*, 2011). The antibacterial and antifungal activity was found to be very low in hexane extract of all the plants. Hexane and aqueous extracts had no antimicrobial activity, methanol and acetone extracts had moderate activity.while ethanol extracts were reported to have high antimicrobial activity against both Gram-positive and Gram-negative bacteria (Medini *et al*., 2014).

5.5.1 Antibacterial activity

The antibacterial activity of flavonoids against both Gram-positive and Gram-negative bacteria including antibiotic resistance bacteria have been reported (Bylka *et al*., 2004). The antimicrobial activity of flavonoids and polyophenolic compounds might be due to their ability to form complex with bacterial cell wall and therefore inhibiting the microbial growth (Sivapriya *et al*., 2011). It was found a regular increase in the zone of inhibition size with the increase in the concentration of extracts for all bacterial strains tested (Ahmed *et al*., 1998; Kanthimathi and Soranam, 2013). Generally, the plant extracts are more effective to gram positive bacteria than the gram negative bacteria (Cutcheon *et al*., 1992). Among gram

positive bacteria *S. aureus* is mainly susceptible (Rahman *et al*. 2011). Similarly, *P. polyphylla* showed its specificity towards gram positive bacteria mostly S. aureus (Panthi and chaudhary, 2006; Chettri *et al*., 2012; Zhang *et al*., 2013). In general, *R. cordifolia* ethanol extract was inhibitory to all *E. coli* strains under study with zone of inhibition more than 6mm (Sawhney *et al*., 2012). The degree of inhibitory action against different organisms is different. Prajapati and Parmar (2011) have reported the zone of inhibition to be 16 mm and 20mm for *K. pneumonia* and *S. aureus* at 150 ug/ml of methanol extract which is higher than result of present study. It has been documented that methanol extract of *R. cordifolia* is inhibitory for *B. cereus, B. subtilis, S. aureus, S. intermedius* etc. (Basu *et al*., 2005). The inhibitory activity of the manjistha is attributed to the known anthraquinones present in the plant (Mishchenko *et al*., 2007; Li *et al*., 2009).

It was noticed that *P. niruri* had highest effect on *S. aureus.* The methanol extract had inhibitory action against all four strains under study except *S. typhi.* The hexane extract did not have any inhibition against the studied organisms. The result of the study is accordance with the study of Sumathi and Parvathi, (2010). The methanol extract was reported to have activity against both gram positive and gram negative bacterial strains - *E. cloacae, S. aureus, P. aeruginosa, E. coli* and *S. viridians*. Maximum Inhibition zone (16mm) was observed in seeds against *Staphylococcus aureus* and minimum in roots (5mm) against *E. coli* (Mathur *et al*., 2012) in the range of 5-16 mm and the present study result is in consistent with it. Similarly, inhibitory action against *S. aureus* was reported but not in *E.coli, Klebsila, Salmonella* by Poh-Hwa *et al*., 2011). In contrast, Sumathi and Parvathi, (2010) did not find any inhibitory effects in *E. coli, K. pneumoniae* and *S. paratyphi* even at 400µg/ml. The methanol extract of *P. niruri* is reported to have strong effect against *Bacillus pumillus, Bacillus ceraus, E. coli* and *Vibrio cholera* at concentration of 750µg/ml/disc (Ajibade *et al*., 2011). The antibacterial acitivty of *P. niruri* is an indicative of presence of metabolic toxins or broad spectrum antimicrobial compounds that act against both gram positive and gram negative bacteria.

5.5.2 Antifungal activity

The antifungal activity of *P. polyphylla* been well studied by Shi *et al.* (2011) which states that Polyphyllin-D and Dioscin of *P. polyphylla* have a strong anti-fungal with MIC of about 2ug/ml and 4ug/ml for *C. albicans*. The present study showed inhibitory action moderately at higher concentration in methanol extract whereas it can be said negligible in hexane extract in comparision to aforementioned report. β-ecdysterone and three pennogenin steroidal saponins isolated from ethanol extract of *P. polyphylla var. yunnanensis* are

reported to have higher antifungal activities against *Saccharomyces cerevisiae* than *Candida albicans* (Zhu *et al*., 2011). The inhibitory action of *R. cordifolia* against *C. albicans* was observed with higher zone of inhibition in methanol extract (17mm) than hexane extract (16mm) at 150 ug/ml. It is suggested that the anthraquinones may be playing a major role in eliciting the antifungal property of the extract (Prajapati and Parmar, 2011). *P.niruri* had exhibited antifungal activity against fungal strains - *A. niger and T. viridae* (Mathur *et al*, 2012). Methanol extract was found to be much effective on fungi *Pythium debaryanum* than butanol extract (Ambikapathy *et al*., 2011).

CHAPTER VI
CONCLUSION

Nepal has always been like the open museum of biodiversity residing in diverse physiographic and climatic variation along the elevation gradient. A huge majority of Nepalese people are still dependent on indigenous use of medicinal plant for their primary health care. It is estimated that over 50% of the plants found in Nepal have some kind of medicinal uses. Only, 10 % of species are reported and listed with medicinal and aromatic properties (Bhattarai and Ghimire, 2006) still there is a world of medicinal flora waiting for the proper utilization, conservation and exploration. In Nepalese industries based on herbal medicines are increasing together with the global competitive market. The number of foreign companies supplying herbal medicines in Nepal was very much progressive from 2007/08 to 2008/09. Presently, the Ayurvedic drugs being supplied in local market are much from 39 different domestic companies than 31 foreign companies (Raut and Khanal, 2011). The prolonged use of commercial drugs has shown many serious complications like allergic response to fatal diseases even cancer. The ever growing global demand for medicinal plants has led many local people to extract medicinal plants as a primary way of livelihood, unfortunately, leading the MAPs to unscientific commercial overexploitation leading to vulnerability. In addition, the changing lifestyles, perceptions, social transformations, and acculturation have eroded the MAPs and its uses. The medicinal plants are being overexploited with no scientific harvest and one-fifth of 50,000 species has been listed as endangered plants. The hypothesis “Conservation by commercialization” should be strictly implemented for sustainable use along with conservation of diversity and economic development (Olsen, 2005; Pyakurel and Baniya, 2011).

In the present study, the qualitative preliminary phytochemical analysis of the plants suggests that methanol extraction is much effective than hexane extraction. The plants under study can be potential natural antioxidants and can be used in food industry. The selected plants can be used as broad spectrum antimicrobial agent against gram positive as well as gram negative bacteria and fungi. The study provides evidence for these plants therapeutic uses in folk and traditional medicine. Hence the systematic and scientific documentation of high valued floras and the identification and purification of pure compounds from these samples hold a promising sector in modern pharmaceutical industry which not only provide the feasible, safety and cheaper medication but can uplift the nation economy as a whole.

RECOMMENDATION

The plants used in folk medicine vary accordingly to places. Medicinal plants in Himalaya region varies in Terai region. The numerous floras are yet not listed and documented nowhere. The documentation of high value plants having each indigenous uses should be carried out. The very few floras have been listed in MAPs of Nepal. Most of medicinal plants are exploited from its wild habitat. The systematic cultivation and conservation of such plants should be carried out. The phytochemical analysis technique, identification of pure biomolecules, standardization and drug formulation process should be made feasible. The anthraquinone from *R. coridifolia* should be researched for development of natural antioxidant, anti-pimple formulation. The bioactive molecules of *P. niruri* can be further researched for them having potential antiviral activities. The steroids of *P. polyphylla* can be pharmacological studied for the development of novel drug treating life threatening disease especially cancer. Besides, food industry is the most potential for optimum use of these plants. The studies of plants under MAPs and other popular medicinal plants can be explored for the promising healthcare system of people and national economy boost.

References

Adhikari MK, Shakya DM, Kayastha M, Baral SR and Subedi MN (2007) *Medicinal Plants of Nepal*, Goverment of Nepal, Ministry of Forests and Soil Conservation, Department of Plant Resources. 1-317

Adwankar MK, Chitnis MP, Khandalekar DD, Bhadsavale CG (1980) Anticancer acvtivity of the extracts of *Rubia cordifolia* Linn. *Ind J Exp Biol*. **18**: 102- 109

Agarwal SK, Singh SS, Verma S and Kumar S (2000) Antifungal activity of anthraquinone derivatives from Rheum emodi. *J. Ethnopharmacol*. **72:** 43-46

Ahmed J, Mehmood Z and Mohammad F (1998) Screening of some Indian medicinal plants for their antimicrobial properties. *J. Ethnopharmacol*. **62**: 183-193

Ajibade VA and Egbebi AO (2011) Effect of alkaloid extract of *Phyllanthus niruri* on rabbits infected with enteropathogenic *Escherichia coli. Int. J of Trop Med & pub Health*.**1**:1

Almey AA, Khan JA, Zahir IS, KS S and KK AR (2010) Total phenolic content and primary antioxidant activity of methanolic and ethanolic extract of aromatic plants' leaves. *Int Food Res J.***17**:7

Amatya KR (2000) Utilization of Himalayan medicinal plant resources-Status, problems and prospects. Proceeding of Nepal-Japan Joint Symposium 2000, Kathmandu, Society for the Conservation and Development of Medicinal Plants Resources, Kathmandu, Nepal. pp 331-352

Amatya KR (2003) Medicinal and aromatic plants trade and promotion.*Plant Resources: Bulletin of Department of Plant Resources.* **22**: 104-107

Ambikapathy V, Gomathi S, and Panneerselvam A (2011) Effect of antifungal activity of some medicinal plants against *Pythium debaryanum* (Hesse). *Asian J Plant Sci Res*. **1** (3): 131-13

Amin ZA, Alshawsh MA, Kassim M, Ali HM and Abdulla MA (2013) Gene expression profiling revelas underlyiing molecular mechanism of hepatoprotective effect of *Phyllanthus niruri* on thioacetamide-induced hepatotoxicity in Sprague Dawley rats. *BMC Complement Altern med.***13**: 160

Amin ZA, Bilgen M, Alshawsh MA, Kassim M, Ali HM, Hadi AHA and Abdulla MA (2012)Protective Role of *Phyllanthus niruri*extract against Thioacetamide-Induced Liver Cirrhosis in Rat Model. Evid Based Complement Alternat Med. 2012:241583.

ANSAB (2012) A field Manual on Management and Cultivation of Satuwa (*Paris polyphylla*).

ANSAB Market Information System. Asia Network for Sustainable Agriculture and Bioresources, Kathmandu, Nepal.

Antarkar SS, Chinwalla T and Bhatt N (1983) Anti-inflammatory activity of *Rubia cordifolia* Linn. In rats.*Ind J Pharmacol.***15** (3): 185-188

Antolovich M, Prenzler PD, Patsalides E, McDonald S and Robards K (2002) Methods for testing antioxidantactivity.*The Analyst.* **127**(1): 183-198

Araujo Junior RF, Soares LA, Costa Porto CR, Aquino RGR, Guede HG, Petrovick PR, Souza TP, Araujo AA and Guerra GCB (2012) Growth inhibitory effects of Phyllanthus niruri extracts in compbination with cisplatin on cancer cell lines. *World J Gastroenterol.* **18**(31): 4162-4168

Arisawa M, Ueno H, Nimura M, Hayashi T and Morita N (1986) Rubiatriol, a new triterpenoid from the Chinese Drug Qián Cáo Gén.*Rubia cordifolia*.*J Nat Prod.* **49**(6):1114-1116

Bagalkotkar G, Sagineedu SR, Saad MS and Stanslas J (2006) Phytochemicals from *Phyllanthus niruri* Linn.and their pharmacological properties: a review. *J Pharm Pharmacol*.**58**: 1559-70

Bajracharya MB (1979) *Ayurvedic Medicinal Plants and General Treatments*. Jore Ganesh Press Pvt Ltd, Kathmandu

Barakoti TP and Shrestha KP (2008) Commercial utilization of allo (Girardinia diversifolia) by the Rais of Sankhuwasabha for income generation.*Banko Janakari*. **18**(1): 18–24

Baral SR and Kurmi PP (2006) A Compendium of Medicinal Plants in Nepal, Chabahil, Kathmandu

Barros ME, Lima R, Mercuri LP, Matos JR, Schor N and Boim MA (2006) Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis. Urol Res. **34(**6): 351–357

Basu S, Ghosh A and Hazra B (2005) Evaluation of the antibacterial activity of *Ventilago madraspatana Gaert.Rubia cordifolia Linn.*and*Lantana camara Linn*.: isolation of emodin and physcion as active antibacterial agents. *J Phyto Res*. **19**(10):888–894**5**

Beringer PM (1999) New approaches to optimizing antimicrobial therapy in patients with cystic fibrosis. *Current Opinion in Pulmonary Medicine*: 371-377

Bhattacharjee R and Sil PC (2007) "Protein isolate from the herb, *Phyllanthus niruri* l. (euphorbiaceae), plays hepatoprotective role against carbon tetrachloride induced liver damage via its antioxidant properties," *Food and Chemical Toxicology*. **45**(5): 817–826

Bhattarai KR and Ghimire M (2006) Commercially important medicinal and aromatic plants of Nepal and their distribution pattern and conservational measure along the elevation gradient of the Himalayas. *Banko Jankari*. **16**(1): 3-13

Bhopale NM, Aswar PB, Banarase NB and Khadabadi SS (2008) Design, development and *in vitro* evaluation of sustain release *Rubia cordifloia* matrix tablet. *Research J. Pharm. and Tech.* **1**(4): 475-477

Bhuju J (2013) Leshmanicidal activity expressed *in vitro* by some Nepalese medicinal plants against *Leishmania donovani.* M. Sc. Thesis submitted in Tribhuvan University, Kirtipur, Nepal

Bulgakov VP, Tchernoded GK, Mischenko NP, Khodakovskaya MV, Glazunov VP, Zvereva EV, Fedoreyev SA and Zhuravlev YN (2002) Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes. *J. Biotechnol.***97**: 213–221

Bylka W, Matlawska I and Pilewski NA (2004) Natural flavonoids as antimicrobial agents. *J. Amer. Nutr. Asso.***7**:24-31

Cabieses F (1993) Apuntes de medicina trational. La Racionalizcion de lo Irracional. Notes on Traditional Medicine. Consejo Nacional de Ciencia Y Technologia CONCYTEC Lima-Peru. 414

Calixto JB, Santos ARS, Cechinel Filho V and Yunes RA (1998) A review of the genus Phyllanthus: their chemistry, pharmacology and therapeutic potential. *Med Res Rev*. **18**: 225-58

CAMP (2001) Conservation, Assessment and Management Prioritization Report. International Development Research Center (IDRC), Canada and Ministry of Forest and Soil Conservation, Kathmandu, Nepal

Castelluccio C, Paganga G and Melikian N (1995) Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. *FEBS Lett*.**368**: 188-192

Chandra R (2000) Lipid lowering activity of *P.niruri*, *Journal of Medicinal & Aromatic Plant Sciences*.**22** (1): 29-30

Chandrasekharnath N, MahalakshmiYV, JayalakshmiL, VenkannaB and Uma A (2013) Screening and isolation of bio active factors from *Commiphora myrrha* and evaluation of their anti microbial activity. *IJERA*. **3**(2):1291-1294

Chang C, Yang M, Wen H and Cheru J (2002) Estimation of total flavonoids content in propolis by two complementary colormetric methods. *J. Food Drug Anala.***10:** 178-82

Chang CC, Lien YC, Karin CS, Liu C and Lee SS (2003) Lignans from Phyllanthus urinaria. *Phytochemistry*.**63:** 825–833

Chang LC, Chavez D, Gills JJ, Fong HHS, Pezzuto JM and Kinghorn AD (2000) Rubiasins A-C, new anthracene derivatives from the roots and stems of *Rubia cordifolia. Tetrahedron Lett.* **41**(37): 7157-7162.

Chatterjee M and Sil PC (2006) Hepatoprotective effect of aqueous extract of *Phyllanthus niruri* on nimesulide-induced oxidative stress in vivo. *Ind J Biochem Biophys*. **43**(5):299-305

Chattopadhyay P, Agrawal SS and Garg A (2006) Liver regenerative affect of *Phyllanthus amarus* Linn.against alcohol induced liver cell injury in partially hepatectomised Albino Rats. *International J. of Pharm*. **2**(4): 426-430

Chaurasia SC and Vyas KK (1977) In vitro effect of some volatile oil against *Phytophthora arasitica* var. *piperina*. J. Res. Indian Med. Yoga Homeopath. 24–26

Cheung YN, Ong CY, Suen YK, Ooi V, Wong NC, Mak CW, Funga KP, Yue B and Kong SK (2005) Polyphyllin D is a potent apoptosis inducer in drug-resistant HepG2 cells. *Cancer Lett*.**217**: 203-211.

Chhetri MS, Timilsina YP, Tripathee HP and Devkota KP (2012) Socio-ecological status and antibacterial activity of *Paris polyphylla* from Panchase area of Kaski District. Nepal Sci Tech **13**(2): 167-174

Cimanga RK, Tona L, Luyindula N, Mesia K, Lusakibanza M, Musuamba CT, Apers S, De Bruyne T, Van Miert S, Hermans N,Totte J, Pieters L, Vlietinck AJ (2004). *In vitro*

antiplasmodial activity of callus culture extracts and fractions from fresh apical stems of *Phyllantus niruri* L (Euphorbiaceae); Part 2.*J. Ethnopharmacol*. 399-404

Cragg GM and Newman DJ (2005) Plants as a source of anti-cancer agents.*J Ethnopharmacol.* **100**(1): 72-79

Crisanto ML, Sunee N, Penkhae W and Ngamtip P (2003) Antimicrobial Activity of Medicinal Plant Extracts against Foodborne Spoilage and Pathogenic Microorganisms. *J. Kas, (Nat.Sci.)* **37**: 460 – 467**95**

Cushnie T and Lamb AJ (2005) Antimicrobial activity of flavonoids.*Int J Antimicrob Agents*. **26**(5): 343-356.

D'Incalci M, Steward WP and Gescher AJ (2005) Use of cancer chemopreventive phytochemicals as antineoplastic agents. *Lancet Oncology*.**6**: 899-904

De Souza TP, Holzschuh MH, Lionco MI, Gonzalez Ortega G and Petrovick PR (2002) Validation of a LC method for the analysis of phenolic compounds from aqueous extract of *Phyllanthus niruri* aerial parts. *J Pharm Biomed Anal.* **30**(2): 351-356

Deng ZC, Huang W, Zhang WS, Meng FY and Wang YY (2007) Research progress on *Paris polyphylla* Smith var. *yunnanensis* (France.) Hand-Mazz.*Chin J Pharm Technol Economics Manag.***1**(2): 57-63

Deoda RS, Kumar D, Kadam PV, Yadav K, Bhujbal SS and Patil MJ (2011) Pharmacognostic and Biological Studies of the Roots of Rubia Cordifolia Linn.(Rubiaceae).*Int. J. Drug Dev. & Res.***3**(3): 148-158

Devkota KP, Khan MT, Ranjit R, Lannang AM, Samreen and Choudhary MI (2007) Tyrosinase inhibitory and antileishmanial constituents from the rhizomes of *Paris polyphylla*. *Nat Prod Res* **21**(4): 321-327

Devkota S (2006) Yarsagumba [Cordyceps sinensis (Berk.)Sacc.]; traditional utilization in Dolpa district, western Nepal.*Our Nature.***4**(1):48-52

Dingman SL (2002) Water in soils: infiltration and redistribution. Physical hydrology, 2nd edit, upper saddle river, New Jersey: Prentice-Hall, Inc., 646

Doughari JH, Human IS Bennade S and Ndakidemi PA (2009) Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. *Journal of Medicinal Plants Research*.**3**(11): 839-848

Dutta IC (2007) Non Timber Forest Products of Nepal: Identification, Classification, Ethnic Uses and Cultivation.Hillside Press.Kathmandu. 484

Edwards DM (1996) The Trade in Non-Timber Forest products from Nepal. *Mountain Research and Development*.**16**(4): 383-394

Ekwenye UN and Njoku NU (2006).Antimicrobial effect of *Phyllanthus niruri* (Chanca Piedra) on three enteropathogens in man.*Int. J. Mol. Med. Adv. Sci.***2**(2): 184-189

Elfahmi, Batterman S, Koulman A, Hackl T, Bos R, Kayser O, Woerdenbag HJ and Quax WJ (2006) Lignans from cell suspension cultures of *Phyllanthus niruri*, an Indonesian medicinal plant. *J Nat Prod.***69**(1): 55-58

Engebretson J (2002) Culture and complementary therapies.*Complement Ther Nurs Ethnopharmacol*. **71**: 23-43.

Fan JT, Kuang B, Zeng GZ, Zhao SM, Ji CJ, Zhang YM and Tan NH (2011): Biologically active arborinane-type triterpenoids and anthraquinones from Rubia yunnanensis.*J Nat Prod.***74**(10):2069–2080

Foo LY (1993) Amariinic Acid and related ellangitanouns from *Phyllanthus amarus.Phytochemistry*.**39:** 219-224

Fotia C, Avnet S, Granchi D andBaldini N (2012) The natural compound alizarin as an osteotropic drug for the treatment of bone tumors. *J Orthop Res.***30:** 1486-1492.

Fratianni F, Tucci M, De-Palma M, Pepe R and Nazzaro F (2007) Polyphenolic composition in different parts of some cultivars of globe artichoke (*Cynara cardunculus* L. var. *scolymus* (L.) *Fiori*).*Food Chem.***104**: 1282-1286

Freitas AM, Schor N and Boim MA (2002) The effect of *Phyllanthus niruri* on urinary inhibitors of calcium oxalate crystallization and other factors associated with renal stone formation. *BJU Int.* **89**(9): 829-834

Gaire, BP and Subedi L (2011) Medicinal Plant Diversity and their Pharmacological Aspects of Nepal Himalayas.*Pharmacognosy J*. **3**(25): 6-17

Galvez CJ, Martin-Cordero P and Houghton AM (2005) Antioxidant activity of methanol extracts obtained from *Plantago* species. *J.Agric.Food Chem*. **53**: 1927-1933

Gao XM, Xu ZM and Li ZW (2000) Traditional Chinese Medicine. Beijing: People's Health Publishing House.

Garbyal SS (2005) Traditional knowledge of plant resources in Dharchula region: Biotechnological potential, conservation and management strategies. Doctor of Philosophy Thesis, submitted in Guru Gobind Singh Indraprastha University, Delhi, India

Gewali MB (2008) *Aspects of traditional medicine in Nepal*, Institute of Natural Medicine. Thesis submitted in University of Toyama, Japan

Gilani AH, Janbaz KH, Zaman M, Lateef A, Suria A and Ahmed HR (1994) Possible presence of calcium channel blocker(s) in *Rubia cordifolia*: an indigenous medicinal plant. *J Pak Med Assoc.* **44**(4): 82-85.

GIZ (2011) Medicinal and aromatic plants: Poverty impact assessment of proposed trade support measures in Nepal's MAPs sector. Kathmandu

Global Industry Analysts Inc (2012) Herbal Supplements and Remedies: A Global Strategic Business Report. Global Industry Analysts, San Jose, California, USA

Gogte VVM (2000) Ayurvedic pharmacology and therapeutic uses of medicinal plants. Bharatiya Vidya Bhavan, Mumbai

Govindarajan R, Vijayakumar M and Pushpangadan P (2005) Antioxidant approach to disease management and the role of 'Rasayana' herbs of Ayurveda. *J Ethnopharmacol.***99**: 165-178

Gresham LJ, Ross J and Izevbigie EB (2008) *Vernonia amygdalina*: anticancer activity, authentication and adulteration detection. *Int J Env Res Public Health.* **5**(5): 342-348

Gupta D and Gulrajani ML (1992) Natural dyeing and their applications on textiles.Department of Textile technology, IIT, Delhi. 27

Gupta PP, Srimal RC, Verma N and Tandon JS (1999) Biological activity of *Rubia cordifolia* and isolation of an active principle. *Pharm. Biol*. **37** (I): pp 46-49

Gutteridge JM and Halliwell B (2000) Free radicals and antioxidants in the year 2000, A historical look to the future. *Ann N Y Acad Sci***899**: 136-147

Halberstein RA and Saunders AB (1978) Traditional Medicinal Practices and Medicinal plant usage on a Bahamian Island. *Cul. Mad.Psychiat.* **2**: 177-203

Haman A, Taie A, El-Mergawi R and Radwan S (2008) Isoflavonoids, flavonoids, phenolic acids profiles and antioxidant activity of soyabean seeds as affected by organic and bioorganic fertilization. *American-Eurasian J. Agric. and Environ. Sci*. **4**(2): 207-213

Harish R and Shivanandappa T (2006) Antioxidant acitivty and hepatoprotective potential of *Phyllanthis niruri.Food Chem*. **95**(2): 180-185

Hasuda T, Hitotsuyanagi Y and Takeya K (2011) Production of monoclonal antibodies against antitumor cyclohexapeptide RA-VII from *Rubia cordifolia* and their characterization. *J Nat Med*. **65**(3): 588–593

Hayashi Y, Matsushima M, Nakamura T, Shibasaki M, Hashimoto N, Imaizumi K, Shimokata K, Hasegawa Y, and Kawabe T (2012) Quercetin protects against pulmonary oxidant stress via heme oxygenase-1 induction in lung epithelial cells. *Biochem Biophys Res Commun.***417**: 169-174

Hazra B, Basu S and Ghosh A (2005) Evaluation of Antibacterial activity of *Ventilago draspatana* Gaertn.1988, *Rubia cordifolia* Linn.and*Lantana camara* linn. Isolation of emodin and physician as active Antibacterial agents.*Phytotherapy Research*. 888-894

HMGN (2002) Nepal Biodiversity Strategy Singhadarbar, Kathmandu

Hoffman PC, Combs DK and Caster MD (1998) Performance of lactating dairy cows fed alfalfa silage or perennial ryegrass silage. *J Dairy Sci*. **81**, 162-168

Holdsworth D and Wamoi B (1982) Medicinal plants of the Admiralty Islands, Papau New Guinea. Part 1.*Int. J.Crude Drug Res.* **20**(4): 169-181

Hostettmann K and Marston A (1995) Saponins Chemistry and Pharmacology of Natural Products. University Press, Cambridge

http://www.who.int/mediacentre/factsheets/fs134/en/(WHO, 2003)

Hua HM, Wang SX, Wu LJ, Li X and Zhu TR (1992) Studies on naphthoic acid esters from the roots of *Rubia cordifolia* L. *Yao Xue Xue Bao*. **27**(4): pp 279-282

Huang D, Ou B and Prior RL (2005) The chemistry behind antioxidant capacity assays.*J. Agric. Food Chem.***53**: 1841–1856

Huang YL, Kou JP, Ma L, Song JX and Yu BY (2008) Possible mechanism of the anti-inflammatory activity of Ruscogenin: role of Intercellular adhesion molecule-1 and Nuclear Factor-*k*B; J. *Pharmacol. Sci*. **108**: 198-205

Humagain K and shrestha KK (2009) Medicinal plants in Rasuwa district, central Nepal: trade and livelihood. *Botanica Orientalis – J Plant Sci*. **6**: 39–46

Ishimaru K, Yoshimatsu K, Yamakawa T, Kamada H and Shimomura K (1992) Phenolic constituents in tissue cultures of *Phyllanthus niruri*. *Phytochem*.**34**: 2015-2016

Itokawa H, Morita H, Takeya K, Tomioka N, Itai A and Iitaka Y (1991) New antitumor bicyclic hexapeptides, RA-VI and -VIII from *Rubia cordifolia*; conformation-activity relationship II. Tetrahedron. **47**(34):7007-7020

Itokawa, H, Qiao, YF, Takeya Kand IitakaY (1989) New triterpenoids from *Rubia cordifolia* var. pratensis (Rubiaceae).*Chem. Pharm. Bull.***37**: 1670-1672

IUCN (2004) National Register of Medicinal and Aromatic Plants (Revised and updated). pp 202

Jagetia GC, Venkatesh P and Baliga MS (2005) Influence of seed extracts of *S. cumini* on mice exposed to different doses of γ radiation*. J. Radiat. Res.***46:** 59-65.

Jain N, Goyal S and Ramawat KG (2011) Evaluation of antioxidant properties and toral phenolic content of medicinal plants used in diet therapy during postpartum healthcare in Rajasthan. *Int J Pharm Sci.* **3**(3): 248- 253

Jokharapurkar AA, Zambad SP, Wanjari MM and Umathe SN (2003) *In vivo* evaluation of antioxidant activity of alcoholic extract of *Rubia cordifolia* Linn. and its influence on ethanol-induced immunosuppression. *Indian J Pharmacol.***35**: 232-236

Joshan RS, Nagarauk R and Anuradha P (2010) Antibacterial properties of extracts of Indian medicinal plants: *Syzygium alternifolium*, *Phyllanthus niruri* and *Rubia cordifolia*. *Biomed Pharmacol J.* **3**(1): 123-128

Joshi AR and Joshi DP (1991) Endemic Plants of Nepal Himalaya: Conservation Status and Future Direction. *Mountain Environment and Development.* **1**(2): 1-35

Justyna SH, Anna K, Jerzy G, Ochocka JR, Monika P, Piotr Sand Ewa L (2011) Cytotoxic activity of Paris *quadrifolia* extract and isolated saponin fractions against human tumor cell lines. *Botanica*. **53**(2): 127-131

K.C M, Phoboo S and Jha PK (2010) Ecological Study of Paris polyphylla Sm. Ecological Society (ECOS), Nepal.**17**: 87-93

Kalt C, Forney F and Martin A (1999) Antioxidant capacity, vitamin C, phenolics and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.***47**: 4638-4644

Kang LP, Ma BP, Zhang J, Xiong CQ, Tan DW and Cong YW (2005) Isolation and identificiation of steroidal saponins from *Paris polyphylla* Smith. *Chin J Med Chem.* **15**(63): 25-31

Kang LP, Yu K, Zhao Y, Liu YX, Yu HS, Pang X, Xiong CQ, Tan DW, Gao Y, Liu C and Ma BP (2012) Characterization of steroidal glycosides from the extract of *Paris polyphylla* var. *yunnanensis* by UPLC/Q-TOF MSE. *J Pharm Biomed Anal.***62**: 235-249

Kanthimathi M and Soranam R (2013) Antibacterial effects of *Emblica officinalis* and *Phyllanthus niruri* crude extracts against bacterial pathogens.*Int J Pharmaceut Clin Sci*. **3**(3): 20-23

Kapur V, Pillai KK, Hussain SZ and Balani DK (1994) Hepatoprotective activity of jigrine on liver damage caused by alcohol, carbon tetrachloride and paracetamol in rats. *Ind J Pharm*. **26**: 35–40

Kapur V, Pillai KK, Hussain SZ and Balani DK (1994) Hepatoprotective activity of jigrine on liver damage caused by alcohol, carbon tetrachloride and paracetamol in rats. *Ind. J. Pharmacol*.**26:** 35-40

Kar A (2007) Pharmaocgnosy and Pharmacobiotechnology (Revised-Expanded Second Edition). New Age International LimtedPublishres New Delhi. 332-600

Karki M, Tiwari BK, Badony, AK and Bhattarai N (2003).Creating Livelihoods Enhancing Medicinal and Aromatic Plants Based Biodiversity-Rich Production Systems: Preliminary Lessons from South Asia. Paper presented: 3rd World Congress on Medicinal and Aromatic Plants for Human Welfare. Chiang Mai: WOCMAP III

Karodi R, Jadhav M, Rub R and Bafna A (2009) Evaluation of the wound healing activity of a crude extract of *Rubia cordifolia* L. (madder) in mice.*Int J App Res Nat Prod.* **2**(2):12-18

Karuppusamy S (2009) A review on trends in production of secondary metabolites from higher plant by *in vitro* tissue, organ and cell culture. *J. Medicinal Plants Research*. **3**(13): 1222-1239

Kasture VS, Desmukh VK, Chopde CT (2000) Anticonvulsant and behavioral actions of triterpine isolated from *Rubia cordifolia* Linn. *Ind J Exp Biol*. **38**: 675-80.

Kaur P, Kaur S, Kumar S and Singh P (2010) *Rubia cordifolia* L. and *Glycyrrhiza glabra* L. Medicinal Plants as Potential Source of COX-2 Inhibitors. *Am. J. Biomed Sci.* **2**(2): 108-120

Kawada M, Ohno Y, Ri Y, Ikoma T, Yuugetu H, Asai T, Watanabe M, Yasuda N, Akao S, Takemura G, Minatoguchi S, Gotoh K, Fujiwara H and Fukuda K (2001) Anti-tumor effect of gallic acid on LL-2 lung cancer cells transplanted in mice. *Anticancer Drugs.***12**: 847-852

Khan M and Wassilew SW (1987) Natural pesticides from the neem tree and other tropical plants. (Eds) Schmutterer H and Asher KRS, Germany: Digitalverlag GmbH. 645-650

Khan N, Karodi, Siddiqui A, Thube S and Rub R (2012) Development of anti-acne gel formulation of anthraquinones rich fraction from *Rubia cordifolia* (Rubiaceae).*Int J App Res Nat Prod.* **4** (4): 28-36

Khanna AK, Rizvi F and Chander R (2002) Lipid lowering activity of Phyllanthus niruri in hyperlipemic rats. *J Ethnopharmacol*. **82**(1): 19-22

Kiemer AK, Hartung T, Huber C and Vollmar AM (2003) *Phyllanthus amarus* has anti-inflammatory potential by inhibition of iNOS, cox-2 and cytokine via the NF-KB pathway. *J. Hepatol.*: 289-297

Kirtikar ID and Basu BD (1994) Indian Medicinal Plants.Published by Lalit Mohan Basu, Allahabad, India. **3**(2): 1684-1690.

Klejdus B, Mikelova R, Petrolova J, Potesi, D, Adam V and Stiborova M (2005) Evaluation of isoflavone aglycon and glycoside distribution in soy plants and soybeans by fast column high-performance liquid chromatography coupled with a diode-array detector. *J Agr Food Chem.***53:** 5848-5852

Kunwar RM, Shrestha KP and Bussmann RW (2010) Traditional herbal medicine in Far-west Nepal: a pharmacological appraisal. *J Ethnobiol Ethnomed.*doi: 10.1186/1746-4269-6-35

Kunwar RM, Uprety Y, Burlakoti C, Chowdhary CL and Bussmann RW (2009)Indigenous use and Ethnopharmacology of Medicinal Plants in Far-west Nepal

Laird SA and Pierce AR (2002) Promoting Sustainableand Ethical Botanicals: Strategies to improve commercial raw material sourcing. Rainforest Alliance, New York

Lange D and Schippmann U (1997) Trade survey of medicinal plants in Germany: a contribution to international plant species conservation. Bonn-Bad Godesberg (Bundesamt für Naturschutz) pp 128 annexes (pp. I-XVI)

Lapomik A, Prosek M and Wondra GA (2005) Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng*. **71**: 214-222

Lapomik A, Prosek M and Wondra GA (2005) Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng.* 71: 214-222.

Lee MS, Yuet-Wa JC, Kong SK, Yu B, Eng-Choon VO, Nai-Ching HW, Chung-Wai TM and Fung KP (2005) Effects of polyphyllin D, a steroidal saponin in *Paris polyphylla*, in growth inhibition of human breast cancer cells and in xenograft. *Cancer Biol Ther* **4**(11): 1248-1254

Lewis WH and Elvin-Lewis MPH (1977) Medicinal Botany Plants Affecting Man's Health. John Wiley & Sons, New York. pp. 515

Li X, Liu Z, Chen Y, Wang LJ, Zheng YN, Sun GZ and Ruan CC (2009) Rubiacordone A: a new anthraquinone glycoside from roots of *Rubia cordifolia. Molecules*. **14**(1):566-572

Li, FR, Jiao P, Yao, ST, Sang H, Qin SC, Zhang W, Zhang YB and Gao LL (2012) *Paris polyphylla* Smith extract induces apoptosis and activates cancer suppressor gene connexin26 expression. *Asian Pac*. *J*. *Cancer Prev*.***13***: 205–209

Liang SY (2000) Flora of China.Science Press, Beijing and Missouri Botanic Garden, St. Louis. 24:90-91.

Lindequist U, Niedermeyer THJ and Julich WD (2005) The Pharmacological potential of mushrooms. eCAM. **2:** 285-299

Liu H, Zhang T, Chen XQ, Huang Y and Wang Q (2006) Steroidal saponins of *Paris polyphylla* Smith var. *yunnanensis*. *Chin J Nat Med.* **4**(4): 264-267

Lobo V, Patil A, Phatak A and Chandra N (2010) Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev.* **4**(8)*:* 118-126

Lodia S and Kansala L (2012) Antioxidant activity of *Rubia cordifolia* against lead toxicity.*Int J pharmaceut sci Res*.**3**: 07

Lopez CM, Nitisinprasert S, Wanchaitanawong P and Poovarodom N (2003) Antimicrobial Activity of Medicinal Plant Extracts against Foodborne Spoilage and Pathogenic Microorganisms. *J Kas (Nat. Sci.)* **37**: 460 – 467

Lucas DM, Still PC, Pérez LB, Grever MR and Kinghorn AD (2010) Potential of plant-derived natural products in the treatment of leukemia and *lymphoma.Curr Drug Targets.* **11**(7): 812-822

Luois M and Paul C (2010) 1 KalaDrug-R SOP#18 (online) www.leishrisk.net/Leishrisk/UserFiles/File/Kaladrug-R/SOPs/lab/labSOP18_IC50tool.pdf

Luyindula N, Tona L, Lukembila S, Tsakala M, Mesia K, Musuamba CT, Cimanga RK, Apers S, De Bruyne T, Pieters T, Vlientick A (2004) *In vitro* antiplasmodial activity of callus extracts from fresh apical stems of *Phyllantus niruri* L (Euphorbiaceous); Part 1. *Pharm. Biol.***42**(7):1-7

Madziga HA, Sanni S and Sandabe UK.(2010) Phytochemical and Elemental Analysis of *Acalypha wilkesiana* Leaf.*J Am Sci.* **6**(11): 510-514

Mahesh B, and Satish S (2008) Antimicrobial activity of some important medicinal plants against plant and human pathogens*. World J Agric Sci.***4**: 839-843

Mainen JM, Donald FO, Pamela KM, and Anke W (2010) Ethnomedicine of the Kagera Region, north western Tanzania. Part 2: The medicinal plants used in Katoro Ward, Bukoba District.*J of Ethnobio and Ethnomed*.**6**:19

Man SL, Gao WY, Zhang YJ, Ma CY, Yang L and Li YW (2011) Paridis saponins inhibiting carcinoma growth and metastasis *in vitro* and *in vivo*. *Arch*. *Pharm*. *Res*. **34**: 43-50.

Manandhar NP (2002) *Plants and people of Nepal.* Timber Press, Portland Oregon, USA. pp.50

Manson MM (2003) Cancer prevention – the potential for diet to modulate molecular signalling.*Trends in Molecular Medicine.***9**: 11–18

Marjorie C (1996) Plant products as antimicrobial agents.*Clin Microbiol Rev*. **12**: 564-582

MarkomM, Hasan M, Daud WRW, Singh H, Jahim JM (2007) Extraction of hydrolysable tannins from *Phyllanthus niruri* Linn.: Effects of solvents and extraction methods. *Sep Purif Technol.* **52**: 487-496

Mathur M, Sharma R, Sharma J, Pareek R and Kamal R (2012) Phytochemical screening and antimicrobial activity of *Phyllanthus niruri* Linn. *ElixirAppl. Botany*.**46**: 8487-8489

Matsuda H, Pongpiriyadacha Y, Morikawa T, Kishi A, Kataoka S and Yoshikawa M (2003) Protective effects of steroid saponins from *Paris polyphylla var. yunnanensis* on ethanol- or indomethacin- induced gastric mucosal lesions in rats: Structural requirement for activity and mode of action. *Bioorg. Med. Chem. Lett*. **13**: 1101-1106

Matsuura S, Takahashi H, Kobayashi K, Trimurningsih SC (2005) Antibabesial and anti-plasmodial compounds from *Phyllanthus niruri*. *J NatProd* .**68**(4): 537-539

McIntyre A (2005) Monographs of Ayurvedic herbs commonly used in the treatment of children. *Herbal treatment of children.* pp 87-135.

Medini F, Fellah H, Ksouri R and Abdelly C (2014) Total phenolic, flavonoid and tannin contents and antioxidant and antimicrobial activities of organic extracts of shoots of the plant *Limonium delicatulum. Journal of Taibah University for Science***8**: 216–224

Meixa W and Yanjin C (1995) Herbs of the genus Phyllanthus in the treatment of Chronic hepatitis B observation with three preparation from different geographic sites. *J. Lab. Clin. Med.* **126**(2): 350

Mellinger CG, Carbonero ER, Cipriani TR, Gorin PA and Iacomini M (2005) Xylans from the medicinal herb Phyllanthus niruri.*J Nat Prod.* **68**(1): 129-132

Mellinger CG, Cipriani TR, Noleto GR, Carbonero ER, Oliveira MB, Gorin PA and Iacomini M (2008) Chemical and immunological modifications of an arabinogalactan present in tea preparations of Phyllanthus niruri after treatment with gastric fluid. *Int J Biol Macromol.* **43**(2): 115-120.

Mentreddy SR (2007) Medicinal plant species with potential antidiabetic properties.*J. Sci. Food Midwifery.* **8**(4): 177-184

Mikage M and Mouri C (2000) Altitudinal variations of berberine content in the bark of *Berberis* plants from west Nepal. *Newsletter of Himalayan Botany.***26**: 16-19

Mikage M, Takano A, Jin H, Tomimori T and Namba T (1987) Studies on the Nepalese crude drugs (VII).On the variation in the morphological appearances and the alkaloid contents of the herbal stem of *Ephedra gerardiana* Wall.according to the differences in habitats. *Shoyakugaku Zasshi*.**41**: 209-214

Mischenko NP, Fedoreyev S, Glazunov VP, Chernoded GK, Bulgakov and Zhuravlev YN (1999) Anthraquinone production by callus cultures of *Rubia cordifolia*. *Fitoterapia* **70**:552-557

Mishra A, Sharma AK, Kumar S, Saxena AK and Pandey AK (2013) *Bauhinia variegata* Leaf Extracts Exhibit Considerable Antibacterial, Antioxidant, and Anticancer Activities. *Biomed Res Int.* doi: **10**.1155/2013/915436

Mital K, Kanani B, Chanda S (2012) Assesement of effect of hydroalcoholic and decotionmethods on extraction of antioxidants from selected Indian medicinal plants Asina Pac J Trop Biomed **2**(3): 195-202

MoFSC (2009) *Nepal fourth national report to the Convention on Biological Diversity.* Kathmandu

More BH, Gadgoli C and Padesi G (2007) Hepatoprotective activity of *Rubia Cordifolia*.*Pharmacologyonline*.**3**: 73-79.

Murugaiyah V and Chan KL (2006) Antihyperuricemic lignans from the leaves of *Phyllanthus niruri*.*Planta Med*. **72**: 1262-7.

Murugaiyah V and Chan KL (2009): Mechanisms of antihyperuricemic effect of *Phyllanthus niruri*and its lignan constituents. *J Ethnopharmacol* **124**(2):233–239

Mustofa, Sholikhah EN and Wahyuono S (2007) *In vitro* and *in vivo* antiplasmodial activity and cytotoxicity of extracts of *Phyllanthus niruri* L. herbs traditionally used to treat malaria in Indonesia. *Southeast Asian J. Trop. Med. Public Health* **38**: 609-615

Muzitano MF, Cruz EA,Almeida AP, Silva SA, Kaiser CR,Guette C, Rossi-Bergmann R, Costa SS (2006) Quercitrin: an antileishmanial flavonoid glycoside from Kalanchoe pinnata.*Planta Med*. **72**: 81–83

Naik AD and Juvekar AR (2003) Effects of alkaloidal extract of *Phyllanthus niruri* on HIV replication, *Indian J. Med. Sci.* **57**: 387-93

Nair R, Kalariya T and Chanda S (2005) Antibacterial Activity of Some Selected Indian Medicinal Flora.*Turk J Biol*. **29:** 41-47

Narendra K, Swathi J, Sowjanya KM and Satya AK (2012) *Phyllanthus niruri*: A review on its ethno bothanical, phytochemical and pharmacological profile. *J Pharm Res*. **5**(9): 4681-4691

Natarraj CG (2000) Role of herbal extracts in HIV infected patients, Proceedings of International Congress on Ayurveda. 207.

Natesh S (2000) *Biotechnology in Horticultural and Plantation Crops,* Malhotra Publishing Natural Product Chemistry. England: John Wiley and Sons. pp 283-359.

Ncube NS, Afolayan AJ and Okoh AI (2008) Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *Afr J Biotech.* **7**(12): 1797-1806

Neraliya S and Gaur R (2004) Juvenoid activity in plant extracts against filarial mosquito *Culex quinquefasciatus*, *Journal of Medicinal and Aromatic Plant Sciences.* **26** (1): 34-38

Newman DJ (2008) Natural products as leads to potential drugs: an old process or the new hope for drug discovery. *J Med Chem*. **51**(9): 2589-2599

Newman DJ, Cragg GM and Snader KM (2003) Natural products as sources of new drugs over the period 1981-2002. *J.Nat Prod.* **66**(7): 1022-37

Nimmi I, Jahan IA, Hossain MH, Uddin MB, Rana MS and Haq MM (2012) A compatative study on antioxidant properties of two phyllanthus species growing in Bangladesh. *J. Pharm. Sci.* **11**(2): 191-197

Nohara T, Ito Y, Seike H, Komori T, Moriyama M, Gomita Y and Kawasaki T (1982) Study on the constituents of *Parisquadrifolia* L. *Chem Pharmaceut Bulletin*. **30**: 1851-1856

Nweze EI, Okafor JI and Njoku O (2004) Antimicrobial activities of methanolic extracts of *Trema guineensis* (Schumm and Thorn) and *Morinda Lucida* Benth used in Nigeria herbal medicinal practice. *J.Biol. Res. Biot*. **2** (1): 36-39

Nyambati GK, Lagat ZO, Maranga RO, Samuel M, Ozwara H(2013 *In vitro* Anti-plasmodial Activity of *Rubia cordifolia, Harrizonia abyssinica, Leucas calostachys* Olive and *Sanchus schweinfurthii* Medicinal Plants. *J Appl Pharmaceut Sci.* **3**(12): 057-062

Ogata T, Higuchi H, Mochida S, Matsumoto H, Kato A, Endo T, Kaji A and Kaji H (1992) HIV-1 reverse transcriptase inhibitor from Phyllanthus niruri. *AIDS Res Hum Retroviruses.* **8**(11): 1937-1944

Ohno Y, Fukuda K, Takemura G, Toyota M, Watanabe M, Yasuda N, Xinbin Q, Maruyama R, Akao S, Gotou K, Fujiwara T and Fujiwara H (1999) Induction of apoptosis by gallic acid in lung cancer cells. *Anticancer Drugs.***10**: 845-851

Ojha JK, Dwivedi KN, Chaurasiya AK (1994) Effect of *Rubia cordifolia*on non healing diabetic foot ulcer. *Nat. Sem. Trad. Med*. Plants in Skin Care. pp 17

Okoli CO, Ibiam AF, Ezike AC, AKah PA and Okoye TC (2010) Evalutaion of antidiabetic potentials of *Phyllanthus niruri* in alloxan diabetic rats. *Afr J Biotech*. **9**(2): 248-259

Olsen CS (2005) Valuation of commercial central Himalayan medicinal plants.*Ambio*.**34**: 607-610

Olsen CS and Larsen HO (2003) Alpine medicinal plant trade and Himalayanmountain livelihood strategies.Geographical Journal.**169**:243–254

Ong RC, Lei J, Lee RK, Cheung JY, Fung KP, Lin C, Ho HP, Yu B, Li M and Kong SK (2008) Polyphyllin D induces mitochondrial fragmentation and acts directly on the mitochondria to induce apoptosis in drug-resistant HepG2 cells. *Cancer Lett.* **261**(2): 158-164

Paithankar VV, Raut KS, Chadre RM and Vyas JV (2011) *Phyllanthus niruri:* A magic herb*. Res in Pharm.* **1**(4): 1-9, 2011

Pande M and Flora SJ (2002) Lead induced oxidative damage and its response to combined administration of α-lipoic acid and succimers in rats. *Toxicology.***17:**187-96.

Panthi MP and Chaudhary RP (2006) Antibacterial activity of selected folklore medicinal plants from west Nepal.*Scientific World*.**4:** 16-21

Paonam P and Sharma GJ (2014) Evaluation of antioxidant components and in vitro free radical scavenging capacities of *Paris polyphylla* L. *J Free Radical Antioxidants.***140:** 363-370

Paonam P, Thokckom DS and Sharma GJ (2013) Free radical scavenging and antioxidant potentials of Panax pseudoginseng Wall. *J Free Radical and Antioxidants*.**139**: 289-294.

Parag RP, Bhuvan PR, Hamsraj AK and Vishal RP (2010) Potent antitumor activity of Rubia cordifolia. *Intern J of Phytomed*. **2**: 44-46

Paramaguru R, Janaki PS, Bavanieswaran M, Rao CV, Rawat AKS and Vijayakumar M (2012) Free radical scavenging and lipid potential of various fractions of peroxidation inhibition *Murraya paniculata*. *Pharmacologia*.**3:** 138-143

Parkin DM (2001) Global cancer statistics in the year 2000.*Lancet Oncol.* **2**(9): 533-543

Parmar P, Bhatt S, Dhyani S and Jain A (2012) Phytochemical studies of the secondary metabolites of *Ziziphus mauritiana* Lam. Leaves. *Int J Cur Pharmaceut Res.* **4**(3):153-155.

Patel PR, Nagari AA, Patel RC, Rathod DK and Patel VR (2011a) In vitro anticancer activity of Rubia cordifolia against Hela and Hep2 cell line. *Int J Pharmacy Pharmcol Sci.***3:** 70-1.

Patel PR, Raval BP, Karanth HA and Patel VR (2011b) Potent antitumor activity of *Rubia cordifolia*. *Int J Phytomed*. **2**: 44-6.

Patwardhan B (2000) Ayurveda: The designer medicine - a review of ethnopharmacology and bioprospecting research. *Indian Drugs.***37**: 213-227.

Perumal SR and Gopalakrishnakone P (2010) Therapeutic potential of plants as antimicrobials for drug discovery.*Evidence-Based Complement Altern Med.* **7**(3): 283-294

Pfoze NL, Kumar Y and Myrboh B (2013) Screening of bioactive phytochemicals obtained from lesser known ethnomedical plants of Senapti district of Manpiur, India. ***Pleione*** **7**(2): 489 - 500

Phillipson JD and Wright CW (1996) Plants With Antiprotozoal Activity: Tease and Evans, Pharmacognosy, 14th edn., WBSaunders Company, London. 612

Phoboo S, Pinto MDS and Barbosa ACL (2013) Phenolic-linked biochemicalrationale for the anti-diabetic properties of *Swertia chirayita* (Roxb. ex Flem.)Karst.*Phytotherapy Res*. **27**(2): 227–235

Pinmai K, Chunlaratthanabhorn S, Ngamkitidechakul C, Soonthornchareon N and Hahnvajanawong C (2008) Synergistic growth inhibitory effects of Phyllanthus emblica and terminalia bellerica extracts with conventional cytotoxic agents: doxorubicin and cisplatin against human hepatocellular carcinoma and lung cancer cells. *World J Gastroenterol.***14**: 1491-1497.

Poh-Hwa T, Yoke-Kqueen C, Bala IJ ad Son R (2011) Bioprotective properties of three Malaysia *Phyllanthus* species: An investigation of the antioxidant and antimicrobial activities.I*nt. Food Res J.* **18**(3): 887-893

Popova MP, Chinou IB, Marekov IN and Bankova VS (2009) Terpenes with antimicrobial activity from Cretan propolis.*Phytochemistry.***70**: 1262-1271

Prajapati SN and Parmar KA (2011) Antiviral and in-vitro free radical scavenging activity of leaves of *Rubia cordifolia.Int JPhytomed*. **3**: 98-107

Pyakurel D and Baniya A (2011) NTFPs: Impetus for Conservation and Livelihood support in Nepal: A Reference Book on Ecology, conservation, Product Development and Economic Analysis of Selected NTFPs of Langtang Area in the Sacred Himalayan Landscape. *WWF*

Qin XJ, Chen CX, Ni W, Yan H and Lie HY (2013) C22-steroidal lactone glycosides from stems and leaves of *Parispolyphylla*var. *yunnanensis*. *Fitoterapia*.**84**: 248-251

Qin XJ, Sun DJ, Ni W, Chen CX, Hua Y and He L (2012) Steroidal saponins with antimicrobial activity from stems and leaves of *Paris polyphylla*var. *yunnanensis*.*Steroids*.**77**: 1242–1248

Rahman SMA, Abd-Ellatif SA, Deraz SF and Khalil AA (2011) Antibacterial activity of some wild medicinal plants collected from western Mediterranean coast, Egypt: Natural alternatives for infectious disease treatment. *Afr J Biotech*. **10**(52): 10733-10743

Rajbhandari TK, Joshi NR, Shrestha T, Joshi SKG and Acharya B (1995) Medicinal Plants of Nepal for Ayurvedic Drugs. Government of Nepal, Department of Plant Resources, Thapathali, Kathmandu

Rao GMM, Rao CV, Pushpangadan P and Shirwaikar A (2006) Hepatoprotective effects of rubiadin, a major constituent of *Rubia cordifolia*. *J Ethnopharmacol.***103**: 484-490

Rashed AN, Afifi FU and Disi AM (2003) Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L. growing in Jordan. *J Ethnopharmacol*.**88**:131-136

Raut B and Khanal DP (2011) Present status of traditional healthcare system in Nepal.*Int J Res Ayur Pharmacy.* **2**(3): 876-882

Ravipati AS, Zhang L, Koyyalamudi SR, Jeong SC, Reddy N, Bartlett J, Smith PT, Shanmugam K, Munch G, Wu MJ, Satyanarayanan M and Vysetti B (2012) Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content. *BMC Complement Altern Med.***12**: 173

Rawal A, Muddeshwar M and Biswas S (2004) Effect of *Rubia cordifolia, Fagonia cretica* linn, and *Tinospora cordifolia* on free radical generation and lipid peroxidation during oxygen–glucose deprivation in rat hippocampal slices.*BiochemBiophys Res Comm.* **324**:588–596

Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM and Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res*. **22**(4): 375-383

Riou V, Vernhet A, Doco T and Moutounet M (2002) Aggregation of grape seed tannins in model wine-effect of wine polysaccharides. *Food Hyrdrolloids*.**16:** 17-23.

Ristimaki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H and Isola J (2002) Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Research*.**62**: 632-635

Robineau L. Ed (1991) Towards a Caribbean pharmacopoeia, TRAMIL-4 Workshop, UNAH, Enda Caribe, Santo Domingo.474

Rokaya MB, Münzbergová Z, Timsina B (2010) Ethnobotanical study of medicinal plants from the Humla district of western Nepal.*J Ethnopharmacol.***130:**485–504.

Ross IA (1999) Medicinal plants of the world -chemical constituents, traditional and modern medicinal uses, Pub: Humana Press Inc., Totowa, New Jersey.249-254

Roy S, Rao K, Bhuvaneswari C, Giri A and MangamooriLN (2010) Phytochemicalanalysis of *Andrographis paniculata* extract and its antimicrobial activity. *World J Microbiol Biotechnol*.**26:** 85-91.

Sabir SM and Rocha JBT (2008) Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct in vitro antioxidant and in vivo hepatoprotective activity against paracetamol-induced liver damage in mice. *Food Chemistry*. **111**(4): 845-851

Santos AR (1994) Analgesic effects of callus culture extracts from selected species of Phyllanthus in mice. *J.Pharm. Pharmacol.* **46**(9): 755-759

Sapkota CR and Adhikari SM (2001) *Ayurvedic Pharmacology: Bheshaja Guna Vinjana*. Singha Durbar Vaidyakhana Vikas Samitee, Kathmandu

Sarker SD and Nahar L (2007) Chemistry for Pharmacy Students General, Organic and Natural Product Chemistry. England: John Wiley and Sons. pp 283-359

Savoia D (2012) Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future Microbial*. **7**(8): 979-90

Sawhney R, Berry V and Kumar A (2012) Inhibitory activity of *Rubia cordifolia*plant extract against ESBL producing urinary E.coli isolates. *J Pharmacy Res*. **5**(3): 1328-1330

Scalbert A (1991) Antimicrobial properties of tannins. *Phytochemistry.* 30:3875-3883

Scartezzini P and Speroni E (2000) Review of some plants of Indian traditional medicine with antioxidant activity. *J Ethnopharmacol.* **71**(2): 23-4

Schippmann U, Leaman and Cunningham AB (2006) Cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. In: Bogers, R.J., L.E. Craker, and D. Lange (eds). Medicinal and aromatic plants.Springer, Dordrecht.Wageningen UR Frontis Series no. 17. (http://library.wur.nl/frontis/medicinal_aromatic_plants/05_schipmann.pdf)

Schmidt B, Ribnick DM, Poulev A, Logendra S, Cefalu WT and Raskin I (2008) A natural history of botanical therapeutics. *Metabolism.***57***:* S3-S9

Schultes RE (1978) The Kingdom of plants. In: Thomson WAR, editor. Medicines from the earth. New York: McGraw-Hill Book Co. pp. 208

Seidl PR (2002) Pharmaceuticals from natural products: current trends. *An Acad Bras Cienc.* **74**(1): 145-150

Senapati SK, Dey S, Dwivedi SK and Swarup D (2001) Effect of garlic (*Allium Sativum* L.) extract on tissue lead level in rats*. J. Ethnopharmacol*. **76**: 229-232

Sertoli A, Francalanci S, Giorgini S (1994) Prevention of allergic contact dermatitis with alternative products. *Contact Dermatitis*. **31**: 322-3

Shahidi F, Janitha PK and Wanasundara PD (1992) Phenolic antioxidants.CRC *Critical Rev. Food Science Nutrition.* **32:** 67-103

Shakil NA, Pankaj, Kumar J, Pandey RK and Saxena DB (2008) Nematicidal prenylated flavanones from Phyllanthus niruri. *Phytochemistry.* **69**(3): 759-764

Sharma P and Shrestha N (2011) Promoting exports of medicinal and aromatic plants (MAPs) and essential oils from Nepal. A report submitted to South Asia Watch on Trade, Economics and Environment (SAWTEE) WTO/EIF Support Programme.

Sharma UR, Malla KB and Uprety RK (2004) Conservation and management efforts of medicinal and aromatic plants in Nepal.*Banko Janakari* **14**(2):3-11

Shi W, Chen L, Heber D and Anderson MH (2011) Compositions useful for the treatment of microbial infections.*US Patent No.* 7,875,598 B2

Shilpa PN, Venkatabalasubramanian S, Devaraj SN (2012a) Ameliorative effect of methanol extract of Rubia cordifolia in N-nitrosodiethylamine-induced hepatocellular carcinoma. *Pharmaceutical Biology*. **50**(3): 376-383.

Shilpa PN, Venkatabalasubramanian S, Devaraj SN (2012b) Induction of apoptosis by methanolic extract of *Rubia Cordifolia* Linn in HEp-2 Cell Line is mediated by reactive oxygen species. *Asian Pacific Journal of Cancer Prevention.***13**: 2753-2758

Shimizu M (1989) Studies on aldose reductase inhibitors from natural products. II. Active components of a Paraguayan crude drug Para-parai mi, *Phyllanthus niruri*. *Chem. Pharm. Bull.* **37** (9): 2531- 2532

Shishodia S and Aggarwal BB (2006) Diosgenin inhibits osteoclastogenesis, invasion and proliferation through the downregulation of Akt, I kappa B kinase activation and NF-kappa B-regulated gene expression. *Oncogene.* **25**(10): 1463-1473

Shrestha K, Rajbhandary S, Tiwari N, Poudel R and and Uprety Y (2004) Ethnobotany in Nepal: Review and perspectives. Ethnobotanical Society of Nepal. 27

Shrestha KK, Sah JP and Ghimire SK (1995) Ecology, Exploitation Trend and Conservation 110 NTFPs: Impetus for Conservation and Livelihood Support in Nepal of Potential High Altitude Medicinal Plants in Gyasumdo Valley, Manang. A report submitted to Annapurna Conservation Area Project (ACAP), King Mahendra Trust for Nature Conservation, Kathmandu, Nepal.

Shrestha KK, Tiwari NN and Ghimire SK (2000) MAPDON- Medicinal and aromatic plant data base of Nepal. In: proceedings of Nepal-japan symposium on conservation and utilization of Himalayan medicinal resources. Department of Plant Resources, Ministry of Forest and Soil Conservation, Government of Nepal, Kathmandu Nepal and society of the Conservation and Development of Himalayan Medicinal Resources, Tokyo, Japan. 53-74

Shrestha PM, Dhillion SS (2003) Medicinal plant diversity and use in the highlands of Dolakha district, Nepal.*J Ethnopharmacol.***86:**81–96

Shrestha TB and Joshi RM (1996) Rare, Endemic and Endangered plants of Nepal.WWF Nepal program, Kathmandu, Nepal

Shu YZ (1998) Recent natural products based drug development: a pharmaceutical industry perspective *J nat product*. **8**(61): 1053-1071

Siddiqui A, tajuddin, Amin KMY, Zuberi RH and Jamal Anwar (2011) Standardization of Majith (Rubia cordifolia Linn).*Res J. Pharm. and Tech.***1**(4): 330-333

Sieradzki K, Wu SW and Tomasz A (1995) Inactivation of the methicillin resistance gene mecA in vancomycin-resistant *Staphylococcus aureus*. *Micro Drug Resist*. **5(**4):253–257

Sieradzki K, Wu SW and Tomasz A (1999) Inactivation of the methicillin resistance gene mecA in vancomycin-resistant Staphylococcus aureus. Micro Drug Resist. **5**(4):253–257

Sigdel SR, Rokaya MB and Timsina B (2013) Plant inventory and ethnobotanical study of Khimti hydropower project. *Scientific World.* **11**(11): 105- 112

Singh R and Geetanjali (2004) Isolation and synthesis of anthraquinones and related compounds of *Rubia cordifolia.J.Serb. Chem. Soc*. **70**(7): 937–942

Singh R, Jain A and Panwar S (2005) Antimicrobial activity of some natural dyes. *Dyes and Pigments*.**66**: 99-102.

Singh SB and Thakur RS (1982) Furostanol saponins from *Paris polyphylla*: Structures of plyphyllin G and H. *Phytochemistry.* **21**(8): 2079-2082

Singh SB, Thakur RS, Schulten HR (1980) Spirostanol saponins from *Paris polyphylla*, structures of polyphyllin C, D, E and F. *Phytochemistry.* **21**(12): 2925-2929

Singh YN (1986) Traditional medicine in Fiji. Some herbal folk cures used by Fiji Indians. *J. Ethnopharmacol.* **15**(1): 57 -88

Sivapriya M, Dinesha R, Harsha R, Gowda SST and Srinivas L (2011) Antibacterial activity of different extracts of sundakai (*Solanum torvum*) fruit coat. *Int. J. Biol. Chem*. **5**: 61-67

Smetanska I (2008) Production of secondary metabolites using plant cell cultures.*Adv. Biochem. Eng. Biotechnol.***111**: 187-228

Sofowora A (1982) Medicinal plants and traditional medicine in Africa. John Wiley, Chichester. 179

Son JK, Jung SJ and Jung JH (2008) Anticancer constituents from the roots of *Rubia cordifolia* L. *Chemical and Pharmaceutical Bulletin*.**56:** 213-216

Sridevi Sivakami PL and Nandhini C (2011) Extraction, isolation of medicinal plant pigments for natural food colours. *Plant Arch*. **11**: 479-84

Srinivasa AKB, Kuruba L, Khan S and Saran GS (2013) Antiurolithiatic Activity of Gokhsuradi Churan, A Ayurvedic Formulation By*In Vitro* Method*. Adv Pharm Bull.* **3**(2): 477-479

Subedi BP (2006) Linking Plant-Based Enterprises and Local Communities to Biodiversity Conservation in Nepal

Subedi BP (2006) Linking Plant-based Enterprises and Local Communities to Biodiversity Conservation in Nepal Himalaya. New Delhi: Adroit Publishers

Sumathi P and Parvathi A (2010) Antimicrobial activity of some traditional medicinal plants.*Journal of Medicinal Plants Research.* **4**(4): 316-321

Sun J, Liu BR, Hu WJ, Yu LX and Qian XP (2007) *In vitro* anticancer activity of aqueous extracts and ethanol extracts of fifteen traditional Chinese medicines on human digestive tumor cell lines. *Phytother*.*Res*.**21**: 1102–1104

Surh YJ (2003) Cancer chemoprevention with dietary phytochemicals.*Natural Reviews in* therapy. *Cancer Res*. **66**:3347–3350.

Syamasundar KV, Singh B, Thakur RS, Husain A, Kiso Y and Hikino H (1985) Antihepatotoxic principles of Phyllanthus niruri herbs. *J Ethnopharmacol.* **14**(1): 41-44

Tabassum S, Mahmood S, Hanif J, Hina M and Uzair B (2012) An Overview of Medicinal Importance of *Swertia chirayita*. *Int J Appl Sci Technol*. **2**(1): 298-304

Talapatra SK, Sarkar AC, Talapatra B (1981) Two pentacyclic triterpenes from *Rubia cordifolia*. *Phytochemistry*. **20**(8): 1923-1927

Tan PY (2009) Optimization of extraction conditions for phenolic compounds from Dukung Anak (*Phyllanthus niruri*) by response surface methodology. Thesis: Malaysia: UCSI University

Taylor RSL, Hudson JD, Manandhar NP andTowers GHN (1996a) Antiviral activities of medicinal plantsof Nepal.*J Ethnopharmacol.***52:**157-163

Taylor RSL, Hudson JD, Manandhar NP andTowers GHN (1996b) Antiviral activities of medicinal plantsof southern Nepal.*J Ethnopharmacol.***53:** 97-104

TeissedrePL and LandraultN (2000) Wine phenolics: contribution to dietary intake and bioavailability. *Food Res Int.***33**: 461–467

TEPC (2009) *Nepal Overseas Trade Statistics,* Kathmandu: Trade Promotion and Export Centre- Nepal Trade and Export Promotion Centre

Thaipong K, Boonprakob U, Crosby K, Cisneros-ZevallosL and Hawkins Byrne (2006)Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J.Food Comp. Anal*. **19:** 669-675

Thakur CP, Kanyok TP, Pandey AK, Sinha GP, Zaniewski AE, Houlihan HH and Olliaro P (2000) A prospective randomized, comparative, open-label trial of the safety and efficacy of paromomycin (aminosidine) plus sodium stibogluconate versus sodium stibogluconate alone for the treatment of visceral leishmaniasis. *Trans R Soc Trop Med Hyg.* **94**(4): 429-431

Thomas J, Townsend JH, Hackney S and Strlic M (2010)A chemiluminescence study of madder lakes on paper. *Polymer Degradation and Stability*.**95:** 2343-2349

Thyagarajan SP, Subremanian S, Thirunalasundari T, Venkateswaran PS and Blumberg BS (1998) Effect of *Phyllanthus amàrus* on chronic carriers of hepatitis B virus. *Lancet*.**2**: 764-766.

Tian Y, Zheng LH, Xu ZY, Sun LQ, Gao CK, Zheng QZ, Zhang ZH and Shu Y (1986) Clinical and pharmacological study of the hemostatic action of *Rhizoma Paridis* by contraction of uterus. *J Tradit Chin Med.* **6**(3): 178-182

Tilbert JC and Kaptchuk TJ (2008) Herbal medicine research and global health, an ethical analysis.*Bulletin of the World Health Organization*.**86**: 594-599

Tiwari NN, Poudel RC and Uprety Y (2004) Study on Domestic Market of Medicinal and Aromatic Plants (MAPs) in Kathmandu Valley. Kathmandu, Nepal: Winrock International. 46.

Tiwari P, Kumar B, Kumar M, Kaur M, Debnath J and Sharma P (2011).Comparative study of antihelmintic activity of aqueous and ethanolic stem exract of *Tinospora cordifolia. Int J Drug Dev Res*. **3**(1):70-83

Tiwari S (2004) National register of medicinal and aromatic plants, IUCN Nepal; Nepal, Ministry of Forests and Soil conservation.202

Todkar SS, Chavan VV. Kulkarni AS (2010) Screening of Secondary Metabolites and Antibacterial Activity of Acacia concinna. *Res J Microbiol*. **5**(10): 974-979

Tona L, Mesia K, Ngimbi NP, Chrimwami B, Cimanga K, de Bruyne T, Apers S, Hermans N, Totte J, Pieters L and Vlietinck AJ (2001) In-vivo antimalarial activity of *Cassia occidentalis*, *Morinda morindoides* and *Phyllanthus niruri*. *Ann Trop Med Parasitol.* **95**(1): 47-57

Tripathi Y, Pandey S and Shukda SD (1993) Anti-platelet activating factor property of *Rubia cordifolia. Ind. J. Exp. Biol*. **31:** 533–535.

Tripathi YB and Sharma M (1998) Comparison of antioxidant action of the alcoholic extract of *R. cordifolia* with Rubiadin. Indian J Biochemistry and Biophysics **35**: 313-316

Tripathi YB, and Singh AV (2007) Role of *Rubia cordifolia* Linn.in radiation protection*. Indian Journal of Experimental Biology*.**45**: 620-625

Trivedi PC (2006) Medicinal Plants, Traditional Knowledge. I. K.International Publishing House Pvt. Ltd., New Delhi

Tse WP, Che CT, Liu K and Lin ZX (2006) Evaluation of the antiproliferative properties of selected psoriasis treating Chinese medicines on cultured HaCaT cells.*J Ethnopharmacol.***108**:133-141

Unander DW (1998) Phyllanthus species: in vitro culture and production of secondary metabolites.In Biotechnology in agriculture and forestry (ed.Y.P.S.Bajaj), Springer-Verlag, Berlin. **37**: 304-318

Upadhyay N, Ganie SA, Agnihotri RK and Sharma R (2013) Studies on Antioxidant Activity and Total Phenolic Content of Tinospora cordifolia (Miers.) Stem Using in Vitro Models. *Am J Phytomed Clin Therap.* **1**(8): 617-627

Venkateswaran SP, Millman I and Blumberg BS (1987) Effects of an extract from Phyllanthus niruri on hepatitis B and woodchuck hepatitis virus: in vitro and in vivo studies. Proceedings of the National Academy of Sciences, USA. **84**(1): 274-288

Wang MX, Cheng HW, Li YJ, Meng Lm and Malik (1994) Efficacy of Phyllanthus species in treating patients with chronic Hepatitis B. *Zhongguo Zhong Yaoza Zhi*. **19**(12): 750-764

Wang Q, Xu GJ and Jiang Y (1990) Analgesic and sedative effects of Chinese drug *Rhizoma Paridis*. *China J Chin Mater Med.* **15**(2): 45-47

Wang T, Liu Z, Li J, Zhong M, Li J, Chen X and Bi K (2007) Determination of protodioscin in rat plasma by liquid chromatography-tandem mass spectrometry. *J Chromatogr B.* **848**(2): 363-368

Wang Y, Zhang Y, Zhu Z, Zhu S, Li Y, Li M and Yu B (2007) Exploration of the correlation between the structure, hemolytic activity and cytotoxicity of steroid saponins. *Bioorg Med Chem.* **15**(7): 2528-2532

Wei W, Li X, Wang K, Zheng Z and Zhou M (2012) Lignans with anti-hepatitis B virus activities from Phyllanthus niruri L. *Phytother Res.* **26**(7): 964-968

Weninger B and Haag-Berrurier M (1982) Plants of Haiti used as antifertility agents. *J. Ethnopharmacol.* **6**(1): 67-84

WHO: WHO traditional medicine strategy 2002-2005. Geneva; 2002

Wong BY, Tan CP and Ho CW (2013) Effect of solid-to-solvent ratio on phenolic content and antioxidant capacities of "*Dukung Anak*" (*Phyllanthus niruri*). *Int Food Res J*. **20**(1): 325-330

Wong SK, Lim YY and Chan EWC (2009) Antioxidant properties of Hibiscus: Species variation, altitudinal change, coastal influence and floral colour change. *J Trop Forest Sc*i.**21:** 307-315.

Wu LJ, Wang SX, Hua HM, Li X, Zhu TR, Miyase T, and Ueno A (1991) 6-methoxygeniposidic acid, an iridoid glycoside from Rubia cordifolia. *Phytochemistry*. **30**(5): 1710-1711.

Wu SS, Gao WY, Duan HQ and Jia W (2004) Advances in studies on chemical constituents and pharmacological activities of *Rhizoma Paridis*. *Chin Tradit Herb Drugs* **35**(3): 344-347

Xu HX, Zeng FQ, Wan M and Sim KY (1996) Anti-HIV triterpene acids from Geum japonicum. *J Nat Prod*. **59**(7):643-645

Yan F, Zhang Q, Jiao L, Han T, Zhang H, Qin L and Khalid R (2009b)Synergistic hepatoprotective effect of Schisandrae lignans with Astragalus polysaccharides on chronic liver in jury in rats. *Phytomedicine*.**16:** 805–813

Yan LL, Zhang YJ, Gao WY, Man SL and Wang Y (2009a) *In vitro* and *in vivo* anticancer activity of steroid saponins of *Paris polyphylla* var. *yunnanensis*. *Exp Oncol* **31**(1): 27-32

Yang H, Ding C, Duan Y and Liu J (2005) Variation of active constituents of an important Tibet folk medicine *Swertia mussotii* Franch.(Gentianaceae) between artificially cultivated and naturally distributed.*J Ethnopharmacol.*98: 31-35

Yusuf M, Shahid M, Khan MI, Khan SA, Khan MA and Mohammad F (2012) Dying studies with henna and madder: A research on effect of tin(II) chloride mordant. *J Saudi chemical society*

Zhang L, Ravipati AS, Koyyalamudi SR, Jeong SC, Reddy N and Smith PT (2011) Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. *J Agric Food Chemistry*. **59**(23): 12361-12367

Zhang L, Ravipati AS, Koyyalamudi SR, Jeong SC, Reddy N, Bartlett J, Smith PT, Cruz M, Monteriro MC, Melguizo A, Jimenez E and Vicente F (2013) Anti-fungal and anti-bacterial activities of ethanol extracts of selected traditional Chinese medicinal herbs. *Asian Pac J Trop Med*. 673-681

Zhang XF, Cui Y, Huang JJ, Zhang YZ, Nie Z, Wang LF, Yan BZ, Tang YL and Liu Y (2007) Immuno-stimulating properties of diosgenyl saponins isolated from *Paris polyphylla*. *Bioorg Med Chem Lett.* **17**(9): 2408-2413

Zhao L and Shi Q (2005) Analysis on the therapeutic effect on Colporrhagia due to drug abortion (240 cases) treated by Gongxuening. J Pract Tradit Chin Med. **21:** 455– 456.

Zhou J (1991) Bioactive glycosides from Chinese medicines. *Mem Inst Oswaldo Cruz*. **86**(2):231–234.

Zhou LL, Lin ZX, Fung KP, Che CT, Zhao M, Cheng CHK and Zuo Z (2012) Ethyl acetate fraction of Radix rubiae inhibits cell growth and promotes terminal differentiation in cultured human keratinocytes. *J Ethnopharmacol*.**142**: 241-247

Zhu L, Tan J, Wang B, Guan L, Liu Y and Zheng C (2011) *In-vitro* Antitumor Activity and Antifungal Activity of Pennogenin Steroidal Saponins from *Paris Polyphylla* var. *yunnanensis.*Iran J Pharmaceut Res. 10 (2): 279-286

Zu Y, Yu H, Lu L, Fu Y, Efferth T, Liu X and Nan Wu (2010) Activities of Ten Essential Oils towards Propionibacterium acne and PC-3, A-549 and MCF-7 Cancer Cells.**15:** 3200-3210

APPENDIX - A

(List of Reagents and Culture media)

1. **Mayer's Reagent:**
   1.358g of mercuric chloride was dissolved in 60ml of water. Similarly 5.0g of potassium iodide was dissolved in 10ml of water separately. The two solutions were mixed up to the final volume 100ml

2. **Wagner's reagent:**2g of iodine and 6g of Potassium iodide was dissolved in 100ml water

3. **Preparation of 1 M Na2CO3 -100 ml**
   10.599 gram of the Na2CO3 (Merk Specialities Pvt. Ltd, Mumbai, India) was carefully weighed and then dissolved in distilled water, and the volume was adjusted to 100 ml at the end.

4. **Preparation of Glacial acetic acid (20%) - 200 ml**
   40 ml of the commercially supplied glacial acetic acid (Thermo Fisher Scientific, India) was taken and mixed with ethanol. Finally the volume was adjusted to 200 ml by the addition of ethanol.

5. **Preparationof Aluminium Chloride (10%) -100 ml**
   10 gram of the commercially supplied aluminium chloride (Merk Specialities Pvt. Ltd, Mumbai, India) was weighed and dissolved in water. Finally the volume was maintained to 100 ml.

6. **Preparation of 1M potassium acetate (CH3COOK) – 100 ml**
   Weigh 9.814 gram of the potassium acetate Merk Specialities Pvt. Ltd, Mumbai, India) and dissolve on water. Fine ally maintain the volume to 100 ml by the addition of water.

7. **Preparation of 0.2mM DPPH solution - 100 ml**
   1, 1- diphenyl-2 picryhydrazyl (DPPH) has the molecular weight of 394.32 gm/mol. Thus, 100 ml of 0.2mM solution of DPPH is prepared by weighing the 7.886 mg of the DPPH carefully and dissolving it on ethanol and finally maintaining the volume to 100 ml by addition of ethanol.

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

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

9. Compositionof Nutrient agar media

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

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	7.4 ± 0.2

10. Compositionof Luria Bertani broth, (LB) Miller media

The composition of Luria Bertani broth, (LB) Miller media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

Components	**gram/L**
Casein enzyme hydrolysate	10
Yeast extract	5.0
Sodium chloride	10.0
Final PH	7.5± 0.2

11. Composition of Mueller Hinton Agar (MHA)

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
Final PH	7.3 ± 0.2

12. Composition of Yeast Extract Peptone Dextrose (YEPD) broth

The composition of YEPD broth (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

Components	**gram/L**
Yeast Extract	10
Peptone	20
Dextrose/Glucose	20

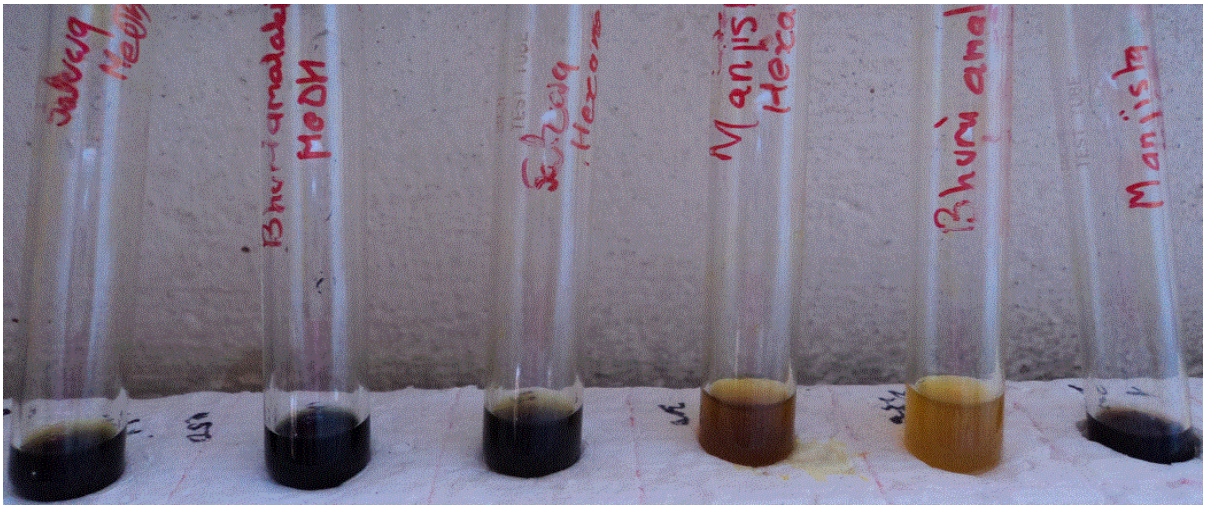
13. Composition of Potato Dextrose Agar (PDA)

The composition of PDA broth (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

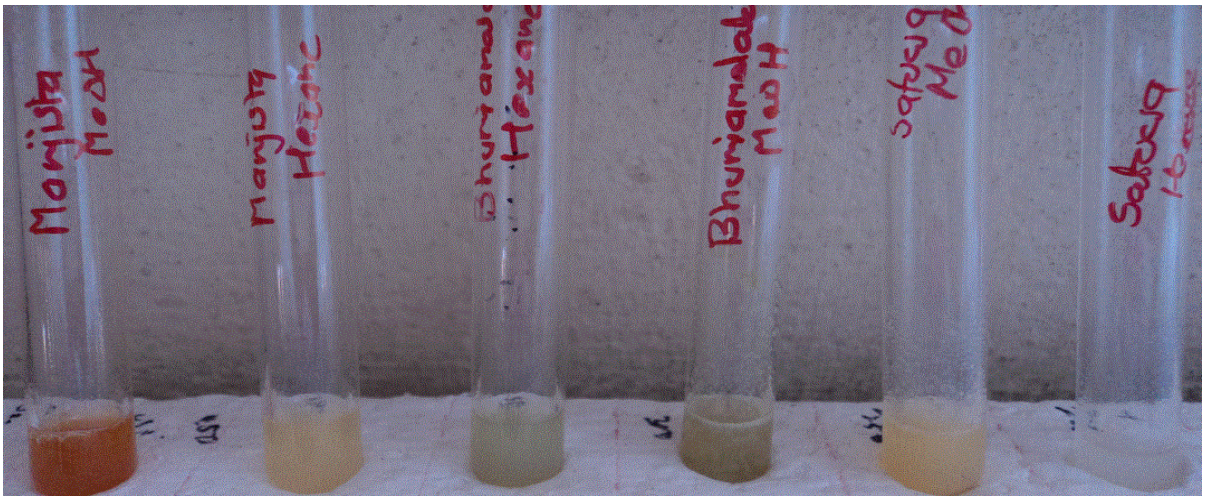
Components	**gram/L**
Potato	200
Agar	2
Dextrose/Glucose	20

APPENDIX - B

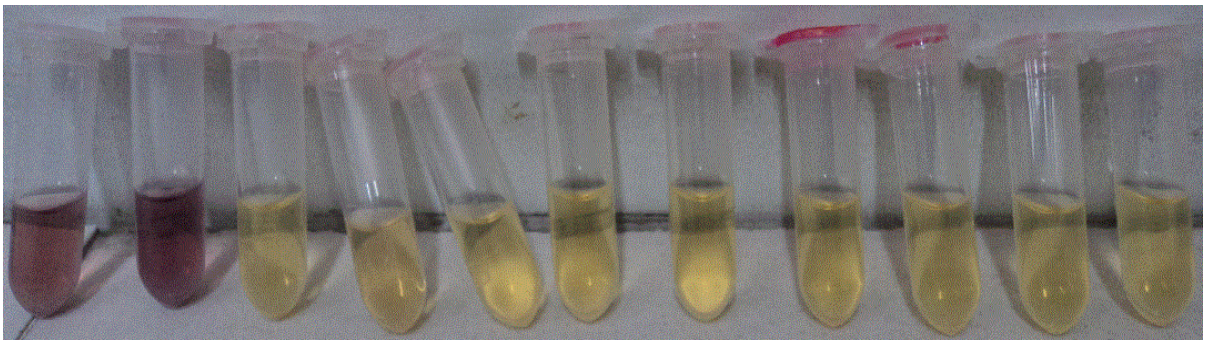
(**Photographs**)



Photograph 1: Preliminary test of Phenol in crude extracts of the plants

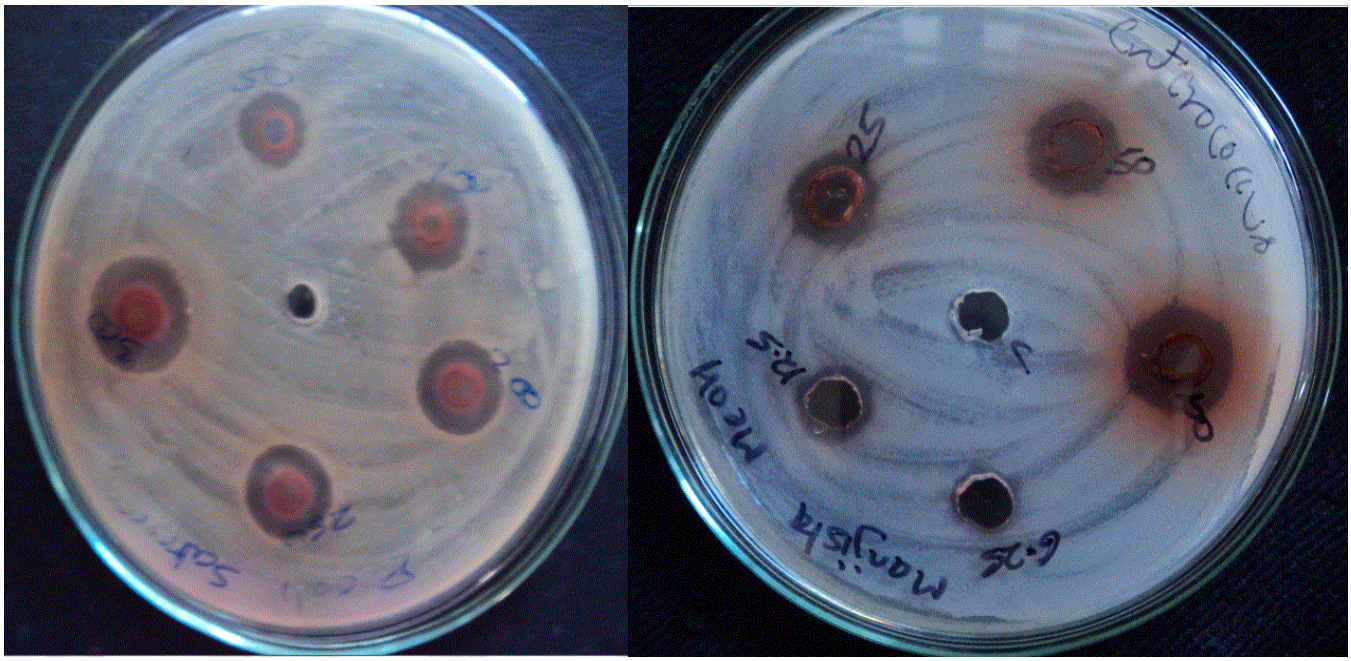


Photograph 2: Preliminary test of Tannins in both extracts of the plants

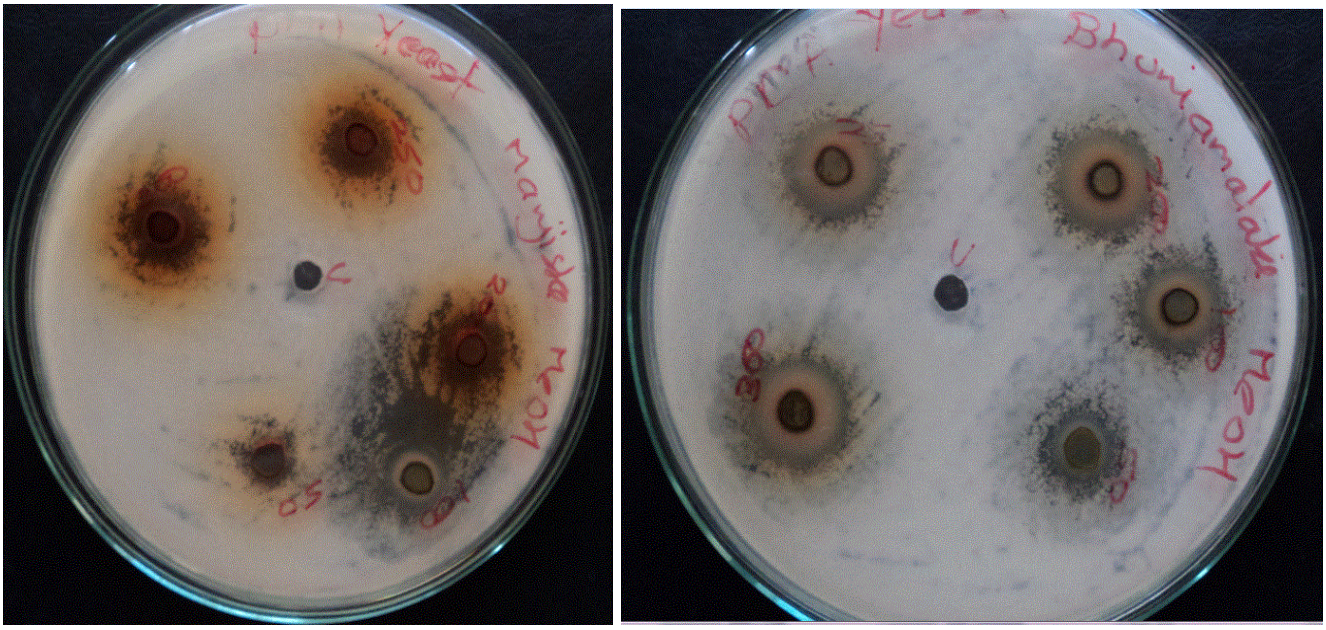


Photograph 3: DPPH based Antioxidant activity of *P. niruri* methanol extract

a b



Photograph 4a: Antifungal susceptibility test of *P. polyphylla* MeOH extract against *P. pastoris*
4b: Antibacterial susceptibility test of *R. cordifolia* MeOH extract against *E. faecalis*



Photograph 5: Antifungal susceptibility test of *R. cordifolia and P. niruri* MeOH against *S. cerevesiae*