PHYTOCHEMICAL ANALYSIS AND INVESTIGATION OF ANTIOXIDANT, ANTIMICROBIAL AND ANTICANCEROUS PROPERTIES OF SIX MEDICINAL PLANTS OF NEPAL

M.Sc. Thesis

(2013)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement for the

Master of Science in Biotechnology

Usha Adhikari

T.U. Regd. No. . 5-2-37-674-2006

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Acknowledgement

At the foremost, I express my sincere heartfelt gratitude with deep appreciation to my supervisors, Prof. Dr. Tribikram Bhattarai, Central Department of Biotechnology and Dr. Smita Shretha, Central Department of Biotechnology TU who bestowed upon me their immense knowledge, motivation and patience throughout the work. Whatever I have done in this research is due to their generous guidance and assistance and I am always thankful to them. I feel blessed to be one of their students.

I owe my profound gratitude to Dr. Rajani Malla, Head of Department, for her valuable advices and constant support. I am also grateful to Dr. Deepak Raj Pant who guided me to identify and collect my sample plants. My heartfelt regards to the respected faculty members- Prof. Dr. Mohan Kharel, Prof. Dr. Krishna Das Manandhar, Dr. Ganga Kharel, Dr. Sampoornanand Jha, Mrs. Jarina Joshi, and Mr. Balhari Poudel. I would also like to remember Late. Mr. Janardan Pandey on this occasion.

My special thanks to Dr. Lakshmaiah Sreerama, St. Cloud University, USA for his words of wisdom. I am also thankful to Dr. Pramod Aryal for his motivation. I would also like to sincerely appreciate and remember the kind help of Dr. Kanti Shrestha, Senior Scientific Officer, Natural Products Research Lab, NAST for providing the rotatory evaporater in her esteemed laboratory. My heartfelt thanks to the NAST family and also Meena Kusi for helping me with the same and providing the Bacterial samples.

Among all, my special thanks goes to Sunita Karki for being there always throughout my research work and Bivek Mathema for his immense support to re-establish cell culture laboratory in our Department.

A special mention of my dear friends- Rajiv Panthi, Jyoti Bhuju, Prabin Shrestha, Sunita Khanal, Bishnu Joshi, Prashanna Maharjan, Nabin Munankarmi, Pratap Khadka, Kushal Shrestha, Santosh Panthi, Nawaneet Mishra, Janardan Khadka, Surendra Raj Sharma . Thank you guys for helping me throughout. Also a heartfelt thanks to all my friends and members of staff at the Central Department of Biotechnology. I owe a special word of thanks to all the lab members, Mr. Mohan Bahadur Shrestha, Mrs Elen Pradhan, Ms Binu Tamang, Mr. Radheshyam Sharma , Subha didi and all teaching and non-teaching staffs of Central Department of Bio-technology for providing me solicitous help during the completion of lab work

Last but not the least; I would like to thank my beloved family for all their love, unconditioned encouragement and support with a special mention to my Father Madhusudan Adhikari. It's for you people that I have been able to be what I am today. Thank you all.

Usha Adhikari

Acronyms

ABTS	2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid)
ATCC	American Type Culture collection
ATP	Adenosine triphosphate
CAT	Catalase
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Effective concentration of extracts to scavange 50% of free radicals
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
F-C	Folin Ciocalteu
FDA	Food and Drug Administration
GAE	Gallic Acid Equivalent
GPx	Glutathione peroxidase
GRx	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	Inhibitory Concentration of drug for killing 50% cells
MCF-7	Michigan Cancer Foundation-7
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration

MRSA	Methicillin Resistant *Staphylococcus aureus*
NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
NCI	National Cancer Institute
NO	Nitric Oxide
NSAIDs	Non steroidal anti inflammatory drugs
PCR	Polymerase chain reaction
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RSA	Radical Scavenging Activity
RT	Room Temperature
SI	Selectivity index
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
TLC	Thin layer Chromatography
TNF	Tumor Necrosis Factor
TPC	Total Polyphenol content
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
ZOI	Zone of Inhibition

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ABSTRACT

This study investigates the medicinal property of some popularly used medicinal plants of Nepal by evaluating their antioxidant, antibacterial and anticancer properties. Six plants namely, *Calotropis procera, Clematis montana, Nardostychs grandiflora, Semecarpus anacardium, Terminalia chebula*, and *Withania somnifera* were selected on the basis of their reported traditional use in the treatment of various ailments. Preliminary phytochemical screening of water and methanol extracts of the plants showed the presence of phytochemicals like alkaloid, steroids, tannins, saponins, polyphenols and terpinoids reducing sugars and proteins. More than 80% of extracts were found to contain high amount of polyphenol and flavonoid. The best antioxidant property was observed in the extract of *Terminalia chebula* comparable to that of standard compounds, Ascorbic acid and Gallic acid. During antibacterial screening all the methanolic extracts and some of the aqueous extracts were found to be effective against the strain of *Staphylococcus aureus* (gram positive) and P*seudomonas aeruginosa* (gram negative). The methanolic extracts of *T. chebula, S. anacardium* and *W. somnifera* and aqueous extract of *T. chebula* showed exciting antiproliferative activity against HeLa cell with IC_{50} value, 30.34±1.44, 43.79±2.88, 93.05±3.88, 34.77±0.63 µg/ml respectively. Cytotoxicity tests on mice peritoneal macrophage revealed the safety of the extracts towards normal cell with selectivity indices greater than 1. The overall comparison of aqueous and methanolic extracts in all assay revealed that the methanolic extracts showed significantly greater activity over aqueous extract as methanolic extracts were found to contain higher polyphenols and flavonoid and other bioactive compounds than that of the aqueous extracts.

Key words: Medicinal plants, ethnomedical, antioxidant, Cytotoxicity, IC_{50}, extrcation, Nepal

Chapter I

Introduction

1.1 General Introduction

Plants are the most important sources of food and medicine since the dawn of human civilization. Until the middle of the 19th century, plants were the main therapeutic agents used by humans, and this herbal medicine is still the foundation of about 75-80% of the whole population, mainly in developing countries, for primary health care. These days, the investigation on medicinal properties of the plants has been rapidly increasing throughout the world due to their potent pharmacological activities, low toxicity and economic viability.

The type of plant and its medical applications depends on the region they are found and traditional Practice introduced by local/ indigenous people. According to one estimate 20,000 to 35,000 species of plants are used as medicines, pharmaceuticals, cosmetics and neutraceuticals by different ethnic group entire the world (Trivedi, 2006). Approximately 700 species have been researched pharmacologically and chemically (Malik *et al*., 2012)

Around 88% of Nepalese population is dependent on traditional plant based medicines (Kunwar *et al*., 2006). Traditional medicine in Nepal comprises those practices based on beliefs that were in existence often for hundreds to thousands of years before the development and spread of modern medicine, and which are still in use today. They are found throughout Nepal, from the plains to the high Himalayas, with the greatest concentration in the cold and arid zones. Nepal recognizes about 1624 plant species as having medicinal and aromatic values, which is high compared to global scenario. A tentative list of the alpine flora of the Nepal Himalaya consists of 1227 species in 317 genera and among them 114 and 45 are medicinal plants respectively from the subalpine and alpine (Kunwar *et al*., 2006). Upto 50% of the Nepal's rural household's income is derived from commercial collection of medicinal and aromatic plants (Kunwar and Bussmann, 2008).

In this modern era of great scientific development, medicinal plants are being exploited as sources of new types of lead molecules for biologic activity. The naturally occurring molecule acts as a template from which novel compounds with improved activity, therapeutic index, or reduced unwanted effects can be designed.

1.2 Free Radicals and Reactive Oxygen

Free radical may defined as a molecule or molecular fragments containing one or more unpaired electrons in its outermost atomic or molecular orbital and are capable of independent existence. It includes reactive oxygen species (ROS) and reactive nitrogen species (RNS) and other non-radical reactive derivatives. They are formed from molecules by the homolytic cleavage of a chemical bond and via redox reactions. The free radicals include hydroxyl (OH·), superoxide ($O_2\cdot^-$), nitric oxide (NO·), nitrogen dioxide ($NO_2\cdot$), peroxyl (ROO·) and lipid peroxyl (LOO·) radicals. Once formed, these highly reactive radicals can start a chain reaction (Bahorun *et. al*., 2006). In addition to these molecules, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH), lead to free radical reactions in living organisms and are called oxidants. A prominent feature of radicals is that they have extremely high chemical reactivity and easily react with various organic substrates such as lipids, proteins and nucleic acids.

ROS and RNS are generated in body by two sources: endogenous or exogenous. Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, aging. While, exogenous ROS/RNS result from air and water pollution, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat), radiation (Valko *et al*., 2007). After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals.

At low or moderate concentrations, ROS and RNS are beneficial to human health. They are necessary for the maturation process of cellular structures and have an important role in host defense system. For example, Phagocytes (neutrophils, macrophages, monocytes) release free radicals to destroy invading pathogenic microbes as part of the body's defense mechanism against disease (Young and Woodside, 2001). They are also involved in number of cellular signaling systems. However, when the free radicals and oxidants are generated in excess or not appropriately controlled, it causes a phenomenon in the body called Oxidative stress. This condition can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA). For example, hydroxyl radical and peroxy nitrite in excess can damage cell membranes and lipoproteins by a process called lipid peroxidation. Studies of chain reactions in purified chemical systems show that a single initiation event can oxidatively damage 200 to 400 lipid molecules before two radicals react to eliminate the unpaired electrons and terminate their action sequence (Jones, 2008). Proteins may also be damaged by ROS/RNS, resulting in structural changes and loss of enzyme activity

(Young and Woodside, 2001). Similarly, Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can cause mutations. Overall, the oxidative stress plays a major role in the development of chronic and degenerative illness such as cancer, autoimmune disorders, aging, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative disorders.

1.3 Antioxidants

An organism has developed an efficient defence system to prevent or to lessen the potential damaging action of ROS (Gutteridge and Halliwell, 2000). This is a complex defence system, made up of various sorts of antioxidants which include a diverse series of substances such as enzymes, vitamins, vitamin-like substance, trace elements, metal-binding proteins and various kinds of repair systems, all with the capacity to cause hinderance or repair the damaging effects of ROS. Broadly, it is categorized into two types; Endogenous and exogenous antioxidants. Endogenous antioxidants are naturally generated in the body and can be classified as enzymatic and non-enzymatic antioxidants. The Enzymatic antioxidants are the enzymes produced in the body, directly involved in the neutralization of ROS and RNS. These include; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). Among these antioxidants, SOD acts as the first line of defense against free radicals, catalyzes the dismutation of superoxide anion radical ($O2\cdot^{-}$) into hydrogen peroxide (H_2O_2),) by reduction (Kohen and Nyska, 2002). The oxidant formed (H_2O_2),) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG) (Martindale and Holbrook, 2002). Glutathione reductase, a favoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. GSH is a tripeptide and a powerful antioxidant present within the cytosol of cells and is the major intracellular nonprotein thiol compound (NPSH). GSH is also capable of scavenging ROS directly or enzymatically via GPx (Droge, 2003).

Non enzymatic antioxidants are the endogenous antioxidants, produced by metabolism in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferring, (Vitamin E and C existing within normal cells) etc.

Exogenous /Nutrient antioxidants are the antioxidant molecules which cannot be produced in the body and must be provided through foods or supplements, which include phytoconstituents and phytonutrients such as vitamin E, vitamin C, carotenoids, trace metals (Selenium, Manganese, Zinc), favonoids, omega-3 and omega-6 fatty acids, etc (Benzie , 2003).

1.4 The Antioxidant process

Antioxidants may exert their activity by several mechanisms. Some of them are: by suppressing the production of active species by reducing hydrogen peroxides, stabilizing transition metal radicals such as copper and iron, termination of chain reaction by scavenging active free radicals and synthesis of other antioxidants or defense enzymes.

1.5 Phytochemicals as Antioxidant

In the modern era of scientific development, many synthetic drugs have been discovered to counteract the deleterious effect of oxidative stress. Although they have been synthesized for the speedy recovery, the chronic administration of such drug lead to more deleterious condition due to the side effect/ incompatibility with the human body. Some synthetic drugs are even capable of producing more reactive radicals. For example, non steroidal anti inflammatory drugs (NSAIDs) are used widely in the treatment of pain, fever, inflammation, rheumatic and cardiovascular disease but chronic use of such drugs causes the generation of free radicals which may results gastric erosions, gastritis or duodenal ulceration and severe complications such as gastrointestinal hemorrhage and perforation (Kamboj, 2000). The plant extract contains variety of organic compounds which provide definite physiological action on human body and these include tannins, alkaloids, terpenoids, steroids and flavonoids, volatile and essential oil, anthocyanins, phenolic acids, hydroxycinnamic acids etc, which are found effective as radical scavengers and inhibitors of lipid peroxidation (Yildirim *et al*., 2001). These chemical constituents present in the herbal medicine or plant are a part of the physiological functions of living flora and hence they are believed to have better compatibility with human body. Among the isolated bioactive compounds, phenols and flavonoids, the major class of secondary metabolites, are proven to be most effective as antioxidants. Recently, these compounds have been proven to be more effective than Vitamin C, E and carotenoids (Dai and Mumper, 2010). The antioxidant activity of these compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans *et al*., 1995). Hence on comparision to the synthetic antioxidants, the phytomedicine has been proved to be safe with better tolerance, relatively less expensive and globally competitive.

1.6 Natural product and Drug Discovery

During the early period when there were no any synthetic drugs, natural products used to be the sole means to treat diseases and injuries. Our ancestors used to chew certain herbs to relieve pain, or wrap certain leaves around wounds to improve healing. Now, by the use of various scientific techniques, humankind has utilized this knowledge in the discovery of an enormous range of natural compounds as a basis for drug development.

Molecular biology and combinatorial chemistry has made possible for the rational design of chemical compounds to target specific molecules. The modern tools of chemistry and biology—in particular, the various 'omics' technologies has allowed scientists to explain the exact nature of the biological effects of natural compounds on the human body, as well as to uncover possible synergies, which holds much promise for the development of new therapies against many devastating diseases, including cancer. Modern analytical and structural chemistry have provided the tools to extract and purify various compounds from the natural product and to determine their structures, which, in turn, has given insights into their action on the human body. The structural analysis of natural compounds and the ability to synthesize them has also allowed chemists to modify them in order to suppress or enhance certain characteristics such as solubility, efficiency or stability in the human body (Newman, 2008).

In 1805, the German pharmacist Friedrich Wilhelm Sertürner (1783–1841) isolated morphine from opium, and it became both the first pure naturally derived medicine and the first to be commercialized, by Merck in 1826. The twentieth century welcomed the antibacterial compound, penicillin, derived from the mold *Penicillium notatum*, which was soon followed by various other anti bacterial compounds that gave physicians an enormously powerful weapon in their battle against infectious diseases.

1.7 Cancer

Cancer has become the major threat across the World and as it is the leading cause of death in economically developed countries and second leading cause of deaths in developing countries (Jemal *et al.,* 2011). Worldwide about 10 million people per year are diagnosed with cancer and more than 6 million die of the disease and over 22 million people in the world are cancer patients (Kaur *et al*., 2011). Another study showed that in 2000, there were 10.4 million new cancer cases and it is expected that this number will be doubled in 2030 (Global cancer control, 2008). Cancer is considered to be one of the most intractable diseases because of the innate characteristics of cancer cells to proliferate uncontrollably, avoid apoptosis, invade and metastasize (Hiss *et al*., 2009). Recent studies have shown strong evidence that biological reactive oxygen species (ROS) such as hydroxyl radicals (OH·) and the superoxide anion ($O2\cdot^{-}$) are involved in the development of cancer. The role of ROS in the pathogenesis of cancer is that these species react with DNA resulting in cell malfunction. Ultimately it can lead to malignancies by accumulation of somatic mutations (Hursting *et al*., 1999). Treatment usually consists of various combinations of surgery, radiation therapy, and chemotherapy but despite these therapeutic options, cancer remains associated with high mortality and becoming the major threat of world. Multi disciplinary scientific investigations are making best efforts to combat this disease, but the efforts are still

insufficient to have control over it and also, the side effects of synthetic drugs is another hurdle associated with this disease. Recently, a greater emphasis has been given towards the researches on complementary and alternative medicine that deals with cancer management. For example, Ayurveda, a traditional medicine of plant drugs, that has been successful from very early times to prevent or suppress various tumors using various lines of treatment (Balachandran and Govindarajan. 2005). Many herbs have been evaluated in clinical studies and are currently being investigated phytochemically to understand their tumoricidal actions against various cancers.

The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. These discoveries prompted the United States National Cancer Institute (NCI) to initiate an extensive plant collection program in 1960 focused mainly in temperate regions. This led to the discovery of many novel chemotypes showing a range of cytotoxic activities, including the taxanes and camptothecins (Cassady and Douros, 1980). Natural products or natural product derivatives comprised 14 of the top 35 drugs in 2000 based on worldwide sales (Butlet, 2004). Two plant derived natural products, paclitaxel and camptothecin were estimated to account for nearly one-third of the global anticancer market or about $3 billion of $9 billion in total annually in 2002 (Oberlines and Kroll, 2004). Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation.

1.8 Plants as Antimicrobial Agent

The antimicrobial qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity, for example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections, use of garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) in more than one type of infection (Heinrich et al., 2004) has been practiced before the people know about the existence of microorganisms. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological test, and a substantial number of new antibiotics introduced on the market are obtained from natural or semisynthetic resources. It has been reported that between the years 1983 and 1994, of 93 new antibacterial agents submitted to analysis by the FDA six were natural products (teicoplanin, mupirocin, miokamycin, carumonam, isepamicin, and RV-11), 45 were semisynthetic products modeled on a natural product lead, and 7 antivirals were synthetic compounds based on natural product models (Cragg and Newman, 1997).

Most of the current antibiotics have considerable limitations in terms of antimicrobial spectrum, side effects, and their over prescription and misuse and has led to increasing

clinical resistance of previously sensitive microorganisms and to the occurrence of uncommon infections (Assob *et al*., 2011). Also, the problem posed by the high cost of these synthetic drugs coupled with their inadequacy in diseases treatment especially in the developing countries has demanded for natural antimicrobial therapeutics as an alternate. As a result, extensive researches have been carried out worldwide these days on the identification and isolation of effective compounds from the plant sources.

Medicinal properties of plants can be attributed to secondary metabolites (Hartmann, 2008). Plant produces high number of secondary metabolites with therapeutic potential either alone or in combination. Antibacterial secondary metabolites are usually classified in three large molecule families: phenolics, terpenes and alkaloids. The phenolics and polyphenols are one of the largest groups of secondary metabolites that have exhibited antimicrobial activity. Important subclasses in this group of compounds include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins (Cragg and Newman, 1997).

Phenols are a class of chemical compounds consisting of a hydroxyl functional group (-OH) attached to an aromatic phenolic group. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Cowan, 1999). Quinones have aromatic rings with two ketone substitutions which have a potential to form irreversible complex with nucleophilic amino acids in proteins (Stern et al., 1996). This could explain their antibacterial properties. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes. Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also phenolic substances composed of a common phenylchromanone structure (C6-C3- C6) with one or more hydroxyl substituents, including derivatives (Birt *et al.,* 2001). Flavones, flavonoids and flavonols have been found, to be effective antimicrobial substances against a wide array of microorganisms in vitro, since they have been known to be synthesized by plants in response to microbial infections (Dixon et al., 1983). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. Lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al., 1996). Tannins are the group of polymeric phenolic substances, found in almost every plant part: bark, wood, leaves, fruits and roots. They are divided into two groups, hydrolyzable and condensed tannins. In plant tissue, tannin have been synthesized and accumulated after microbial attack. Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins, because of a property known as astringency. Coumarins are benzo-α-pyrones and could be categorised as: simple coumarins and cyclic

coumarins (furanocoumarins and pyranocoumarins) (Ojala, 2001). Coumarins have been found to stimulate macrophages, which could have an indirect negative effect on infections (Cowan, 1999).

Terpenes are a large and varied class of organic compounds built up from isoprene subunits, while the terpenoids are oxygen-containing analogues of the terpenes. According to number of isoprene subunits, there are monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) and polyterpenes (Kovacevic, 2004). Monoterpenes, diterpenes and sesquiterpenes are the primary constituents of the essential oils. The mechanism of antibacterial action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Cowan, 1999).

Alkaloids, the heterocyclic nitrogen compounds which are derived from amino acids, and the nitrogen gives them alkaline properties. The mechanism of antibacterial action is attributed to their ability to intercalate with DNA, inhibition of enzymes (esterase, DNA-, RNA-polymerase), inhibition of cell respiration (Cowan, 1999).

1.9 Research Hypothesis

- The plants selected in this study have been used for the treatment of various ailments from a long time in traditional medicine, so they should contain pharmacologically active compounds and could be a safer alternate for the modern synthetic medicine in the treatment of life threatening disease like cancer.
- The claimed medicinal property of these plants must be based on their phytochemical content, most importantly polyphenols and flavonoids.

1.10 Work plan

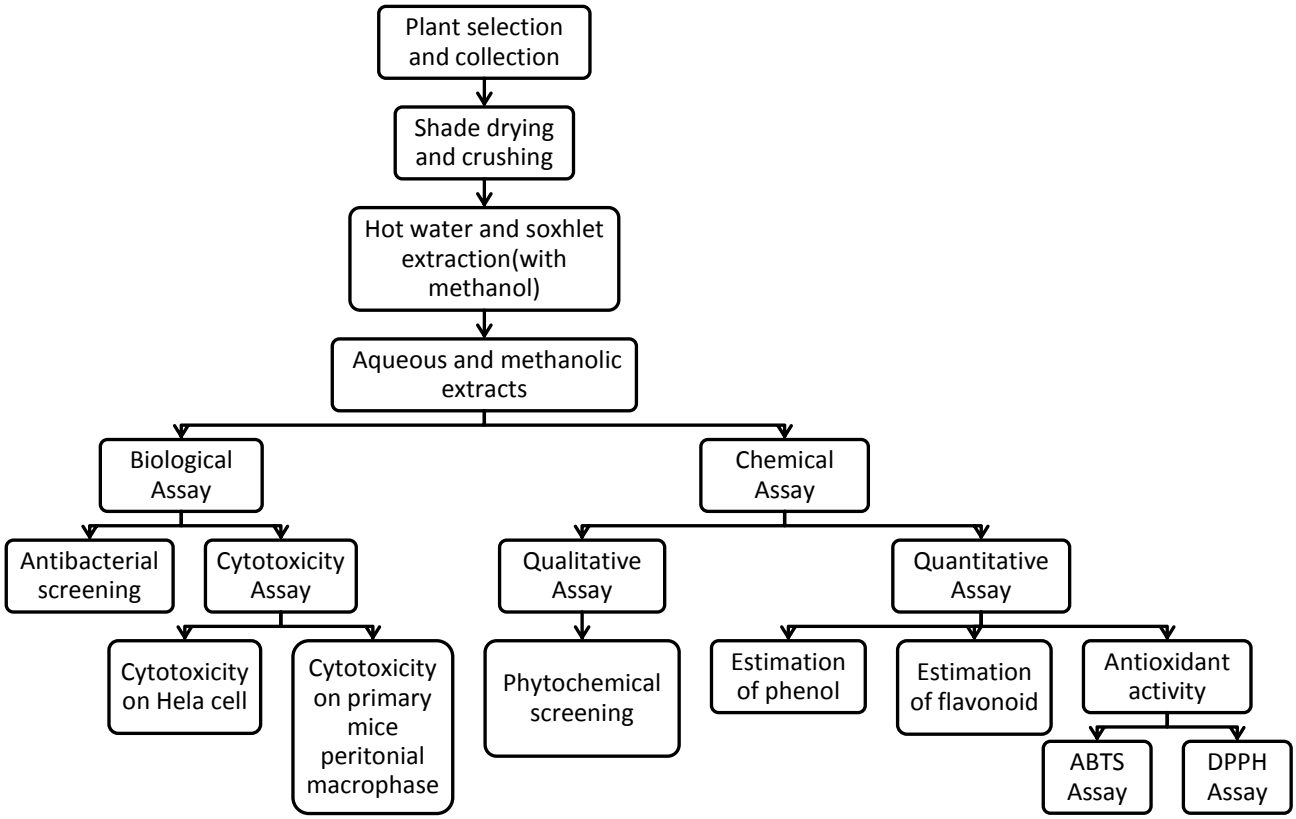


Figure 1 Flow chart showing the work plan of the Research

1.11 Research Objectives

1.11.1 General Objectives

Study and evaluation of six medicinal plants of Nepal (*Calotropis procera (*Aank)*, Clematis montana* (Junge lahara)*, Nardostychs grandiflora* (Jatamansi)*, Semecarpus anacardium* (Bhalayo)*, Terminalia chebula* (Harro) and *Withania somnifera (Ashwogandhaa))* for their in *vitro* antioxidant, antimicrobial and anticancerous activity.

1.11.2 Specific Objectives

1. Aqueous and methanolic extraction of phytochemical compounds from selected medicinal plants
2. Phytochemical Screening of major bioactive compounds in the extracts
3. Estimation of potent bioactive compounds like polyhenol and flavonoid
4. Screening of the extracts for their antioxidant and antibacterial activity
5. Assessment of cytotoxicity of the extracts on cancer cell line (HeLa cell) and on primary mouse peritoneal macrophages

1.12 Rationale

It has been hypothesized that each plant and their parts used in traditional medicine may possess the potent bioactive compounds. They can be used either singly or in combination with other chemotherapeutic agents for the treatment of various diseases including the disease like cancer and HIV. And also, due to their diverse structures and better compatibility to the human body, they can be used as a safer alternate of the modern synthetic medicine, which is often criticized for their severe side effects/ toxicity and short life span. Due to wide range of geographical distribution and diverse climate, Nepal owes a variety of flora with high medicinal values. Many of the plant extracts have been used in various Ayurvedic formulations, upon which more than 80 percent of Nepalese people depend on for their primary health care. Despite having variety of flora and knowledge of traditional medicine in Nepal, only a handful of researches have been carried out on their scientific validation. So concerning on the mentioned hypothesis and high therapeutic potential of Nepalese plants, this research has been focused on scientific evaluation of six popularly used medicinal plants of Nepal for the preliminary phytochemical screening and in vitro screening as a potent antibacterial, antioxidant and anticancerous agent which can further bring a new possibility in drug discovery.

1.13 Scope

1. This crude extract based research work will promote further research for fractionation and isolation of bioactive compounds and their analysis.
2. This study could open a door for discovery of a new drug against cancer and others.
3. This research will shed light on the importance of the medicinal plants under study and promote their conservation.
4. This research work will promote on further exploration of new medicinal plants of Nepal for their medicinal values
5. This research will give a scientific validation on the use of selected plants as medicine by local people.

Chapter II

Literature Review

2.1 Medicinal Plants of Nepal

Nepal lies on the central region of the great Himalayan range. Altitudes vary from less than 60m in the lowland of Terai in the South to the crest of the Himalaya reaching 8848m in the North. Nepal is blessed with most varied climatic conditions and diverse soil, suitable for the growth of veriety of plant species. Himalayan region shows the highest richness for endemic species and medicinal herbs (Kunwar *et al*., 2006) and plays fundamental role to fulfill the medicinal demand of Nepalese society. The medicinal and aromatic plant species richness increases along increasing altitude up to 2000m and then continues to decline (Kunwar and Bussmann, 2008) as the high altitude medicinal plants contain secondary metabolic compounds as an adaptive strategy to reduce the damaging effects (Mikage and Mouri, 2000; Iwashina *et al*., 2004). It has been estimated that the Himalayan region harbors over 10,000 species of medicinal and aromatic plants, supporting the live hoods of about 600 million people living in the area (Shengji, 2001). It has been reported that Neplease medicinal plants account about 20–28% of the local flora, but the field visit study done by Kunwar and Bussmann in seven different districts of west Nepal found that up to about 55% of the flora of the study region had medicinal value which indicates that there are more ethnobotanically and ethnomedicinally importantplant species in the Nepal Himalayas than previously estimated and a vast amount of undocumented knowledge about important plant species needs to be explored and documented (Kunwar and Bussmann, 2008). Use of Nepalese Himalayan medicinal plants is not only limited to indigenous use in Nepal Himalaya but also regarded as chief ingredients in Eastern medicinal system including Ayurveda of Indian subcontinent, Traditional Chinese Medicine etc. Each year thousands of tons of raw material are exported, mostly to India, and also to other Asian, European and American countries.

There are more than 590 studies related to ethnobotany published based on investigations in Nepal (Rokaya *et al.*, 2010). The first scientific study of Nepalese useful and medicinal plants was conducted by Francis Buchanan, who collected plants from 1802–1803. He was followed by Nathaniel Wallich 1820–1821 (Rajbhandari, 2001). Don and Wallich also collected plants in the Nepal Himalayas and recorded their uses (Kunwar and Bussmann, 2008). Since the 1980s, extensive ethnobotanical studies have

been conducted and thousands of medically important plant species of Nepal has been documented.

2.2 Description of the medicinal plants under the study

2.2.1 *Calotropis procera*

Taxonomic Position:

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Gentianales

Family: Asclepiadaecae

Genus: *Calotropis*

Species: *procera*

Botanical name: *Calotropis procera Aiton*

Asclepias procera Aiton

Common name: Aank

Distribution in Nepal: 300-1200 m, east to west

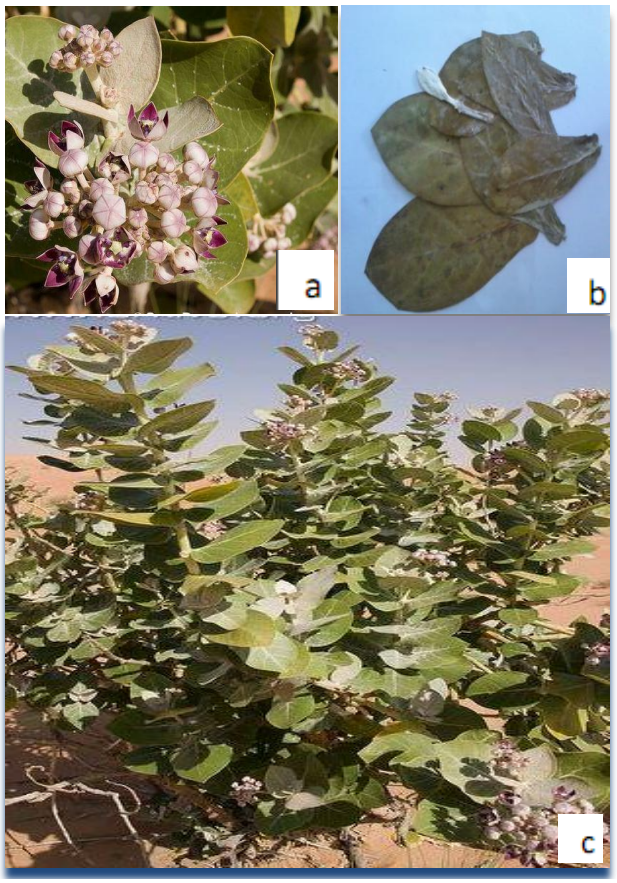


Figure 1 Calotropis procera Aiton. a. flower, b. dried leaves and c. whole palnt

Calotropis procera Family Asclepiadaecae is an erect, tall, large, soft-wooded, evergreen perennial shrub or small tree that grows to a height of 5.4m, with milky latex throughout. It is found in most parts of the world with a warm climate in dry, sandy and alkaline soils. The inner bark of Calotropis is used to make strong fibers called madar which are used in the manufacture of weave carpets, ropes, sewing thread and fishing nets.

2.2.1.1 Ethnomedicinal uses

Traditionally, the plant has been used as an antifungal (Larhsini *et al*., 1997), antipyretic and analgesic agent (Mohsin *et al*., 1989). The dried leaves used as an expectorant, and anti-inflammatory, for the treatment of paralysis and rheumatic pains (Sebastian and

Bhandari, 1984). The dried latex and dried root are used as an antidote for snake poisoning. It is also used as an abortifacient (Basu *et al*., 1992), for the treatment of piles (Gupta *et al*., 1996) and intestinal worms (Singh *et al*., 1980). The fowers are used as a milk drink to treat a variety of complaints including coughs and catarrh, asthma and indigestion, as well as cholera. The tender leaves of the plant are also used to treat migraine. The capsulated root bark powder is effective against diarrhoea and asthma (Singh *et al*., 1980). An infusion of bark powder of *C. procera* is used to treat leprosy and elephantiasis (Sing *et al*., 2002). Extracts, chopped leaves, and latex have shown great promise as nematicides, *in vitro* and *in vivo* (Anver and Alam, 1992; Charu and Trivedi, 1997). Local administration of the latex has been reported to elicit an inflammatory response that is mediated through histamine and prostaglandins (Shivkar and Kumar, 2003; Kumar and Shivkar, 2004). It has been reported that the latex exhibit potent anti-inflammatory, antidiarrheal, analgesic, antipyretic and schizonticidal activities (Dewan *et al*., 2000). It was reported that *C. procera* used in traditional medicine as a purgative, anthelmintic, anticoagulant, anticancer as well as antipyretic, analgesic, antimicrobial and antiseptic for skin infection (Jain *et al*., 1996). The root of the weed is used as a carminative in the treatment of dyspepsia and also used by various tribes of central India as a curative agent for jaundice (Samvatsar and Diwanji, 2000).

2.2.1.2 Chemical constituents

Pharmacologically active substances such as calotropin, uscharine, calotoxin, calactin, uscharidin and calotropagenin etc. are isolated from the leaves and latex of *C. procera* plant (Alam and Ali 2009). The phytochemical studies on the aerial parts of the plant showed the presence of alkaloids, cardiac glycosides, tannins, flavonoids, sterols and/or triterpenes (Mossa *et al*., 1991). Pentacyclic triterpenes (Ansari and Ali, 1999; Khan *et al*., 1988), alkaloids, cardenolides (Seiber *et al*., 1982), phytosterols (Khan and Malik, 1989), and triterpenoids saponins have been isolated from the roots of *C. procera*. Fatty acid composition in the extract of *C. procera* was analyzed which has 7 saturated and 11 unsaturated fatty acids (Khanzada *et al*., 2008). The chemical constituents of the seeds of *C. procera* were investigated and reported the occurrence of coroglaucigenin, frugoside, corotoxigenin and calotropin (Rajagopalan *et al*., 1995). Besides uscharine another cardenolide namely voruscharine was identified in its latex. The constitution of calotropagenin and voruscharine were determined and reported (Hesse and Ludwing, 1960). Pyrocatechuic acid α- amyrin, ß-amyrin, taraxasterol, ψ-taraxastrol, ß-sitosterol, taraxasteryl acetate, taraxasteryl benzoate, α- amyrin benzoate, ß-amyrin benzoate, ß-amyrin acetate, acetic acid and isovaleric acid, taraxasterol isovalerate, benzoyllineolone and benzoylisolineolane were isolated from the root bark, leaf and latex of *C. procera*. Uzarigenin, syrigenin and proceroside were also isolated from the latex (Brueschweile *et al*.,1969). The presence of D-glucose, D-arabinose, D-glucosamine and α-rhamnose in

the aqueous extract of the leaves of *C. procera* were reported while α and ß-amyrin and ß-sitosterol were identified in the unsaponifiable matter of the petroleum ether extract of same species (Pant and Chaturvedi, 1989). Two flavonol glycosides were identified and isolated from the leaves of *C. procera* (Alam and Ali 2009). The essential elements Al, As, Cu, Ca, Cr, Cd, Fe, K, Mn, Na, Pb, and Zn have been reported from this medicinal plant in variable range with 27-32% total protein. The latex contains a powerful bacteriolytic enzyme, a very toxic glycoside calactin (the concentration of which is increased following insect or grasshopper attack as a defense mechanism), calotropin D I, calotropin D II, calotropin F I, calotropin F II and a non toxic protealytic enzyme calotropin (2 %-3 %). This calotropin is more proteolytic than papain, and bromelain coagulates milk, digests meat, gelatin and casein.

2.2.1.3 Antioxidant activity

The antioxidant potential of the methanolic and aqueous extract of *C. procera* leaves was determined on the basis of their stable DPPH free radical scavenging activity. Strong antioxidant activity was reported with IC_{50} value 110.25 µg/ml. However the aqueous extract showed mild antioxidant activity (Yesmin *et al*., 2008). Antioxidant effect of latex of *C. procera* against alloxan-induced diabetic rats was evaluated (Roy *et al*., 2005) which produced an increase in the hepatic levels of the endogenous antioxidants, namely superoxide dismutase (SOD), catalase and glutathione, while it brought down the levels of thiobarbituric acid-reactive substances (TBARS) in alloxan-induced diabetic rats. The efficacy of the plant as an antioxidant agent was comparable to the standard drug, glibenclamide.

2.2.1.4 Anticancerous activity

Recently the cardiotonic steroid UNBS1450 (derived from 2-oxovoruscharin) from *C. procera* was shown to exert an anti cancer activity. UNBS1450 has been proven to be a potent sodium pump inhibitor, showing anti-proliferative and cell death-inducing activities. This anti-cancer potential of UNBS1450 is achieved by disorganization of the actin cytoskeleton after binding to the sodium pump at the cellular membrane, by inducing autophagy-related cell death, by repressing NF-KB activation as well as by down-regulating c-Myc in cancer cells (Mathur *et al*., 2009). The anticancer property of the dried latex of *C. procera* was evaluated in the X15-myc transgenic mouse model of hepatocellular carcinoma and elucidated its mechanism of action in cell culture. Latex treatment of mice showed a complete protection against hepatocarcinogenesis. The serum VEGF (Vascular endothelial growth factor) level was significantly lowered in the treated mice as compared to control animals similarly, Cell culture studies revealed that the methanolic extract of the latex as well as its fraction induced extensive cell death in both Huh-7 (hepatoma cells) and COS-1 (non-hepatoma cells) while AML12 (non

transformed hepatocytes cells) were spared. This was accompanied by extensive fragmentation of DNA in Huh-7 and COS-1 cells. No change in the levels of canonical markers of apoptosis such as Bcl2 and caspase 3 was observed (Smit *et al*., 1995, Choedon *et al*., 2006). Anti tumor studies with extracts of *C. procera* root employing Hep2 cancer cells and their possible mechanism of action was observed, results indicated that the root extracts of the plant inhibited the proliferation of Hep2 cancer cells via apoptotic and cell cycle disruption based mechanisms (Mathur *et al*., 2009).

2.2.1.5 Antibacterial activity

The antibacterial activity of methanolic and aqueous leaf extracts were tested by agar diffusion method at the concentration of 500µg/well. Crude methanol extract showed antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Plesiomonas shigelloides, Shigella dysenteriae,* and *Vibrio cholerae* on the other hand aqueous extract showed antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus pyogenes, Plesiomonas shigelloides, Shigella dysenteriae, Vibrio cholerae, Shigella Flexner, Shigella sonnei* and *Pseudomonas aeruginosa* (Yesmin *et al*., 2008). The anti-bacterial activity of a new cardenolide, of *C. procera* was evaluated which has been found to be active against *Pseudomonas pseudomallei*, a causative agent of melioidosis (Ali *et al*., 1993). The anti-fungal activity was screened by agar dilution method using organic solvent extracts of the stem bark of *C. procera* which significantly inhibited growth of *Trichophyton rubrum* and *Microsporum gypseum* (Hassan *et a*l., 2006). The antimicrobial effect of ethanol, aqueous and chloroform extracts of leaf and latex of *C. procera* were studied on five bacteria namely, *Escherichia coli, Staphylococcus aureus, Staphylococcus albus, Streptococcus pyogenes, Streptococcus pneumoniae* and three fungi: *Aspergillus niger, Aspergillus flavus, Microsporium boulardii* and one yeast *Candida albicans* using agar well diffusion and paper disk methods (Kareem *et al*., 2008). The results revealed that ethanol was the best extractive solvent for antimicrobial properties of leaf and latex of *C. procera* followed in order by chloroform and aqueous. The growth of six bacterial isolates was inhibited by the three extracts except *P. aeruginosa* and *S. pyogenes* that were not inhibited by the aqueous extracts of both leaf and latex of *C. procera*. Similarly, the growth of four test fungi were inhibited by ethanol and chloroform extracts while the aqueous extract was the least effective on the test fungi.

2.2.1.6 Safety evaluation/ Toxicity

The plant is toxic and is one of the few plants not eaten by grazing animals. Due to its toxicity, the latex extracted from the stem has traditionally been used to make poison arrows. The latex is cardiotoxic with the active ingredient being calotropin which is also highly toxic to human eyes and produces sudden painless dimness of vision with

photophobia (Basak *et al*., 2009). The adverse effects of *C. procera* consumption are reported to cause blisters, lesions and eruptions when taken by patients for the treatment of joint pains and gastrointestinal problems (Meena., 2011). The preparations of *C. procera* need to be used under the careful surveillance of a trained medical practitioner.

2.2.2 *Clematis montana*

Taxonomic Position:

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Ranunculales

Family: Ranunculaceae

Genus: *Clematis*

Species: *montana*

Botanical name: *Clematis montana*

Common name: Junge lahara

Figure 2.2 *Clematis montana* a. plant in Rhododendron tree, b. flower, c. leaves and d. dried leaves

Distribution in Nepal:, 2500-3500, east to west

Clematis montana is a woody climber distributed in temperate Himalaya up to an altitude of 4000mt. *Clematis* is a botanical source of various pharmaceutically active components, which has long been used in conventional medicine since the beginning of Chinese civilization. Pharmacologically important Clematis species are: *Clematis chinensis, C. hexapetala* and *C. mandshurica*, *C. armandii* and *C. Montana* (Hao *et al*., 2013).

2.2.2.1 Ethnomedicinal uses

Traditionally, this plant is administered orally to treat sexually transmitted infection, podagra, rheumatoid arthritis, bone disorder, chronic skin diseases, and is used as a diuretic agent. Roots, rhizomes and stems of some *Clematis* species are used to disperse wind-damp and unclog channels. In folk medicine, *Clematis* is applied to body surfaces for blisters and is also used as a cataplasm to treat purulent infections and ulcers (Hao *et*

al., 2013). Different *Clematis* species may have dissimilar drug effects. The leaves of *Clematis montana* are used for skin diseases (Bahuguna *et al*., 1989).

2.2.2.2 Chemical constituents

To date, at least 30 *Clematis* species have been characterized for their chemical components. The aglycone isolated from *Clematis* plants is of five-ring triterpenoid oleanane type, including oleanolic type, olean3β, 28-diol type (B), hederagenin type (C) and hederagenin-11,13-dien type (D). The conjugated glycosyl groups include glucose (Glc, D configuration), rhamnose (Rha, L configuration), galactose (Gal, D configuration), arabinose (Ara, L configuration), xylose (Xyl, D configuration) and ribose (Rib, D configuration). Since 2009, 19 novel triterpenoid saponins have been isolated from *Clematis* plants (Hao *et al*., 2013). Till now, more than 50 oleanolic type saponins, more than 40 hederagenin type saponins and two gypsogenin saponins have been found in *Clematis* plants (Sun and Yang. 2009, Hai *et al*., 2012). Most of these saponins are bidesmosidic, i.e C-3 and C-28 with the oligosaccharide chains. The mono desmosidic saponin is less common. Some oligosaccharide chains are substituted with acetyl, caffeoyl (CA), isoferuloyl (IF), p-methoxy cinnamyl (MC) and 3,4-dimethoxy cinnamyl (DMC) groups. In addition, more than 20 secondary glycosides have been found (Sun and Yang, 2009), which lose their C-28 oligosaccharide chains after hydrolysis.

Additionally, More than 50 flavonoid compounds have been isolated or detected from *Clematis* (Sun and Yang, 2009, Du *et al.,* 2010, Sakaguchi *et al.,* 2012), the aglycones of which are mainly, apigeni kaempfeml, luteolin and quercetin. Twenty-four lignans isolated from Clematis are mainly eupomatene lignans, cyclolignans, monoepoxylignans, bisepoxylignans and lignanolides (Sun and Yang, 2009). Six coumarin compounds have been isolated (Sun and Yang, 2009; Li et al., 2009). Eleven alkaloids isolated from Clematis fall into two categories: aporphine and terpenoid alkaloid. Many other compounds were also isolated from various Clematis species, such as phenolic glycosides (Yan *et al*., 2009) volatile oils, triterpenes, (Yang *et al*., 2009), macrocyclic compounds and others (Wang *et al*., 2009).

The antioxidant, anticancer and antibacterial property of this plant has not been reported yet.

2.2.3 Nardostachys grandiflora/jatamansi

Taxonomic Position:

Kingdom: Planate

Division: Mangnoliophyta

Class: Mangnoliopsida

Order: Dipsacales

Family: Valerianaceae

Genus: Nardostachys

Species: grandiflora/ jatamansi

Botnical name: *Nardostachys jatamansi* DC

Nardostachys grandiflora DC.

Distribution in Nepal: 3200-5000 m, east to west

Figure 2.3 *Nardostychs* jatamansi DC. a. flower, b. dried plant with rhizome and c. plant in natural habitat

N. Jatamansi DC. is perennial herb whose rhizome and roots is mainly used as drug. The plant is about 10 to 60 cm in height and with stout and long woody root stocks. Dark grey rhizomes are crowned with reddish brown tufted fibers. Internally they are reddish brown in color. It has an agreeable odor with bitter aromatic taste and is used as substitute for valerian. It yields up to 2 percent of an essential oil (spikenard oil) with a pleasant odor. It is one of the major income generating resources of the rural population. Rhizome of Jatamansi has high medicinal value and the essential oil extracted from the rhizomes is used in perfumery. Jatamansi is distributed in sub-alpine to alpine regions in dry, open conifer forests, rocks, edges, small depressions, scrubs and in open meadows mostly on north facing slopes. In Nepal, it is found in the eastern and central regions but abundantly in the western region growing on open, stony and grassy slopes, and on the turf of glacial flats (HMG/Nepal 1976).

2.2.3.1 Ethnomedicinal uses

The plant has been valued for centuries in Ayurvedic in Indian, Unani in ancient Greek and Arab, and in ancient Egypt and Rome for its medicinal values. *N. jatamansi* is also

used to season foods in Medieval European cuisine, especially as a part of the spice blend used to flavor. Hippocrates used in sweetened and spiced wine drink.

N. jatamansi DC has been prescribed in Ayurveda since 800 B.C. for a diversified group of ailments such as hysteria, cholera, palpitations, epilepsy, mental disorders, insomnia, hyperlipidemia, hypertension and heart diseases (Subashini *et al.,* 2007) and similar disorders. Root and rhizome has bitter taste, and serve as antispasmodic, diuretic, nerve sedative, nerve stimulant, and tonic, carminative, sedative to spinal cord, promotes appetite and digestion. Different formulations works as a different mode of actions in various ailments as: Medhya (Brain tonic), Rasayana (Rejuvenative to the mind), Nidrajnana (Promotes sleep), Manasrogaghna (Alleviates mental diseases), Pachana (Digestive), Kasaswasahara (Alleviates coughs and breathing difficulties), Kushtaghna (Stops skin diseases and itching), Daha prashamana (Stops burning sensations), Varnya (Benefits complexion) and Roma sanjanana (Promotes hair growth). In the Unani system of Medicine, *N. jatamansi* DC is used as hepatoprotective, cardio tonic, diuretic and analgesic (Ali *et al*., 2000). It has protective effect in Parkinsonisn, cerebral ischemia, and liver damage (Ali et al., 2000). Oil of the rhizome is also recommended in scorpion sting (Sharma and Singh, 2011).

2.2.3.2 Chemical constituents

The chemical composition of *N. Jatamansi* DC is highly complex containing volatile essential oil and other biological active compounds. The main active constituents in the plant material are sesquiterpenes, lignans, neolignans and coumarins (Chatterjee *et al*., 2005) which are obtained from the essential oil of rhizome. Jatamansone or valeranone is the principal sesquiterpene (Rucke *et al*., 1978). Other sesquiterpenes include nardostachone, dihydrojatamansin, jatamansinol, jatamansic acid (Rucker *et al*., 1993), jatamansinone, jatamansinol, oroseolol, oroselone, seselin, valeranal, nardostachyin (Harigaya *et al*., 2000), nardosinone, spirojatamol (Bagchi *et al*., 1990), jatamol A and B (Bagchi *et al*., 1991), calarenol, seychellene, seychelane, coumarin: jatamansin or xanthogalin (Sastry *et al*., 1967). More over roots contain Valeranone, valeranal, nardone, calarenol, nardostechone,n- hexacosanyl arachidate, 8 n-hexaconsanol, calarene, n- hexacosane, n- hexacosanyl isovalerate, ß – sitosterol. norseychelanone, seychellen, patchouli alcohol and ß – patchoulenese (Sastry *et al*., 1967). Roots oil conatins Terpenic coumarins, oroselol, jatamansin, hydrocarbons, ß - eudesmol, elemol, ß - sitosterol, angelicin, jatamansinol (Rahman *et al*., 2011). A new sesquiterpene acid, nardin and new pyranocoumarin: 2', 2'-dimethyl-3'-methoxy-3', 4'-dihydropyranocoumarin have been reported (Chatterjee *et al*., 2005). Actinidine, an alkaloid has also been reported.

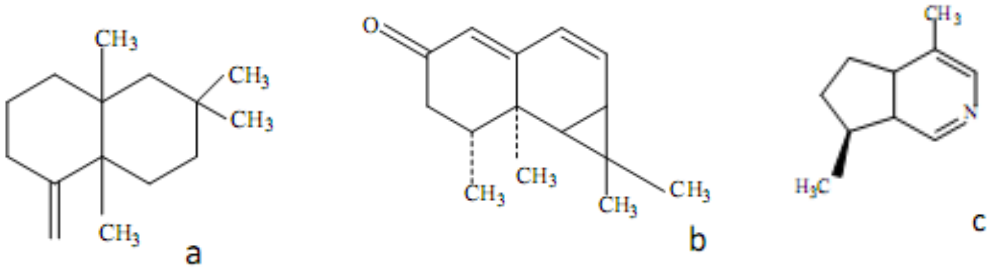


Figure 2.4 Chemical structures of a. Jatamansone, b.Nardostachone and c. Actinidine

2.2.3.3 Antioxidant activity

The medical claim attributed to this plant is mainly due to the anti oxidative role exhibited by the active constituents of this plant. Hydroalcoholic extract of *N. jatamansi* rhizomes exhibited high reduction capability and powerful free radical scavenging, activity especially against DPPH and superoxide anions as well as a moderate effect on NO. The extract also showed the peroxidation inhibiting activity as demonstrated in the linoleic acid emulsion system (Sharma and singh, 2011). This plant has also shown Cardio protective activity which was proven to be due to its anti lipid peroxidase propertiy. The extract also restored the oxidative enzyme activity (superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase) and lipid peroxides to near normal levels (Subashini *et al*., 2006). The activities of glutathione-dependent enzymes, catalase and superoxide dismutase, were dose-dependently restored by *N. jatamansi* extract (Ahmad *et al*., 2006).

2.2.3.4 Antimicrobial activity

Jatamansi oils has been reported to exhibit antibacterial activity with inhibition zones ranging from 11-24 mm for both gram positive and gram negative bacteria (Bharti *et al*., 2012). In another study also, oil of jatamansi showed antibacterial activity against both Gram positive and Gram negative bacteria (*Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae, Salmonella typhimurium, Pseudomonas aeruginosa, Pseudomonas fluorescens,* and *Enterobacter aerogenes*). Among Gram positive bacteria, maximum antibacterial activity was observed against *B. subtilis* followed by *S. aureus* (Parveen *et al*., 2011). The plant has also proven its activity against various fungal strains as its oil had shown fungistatic or fungicidal efficacy against *Aspergillus flavus, A. fumigatus, A. sulphureus, Mucor fragilis* and *Rhizopus stolonifer* depending upon the concentrations (Sarbhoy *et al*., 1978).

2.2.4 *Semecarpus anacardium*

Taxonomic Position:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Sub class: Rosidae

Order: Spindales

Family: Anacardiaceae

Genus: *Semecarpus*

Species: *anacardium*

Figure 2.5 Semecarpus anacardium Linn.f. a. plant with nuts b. dried nuts

Botanical name: *Semecarpus anacardium* Linn.f.

Common name: Bhalaayo

Distribution in Nepal: 200-1400m, east to west

Semecarpus anacardium is a deciduous tree belonging to Anacardiaceae family and is distributed in tropical and temperate regions and is well-known for its medicinal value in Ayurvedic and Siddha system of medicine. The seed is commonly known as 'marking nut and in local term it is called Bhalayo. Bark is grey in colour and exudes an irritant secretion on incising.

2.2.4.1 Ethnomedicinal uses

Semecarpus anacardium is a one of most popular medicinal plant in world of Ayurveda. Charak, Sushrut and Vagbhatt, the main three treatises of Ayurveda have described the medicinal properties of *S. anacardium* and its formulation. The fruits, their oil and the seeds have great medicinal value, and are used to treat the wide range of diseases. The nut is very toxic to be used without purification, for which the number of detoxification methods have been recorded and most common detoxification method involves rubbing of *S. anacardium* seeds with brick powder and then washing with warm water. The second common recommended method is to tie the seeds in muslin cloth and suspended it in a vessel containing coconut water, then heated for about 3 hrs continuously (Paras and Sharma, 2013). A decoction of bruised fruit (1 to 8) in a dose of 25 ml was given for asthma. A decoction with milk and purified butter, in gradually increasing doses, is given in peripheral neuritis, sciatica and paralysis (Majumdar *et al*., 2008). Detoxified nut were used in Ayurveda for skin diseases, tumors, malignant growths, fevers haemoptysis, excessive menstruation, vagina discharge, deficient lactation, constipations intestinal parasites. It has been used therapeutically in neurological disorders, ulcers (Sharma and singh, 1981). Purified butter, cooked with the paste and decoction of nut, mixed with sugar, was administered for treating tumors

(Khare, 1982). Various formulations of the nut is used as as blood purifiers and haematinic tonics, gastrointestinal disorder, fevers etc (Majumdar *et al*., 2008). In Unani medicine, different formulations are used for different diseases like; majoon-e-Asal-e-Balaadur is prescribed for neurasthenia, Majoon-e-Balaadur for dementia, amnesia, Raughan-e-Balaadur externally in paralysis, hemiplegia, and Bells palsy. Angaruya-e-Kabir is also prescribed for neurological affection (Khare, 1982). Ash of the plant in combination with other drugs is used in snake bite and scorpion sting (Adhikari *et al*., 2007).

2.2.4.2 Chemical constituents

Till now, many medically important compounds have been identified from the *semecarpus anacardium* nut. The most significant of which are phenolic compounds (bhilavanols), glycosides, steroids, and biflavonoids. The two main phenolic compounds are Bhilavanol A (monoenepentadectyl catechol I) Bhilavanol B (dienepentadectyl catechol II) (Lamture *et al*., 1982) and the glucoside exclusively found in this plant is Anancardoside (Gil *et al*., 1995). The most important Biflavanoids are: semecarpuflavanone, jeediflavanone, galluflavanone, nallaflavanone, semecarpetin, tetrahydroarmentoflavone, tetrahydrobustaflavone and anacarduflavanone (Murthy, 1985). The kernel is also found to contain phenolic glucoside (Vaishnav *et al*., 1983).

2.2.4.3 Antioxidant activity

Semecarpus anacardium has been reported in various studies to possess potent antioxidant activity. Administration of the aqueous extract of the nuts to lymphoma-transplanted mouse lead to increase in the activities of antioxidant enzymes, whereas LDH activity is brought down significantly indicating a decrease in carcinogenesis (Verma and Vinayak, 2009). The ethanol extract of nut has been found to exhibit antioxidant activity by scavenging free radicals, DPPH and ABTS with IC_{50} values, 88.73 ± 2.26 and 81.65 ± 1.57 µg/ml respectively and also showed metal chelating activity (72.37 ± 2.26 µg/ml) compared with antioxidant controls, ascorbic acid and BHA respectively (Barman *et al*., 2013). In another experiment when *S. anacardium* seed oil and extra virgin olive oil were compared for their radical scavenging activity toward DPPH radical and galvinoxyl radical (by electron spin resonance spectrometry), *S. anacardium* seed oil exhibited a stronger radical scavenging Activity (Ramad *et al.,* 2010).

2.2.4.4 Anticancerous activity

In traditional medicine, *S. anacardium* nut is highly valued for the treatment of tumors and malignant growth and several studies have been done on antitumor activity of the crude extracts and the isolated compounds *in vitro* and *in vivo* mice models. The chloroform extract of its nut possess anti tumour action with increased life span against

leukaemia, melanoma and glioma (Chitinis *et al*., 1980). An Ayurvedic drug containing *S. anacardium*, Amurarohitaka, Glycyrrhiza glabra and copper powder were reported to inhibit breast tumour development in mice by significantly extending the survival period. This drug was also found to be efficient in clinical trials (Singh, 2002). Premalatha, Sachdanandam and few other scientists had studied time and again, the biochemical basis of anticarcinogenic potency of nut using hepatocellular carcinoma as cancer model in rats. Extensive analysis on effect against biochemical abnormalities during cancer showed that the drug modulates the abnormalities of all biochemical pathways including carbohydrate, lipid, cytochrome P-450 mediated microsomal drug metabolism, cancermarkers and membrane proteins during cancer progression (Veena *et al*., 2006; Premalatha and Sachdanandam, 1999; Smit *et al*., 1995). Anacartinforte, a preparation from *S.anacardium* has been used as an anticancer drug for several decades since it is giving health improvement with alleviation or disappearance of troublesome symptoms. It provides clinical benefit with an extension of survival time in various cancers including oesophageal, chronic myeloid leukaemia, urinary bladder and liver cancer (Premalatha and Sachdanandam, 1999). The milk extract of nut produces regression of hepatocarcinoma by stimulating host immune system (Premalatha and Sachdanandam, 1998) and normalizing tumour markers including alpha-fetoprotein levels (Premalatha *et al*., 1999). This preparation stabilizes the lysozomes, and normalizes glycoprotein and mineral content in the body during cancer progression (Premalatha and Sachdanandam, 1998; Premalatha *et al*., 2000). It also corrects hypoglycaemia (Premalatha *et al*., 1997) and controls abnormal lipidperoxidation by the maintenance of antioxidant defense status (Premalatha *et al*., 1997). In the microsomes, it acts as a bifunctional inducer of both phase I and II biotransformation enzymes and prevents tumour initiation by preventing carcinogen activation (Premalatha, Sachdanandam, 2000).

2.2.4.5 Antibacterial activity

The petroleum ether nut extract of *S. anacardium* exhibited antibacterial property against both gram positive and gram negative bacteria by agar well method, E coli (19 mm), *Micrococcus luteus* (23 mm), *Salmonella typhi* (26mm), *Bacillus subtilis* (14 mm) and *Klebsiella pneumoniae* (22 mm) (Bagewadi *et al*., 2012).

2.2.4.6 Safety evaluation/Toxicity

Semecarpus anacardium is classified in Ayurveda under the category of toxic plants and it should be used with caution. Individuals showing allergic reactions should stop and avoid the usage of this nut. It should not be used in pediatric age group, very old persons, pregnant women and individuals of predominant pitta constitution and in certain diseased condition suhch as, renal function disorder, past histry of Bhallataka intolerance etc. The use of the same should be restricted in summer season. The

commonly seen Bhallataka related adverse effects are: generalized itching, stomatitis, gastritis, urethritis, etc and antidodes are For its allergic reactions like rash, itching and swelling, the antidotes used externally are coconut oil, rala ointment, ghee, coriander leaves pulp or butter mixed with musta (Cyperus rotundus). The salt and spices should be strictly restricted and during Bhallataka treatment, it is recommended to avoid exposure to sun, heat and excessive sex. The oily part of the nuts is toxic and its degree of removal is proportional to its safety margin. The maximum tolerated dose of a 50% alcoholic extract of the fruit when given intraperitoneally to mice was found to be 250 mg/kg body weight (Kumar *et ai.* 2007).

2.2.5 *Terminalia chebula*

Taxonomic Position:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: *Terminalia*

Species: *chebula*

Botanical name: *Terminalia chebula* Retz.

Common name: Harro

Figure 2.6 Terminalia chebula Retz. Plant with fruit

Distribution in Nepal: 150-1100m, east to west

Terminalia chebula (haritaki) is called the "king of medicines" and is always listed first in Ayurvedic medicine because of its extraordinary healing powers (Lee *et al*., 2005). It is one of the most important medicinal plants used in medicines of ayurveda, siddha, unani and homeopathy because of having a number of pharmacological properties.

2.2.5.1 Ethnomedicinal uses

T. chebula is one the most popularly used plant as folk medicine in Southeast Asia. The fruit of this plant is extensivey used in ayurvedia, siddha, unani and homeopathic medicines with variety of formulations. It is one of the main ingredients of Triphala, herbal preparation of three fruits from plants; *Terminalia chebula, Terminalia bellerica* and *Emblica officinalis*, which is considered as the most versatile of all herbal formulations (Sharma *et al*., 2012) and also prescribed as a cardiotonic and for candid infection (Kaur *et al*., 2005). The fruits of *T. chebula* are used both externally as well as

internally for various medicinal purposes. Externally, the paste of fruits effectively cleanses the wounds and ulcers, reduces the swelling, hastens the healing and also prevents accumulation of pus in skin diseases. The oil extracted from the fruit is extremely helpful in healing of wounds especially in burns. The paste of fruit is also applied in conjunctivitis for relief due to its anti-inflammatory property. The gargles with its decoction give excellent results in problems of the throat. Triphala can be used externally for hair wash, for brushing the teeth in pyorrhea or bleeding gums, and its decoction for washing the chronic, non-healing wounds and ulcers. The powder of the fruit is used as a tooth powder to strengthen the gums and its aqueous extract is recommended by the dentists in tooth ache due to its anticaries property (Usha *et al*., 2007).

Internally, it is used for the treatment of variety of diseases like Common gastrointestinal ailments, tumours, ascites, piles, enlargement of liver and spleen, worms, colitis etc. According to Vagbhata, when haritaki powder fried in ghee is regularly consumed with sufficient ghee in food, it promotes longevity and boosts energy. The mixture of Triphala powder and haridra is a well known adjunct in diabetes. It is adjuvant in hemorrhages due to its astringent nature and good for chronic cough, chorizo, sore throat as well as asthma. Also it is useful in renal calculi, dysurea, and retention of urine and skin disorders like allergies, urticaria and other erythematous disorders (Chang and Lin, 2010). The decoction of haritaki or triphala is given along with honey in hepatitis. Haritaki powder with honey and ghee is also effective remedy for anemia. In obesity, its decoction with honey reduces the excessive body fats. It is used in nervous weakness, nervous irritability. It promotes the receiving power of five senses organs and also believed that its regular use improves memory power.

2.2.5.2 Chemical constituents

Attractive number of studies has carried out time and again to study the essential compounds responsible for the medicinal value of *T. chebula*. The compounds identified are predominantly phenolics, particularly hydrolysable tannins, flavonoids, and other like sterols, anthraquinones, terpinenes, terpinoles, amino acids, fructose, resin, fixed oils etc. It is fairly rich in different tannins (approximately 32%) and its tannin contents largely depend on its geographic location (Kumar, 2006). The tannins of *T. chebula* are of pyrogallol (hydrolysable) type. Group of researchers (Juang et al., 2004) have isolated 14 components of hydrolysable tannins (gallic acid, chebulagic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulinic acid, 1,2,3,4,6-penta-O-galloyl-β-D-glucose, 1,6-di-o-galloyl-D-glucose, casuarinin, 3,4,6-tri-o-glloyl-D-glucose, terchebulin) from *T. chebula* fruits by HPLC and capillary electrophoresis technique. Some of the other minor constituents were polyphenols such as corilagin, galloyl

glucose, punicalagin, terflavin A, maslinic acid (Williumson, 2002). Besides, fructose, amino acids, succinic acid, betasitosterol, resin and purgative principle of anthraquinone are also present (Tubtimdee and Shotipruk, 2011 and Thakur *et al*., 2008). Flavonol, glycosides, triterpenoids, coumarin conjugated with gallic acids called chebulin as well as other phenolic compounds were also isolated (Rangsriwong, 2009, Muhammad *et al*., 2012). Twelve fatty acids were isolated from *T.chebula* of which palmitic acid, linoleic acid and oleic acid were the main constituents (Zhang *et al.,* 1997). Triterpenoid glycosides such as chebulosides I and II, arjunin, arjunglucoside, 2α-hydroxyursolic acid and 2α-hydroxymicromiric acid also have been reported (Mammen *et al*., 2012). The plant is found to contain phloroglucimol and pyrogallol, along with phenolic acids such as ferulic, p-coumaric, caffeic and vanillic acids and oil extracted from kernels yielded palmitic, stearic, oleic, linoleic, behenic and arachidic acids (Khare, 2004). Recent studies have shown that *T. chebula* contains more phenolics than any other plants (Saleem *et al*., 2002).

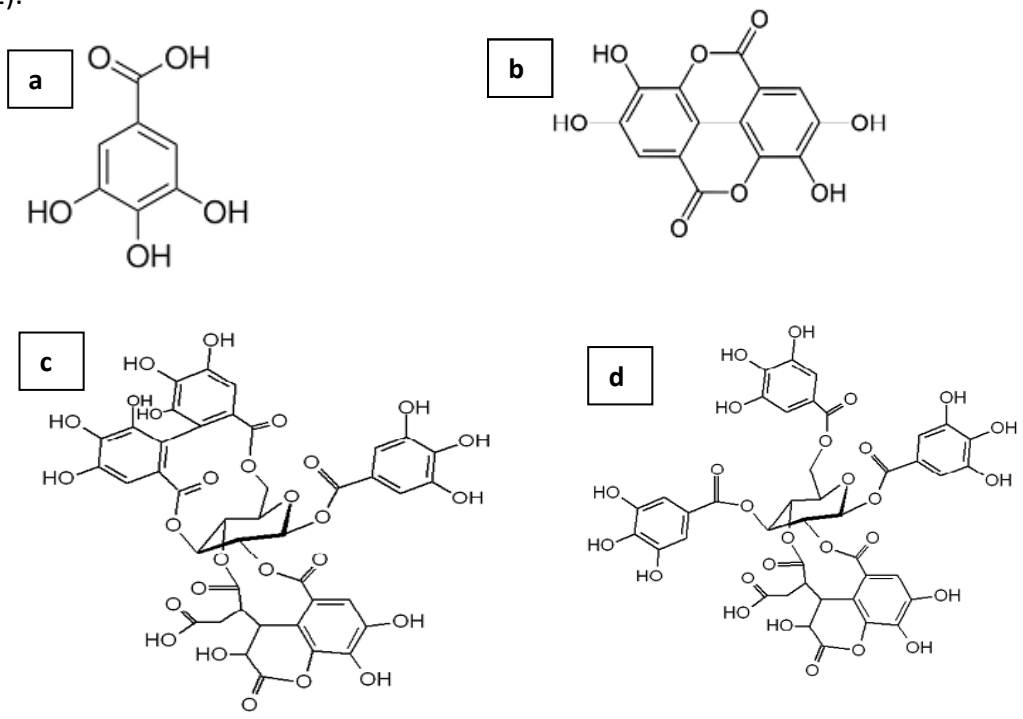


Figure 2.7 Compounds from T. chebula fruit extract: (a) Gallic acid, (b) Ellagic acid, (c) Chebulagic acid and (d) Chebulinic acid

2.2.5.3 Antioxidant Activity

T. chebula is an excellent antioxidant and its antioxidant property is attributed to its high phenolic content (Chang and Lin, 2010). In a study, 6 extracts (Methanol, chloroform, Acetone, n-Butanol, organic aqueous extract and water extracts) and 4 pure compounds (casuarinin, chebulanin, chebulinic acid and 1,6-di-O-galloyl-b-D-glucose) of *T. chebula* exhibited anti-lipid peroxidation, anti-superoxide radical formation and free radical scavenging activities at different magnitudes of potency (Cheng *et al*., 2003). Administration of aqueous extract *of T. cheubla* effectively modulated oxidative stress

and enhanced antioxidant status in the liver and kidney of aged rats (Mahesh *et al.,* 2009). The aqueous extract of *T. chebula* protected the antioxidant enzymes from reactive oxygen species produced by gamma radiation in the rat liver microsomes and michondria (Naik *et al*., 2004). Methanolic extract (75%) inhibited lipid peroxidation and scavange hydroxyl and superoxide raadicals with IC_{50} values, 85.5±6.5, 165.5±8.5 and 20.5±3.2 µg/ml respectively (Sabu and Kuttan, 2002). Triethylchebulate is an aglycone isolated from the fruits of *T.chebula* which significantly inhibited FeSO4/Cys-induced microsomes lipid peroxidation and protected both H2O2-induced RBCs hemolysis and RBCs autohemolysis in dose dependent manner (Chen *et al*., 2011).

2.2.5.4 Anticancerous Activity

Methanolic extract of *T.chebula* and its isolated compounds, gallic acid, 1,2,3,4,6-penta-O-gal- acid showed moderate in vitro cytotoxicity against cultured human tumor cell lines including A-549, SK-OV-3, SK-MEL-2, XF-389, and HCT-195 (Lee *et al.*, 1995). A 70% methanol extract of T. chebula fruit, was studied for its effects on growth in several malignant cell lines including a human (MCF-7) and mouse (S115) breast cancer cell line, a human osteosarcoma cell line (HOS-1), a human prostate cancer cell line (PC-3) and a non-tumorigenic, immortalized human prostate cell line (PNT1A) using assays for proliferation ([^{3}H]-thymidine incorporation and coulter counting), cell viability (ATP determination) and cell death (flow cytometry and Hoechst DNA staining). In all cell lines studied, the extract decreased cell viability, inhibited cell proliferation, and induced cell death in a dose dependent manner and also found that chebulinic acid, tannic acid and ellagic acid were the most growth inhibitory phenolics of *T. chebula* with respective IC_{50} values on HOS-1 cell lines are, 53.2µM±0.16, 59.0µg/ml±0.9 and 78.52µM±0.24 (Saleem e*t al*., 2002). Acetone extract of fruit has been reported to contain phytochemicals with promising antimutagenic and anticarcinogenic properties (Arora *et al*., 2003) however, the extract at 50 µg/mL had shown the significantly increased percentage of cell viability of primary human skin fibroblast (Itsarasook *et at*., 2012).

2.2.5.5 Antibacterial Activity

Various extracts *of T. chebula* exhibit antibacterial activity against wide range of bacterial species including both gram positive and gram negative human pathogenic bacteria (Khan and Jain, 2009; Malckzadeh *et al*., 2001). Gallic acid and its ethyl ester isolated from ethanolic extract fruit showed antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (Sato *et al*., 1997). An ethanol extract of *T. chebula* fruit was found to be highly effective against clinically important standard reference bacterial strains of both gram-positive and gram-negative bacteria such as, *Salmonella typhi* SSFP 4S, *Staphylococcus epidermidis* MTCC 3615, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* MTCC 441 and *Pseudomonas aeruginosa* ATCC 27853 suggesting its broad

spectrum antimicrobial activity (Kannan *et al.,* 2009). Several biologically active components isolated from butanol fraction of fruit extract of *T. chebula* were tested against six intestinal bacteria in which, Ethanedioic acid showed strong and moderate inhibitory activity against *Clostridium perfringens* and *Escherichia coli*, with no adverse effects on the growth of the four tested lactic acid-producing bacteria. Similarly, ellagic acid exerted a potent inhibitory effect against *C. perfringens* and *E. coli,* but little or no inhibition was observed for behenic acid, β-caryophyllene, eugenol, isoquercitrin, oleic acid, α-phellandrene, β-sitosterol, stearic acid, α-terpinene, terpinen-4-ol, terpinolene, or triacontanoic acid (Kim *et al*., 2006). Aqueous, ethanol and acetone fractions of the fruit extracts were tested against 52 multidrug-resistant uropathogenic bacteria and among them the ethanol extract of demonstrated a strong antimicrobial activity against all the test isolates and found to be most effective over others (Bag *et al*., 2012).

2.2.5.6 Safety evaluation/Toxicity

Aqueous, ethanol, and ethyl acetate extracts of *T. chebula* fruits also demonstrated no cellular toxicity on sheep erythrocytes as well as acute oral toxic effects on rats at recommended and higher doses (Panunto, 2011; Ji-hoon *et al*., 2012). Besides, hydroalcoholic extract of *T. chebula* fruits demonstrated cytochrome P-450 inhibition potential in rats (Ponnusankar, 2011). The hydrolysable tannins obtained from *T. chebula* fruits also showed antimutagenic activity against direct acting mutagens like sodium azide and 4-nitro-O-phenylene diamine. These findings indicated that it is a safe substance to be used as drug ordinarily.

2.2.6 *Withania somnifera*

Taxonomic Position:

Kingdom: Plantae

Division: Tracheophyta

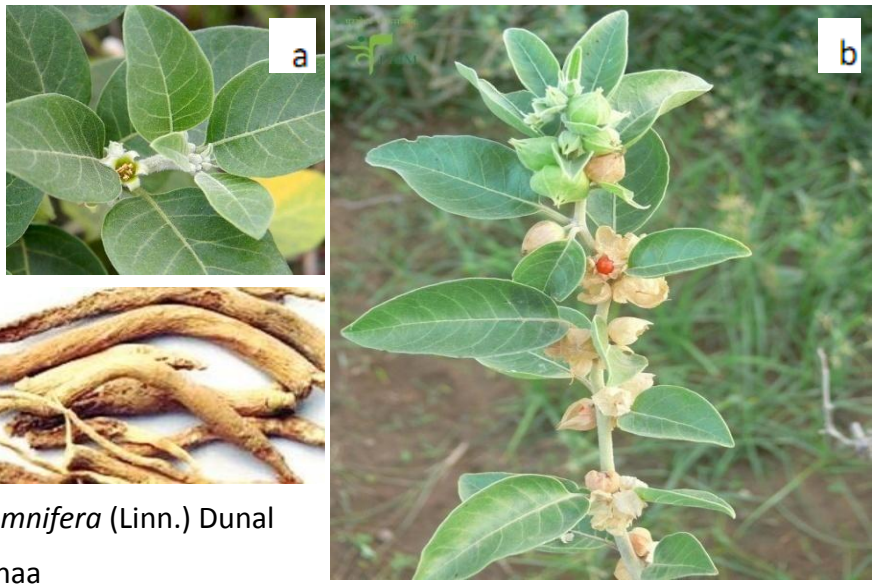
Class: Magnoliopsida

Order: Solanales

Family: Solanaceae

Genus: Withania

Species: somnifera



Botanical name: *Withania somnifera* (Linn.) Dunal

Common name: Ashwogandhaa

Distribution in Nepal: Cultivated, 150-1500 m.

Figure 2.8 Withania somnifera (linn.). Dunal. a. leaves, d, whole plant and c, dried roots

Withania somnifera is a small, erect, evergreen woody shrub belongs to Solanaceae family that grows or reaches about 30-150cm in height. *W.* somnifera is called Aswagandha in Sanskrit which means, "Horse's smell (Aswa=horse, gandha=smell) originatimg from the smell of its root. The species name, somnifera means "sleep-inducing "in Latin. The roots are the main part of the plant that are widely used as therapeutic agents which are the constituent of over 200 formulations in Ayruvedha, Siddha and Unani medicine, which are used in the treatment of various physiological disorders (Sharma *et al*., 2011).

2.2.6.1 Ethnomedicinal uses

In Ayurveda, *Withania* is widely claimed to have potent aphrodisiac, sedative, rejuvenative and life prolonging properties. It has been reported that all of the major parts of this plant such as the roots, fruits and leaves provide potential benefits for human health because of their high content of polyphenols and antioxidant activities (Mandal *et al*, 2012). Numerous studies indicated that ashwagandha possesses antioxidant, antitumor, antistress, anti inflammatory, immunomodulatory, hematopoetic, anti-ageing, anxiolytic, anti depressive properties and also influences various neurotransmitter receptors in the central nervous system (Sharma *et al*., 2011).The plant was traditionally used to promote youthful vigor, endurance, strength, and health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells system (Scartezzini and Speroni, 2000). The roots are used as a nutrient and health restorative in pregnant women and old people. The decoction of the roots boiled with milk and ghee is recommended for curing sterility in women. *W.somnifera* thickens and increases the nutritive value of the milk when given to nursing mothers. The roots are also used in constipation, senile debility, rheumatism, asthma, bronchitis, edema, leucoderma, anorexia, anemia, exhaustion, aging, infertility, impotence, repeated miscarriage, paralysis, multiple sclerosis, immune- dysfunction general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea (Sharma *et al*., 2011). It has been used owing to its antioxidant property to treat rheumatism and neurodegenerative disorders (RajaSankar *et al*., 2009). The Japanese patent applications are related to the use of the herb as a skin ointment and for promoting reproductive fertility. In US, the New England Deaconess Hospital, has taken a patent on an Ashwagandha formulation claimed to alleviate symptoms associated with arthritis in mice (Panda and Kar 1997).

2.2.6.2 Chemical constituents

The chemistry of Withania species has been extensively studied and several groups of chemical constituents such as steroidal lactones, alkaloids, flavonoids, tannin etc. have been identified, extracted, and isolated (Rahman *et al*., 1991 and Rahman *et al*., 1993). The pharmacological effects of W. somnifera are attributed to the presence of steroidal lactones (withanolides, withaferins), alkaloids (isopelletierine, anaferine), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X). This plant is also rich in iron (Hossein *et al*., 2009). The active compounds reported in W.somnifera include withaferin, a phytosteroid (Lavi *et al*., 1965), sitoindosides VII–X, 5-dehydroxywithanolide-R, withasomniferin-A, 1-oxo-5β, 6β-epoxy-witha-2-ene-27-ethoxy-olide, 4-(1-hydroxy-2, 2dimethylcyclpropanone)-2,3-dihydrowithaferinA, 2,3-dihydrowithaferin A, 24,25-dihydro-27-desoxywithaferinA, physagulin D (1→6)-βD-glucopyranosyl-(1→4)- βD-glucopyranoside, 27-O-βD-glucopyranosylphysagulin D, physagulin D, withanoside I–VII, 27-O-βD-glucopyranosylviscosalactone B, 4,16-dihydroxy-5β,6β-epoxyphysagulin D, viscosalactone B and diacetylwithaferin A (Ganzera *et al*., 2003; Kaur *et al*., 2003 and Matsuda *et al*., 2001).

The withanolides have C28 steroidal nucleus with C9 side chain, with a six membered lactone ring. A sitoindoside is a withanolide containing a glucose molecule at carbon 27 (Ganzera *et al.,* 2003). Much of Ashwaganda's pharmacological is due to presence of two main withanolides: withaferin A and withanolide D (Mirjalili *et al*., 2009). Apart from these contents plant also contain constituents like withaniol, acylsteryl glucosides starch, reducing sugar, hantreacotane, ducitol, a variety of amino acid including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cystine, tryptophan, and high amount of iron. The reported alkaloids are anaferine [bis (2-piperidylmethyl) ketone], isopelletierine, tropine, pseudotropine, 3α-tigloyloxtropine, 3-tropyltigloate, cuscohygrine, dl-isopelletierine, anahygrine, hygrine, mesoanaferine, choline, somniferine, withanine, withananine, hentriacontane, visamine, withasomine, pseudowithanine and ashwagandhine. Withaniol (mixture of withanolides) and number of withanolides including withaferine-A, withanolide N and O, withanolide D, withanolide p and 8, withanolide Q and R, withanolide y, 14α- hydroxyl steroids and withanolides G, H, I, J, K and U (Kirson and Glotter.,1980). Seven new withanolide glycosides called withanosides I, II, III ,IV, V, VI and VII had been isolated and identified (Matsuda *et al.,* 2001).

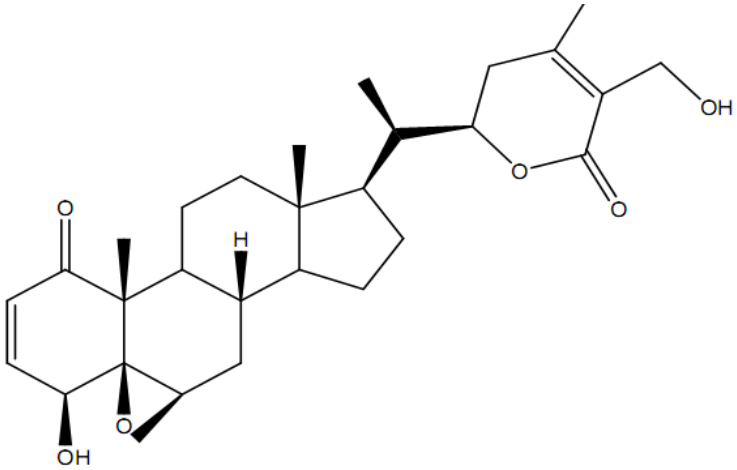


Figure 2. 9 Structure of Withaferin A

2.2.6.3 Antioxidant Activity

70% methanolic extract of *W. somnifera* root showed strong antioxidant activity by scavanging hydroxyl radical, superoxide radical, nitric oxide, singlet oxygen radicals, hypochlorous acid and inhibition of lipid peroxidation with IC_{50} values, 1808.69 ± 391.16, 650.37 ± 107.18, 405.91 ± 145.84, 234.49 ± 37.69, 328.99 ± 35.92, 284.13 ± 146.66 µg/ml respectively which was quiet comparable with standard antioxidant compound (Mandal *et al*., 2012). The active principles of *W. somnifera* (withanolides) increased the levels of the major free-radical scavenging enzymes; superoxide dismutase, catalase and glutathione peroxidase, in the rat brain frontal cortex and striatum with corresponding protective effect on neuronal tissue, suggesting that the antioxidant effect *of W. somnifera* in the brain may be responsible for its diverse pharmacological properties (Bhattacharya, 1997). Similarly, oral administration of *W. somnifera* extracts prevented an increase in lipid peroxidation in mice and rabbits (Dhuley, 1998).

2.2.6.4 Anticancerous activity

Research on animal cell cultures has shown that the herb decreases the levels of the nuclear factor kappaB, suppresses the intercellular tumor necrosis factor, and potentiates apoptotic signalling in cancerous cell lines (Ichikawa, 2006). One of the most exciting of the possible uses of Ashwagandha is its capacity to fight cancers by reducing tumor size. Following administration of Ashwagandha over a period of seven months, the histological appearance of the lungs of animals which received the herb was similar to those observed in the lungs of control animals.

Withaferin A, showed significant antitumor & radiosensitizing effects in experimental tumors without any toxicity & inhibiting tumor growth increasing survival in swiss albino mice inoculated with Ehrlich ascites carcinoma (ESC) (Devi *et al*., 1995; Devi, 1996). The

administration of Ashwagandha Rasayana significantly reduced the lung tumor nodule formation and also reduced leucopenia induced by cyclo-phosphamide treated experimental animals, indicatingits usefulness in cancer therapy (Menon *et al*., 1997; Davis and Kuttan, 1998). Ethanol extract of WS root (400 mg/kg and up, daily for 15 days) after intra-dermal inoculation of 5x105 cells of S-180 in BALB/c mice produced complete regression of tumor after the initial growth. A 55-percent complete regression was obtained at 1000 mg/kg; however, it was a lethal dose in some cases (Devi *et al*., 1992).

2.2.6.5 Antibacterial activity

Being rich source in phytochemicals, different extracts of different parts of *W. somnifera* possess potent antimicrobial activity against wide range of bacterial and fungal species. Mechanisms of action can largely be attributed to cytotoxicity, Gene silencing and immunopotentiation (Mwitari *et al*., 2013). Alkaloid extracts of different parts (root, stem, leaf & fruits) of *W. somnifera* showed significant antibacterial activity *against Enterobacter aerogens, Bacillus subtilis, Klebsiella pneumoniae, Raoultella planticola* and *Agrobacterium tumefaciens* (Singh and kumar, 2011). Aqueous root extract of the plant was found to possess strong antibacterial activity against methicillin resistant Staphylococcus aureus (MRSA) as revealed by the in-vitro agar well diffusion assay. Two TLC spots were found to be bioactive against the pathogen with minimum inhibitory concentrations of 2.3µg/µl and 5.2µg/µl respectively. One spot was of alkaloids and the other one was a mixture of essential oil and phenolics. Oral treatment of the *W. somnifera* (ethanol root extract) with doses 50 and 100mg/kg plant extract caused significant benefit results in Guinea pig infected with *E. coli* by the correction of some hematological and biochemical parameters also try to suppressed inflammatory cytokine response representing TNF-alpha (Boshy *et al.,* 2013)

2.2.6.6 Safety evaluation/Toxicity

For *W. somnifera*, no systematic study was found which included acute, sub-acute, sub-chronic or chronic toxicity of its root powder, whole plant powder, or different extracts of the plant (e.g.,water, alcohol, petroleum ether, purified alkaloids, and glycosides.It is generally safe when taken in the prescribed dosage range (Aphale *et al*., 1998). Large doses have been shown to cause gastrointestinal upset, diarrhea, and vomiting. A typical dose of ashwagandha is 3-6 grams daily of the dried root, 300-500 mg of an extract standardized to contain 1.5 percent withanolides, or 6-12 ml of a 1:2 fluid extract per day. Large doses of ashwagandha may possess abortifacient properties; therefore, it should not be taken during pregnancy. Since ashwaganda acts as a mild central nervous system depressant, patients should avoid alcohol, sedatives, and other anxiolytics while taking ashwagandha.

2.3 Cytotoxicity test:

In past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). Cytototoxicity assays have been developed which use different parameters associated with cell death and proliferation, typically based on three basic parameters (i) measurement of cellular metabolic activity, (ii) measurement of membrane integrity (iii) measurement of cell number (Weyermann et al., 2005).

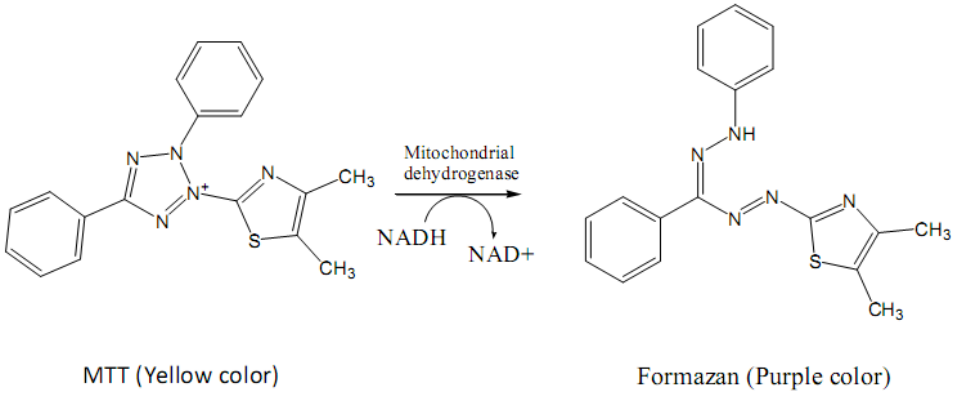


Figure 2.10 Convertion of MTT into Formazan by mitochondrial dehydrogenase

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], a yellow tetrazole, is a colorimetric assay based on the metabolic activity of viable cells. The yellow tetrazole is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. The purple MTT formazan crystals are insoluble in aqueous solutions are dissolved in solvent (usually, dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) to a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer depending upon the solvent used (Mosmann, 1983). When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance (Mosmann, 1983). The use of the MTT method does have limitations influenced by: (1) the physiological state of cells and (2) variance in mitochondrial dehydrogenase activity in different cell types. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors

and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves.

2.3.1 HeLa cells

HeLa cells originated as cervical tumor cells taken from Henrietta Lacks, who later died from the disease in 1951. They have been used in over 60,000 studies and in two Nobel-Prize-winning projects leading to important discoveries, such as the development of the polio vaccine. HeLa are considered "immortal": they do not die of old age and can divide an unlimited number of times as long as basic cell survival conditions are met (i.e. being maintained and sustained in a suitable environment). There are many strains of HeLa cells as they continue to evolve by being grown in cell cultures, but all HeLa cells are derived from the same tumour cells removed from Lacks. It has been estimated that the total mass of HeLa cells today far exceeds that of the rest of Henrietta Lacks' body.

HeLa cells have also been used successfully as a model system in various researches including drug testing. For this research, the ATCC culture of the HeLa cell was purchased from Everest Biotech pvt. LTD, Khumaltar, Lalitpur, Nepal.

Chapter III

Materials and Methods

3.1 Collection of Plant materials

The plants were freshly collected from the different parts of Nepal**.** The parts of the plants were selected on the basis of their reported uses in ethno medicine and in different literature. The plants thus collected were identified with the help of Annoted Checklist of Flowering Plants in Nepal (Press *et al*., 2000) and authenticated by Dr. Deepak Raj Pant, Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal.

Table 3.1 Collection site and Parts of plants selected

Scientific name	Vernacular Name	Collection site	Parts of plant used	Time of collection
C. procera	Seto Aank	Makwanpur	Leaf	July
Cl. Montana	Junge lahara	Rasuwa	Leaf	August
N. jatamansi	Jatamansi	Rasuwa	Rhizome	August
S. anacardium	Bhalayo	Makwanpur	Fruit	December
T. chebula	Harro	Makwanpur	Fruit	December
W. somnifera	Aswagandha	Janakpur	Root	August

3.2 Phytochemical Extraction

The plant materials were shade dried at room temperature for 10-15 days and grinded to fine powder using the mechanical mixer grinding and subjected for extraction. The extraction technique followed was Soxlation method. In this method, 20g powder was filled in a cellulose thimble and fed to the soxhlet chamber. The soxhlation apparatus was fitted with its RB (round bottom) flask containing 200ml of absolute methanol and condenser, at the bottom and top respectively. Soxhlation was carried out for 38 hours at 60-70°C using a heating mantle. The extract collected in the RB flask after soxhlation was filtered and then concentrated at reduced pressure at 40°C using a rotary evaporator (Hanshin Scientific Co., Korea) to yield crude extract.

Similarly for aqueous extraction, hot water extraction was done by taking 20g of powder in a conical flask with 200ml of water at 50°C with intermittent shaking for 24 hours. The

mixture was centrifuged and subjected for further round of extraction till colorless. After filtration of the extract, the solvent was evaporated in water bath at 50°C to obtain solid mass of the extract. The obtained solvent free extracts were stored at 4°c until use. The percentage yield of the extract was calculated by using following formula:

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

3.3 Phytochemical screening

The crude extracts (aqueous and methanolic) were subjected to preliminary phytochemical screening to detect the major phytoconstituents present in them. The analysis was carried out using standard qualitative methods as described previously by Harborne and Baxter, 1995; Todkar *et al.*, 2010. The following tests were carried out on extracts to detect various phytoconstituents present in them.

3.3.1 Detection Alkaloids

100mg of solvent free extracts were boiled with 2ml of 1% HCl, filtered, divided into two equal parts and subjected for following tests:

3.3.1. a Mayer's Test

Filtrate was treated with Mayer's reagent (Potassium Mercuric Iodide) by adding few drops from the side wall of test tube. Formation of a yellow colored precipitate was noted as the presence of alkaloids.

3.3.1.b. Wagner'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 Saponins

Frothing test: The extracts of 50 mg were first dissolved in 1ml of water and filtered. The filtrate was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed for a stable persistent froth.

3.3.3 Detection Terepenoids and Steroids

3.3.3.a Libermann- Buchard test

To the solution containing equal volume of methanolic extracts and chloroform, 2-3 drops of acetic anhydrate was added. After that few drops of Conc. Sulphuric acid was added from the sides of the test tube. Formation of a brown ring at the junction of two

layers and the upper layer turns green which shows the presence of Steroids and formation of deep red colour indicates the presence of Triterepenoids.

3.3.3.b Salkowski test

5ml of the extracts were treated with 2ml of Chloroform with 3ml of conc. Sulphuric acid. The mixture was shaken well and allowed standing for some time. Red color appeared at the lower layer indicated the presence of Steroids and formation of yellow colored lower layer indicated the presence of Terpenoids.

3.3.4 Detection of Tannins

3.3.4.a Ferric Chloride Test

To 3ml of the extract solution (10mg/ml), 2ml of 1% ferric chloride solution were added. Appearance of a transient greenish to black color was regarded as the presence of tannins.

3.3.5 Detection of Flavonoids

3.3.5.a Shinoda test

2ml of each extracts were treated with few drops of Conc. HCl and Magnesium turnings. Presence of Flavonoid was indicated if pink or red color developed within 3 minutes.

3.3.5.b Alkaline reagent test

To the 5ml test solution, 2ml of 2% of NaOH was added. Formation of an intense yellow colour, which turns to Colourless on addition of few drops of dil. acid, indicates presence of Flavonoids.

3.3.6 Detection of Phenols

Bromine water test: 3ml of extract solution (10mg/ml) was treated with 1ml of bromine water. Formation of yellow precipitate indicates presence of Phenols (De *et al*., 2010).

3.3.7 Detection of Carbohydrates and Glycosides

0.2g dry extracts were dissolved in 10ml distilled water, filtered and subjected for following tests:

3.3.7.a Molisch's test

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.b Fehling's test

To 2ml of Fehling's solution (1ml of Fehling's A and 1 ml of Fehling's B solution), 2ml of filtrate was added, mixed well and placed in a boiling water bath. Appearance of yellow or red color precipitate indicates the presence of reducing sugars.

3.3.8 Detection of proteins and amino acids

Aqueous fraction of the extracts were obtained by dissolving 50mg of extracts in 5ml distilled water, filtered and subjected for following tests:

3.3.8.a Biuret Test

Few drops of 2% copper sulphate+ 1ml of 95% ethanol was added to 2ml of the filtrate followed by addition of KOH pellets in excess and presence of protein is confirmed by appearance of pink color in the ethanolic layer.

3.3.8.b Ninhydrin test

To the 2ml of filtrate, 2 drops of Ninhydrin reagent was added and presence of amino acid is confirmed by appearance of characteristic purple color.

3.4 Antibacterial assay (NCCLS, 1999)

The effect of various plant extracts on the different bacterial strains were assayed by Agar well diffusion method and further confirmed by Disc diffusion method. The minimum concentrations of the plant extracts to inhibit the microorganisms were also determined by micro dilution method using plant fractions serially diluted in sterile nutrient broth (NCCLS, 1999).

3.4.1 Microorganisms used

The screening of the antimicrobial activity of crude extract was carried out individually on active cultures of six standard (ATCC) strains of bacteria. These include a single strain of gram positive bacteria (*Staphylococcus aureus* ATCC 25923) and five strains of gram negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurim* ATCC 14028, *Serratia marcesens* ATCC 13880). These microorganisms were provided by Nepal Academy of Science and Technology (NAST), Lalitpur Nepal.

3.4.2 Preparation of Extract

50mg/ml concentration of crude extracts was prepared by dissolving 100mg of extract in 200 ml of DMSO. The resulting solution was stored in refrigerator At 4°C until use.

For the disc diffusion assay, filter paper was cut to make disc of 6mm diameter then the discs were soaked with 10µl of different extract solution (50mg/ml) and allowed to dry in laminar hood which was ready to use.

3.4.3 Antibacterial screening via Agar well diffusion and Disc diffusion Technique

At first, sterile and uniform thickness (4mm) of Muller Hinton Agar (MHA) plates were prepared. Then the bacteria inoculums were prepared by adding an overnight culture of the organism in Nutrient Broth (NB) at 37°C for 12-18 hours. The inoculums were then compared with Mc Farland standard 0.5 (approximately 10^8 CFU/ml). The suspension were then diluted 1:100 in MH broth to obtain 10^6 CFU/ml. The freshly prepared inoculums of each bacterium were swabbed uniformly all over the surface of the MHA plate using sterile cotton swab and allowed to diffuse for 15 minutes at room temperature in laminar hood.

For the agar diffusion method, 8 wells of 6mm diameter were bored in the inoculated medium with the help of sterile cork-borer of 6mm diameter. In each plate, six different extracts (10µl), a positive control (Gentamycin, 50mcg) and a negative control/ solvent control (DMSO) were added into eight wells. The plates were then incubated at 37°C for 12- 18 hours in upright position.

For the disc diffusion method, the inoculated plate was taken and in each plate, eight discs were placed at equidistance. Out of eight discs, one is negative control (DMSO) one is positive control (Gentamycin, 50mcg) and six discs, each impregnated with different extract. Each disc was pressed down to ensure complete contact with the agar surface and then incubated at 37°C for 12-18 hours in inverted position.

After the incubation, the plates were observed for the formation of halozone around the well/ disc which corresponds to the antibacterial activity of the tested compound then the zone of inhibition (ZOI) or the diameter of halozone were recorded.

3.4.4 Determination of Minimum Bactericidal Concentration (MBC)

The minimum Bactericidal Concentration (MBC) was determined by micro dilution method using serially diluted plant extracts according to the NCCLS protocol. The aqueous and methanol extracts were diluted by two fold to get series of concentrations from 1 to 32mg/ml in freshly prepared sterile nutrient broth. Fifty microliter of the microorganism suspension (correspond to 10^6 CFU/ml) was added to each of the sample dilutions. These were incubated for 18 hours at 37°C and each tube content was subcultured in fresh nutrient agar separately and minimum bactericidal concentration was determined that showed no growth at all.

3.5 Determination of Total Polyphenol Content

The total polyphenol contents of the extracts (Aqueous and methanolic) were determined by Folin-Ciocalteu method (Roy *et al.,* 2011; Ainsworth and Gillespie 2007). At first the crude extract was dissolved in absolute methanol to make concentration of 5mg/ml. 0.5ml of each extract was separately mixed with Folin–Ciocalteu (F-C) reagent (5ml,1:10 diluted with distilled water) and aqueous Na2CO3 (4ml, 1M) solution. The reaction mixture was allowed to stand for 15min at room temperature. The absorbance of the reaction mixture was measured at 765nm using a UV–visible spectrophotometer. For blank, 0.5 ml of absolute methanol was added instead of extract solution in above reaction mixture and Gallic acid solution (concentration ranging from 25-250µg/ml) was used as standard. The total polyphenol content was expressed in terms of milligram of gallic acid equivalent per gram of dry mass (mgGAE/g) using galllic acid standard curve.Three replicates were performed for each sample concentration to check the reproducibility of the experimental result and to get more accurate result.

3.6 Determination of Total Flavonoid Content

The aluminium chloride colorimetric method was used for flavonoid content determination (Chang *et al.,* 2002, Roy *et al*., 2011). Each plant extracts was dissolved in absolute methanol at the concentration of 5mg/ml. Then, 0.5 ml of each sample solution was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The resulting solutions were shaken and allowed to stand at room temperature for 30 min. The absorbance of the reaction mixture (yellow colored complex) was measured at 415 nm. Quercetin was used as standard (10-100µg/ml) and blank was prepared by adding all the reagents except the plant sample. The total flavonoid content were then expressed in terms of quercetin equivalent per gram of dry mass ie, milligrams of quercetin per gram dry weight of the plant. All these tests were carried out in triplicates (n=3).

3.7 Determination of Antioxidant Activity

The antioxidant activity of the plant extracts were determined by two different methods, namely: ABTS Radical Cation Decolorization Assay (RE *et al*., 1998, Thaipong *et al.,* 2006)and DPPH free radical scavenging activity (Thaipong *et al*., 2006, Brand-Williams *et al*. 1995) with some modification.

3.7.1 Radical Cation Decolorization (ABTS) Assay

First of all ABTS: 2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonicacid) was dissolved in Na. acetate buffer (P 6.5) prepared in 70% methanol to a concentration of 7mM. ABTS

radical cations (ABTS•[1]) were generated by Reacting ABTS stock solution with 2.45mM potassium Persulfate (final concentration). The mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The cation solution thus formed was diluted with methanol to an absorbance of 0.7(±0.02) at 734 nm and equilibrated at 30°C.

To the 1ml of diluted ABTS solution, 10µl of different concentration of sample solution was added and the absorbance of the resulting solution was measured at 734nm after 6 minute. Gallic acid and Ascorbic acid were used as positive control. Sample blank and control were the solvent (DMSO) and ABTS cation solution respectively.

The extent of decolarization or the radical scangavanging activity (RSA) was calculated as:

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

Abs: Absorbance

3.4.2 DPPH free radical scavenging activity

The stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to assay the antioxidant activity of crude extracts by measuring the DPPH scavenging activities of the plant extracts by UV spectrophotometer at 517 nm. A solution of 1mM DPPH· was prepared in 80% (v/v) methanol by stirring for about 40 min. Absorbance of the solution was adjusted to 0.7(±0.02) at 517nm using fresh 80% (v/v) methanol. Then, 10 µL of standard or sample solution were mixed with 1mL of DPPH· solution and incubated for 30min in the dark covered with aluminum foil at the room temprature. Then after, the absorbance of each test was measured at 517nm. Gallic acid and ascorbic acids were used as positive control and sample blank and control solutions were the solvent and DPPH solution respectively.

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

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

In both the cases, the EC_{50} value (concentration of extracts that show 50% Radical scavenging activity) of each plant extract was calculated by the formula in Microsoft Excel 2007 software as described by Prof. Dr. Louis Maes and Prof. Dr. Paul Cos (Louis and Paul, 2010).

$$EC_{50} = \exp\left[\ln(\text{conc} > 50\%) - \left(\frac{\text{signal} > 50\% - 50}{\text{signal} > 50\% - signal < 50\%}\right) \text{X} \ln\left(\frac{\text{conc} > 50\%}{\text{conc} < 50\%}\right)\right]$$

exp: exponential function, ln: natural log function both used in Microsoft Excel 2007 software. Signal >50%: RSA value just above 50%, signal <50%: RSA value just below 50%. Conc >50%: concentration of extracts/control for signal >50% and conc <50%: concentration of extracts/control for signal <50%.

All the tests were performed in triplicates **(n=3)**

3.5 Assay for anticancerous/cytoxicity activity

3.5.1 HeLa Cell culture

HeLa cell line, Fetal Bovine Serum (FBS), Antibiotics (penicillin and streptomycin) were purchased from Everest Biotechnology centre, Khumaltar, Lalitpur Nepal. Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (cRPMI) at 37°C in a humidified CO_2 incubator containing 5% CO_2. These cell lines were cultured and subcultured in cRPMI-1640 media and were cryopreserved for further use. For the cytotoxicity assay, when the cell confluency reached > 80%, cells were washed with PBS (P 7.4), Trypsinized and harvested from T-25 flasks using 0.25 % trypsin/ EDTA solution. Then the cells were sub-cultured in 96 well plates (Merck, Germany).

3.5.2 Macrophage isolation and culture

In order to study the toxicity of extracts on the normal cell, the isolated peritoneal macrophage of a healthy mice was treated with different concentration of the extracts (only those which showed good anticancerous activity against HeLa cell) and compared with the cancerous cell.

The mice peritoneal macrophage was isolated according to the method described by Zhang *et al*., 2008. Briefly, a BALB/C mouse was injected with 2% starch in its peritoneal cavity and left for 2 days. The mouse was then euthanized and its peritonial cavity was exposed by making short, horizontal incision at the abdominal region. 10ml harvest medium (cold iRMPI) was injected in peritoneal cavity with syringe and using the same syringe, the peritoneal fluid was aspirated along with the harvest medium. The fluid was then centrifuged at 4°C at 1000 rpm for 10 minutes. The pellet was washed with iRMPI for two times and finally resuspended in 1ml cRPMI. The macrophage was counted using Neubauer's chamber and the volume was made upto 4ml adding cRPMI and the cell suspension was then transferred to the T25 culture flask and incubated for 1 hour at 37°C in a humidified CO_2 incubator containing 5% CO_2. Non adherent cells were removed by gently washing three times with warm PBS. For execution of cytotoxicity assay, the cells were detached from flasks using 0.25 % trypsin/ EDTA solution and seeded in 96-well tissue culture plates.

3.5.3 MTT Assay

Cytotoxicity of methanolic and aqueous extract of plants were assessed in HeLa cells as well as mice peritoneal macrophages using the MTT reagent (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This method is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases. The cells were seeded in 96-well plates at a density of 10^4 cells/well/100 µl and incubated for 24 h at 37°C and 5% CO_2. After incubation, the cells were washed with fresh RPMI-1640 medium (without serum) to remove non-adherent/ dead cells and 100µl of fresh complete medium was dispensed containing different concentration of plant extracts (6.25, 12.5, 25, 50, 100, 200 µg/ml). DMSO (Merck, Germany) of concentration 0.2 % (v/v), was used as a negative control. After completion of incubation, 50µl of 0.5% MTT was added to each well and plates were further incubated for 4 hours and 100µl of DMSO was added in each well to dissolve blue formazan formed by reduction of yellow tetrazolium salt within the cells. The absorbance of the plate was read at 540 nm in Microplate Elisa Reader. The experiments were performed in triplicate and a well with cells in media only (without extract) was used as control and well with media only served as blank in the experiment. Fluorouracil, an anticancer drug, was used as a positive control in this study. Results were expressed as percentage reduction in cell proliferation, compared with controls (cells without extracts).

$$Percentage\ Inhibition\ (PI) = 100 - \left(\frac{At - Ab}{Ac - Ab}\right) X100$$

Where,

At= Absorbance value of test compound
Ab= Absorbance value of blank
Ac=Absorbance value of control

The cytotoxic concentration/ Inhibitory concentration of the extracts required to inhibit the 50% of HeLa cells proliferation (IC_{50}) and 50% of mouse peritoneal macrophages proliferation (IC_{50}) were calculated by using formulae as described by Prof. Dr. Louis Maes and Prof. Dr. Paul Cos (Louis and Paul, 2010).

$$\mathrm{IC}_{50} = \exp\left[\ln(\mathrm{conc} > 50\%) - \left(\frac{\mathrm{signal} > 50\% - 50}{\mathrm{signal} > 50\% - signal < 50\%}\right) \mathrm{X} \ln\left(\frac{\mathrm{conc} > 50\%}{\mathrm{conc} < 50\%}\right)\right]$$

exp: exponential function, ln: natural log function both used in Microsoft Excel 2007 software. Signal >50%: PI (Percentage Inhibition) value just above 50%, signal <50%: PI just below 50%. Conc >50%: concentration of extract for signal >50% and conc <50%: concentration of extract for signal <50%.

All the tests were performed in triplicates **(n=3)**

3.5.6 Determination of cell count and viability

Neubauer's chamber was used to determine the cell count throughout the experimental procedures. Neubauer's chamber is a thick glass microscope slide with a rectangular indentation engraved with a laser-etched grid of perpendicular lines consisting 9 large squares (further divided in 16 smaller squares) each measuring 1mm x 1 mm in area and 0.1 mm in depth equating to a volume of 1 mm^3. For determining the cell count 10 µL of well homogenized cell suspension was loaded in the counting chamber and covered with Neubauer's coverslip and observed in light microscope. Total live cell count was determined usin the formula:

$$Cell\ number = \frac{Total\ no.of\ live\ cells\ counted\ in\ 4\ corner\ squares}{4} X\ dilution\ factor X 10^4$$

The dead and live cells were separated by staining with 0.4% Trypan blue. Trypan blue is a vital dye. Its reactivity is based on negatively charged chromophore which do not interact with the cell unless the membrane is damaged (Masters, 2000). Dead cells were stained blue while live remained colorless.

3.5.7 Determination of survival index

The cytotoxicity of crude extracts and drugs on the mice peritonial macrophages was compared with the activity against cancerous cell line, by using the selectivity index (SI). SI is the ratio of cytotoxicity towards normal cell (IC_{50}) to inhibitory concentration of the cancer cell (IC_{50}). A value greater than 1 indicates the treatment is more selective towards the cancer cell.

$$\text{Selectivity Index (SI)} = \frac{\text{Cytotoxicity to Macrophages}(IC_{50})}{\text{Cytotoxicity to HeLa cell } (IC_{50})}$$

3.6 Statistical Analysis

All the experiments were performed in triplicates and the data represent the mean ± standard deviation from three independent assays. Microsoft Excel 2007 software was used for the calculation of phenol/flavonoid content, calculation of Inhibitory concentration values, EC_{50}, (IC_{50}) and (IC_{50}) for antioxidant, anticancer and cytoxicity assay respectively and generation of graphs and charts and for statatistical analysis GraphPad Prism V.5.0 was used. The confidence interval was taken to be 95% for paired two tailed t-test to compare the methanolic and aqueous values of extracts. The p values showing less than 0.05 was considered to be significant.

Chapter IV

Results

4.1 Phytochemical Extraction

Due to the use of two different solvent and different parts of plants, the percentage yield, texture, and consistency of the resulting extract showed some variations. The percentage yield of the extract varied from 9.3% to 44%, *Terminalia chebula* yielding the highest percentage while *Withamnia somnofera* had the lowest yield in both aqueous and methanolic extract. The consistency of most of the extracts was very sticky and oily, some being solid and some semi solid in appearance except for *Clematis montana,* which had almost liquid like appearance even after in vacuo concentration in the evaporator. The percentage yield and some characteristics of the resulting extracts have been tabulated in Table 4.1.

Table 4.1 Physical characterization (Color and consistency) and percentage yield of the crude methanolic and aqueous extracts

Plant	Solvent	Characteristics Of Extracts		Dry Wt. taken (gm)	Wt. of Extract (gm)	Percenrage yield (%)
		Color	Consistency			
Cl. montana	Methanol	Dark green	Liquid	25	6.75	27
	Aqueous	Dark green	Liquid	10	4.05	40.5
C. procera	Methanol	Dirty green	Semi solid	20	2.49	12.45
	Aqueous	Dark green	Semi solid	10	2.02	20.2
N. grandiflora	Methanol	Brown	Sticky with aroma	20	3.58	17.9
	Aqueous	Light Brown	Sticky with aroma	10	1.1	11
S. anacardium	Methanol	Blackish	Very oily	25	2.33	9.32
	Aqueous	brown	Sticky	10	0.93	9.3
T. chebula	Methanol	Dark brown	Solid	20	8.8	44
	Aqueous	Dark brown	Solid	10	4.2	42
W. somnifera	Methano	Brownish yellow	Sticky	20	2.02	10.1
	Aqueous	Light yellow	Sticky	10	0.94	9.4

4.2 Phytochemical screening

Preliminary Phytochemical screening of the methanolic and aqueous extracts revealed the presence of different kind of chemical groups that are summarized in table 4.2 (Shown in Appendics). The extracts showed the presence of different pharmacologically active components as, Alkaloid, saponins, steroid, tannin, Reducing sugars, Proteins, phenolic compounds etc. Among the extracts, *T.chebula* was only the plant that in which almost all the chemical groups were detedced except for Ninhydrin test. Many chemicals were not detected in the aqueous extracts of *C. procera, N. jatamansi* and *W. somnifera.* Methanolic extract of *W. somnifera* was detected to contain highest alkaloid than other plant extracts. Those extracts that didn't show positive test for both the test of single chemical group was considered as negative.

4.3 Antimicrobial screening

The antimicrobial activity of plant extracts were studied by Disc diffusion method and further confirmed by agar diffusion method. Both test showed similar type of result however, better visual result was obtained from agar diffusion assay. Among all the pathogens, only two bacterial strains (*Staphylococcus aureus* and *Pseudomonas aeuroginosa)* were found to be inhibited by the plant extracts rest of the Bacteria were resistant to the extracts. *T. chebula* showed potent anti bacterial activity against both of the pathogens with highest zone of inhibition and lowest value of MBC.

Table 4.3 Antimicrobial activity of aqueous and methanolic extracts of the plants

Plants	**Solvent**	**Zone of Inhibition (mm) Diameter of well= 6mm**					
	Bacterial strains	***S. aureus***	***E. Coli***	***K. Pneumonia***	***P. aeuroginosa***	***S. typhimurim***	***S. Marcesens***
	ATCC no.	**25525**	***25922***	***700603***	**27853**	**14028**	**13880**
Cl. Montana	Methanol	11	0	0	11	0	0
	Aqueous	10	0	0	0	0	0
C. procera	Methanol	8	0	0	0	0	0
	Aqueous	0	0	0	0	0	0
N. jatamansi	Methanol	10	0	0	11	0	0
	Aqueous	0	0	0	0	0	0
S. anacardium	Methanol	9	0	0	10	0	0
	Aqueous	0	0	0	0	0	0
T. chebula	Methanol	20	0	0	18	0	0
	Aqueous	18	0	0	15	0	0
W. somnifera	Methanol	12	0	0	12	0	0
	Aqueous	0	0	0	0	0	0
Gentamycin		31	19	10	30	21	30
DMSO		0	0	0	0	0	0

The minimum bactericidal concentration of the plant extracts, which are able to produce zone of inhibition during screening process are tabulated in table 4.4.

Table 4.4 Minimum Bactericidal concentration of extracts against Susceptible Bacteria

Plants	**Minimum Bactericidal Concentration (MBC) (mg/ml)**			
Bacteria	***S. aureus* (ATCC 25525)**		***P. aeuroginosa* (ATCC 27853)**	
	Methanol	**Aqueous**	**Methanol**	**Aqueous**
Cl. Montana	8	>10	8	>10
C. procera	8	>10	4	>10
N. jatamansi	4	>10	8	>10
S. anacardium	8	>10	8	>10
T. chebula	2	2	2	4
W. somnifera	2	>10	8	>10
Gentamycin**(µg/ml)**	1.5	2	0.5	0.5

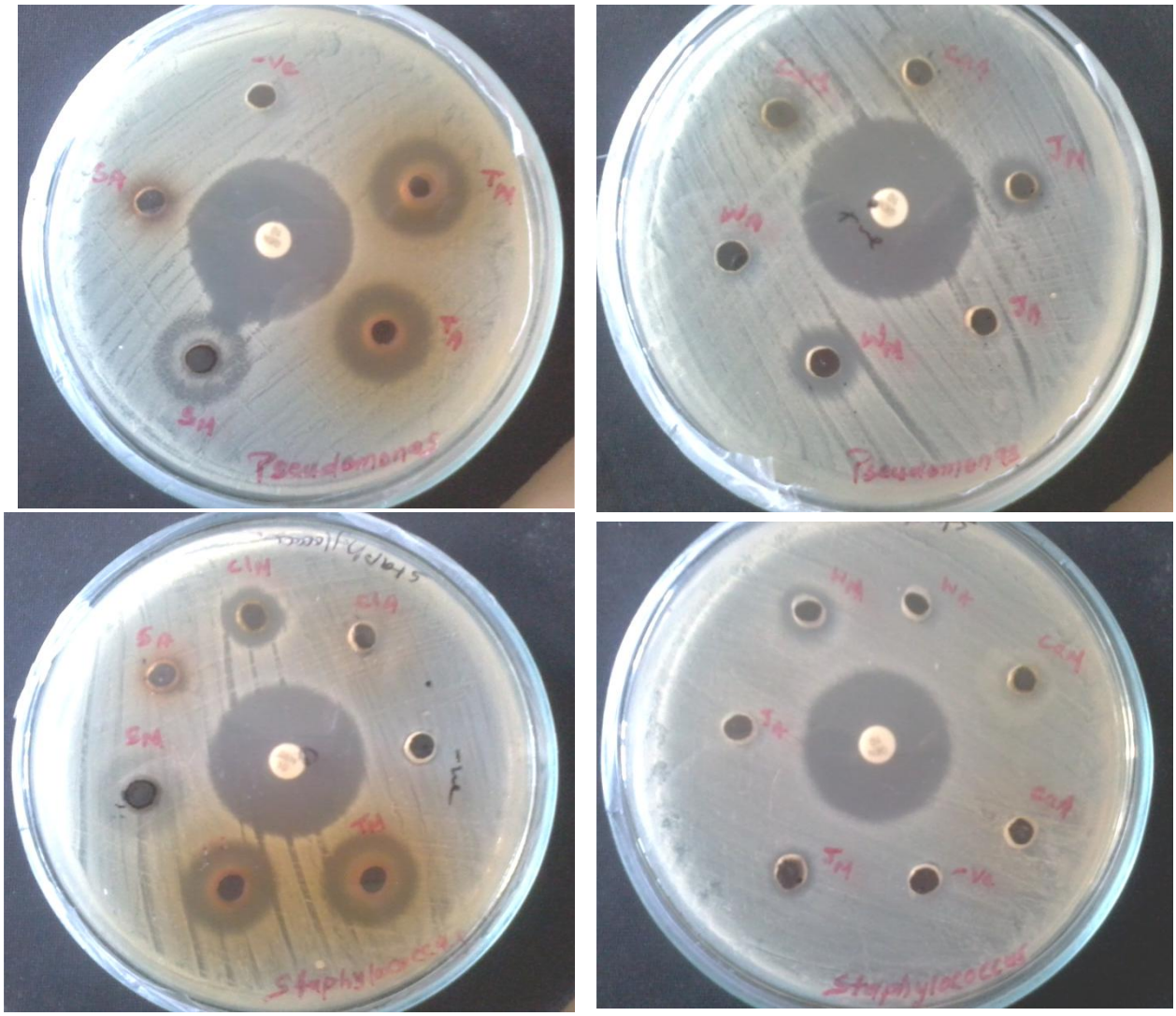


Figure **4.1** Antimicrobial screening of plant extracts showing Zone of Inhibition for *P. aeuroginosa and S. aureus*

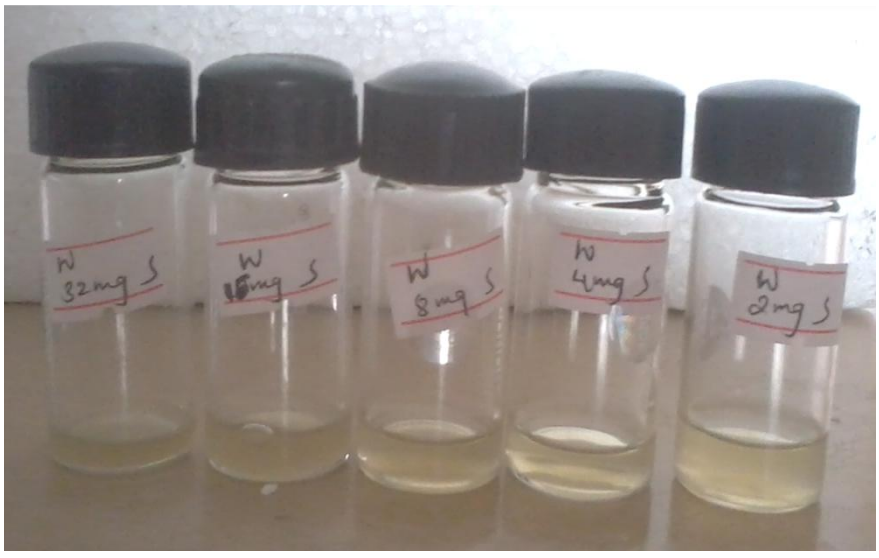


Figure 4.2 Determination of Minimum Inhibitory Concentration

4.4 Determination of Total Polyphenol Content

The total phenolic content of the crude aqueous and methanolic 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 (0-250µg/ml, y = 0.010x + 0.016, R^2 = 0.993).The total phenol content varied from 43.69 ± 1.32 to 888.79 ± 11.6 mg GAE/g dry wt. *Terminalia chebula* was found to contain very high amount of polyphenol in both aqueous (860± 10.8 mg GAE/g dry wt) and methanloic (888.79 ± 11.6 mg GAE/g dry wt.) extracts. *Withamnia somnifera* showed lowest content of polyphenol in both aqueous (43.69697 ± 4.34 mg GAE/g dry wt.) and methanolic (63.27 ± 3.53 mg GAE/g dry wt.) extracts. The amount of polyphenol was found to be higher in methanolic extracts than the aqueous extract except for *Clematis montana and Nardostachys jatamansi.* In *Cl. montana* the aqueous extract of which contained 252.7273 ± 7.7 mg GAE/g dry wt. and the methanolic extract contained 164.06 ± 6.5 mg GAE/g dry wt. whereas the polyphenol content of both the extracts of *Nardostachys jatamansi* was found to be almost same. Total polyphenol content of all the extracts are shown in a figure 4.3.

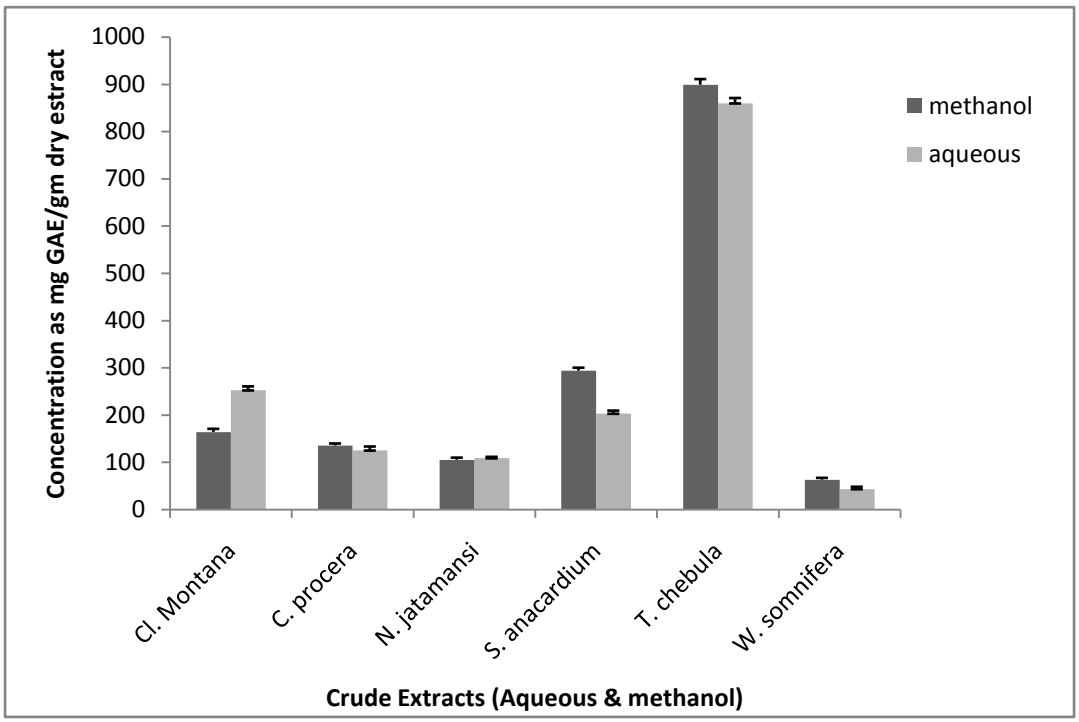


Figure 4.3 Comparision of total phenolic content of aqueous and methanolic extracts

Error bars represents the standard deviation from mean values

4.5 Estimation of Total Flavonoid Content

The total flavnoid content of both the aqueous and methanolic extracts of all the six plants are listed in the table below. The result were expressed in terms of Quercetin equivalent (mg Quercetin /gm dry mass). The calibration curve was generated by using Quercetin (0-100µg/ml) with equation y = 0.008x - 0.056 (R^2 = 0.992) and using this equation, the flavonoid content of the plants were estimated, the value of which vaired from 185.5 ± 9.98 to 4.75 ± 0.11. Methanolic extract of *Terminalia chebula* showed the highest flavonoid content of 185.5 ± 9.98 mg Quercetin /gm dry mass whereas the aqueous extracts of *S. anacardium, N. jatamansi* and *W. somnifera* showed very low amount of flavonoid content. The methanolic extracts have shown greater content of Flavonoid than that of aqueous extract except for *Clematis montana.*

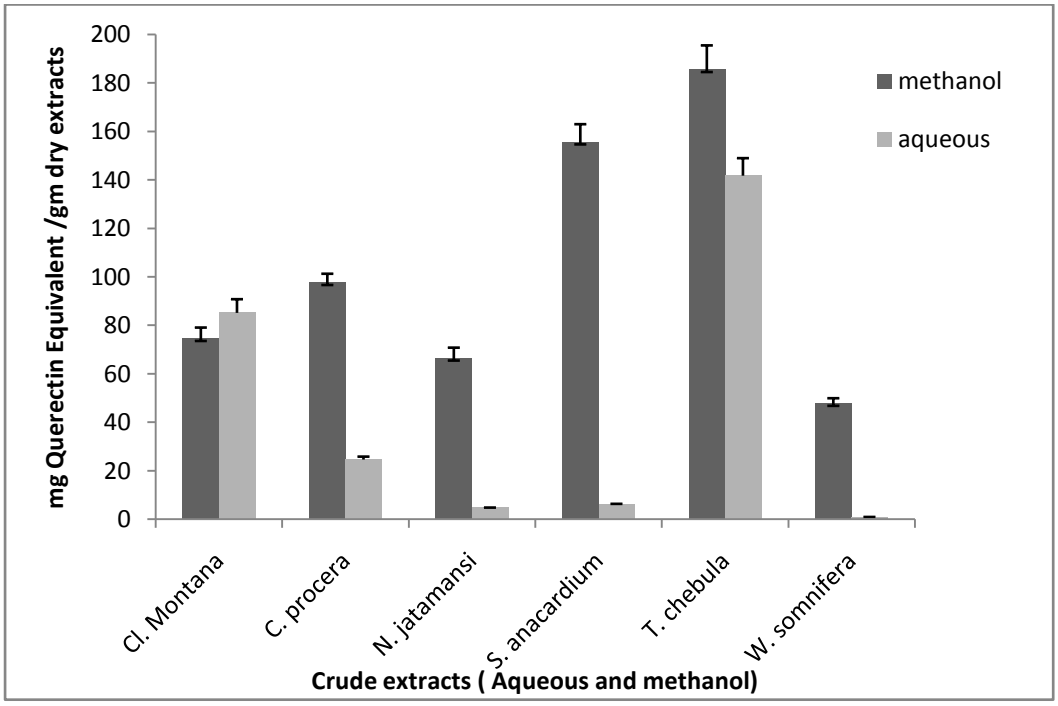


Figure 4.4 Comparision of total flavonoid content of aqueous and methanolic extracts

Error bars represents the standard deviation from mean values

4.6 Determination of Antioxidant Activity

4.6.1 ABTS Radical Cation Decolorization Assay

Total antioxidant activity was determined by ABTS Radical Scavenging assay/cation decolarization assay and the efficacy of antioxidant capacity of all the extracts were compared with two standard compounds, Gallic acid and Ascorbic acid. ABTS•+ is a blue colored chromophore which is reduced to ABTS in a concentration dependent manner upon addition of extract. All the extract showed radical scavenging activity in concentration dependent manner. The EC_{50} values of standard Gallic acid and Ascorbic acid was found to be 0.036 mg/ml and 0.17 mg/ml respectively. Among all the plant extracts, *T. chebula* showed the lowest EC_{50} value, indicating potent antioxidative capacity, comparable to that of standard compounds. The EC_{50} value of methanolic and aqueous extracts of *T. chebula* are 0.10 and 0.170 respectively which is lower than that of the Ascorbic acid. The EC_{50} value of the extracts of *Cl. montana, C.procera, N. jatamansi* and *W. somnifera* was found to be very high compared to standard among which *W. somnifera* showed the exceedingly high EC_{50} value. The EC_{50} values of all the extracts and standards are shown in table 4.5.

Table 4.5 EC_{50} values of extracts and standards in ABTS cation decolorization assay

Plants	**EC_{50} values of Methanolic extract (mg/ml)**	**EC_{50} values of Aqueous Extract(mg/ml)**
Gallic acid	0.036	0.036
Ascorbic acid	0.17	0.17
Cl. Montana	4.340	2.35
C. procera	5.86	4.23
N. jatamansi	6.52	9.66
S. anacardium	0.98	0.90
T. chebula	0.10	0.17
W. somnifera	15.6	24.12

4.6.2 DPPH free radical scavenging activity

The hydrogen donating or free radical scavenging ability of extracts was examined using the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH). The antioxidant properties of

these extracts were found to be concentration dependent and their antioxidant property was compared with standard compounds: Gallic acid and ascorbic acid, the EC_{50} values of which were found to be, 0.09mg/ml and 0.23mg/ml respectively. Among all the extracts, *T. chebula* showed the highest DPPH free radical scavenging activity with EC_{50} values 0.31 mg/ml and 0.34 mg/ml for methanolic and aqueous extract respectively.The scavenging activity of *C. procera, N. jatamansi, and W. somnifera* was found to be very low with their EC_{50} value exceeding 10mg/ml. Both the extract of *S. anacardium* and aqueous extract of *Cl. montana* showed moderate DPPH scavenging activity. The EC_{50} values of all the extracts and standards are listed in table 4.6.

Table 4.6 EC_{50} values of extracts and standards in DPPH Assay

Plants	**EC_{50} values of Methanolic extract (mg/ml)**	**EC_{50} values of Aqueous Extract(mg/ml)**
Gallic acid	0.09	0.09
Ascorbic acid	0.23	0.23
Cl. Montana	12.22	4.82
C. procera	12.85	15.76
N. jatamansi	14.50	16.34
S. anacardium	1.62	2.71
T. chebula	0.31	0.34
W. somnifera	31.02	47.36

4.7 Anticancerous activity on HeLa Cell

The cytotoxicity of all the plant extracts were studied by MTT assay on cancerous cell line (HeLa cell) with the treatment concentration, 0 to 200µg/ml with two fold dilution. The dose dependent inhibition was observed only on the methanolic extracts of *T. chebula, S. anacardium* and *W. somnifera* and aqueous extract of *T. chebula.* The cytotoxic activity of rest of the extracts couldn't be determined as they didn't show inhibition in dose dependent manner. Among all the methanolic extracts, *T. chebula* showed the high cytotoxic activity with IC_{50} value 30.33±1.4 µg/ml followed by *S. anacardium* and *W. somnifera* with their respective IC_{50} values 43.79±2.8 and 93.05 ± 3.8µg/ml. Among the aqueous extract, the cytotoxic activity could be determined only of the extract of *T. chebula* with its IC_{50} value 34.77± 0.6µg/ml. Fluorouracil was used as the standard drug taken was which showed the IC_{50} value 17.25±1.2µg/ml.

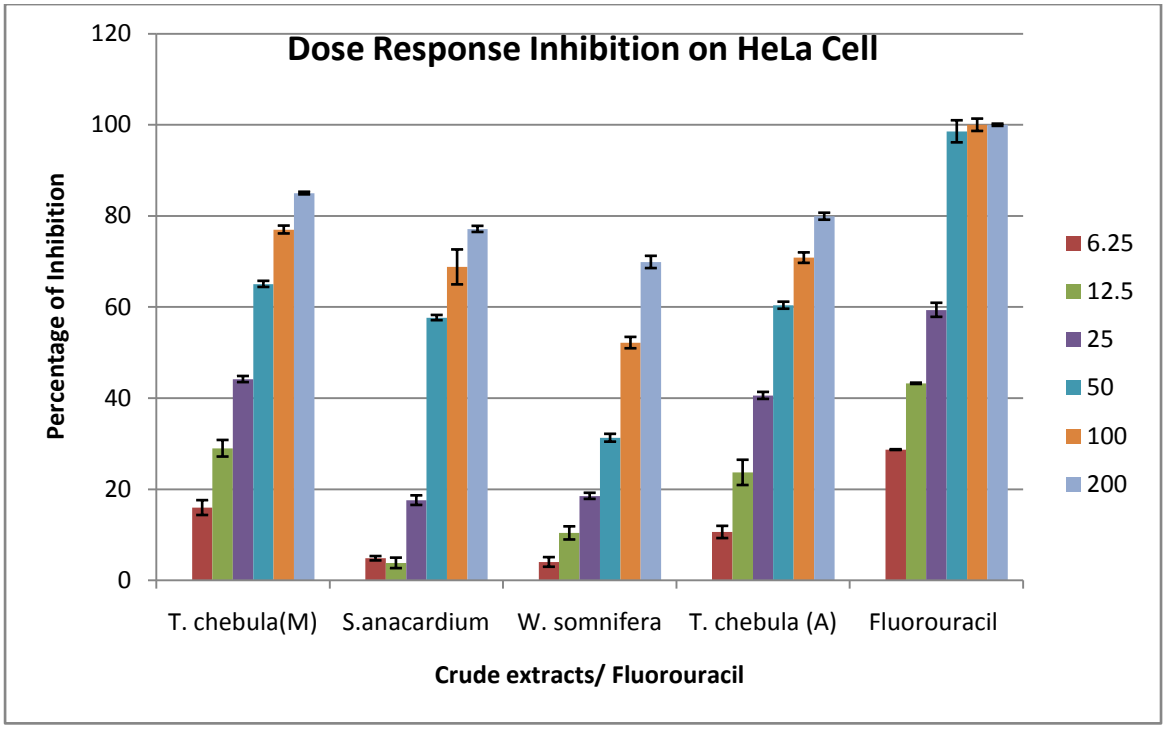


Figure 4.5 Inhibition of HeLa cell proliferation at different concentration of extracts/ Drug

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

4.8 Cytotoxicity on mouse peritoneal Macrophase

The extracts that were able to show anticancerous activity were tested for their cytotoxicity on normal cell using primary mice peritoneal macrophages. The result showed that the IC_{50} values (Concentration of the extract required to kill 50% of the macrophages cell population) (IC_{50}) of all the tested extracs were higher than that of their respective anticancerous activity indicating that they are relatively non toxic to the normal cells.

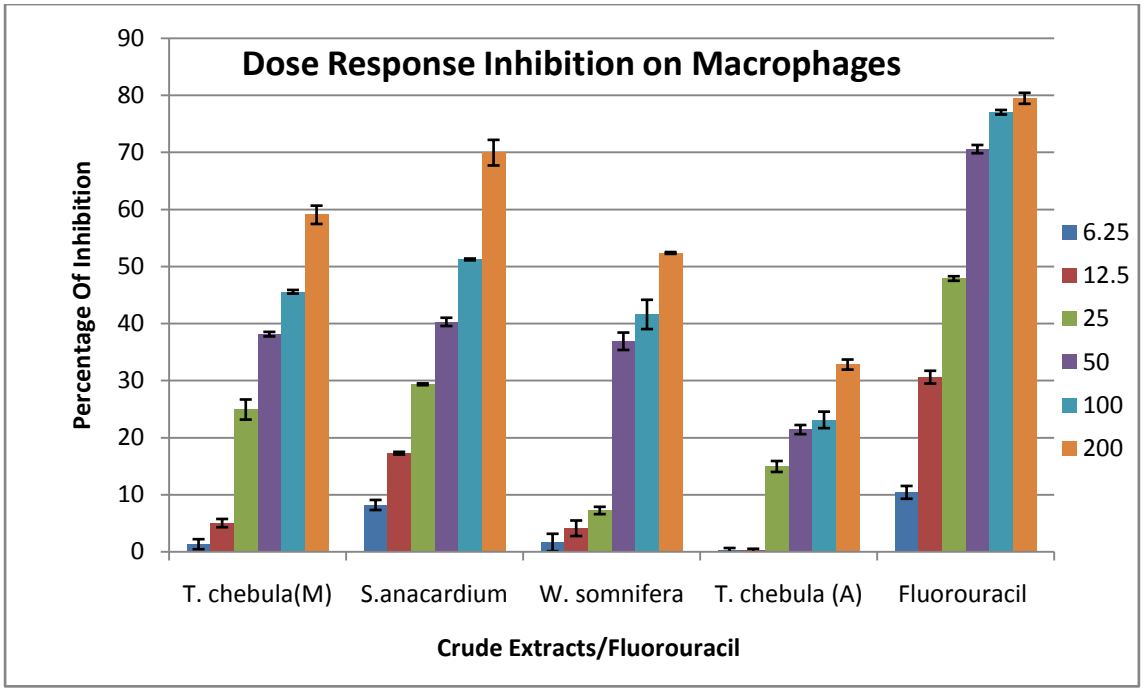


Figure 4.6 Effect of extracts/drug at different concentration on mouse peritonial macrophages

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

The Aqueous extract of *T. chebula* was found to be least cytotoxic with IC_{50} value higher than the maximum treatment concentration (>200µg/ml) followed by the methanolic extract of *W. somnifera, T. chebula* and *S. anacardium* with IC_{50} values, 171.58±2.0, 125.5±1.9, and 92.4 ± 2.5. The reference drug, Fluorouracil was found to be comparatively toxic than any other extracts with least IC_{50} value of 26.65 ± 0.8µg/ml.

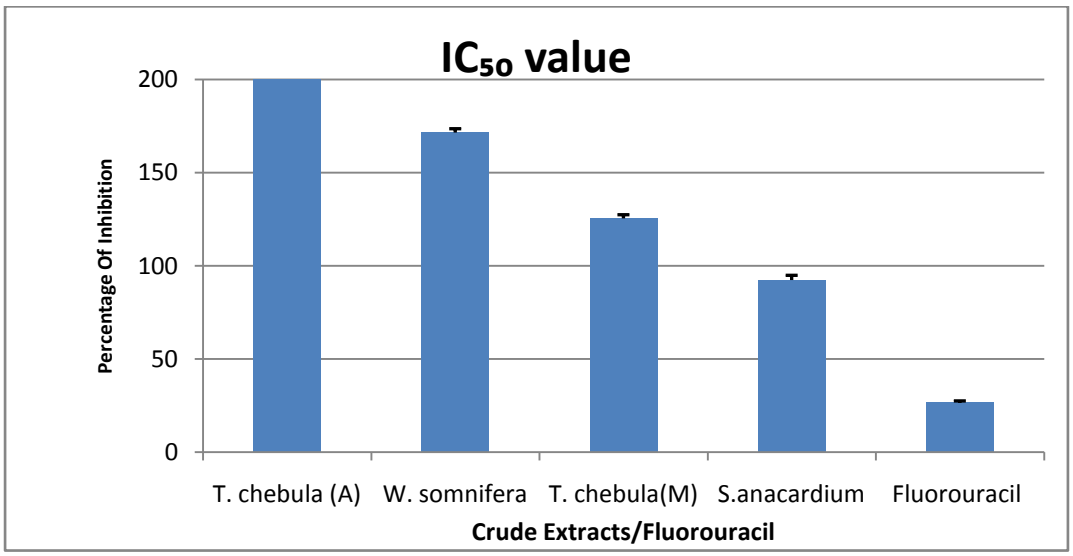


Figure 4.7 Comparison of IC_{50} values of crude extracts and drug on mice peritoneal macrophages

Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

4.9 Efficacy comparison on the basis of selectivity indices (SI)

Table 4.7: In virto cytotoxic activity of extracts against HeLa cell $(IC_{50})^H$ and Mice peritonial macrophage $(IC_{50})^M$ and their respective selective indices

Extracts/Drug	Cytotoxicity on HeLa Cell $(IC_{50})^H$ (µg/ml)	Cytotoxicity on Macrophages $(IC_{50})^M$ (µg/ml)	Selectivity index (SI)
Fluorouracil	17.25 ± 1.2	26.65 ± 0.8	1.54
S.anacardium	43.79 ± 2.8	92.40 ± 2.5	2.11
T. chebula(M)	30.34 ± 1.4	125.5 ± 1.9	4.13
T. chebula (A)	34.77 ± 0.6	>200	>5
W. somnifera	93.05 ± 3.8	171.58 ± 2.0	1.84

The cytotoxicity of extracts on mice peritoneal macrophage was compared with the cytotoxic activity against cancer cell , by using the selectivity index (SI) to compare the efficacy of the treatments.The selectivity indices of all the extracts was found to be higher than 1 indicating their selectivity towards cancer cell than that of normal cell. Among the extracts, the Aqueous extract of *T. chebula* showed more than 5 times more selectivity towards anticancer activity. The methanolic extracts of *T. chebula, S. anacardium* and *W. somnifera* were found to be 4.13, 2.11, and 1.84 times selective for cancer cell respectively. The drug, Florouracil showed 1.54 times more selectivity towards cancerous activity, the value of which is lower than that of both the extracts of *T. chebula.*

Stastistical Analysis

While comparing between the aqueous and methanolic extracts of all the tested plants, the antibacterial effect on two bacteria was found to be highly significant (p=0.005). Accordingly, significant difference (p=0.0357) was observed comparing the datas of total flavonoid content between the different extracts i.e. aqueous and methanolic. But the phenolic content datas showed no any significant differences (p=0.6978) between the TPC values of aqueous and methanolic extracts.

Chapter V

Discussion

Plants have been known to treat various diseases in Traditional systems of medicine. Therefore, the researchers today are emphasizing on evaluation and characterization of various plants and plant constituents against a number of diseases based on their traditional claims. This research work also attempts to study some of medicinal plants of Nepal for their antioxidant, antibacterial and anticancer property. In this work, six medicinally important plants were selected and some phytochemical data have been generated that can help to justify the traditional claim about their medicinal values. All the plants under the study were freshly collected from the field and it is important to mention that out of six two of the plants namely, *Calotropis procera* and *Nardostychs jatamansi* were young during collection. The parts of the plants for study were shed dried to preserve the bioactive compounds and subjected for extraction.

Extraction, in pharmaceutical term, involves the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. The extract thus obtained, contains complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans (Handa *et al*., 2008). The basic parameters influencing the quality of extract are, plant part used as starting material, solvent used for extraction and extraction procedure (Ncube et al., 2008). Here the part of plant to be study was selected on the basis of reported use of plant in traditional medicine. Two solvent systems (water and methanol) was selected as Water is universal solvent which is primarily used by traditional healers and has no toxicity, on the other hand methanol is more polar than water and ethanol and believed to extract nearly all the active constituents from the plant material. Another advantage of using methanol as extracting solvent is that it evaporates easily resulting in quick concentration of the extracts. The extraction process was completed by means of soxhlation. During soxhlet extraction, the sample is continually exposed to fresh solvent, which improves the efficiency of the method extracting maximum of the phytochemicals (Ncube *et al*., 2008). The variations in quantity and secondary metabolite composition of the extracts in different extraction method depends on, nature of solvent, time and temperature of the extraction, purity of solvent and Polarity.

Phytochemical analysis conducted on the plant extracts revealed the presence of several constituents which are known to exhibit medicinal as well as physiological activities. The major phytochemicals that are found are; alkaloid, phenols, tannins,

flavonoids, saponins, steroids, terpenoids, reducing sugars and proteins. The medicinal properties of any plants have been attributed to the type and the amount of these phytochemicals possessed by them.

Alkaloids have been associated with medicinal uses for centuries and several workers have reported the analgesic, antispasmodic and antibacterial (Okwu and Okwu, 2004) properties of alkaloids and one of their common biological properties is their cytotoxicity (Nobori *et al*., 1994). High content of alkaloids lead to the high risk of poisoning by the plant. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites and natural antioxidant mainly come from plants in the form of phenolic compounds such as flavonoid, phenolic acids, tocopherols etc (Ali *et al*., 2008). They possess biological properties such as antiapoptosis, antiaging, anticarcinogen, antiinflammation, antiatherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities (Han *et al*., 2007). Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds (Brown and Rice-Evans, 1998). Similarly, 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). They also are effective antioxidant and show strong anticancer activities (Salah *et al*., 1995). Terpenoids and tannins are attributed for analgesic and anti-inflammatory activities. Apart from this tannins contribute property of astringency i.e. faster the healing of wounds and inflamed mucous membrane (Okwu and Josiah, 2006). Tannins bind to proline rich protein and interfere with protein synthesis. The plant extracts were also revealed to contain saponins which are known to produce inhibitory effect on inflammation, show hemolytic activity, cholesterol binding properties and bitterness (Sodipo et al., 2000). Steroids have been reported to have antibacterial properties and they are very important compounds especially due to their relationship with compounds such as sex hormones (Santhi et al., 2001). Levels of secondary metabolites are both environmentally induced as well as genetically controlled (Savithramma et al., 2011) and this might be the reason in the variability of different secondary metabolites in different plants and this also depend upon age of the plants as older plant possess higher amount of secondary metabolite than the younger.

Many methods have been used to determine the antioxidant activity of natural products (Antolovich *et al*., 2002). No single assay however can establish the full antioxidant potential of compounds, because multiple reactions and mechanisms are involved in anti oxidative processes (Chu *et al*., 2000). Therefore, at least two different methods

should be applied in order to evaluate the antioxidant capacity of extracts of fruits, vegetables, and other plant products (Du *et al*., 2009). Therefore, in this study, the antioxidant potency of the extracts was evaluated using ABTS and DPPH assays. The ABTS assay is based on the generation of a blue/green cation radical (ABTS•+) by oxidation with potassium persulphate/manganese dioxide with the loss of a electron by the nitrogen atom of ABTS. In the presence of hydrogen donating antioxidant, the nitrogen atom quenches the hydrogen atom, yielding the decolorized solution and the antioxidant capacity of any compound is determined by the monitoring the extent of decolorization at 743nm, which is the absorption maxima of ABTS radical (Re *et al*., 1998). DPPH• is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH• does not dimerize, as happens with most free radicals. The delocalisation on the DPPH• molecule determines the occurrence of a purple color, with an absorption band with a maximum around 517nm. When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the purple colour. Therefore, the absorbance reduction depends linearly on the antioxidant concentration (Thaipong *et al.,* 2006). On comparing these two methods, ABTS assay is applicable to both hydrophilic and lipophilic antioxidant systems; whereas DPPH assay uses a radical dissolved in organic media and is, therefore, applicable to hydrophobic systems (Kim *et al*., 2002). Antioxidant capacity of plants may differ depending on the growing season, geographical origin, age of plant etc (Chun *et al*., 2005). A previous study reported that antioxidant capacity depends on the solvent used (Pe ´ rez-Jime ´nez and Saura-Calixto, 2006). In this study, for the extraction of the antioxidants, methanol and water were used. Especially the fat soluble antioxidants might not have been extracted to the full extent.

Among the extracts, *T. chebula* showed strong antioxidant activity in both ABTS and DPPH Assays with minimum EC_{50} value, comparable to that of standard compounds Gallic acid and Ascorbic acid. In this study, extracts of *C. procera* was showed poor antioxidant activity with the EC_{50} value 15.76 and 12.85mg/ml in aqueous and methanolic extracts by DPPH Assay but in a similar kind of study, (Yesmin *et al.,* 2008), the methanolic extract of plant showed strong antioxidant capacity with EC_{50} 110.25µg/ml. Many factors play role in the difference in the value and in this study, the main reason may be the physiological state of the plant during collection.

Antioxidant capacity of the medicinal plants has been found to be correlated with its flavonoid and phenol content as higher the phenol and flavonoid possessed by the plant, higher is its antioxidant activity (Chun et al., 2003; Kim et al., 2003). Similar results have been observed in this study as plants possessing higher amount of phenols and flavonoid have shown higher antioxidant activity. Phenols are compounds that have the ability to

destroy radicals because they contain hydroxyl groups. These important plant components give up hydrogen atoms from their hydroxyl groups to radicals and form stable phenoxyl radicals; hence, they play an important role in antioxidant activity. The overall antioxidant capacity of phenolic compounds can be described due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have metal-chelating potential (Rice-Evans *et al*., 1996). Therefore, determination of the quantity of phenolic compounds is very important in order to determine the antioxidant capacity of plant extracts.

Among the extracts under study *T. chebula* showed very high content of total phenolics which was in accordance with previous studies which has concluded that *T. chebula* contains more phenolics than any other plants (Saleem *et al* 2002). More than 80% of plant extracts showed TPC (Total phenol content) value greater than 100 GAE per gram extract which gives a valid reason for their use in traditional medicine. But as compared to TPC the flavonoid content data were very poor. Only 40% of extracts showed significant amount of flavonoid content while in case of aqueous *W. somnifera* extract, the resulting solution appeared milky and the data shown was the value obtained after centrifugation which was 0.92 ± 0.07 mg of Quercetin/gm of extract, which may or may not be its actual flavonoid content. The low falvonoid content despite their richness in polyphenol content might be because the plants extracts were rich in polyphenols other than flavonoid such as Tannins, phenolic acids, and other non flavonoid polyphenolics. The other reasons may be that some of the flavonoids in plants exist as glycosides so that their hydroxyl group remains unavailable for recation with $AlCl_3$. On overall comparison to the aqueous and methanolic extracts, phenol and flavonoids were found to be higher in methanolic extract than that of aqueous and so do their antioxidant capacity. The same pattern was observed on the study by Eghdami and Sadeghi on comparing the efficacy of methanol extract over aqueous extract as antioxidant (Eghdami A and Sadeghi F, 2010). Also, the scavenging of ABTS by the extracts was found to be higher than that of DPPH. Several factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals (Yu et al., 2002). Similarly Wang *et al*. found that some compounds that have ABTS.+ scavenging activity could not scavenge DPPH (Wang et al., 1996). The antioxidative potential of the extracts were compared with the pure compounds, Gallic acid and ascorbic acid and among the extracts, only *T. chebula* showed the comparable scavenging activity with the pure compounds.

Antimicrobial screening of the extracts was first done by disc diffusion and further verified by agar well diffusion methods. Both the assay showed similar result by forming clear zones around the positive sample site. The inhibition produced by the plant

extracts against particular organism depends upon various extrinsic and intrinsic parameters. Due to variable diffusability in agar medium, the actual antibacterial efficacy may not be demonstrated by ZOI. Therefore MBC value has also been computed in this study which is the lowest concentration of antibacterial substance required to produce a sterile culture (NCCLS, 2000). Among all the bacteria tested, only two strains of bacteria namely, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were found to be inhibited by majority of plant extracts while other remained resistant at same dose of extract. All the bacteria under study are pathogenic but *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the two bacterial strains which are exhibiting most forms of resistance and being major threat around the world. Since these two strains were inhibited by the extracts, further research in these extracts may lead to the discovery of new antibacterial drug against resistant pathogens. Among all the extracts, *T. chebula* was found to most potent antimicrobial agent with MBC value 2mg/ml for both strains followed by methanolic extracts of *W. somnifera* and *N. jatamansi.* The antibacterial potential of extracts can be attributed to their high polyphenol and flavonoid content (Cowan, 1999), however, *W. somnifera* and *N. jatamansi* showed better antibacterial activity than the extracts of *S. anacardium*, *Cl. montana* and *C. procera* which had higher polyphenol and flavonoid content. This indicates that phytoconstituents other than polyphenols might have shown antibacterial activity in those plant extracts.

Plant extracts and their bioactive constituents exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane which has as a consequence a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components and destruction or inactivation of genetic material. In general, the mechanism of antimicrobial action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou et al., 2008).

The preliminary anticancer screening of all the extracts was done against human cervical cancer cells (HeLa cell). Only the extracts of of *T. chebula* (both aqueous and and methanlic), methanolic extracts of *S. anacardium* and *W. somnifera* showed the dose response inhibition of HeLa cell, while other extracts failed to show concentration dependent cytotoxicity or growth inhibitory activity even at the highest concentration of 200 µg/ml. *T. chebula* which had shown excellent antioxidant and antimicrobial activity, also showed good antiproliferative activity in a dose dependent manner with IC_{50} value 30.34 ± 1.44 and 34.77 ± 0.632 µg/ml for methanolic and aqueous extract respectively. This value is found to be lower than that of the cytotoxicity values of isolated phenolics (gallic acid, ethyl gallate, luteolin, and tannic acid) of this plant against varoius malignant cell lines (Saleem et al., 2002). This suggests that phytoconstitutents

of *T. chebula* combinely show better anticancerous activity than in isolated form. However the difference in the values may be due to the difference in cell lines used, exposure time of drug during experiment, method for analysis etc. *S. anacardium* also showed good antiproliferative activity with IC_{50} value 43.79±2.88 µg/ml. However, a previous study of methanolic extract of *S. anacardium* nut on the human epidermoid larynx carcinoma cell line (Hep 2), showed poor anticancerous activity (IC_{50} value, 468 µg/ml) (Patel et al., 2009) as compared to this study. Similarly, for *W. somnifera*, highest IC_{50} value was obtained among the four extracts which was 93.05±3.88 µg/ml. Several mechanisms of anticarcinogenic property of *W. somnifera* have been reported in previous studies. Research on animal cell cultures has shown that the herb decreases the levels of the nuclear factor kappaB, suppresses the intercellular tumor necrosis factor, and potentiates apoptotic signalling in cancerous cell lines (Ichikawa *et al*., 2006). One of the most exciting of the possible uses of Ashwagandha is its capacity to fight cancers by reducing tumor size (Singh *et al.,* 2010). The positive control, 5-fluorouracil also impaired good cytotoxic and dose dependent inhibition of cell proliferation with IC_{50} value 17.25±1.22 µg/ml.

In any drug discovery process, the important aspect to be considered is the safety of the compound under study. Medicinal plants can be considered as a safer alternate of the synthetic drug, but their safety cannot always be guaranteed. For example the pharmacologically active compound which showed toxicity against tumor cells, might be toxic to normal cells too including normal body tissue or immune cells. Hence, any drug or drug formulation has to go through the toxicity tests in order to render them safe and benefit from them. The preliminary safety of the extracts under this study was assessed by their toxicity against primary mice peritoneal macrophages via MTT assay. For this toxicity assay, only those extracts were taken which showed anti proliferative activity against HeLa cells.

Aqueous extract of *T. chebula* showed the least toxicity among the extract under study with only 33% inhibition of macrophages at maximum treated concentration of 200µg/mL. A slightly higher toxicity was observed in the methanolic extracts of this plant with CC_{50} value 125.5±1.96 µg/mL. On the basis of selectivity index, this plant can be considered to be safe with more than four times selective towards cancer cell. The other study on normal human cell has also declared this plant extract safer with no toxicity as all concentrations of the extract (0-50 µg/mL) significantly increased the viability of primary human skin fibroblasts (Itsarasook et al., 2012). As compared to other, *S. anacardium* showed more toxicity towards macrophage cell with CC_{50} value 92.40 ± 2.54 µg/mL, however on the basis of selectivity index, the extract is 2.11 times more selective towards cancer cell than normal macrophase and can be considered as a safe compound to be used as drug. On a previous study also, the methanolic extract of the nut showed no toxicity against Vero cell (Patel et al., 2009). *W. somnifera* was found to

be relatively safer with CC_{50} value 171.58 ±2.04 µg/mL and SI value 1.84. The reference drug, 5-Fluorouracil showed high levels of toxicities at lower doses as compared to the extracts. CC_{50} value of which was 26.65 ± 0.87 µg/mL. But its selectivity index showed the affinity more towards cancer cell. The selectivity index greater than 1 for all the tested extracts reveal the safety of the extracts however further investigation are required before coming to a discrete conclusion about their safety.

Chapter VII

Conclusion

Nepal contains diverse physiographic and climatic variation along the elevation gradient and harbors different ecosystems with valuable floral wealth. Among total floral wealth of Nepal, about 10 % of species are reported with medicinal and aromatic properties (Bhattarai and Ghimire, 2008). Due to insufficient supplement of modern allopathic medicine and the traditional believe of ethnomedicinal therapy, still vast majority of Nepalese people are dependent on indigenous use of medicinal plant for their primary health care. Although large number of plants of Nepal has been documented with high medicinal value, vast majority of these medicinal plants are still far behind of pharmacological researches in order to prove their therapeutic potential scientifically. Therefore concise and continues research with advanced instruments is necessary to explore their pharmacological property which may act as milestone to decrease the resistance and adverse effect problem associated with modern allopathic medicine.

This present study illustrates the pharmacological properties of six medicinal plants and found that some of these plants could be an excellent source of antioxidant compounds which indicate the possible use of these plants in several chronis disorders like Alzheimer's disease, Parkinson's disease, atherosclerosis, diabetetes and many more. The plants were also found to show antibacterial actions against the bacteria which belong to one of the most resistant forms emerging worldwide (*S. aureus and P. aeruginosa)*. And some of the extracts were able to show antiproliferative activity against cancer cell at low dose. Further investigations are required to isolate and identify the active compounds in the extracts. This study also reveals the fact that in any drug discovery process it is better to use range of solvents (from polar to non polar) in order to extract different bioactive compounds within them since using only two solvent system in this study, the extracts has shown significance difference in their pharmacologic activity. It is important to note that all the extracts in this study are in crude form and further research on the isolated compounds from these extracts may be a breakthrough for a drug discovery process especially against life threatening disease like cancer. This study also recommends for need of extensive study on effective, safe, cheap and non-toxic drug formulations, which would not only increase value to our resources but also generates a rational approach to exploit our resources.

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.pp

Ahmad M, Yousuf S, Khan B, Hoda MN, Ahmad MA, Ishrat T, Agarwal AK and Islam F (2006) Attenuation by Nardostachys jatamansi of 6-hydroxydopamine-induced parkinsonism in rats: behavioral, neurochemical, and immunohistochemical studies. *Pharmacol and Biochem Behav.* **83**(1): 150-60

Ainsworth EA and Gillespie KM (2007) Estimation of total phenolic content and other oxidation substrates in plant tissue using Folin-Ciocalteu reagent. *Nat Protoc.* **2**(4): 875-877

Alam P and Ali M (2009) Phytochemical investigation of Calotropis procera Ait roots. *Indian J Chem*. **48**: 443-446

Ali S, Ansari KA, Jafry MA, Kabeer H and Diwakar G (2000) Nardostachys jatamansi protects against liver damage induced by thioacetamide in rats. *J Ethnopharm.* **71**(3): 359-363

Ali SN, Kazmi SU, Akhtar N and Malik A (1993) An Antitumor Cardenolide with Inhibitory Activity against Pseudomonas pseudomallei. *Pak J Pharm Sci*. **6**(1): 61-66

Ali SS, Kasoju N, Luthra, Singh A, Sharanabasava H, Sahuand A and Bora U (2008) Indian medicinal herbs as source of antioxidants. *Food Res. Int.* **41**:1-15

AMERICAN ASSOCIATION FOR CANCER RESEARCH (WWW.AACR.ORG)

Ansari SH and Ali M (1999) New oleanene triterpenes from root bark of Calotropis procera. *J Med Aromatic Plant Sci. **21***: 978-981

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

Anver S and Alam MM (1992) Effect of latex seed dressing on interacting root-knot and reniform nematodes. *Afro-Asian J Nematology.* **2**:17-20

Aphale AA, Chhibba AD and Kumbhaakarna NR (1998) Sub acute toxicity study of the combination of ginseng (Panex ginseng) and ashwagandha (Withania somnifera) in rats: a safety assessment. *Ind J Physiol Pharmacol*. **42**: 299-302

Arora S, Kaur K, Kaur S (2003) Indian medicinal plants as a reservoir of protective phytochemicals. *Teratogenesis, Carcinogenesis, and Mutagenesis.* **23**(1): 295-300

Assob JC, Kamga HL, Nsagha DS, Njunda AL, Nde PF, Asongalem EA, Njouendou AJ, Sandjon B, and Penlap VB (2011) Antimicrobial and toxicological activities of five medicinal plant species from Cameroon Traditional Medicine. *BMC Complementary and Alternative Med.* **11**: 70-77

Bag A, Bhattacharyya SK, Pal NK and Chattopadhyay RR (2012) In vitro antimicrobial potential of Terminalia chebula fruit extracts against multidrug-resistant uropathogens. *Asian Pacific J Trop Biomed.* **2**(3): 1883-1887

Bagchi A, Oshima Y and Hikino H (1990) Spirojatomol, a new skeletal sesquiterpenoid of Nardostachys jatamansi roots. *Tetrahedron.* **46**: 1523-1530

Bagchi A, Oshima Y and Hikino H (1991) Jatamols A and B: sesquiterpenoids of Nardostachys jatamansi roots. *Planta Med.* **57**(3): 282-283

Bagewadi ZK, Siddanagouda RS and Baligar PG (2012) Phytochemical Screening and Evaluation of Antimicrobial Activity of Semecarpus anacardium Nuts. *Int J Pharm and Pharmaceutical Tech.* **1**(2): 68-74

Bahorun T, Soobrattee MA, Luximon-Ramma V and Aruoma OI (2006) Free radicals and antioxidants in cardiovascular health and disease.*Internet J Med Update*. **1**: 1-17

Bahuguna RP, Jangwan JS, Kaiya T and Sakakibara J (1989) Clemontanoside-A, a Bisglycoside from Clematis montana. *Phytomed.* **28**(9): 2511-2513

Balachandran P and Govindarajan R (2005) Cancer-an ayurvedic perspective. *Pharmacol Res.* **5**1: 19–30

Barman N, Sharma A and Kumar A (2013) Radical Scavenging and Antioxidant Potential of Nuts and Leaves Extracts of Semecarpus anacardium (L.) *Am J plant Sci.* **4**: 1679-1683

Basak KS, Bhaumik A, Mohanta A and Singhal P (2009) Ocular toxicity by latex of Calotropis procera (Sodom apple)*. Ind J Ophthalmol*. **57**(3): 232-234

Basu A, Sen T, Ray RN and Chaudhuri A (1992) Hepatoprotective effects of Calotropis procera root extract on experimental liver damage in animals. *Fitoterapia.* **63**(6): 507-514

Benzie IF (2003) Evolution of dietary antioxidants. *Comp Biochem Physiol*. **136**: 113-126

Bertino JR (1997) Irinotecan for colorectal cancer*. Semin Oncol.* **24**: S18-S23

Bharti P, Bai S, Seasotiya L, Malik A and Dalal (2012) Antibacterial activity and Chemical Composition of Essential Oils of Ten Aromatic Plants against selected Bacteria. *Int J Drug Dev & Res*. **4**(4): 342-351

Bhattacharya SK, Satyan KS and Chakrabarti A (1997) Effect of Trasina, an Ayurvedic herbal formulation, on pancreatic islet superoxide dismutase activity in hyperglycaemic rats. *Indian J Exp Biol*. **35**: 297-299

Bhattarai KR and Ghimire M (2008) commercially important medicinal and aromatic plants of Nepal and their distribution pattern and conservation measure along the elevation gradient of the Himalayas. *Banko Janakari*. **16**(1): 3-13

Birt DF, Hendrich S and Wang W (2001) Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol &*Therapeutics. **90:** 157–177

Boshy MS, Abdalla OM, Risha A, Moustafa F (2013) Effect of Withania somnifera Extracts on Some Selective Biochemical, Hematological and Immunological Parameters in Guinea Pigs Experimental Infected with E.coli. *Veterinary Sci*

Brand-Williams W, Cuvelier ME and Berset C (1995) Use of free radical method to evaluate antioxidant activity. *Lebensmittel Wissenschaft und Technologie*. **28**: 25–30

Brown JE and Rice-Evans CA (1998) Luteolin rich artichoke extract protects low density lipoprotein from oxidation in vitro. *Free Radical Res.* **29**: 247-255

Brueschweiler F, Stockel K and Reichstein T (1969) Calotropis – Glycoside, vermutliche Teilstruktur. *Helv Chim Acta.* **52**: 2276-2303

Butlet MS (2004) The role of natural product chemistry in drug discovery. *J Nat Prod*. **67**: 2141-2153

Cassady JM and Douros JD (1980) *Anticancer Agents Based on Natural Product Models*. Academic Press, New York.pp

Chang C, Yang M, Wen H & Chern J (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food & Drug Analysis* **10**(3): 178–182

Chang CL and Lin CS (2010) Development of antioxidant activity and pattern recognition of Terminalia chebula Retzius extracts and its fermented products. *J HungKuan*. **61**: 115-129

Chanphen R, Thebtaranonth Y, Wanauppathamkul S, Yuthavong Y (1998) Antimalarial principles from Artemisia indica. *J Nat Prod.* **61**: 1146-1147

Charu J and Trivedi PC (1997) Nematicidal activity of certain plants against root-knot nematode, Meloidogyne incognita, infecting chickpea, Cicer arietinum. *Annals of Plant Protection Sci*. **5**(2): 171-174

Chatterjee B, Basak U, Datta J, Banerji A and Neuman T (2005) Studies on the Chemical Constituents of Nardostachys jatamansi DC (Valerianaceae). *Cheminform.* **36**: 17-21

Chen X, Sun F, Ma, Wang J, Qin H and Du G (2011) In vitro evaluation on the antioxidant capacity of triethylchebulate, an aglycone from Terminalia chebula Retz fruit. *Ind J Pharmacol.* **43**(3): 320-323.

Cheng HW, Lin TC, Yu KH, Yang CM and Lin CC (2003) Antioxidant and free radical Scavenging activities of Terminalia chebula. *Biol Pharm Bull*. **6**(9): 1331-1335

Chitinis MP, Bhatia KG, Phatak MK and Kesava Rao KV (1980) Antitumour activity of the extract of Semecarpus anacardium L. nuts in experimental tumour models. *Indian J Exp Biol*. **18**: 6–8

Choedon T, Mathan G, Arya S, Kumar VL and Kumar V (2006) Anticancer and cytotoxic properties of the latex of Calotropis procera in a transgenic mouse model of hepatocellular carcinoma. *World J Gastroentero.* **12**: 2517-2522

Chu YH, Chang CL and Hsu HF, (2000) Flavonoid content of several vegetables and their antioxidant activity. *J Sci of Food &Agri*. **80**(5): 561–566.

Chun OK, Kim DO, Moon HY, Kang HG and Lee CY (2003) Contribution of individual polyphenolics to total antioxidant capacity of plums. *J Agri & Food Chem.* **51**: 7240–7245

Chun OK, Kim DO, Smith N, Schroeder D, Han JT and Lee CY (2005) Daily consumption of phenolics and total antioxidant capacity from fruits and vegetables in the American diet. *J Sci of Food & Agri*. **85:** 1715–1724

Cook JA and Mitchell JB (1989) Viability measurements in mammalian cells systems. *Anal.Biochem*. **179**: 1-7

Cowan MM (1999) Plant Products as Antimicrobial Agents. *Clin Microbiol Rev.* **12**(4): 564–582

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

Cragg GM, Newman DJ and Snader KM (1997) Natural products in drug discovery and development. *J Nat Product.* **60**: 52-60.

Creemers GJ, Bolis G, Gore M, Scarfone G, Lacave AJ, Guastalla JP, Despax R, Favalli G, Kreinberg R, VanBelle S, Hudson I, Verweij J and Huinink WWT (1996) Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: results of a large European phase II study. *J Clin Oncol*. **14**: 3056-3061

Dai J and Mumper RJ (2010) Plant phenolics: Extraction, analysis, and their antioxidant and anticancer properties. *Molecules*. **15**: 7313-7352

Das K, Tiwari RKS and Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *J Med Plants Res*. 2010; **4**(2): 104-111.

Davis L and Kuttan G (1998) Suppressive effect of cyclo-phosphamide-induced toxicity by Withania somnifera extract in mice. *J Ethnopharmacol.* **62**(3): 209-214

De S, Dey YN and Ghosh AK (2010) Phytochemical Investigation and Chromatography Evaluation of the different Extracts of Tuber of Amorphaphallus Paeoniifolius (Araceae*). Int J Pharm Biomed Res.* **1**(5): 150-157

Deng SG, Wu ZF, Li WY, Yang ZG, Chang G, Meng FH and Li-Li M (2004) Safety of Curcuma aromatica oil gelatin microspheres administered via hepatic artery. *World J Gastroenterol*. **10:** 2637-2642

Devi PU (1996) Withania somnifera Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. *Ind J Exp Biol*. **34**(10): 927-932

Devi PU, Sharada AC and Solomon FE (1995) In vivo growth inhibitory and radiosensitizing effects of withaferin A on mouse Ehrlich ascites carcinoma. *Cancer Lett*. **16**(1-2): 189-193

Devi PU, Sharada AC, Solomon FE and Kamath MS (1992) In vivo growth inhibitory effect of Withania somnifera (Ashwagandha) on a transplantable mouse tumor, Sarcoma 180. *Ind J Exp Biol.* **30**: 169-172

Dewan S, Kumar S and Kumar VL (2000) Antipyretic effect of latex of Calotropis procera. *Ind J Pharmacol*. **32**: 252-256

Dewanjee S, Mandal V, Sahu R, Dua TK, Manna A and Mandal SC (2009) Antiinflammatory activity of a polyphenolic enriched extract of Schima wallichii bark. *Nat Prod Res.* **31**: 1-8

Dhuley JN (1998) Effect of ashwagandha on lipid peroxidation in stress-induced animals. *J Ethnopharmacol.* **60**: 173-178

Dixon RA, Dey PM and Lamb CJ (1983) Phytoalexins: enzymology and molecular biology. *Adv Enzymol*. **55**: 1–69

Droge W (2002) Free radicals in the physiological control of cell function. *Physiol Rev*. **82**(1): 47-95

Du ZZ, Yang XW, Han H, Cai XH and Luo XD (2010) A new flavone C-glycoside from Clematis rehderiana. *Molecules.* **15**(2): 672–679

Du G, Li M, Ma F and Liang D (2009). Antioxidant capacity and the relationship with polyphenol and vitamin C in Actinidia fruits. *Food Chem.* **113**(2): 557–562

Eghdami A and Sadeghi F (2010) Determination of Total Phenolic and Flavonoids Contents in Methanolic and Aqueous Extract of Achillea Millefolium. *Org Chem J*. **2**: 81-84

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

Ganie SA, Haq E, Masood A, Hamid A and Zargar MA (2011) Antioxidant and protective effect of ethyl acetate extract of podophyllum hexandrum rhizome on carbon tetrachloride induced rat liver injury*. Evid Based Complement Alternat Med*.

Ganzera MI, Chodhary IA and Khan H (2003) Quantitaive HPLC analysis of withanolides in Withania somnifera. *Fitoerapia.* **74**(1-2): 68-76

Gil RR, Lin LZ, Cordell GA, Kumar MR, Ramesh M, Reddy BM and Appa Rao AVN (1995) Anacardoside from the Seeds of Semecarpus anacardium. *Phytochem.* **39(**2): 405-409

Gilani SN, Khan AU and Gilani AH (2010) Pharmacological basis for the medicinal use of Zanthoxylum armatum in gut, airways and cardiovascular disorders*. Phytother Res*. **24**: 553-558

Global cancer control (2008) A Call to Action from the Global Cancer Community*. Draft World Cancer Declaration.*

Gupta DK, Ali M and Bhutani KK (1996) Triterpenoids from Calotropis procera root bark. *Ind J Chem.* **35**(10): 1079-1084

Gutteridge JM 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

Hai W, Cheng H, Zhao M, Wang Y, Hong L, Tang H and Tian X (2012) Two new cytotoxic triterpenoid saponins from the roots of Clematis argentilucida*. Fitoterapia*. **83**(4): 759–764

Han X, Shen T and Lou H (2007) Dietry polyphenols and their biological significance. *Int J Mol Sci*. **12:** 950-988

Handa SS, Khanuja SPS, Longo G and Rakesh DD (2008). Extraction Technologies for Medicinal and Aromatic Plants. *Int centre for sci & high tech.* **15:** 21-25

Hao DC, Gu X, Xiao PG & Peng Y (2013) Chemical and biological research of Clematis medicinal resources. *Chin Sci Bull*. **58**: 1120-1129

Harborne JB and Baxter H (1995*) Phytochemical dictionary: a handbook of bioactive compounds from plants*, 4 John St. London, Taylor & Francis Ltd.

Harigaya Y, Chatterjee A, Basak B, Saha M, Dutta U, Mukhopadhyay C, Banerji J and Konda Y (2000) Structure and stereochemistry of nardostachysin, a new terpenoid ester constituent of the rhizomes of Nardostachys jatamansi*. J Nat Prod.* **63**(11): 1531-1533

Hartmann T (2008) The lost origin of chemical ecology in the late 19th century. *Proc Nat Ac Sci USA.* **105**(12): 4541-4546.

Harvey AL (1999) Medicines from nature: are natural products still relevant to drug discovery. *Trends Pharmacol Sci***. 20**: 196-198

Hassan SW, Bilbis FL, Ladan MJ, Umar RA, Dangoggo SM and Saidu Y (2006) Evaluation of Antifungal Activity and Phytochemical Analysis of Leaves, Roots and Stem Barks Extracts of Calotropis procera (Asclepiadaceae). *Pak J Biol Sci*. **9**(14): 2624-2629.

Hazra B, Sarkar R,Biswas S and Mandal N (2010) Research article Comparative study of the antioxidant and reactive oxygen species scavenging properties in the extracts of the fruits of Terminalia chebula, Terminalia belerica and Emblica officinalis. *BMC Complementary & Alternative Med.* **10:** 1123-1145

Hesse G and Ludwing G (1960) African arrow poisons XIV. Voruscharine, a second sulfur-containing heart poison from Calotropis procera L. *Justus Lieb Ann Chem.* **632**: 158-171

Hiss DC and Gabriels GA (2009) Implications of endoplasmic reticulum stress, the unfolded protein response and apoptosis for molecular cancer therapy. Part I: targeting p53, Mdm2, GADD153/CHOP, GRP78/BiP and heat shock proteins. *Expert Opin Drug Discov*. **4**: 799-821

HMG/Nepal (1976) *Catalog of Nepalese Vascular Plants*, Ministry of Forest, Department of Medicinal Plants, Thapathali, Kathmandu, Nepal.

Hossein MM, Moyano E, Bonfill M, Cusido RM, and Palazón J (2009) Steroidal Lactones from Withania somnifera, an Ancient Plant for Novel Medicine . *Molecules.* **14**: 373-2393

Hursting SD, Slaga TJ, Fischer SM, DiGiovanni J and Phang JM (1999) Mechanism based cancer prevention approaches: targets, examples, and the use of transgenic mice. *J National Cancer Ins*. **91**(3): 215–224.

Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair MG and Aggarwal BB (2006) *Mol Cancer Therapeutics,* 1434-1445 pp.

Itsarasook K, Ingkaninan K, Chulasiri M, and Viyoch J (2012) Antioxidant Activity and Cytotoxicity to Human Skin Fibroblasts of Terminalia Chebula Fruit Extract. *Int Conf on Biol & Life Sci*. **40**: 43-46

Iwashina T, Omori Y, Kitajima J, Akiyama S, Suzuki T and Ohba H (2004) Flavonoids in translucent bracts of the HImalayan Rheum nobile (Polygonaceae) as ultraviolet shields. *J Plant Res*. **117**(2): 101-107

Jain SC, Sharma R, Jain R and Sharma RA (1996) Antimicrobial activity of Calotropis procera. *Fitoterapia*. **67**: 275-276.

Jayaprakasam B, Zhang Y, Seeram N, Nair M (2003). *Life Sci*. **74**: 125-132

Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D (2011) Global cancer statistics. *CA Cancer J. Clin.* **61**: 69-90

Ji-hoon K, Yun-chang K, Chung-Oui H, Sung-Yong Y, Woojin J and Lee K (2012) Mutagenicity and oral toxicity studies of Terminalia chebula. *Phytother Res*. **26**: 39-47

Jones DP (2008) Radical-free biology of oxidative stress. *AJP-Cell Physiol.* **295**: C849–C868

Juang LJ, Sheu SJ and Lin TC (2004) Determination of hydrolyzable tannins in the fruit of Terminalia chebula by high-performance liquid chromatography and capillary electrophoresis*. J Sep Sci.* **7**(9): 718-724

Kamboj VP (2000) *Herbal medicine*, Current Sci, 7835-7839 pp.

Kannan P, Ramadevi SR and Hopper W (2009) Antibacterial activity of Terminalia chebula fruit extract. *African J Microbiol Res*. **3**(4): 180-184

Kareem SO, Akpan I and Ojo OP (2008) Antimicrobial Activities of Calotropis procera on Selected Pathogenic Microorganisms. *African J Biomed Res*. **11**(1): 105-110.

Kasabri V, Flatt PR and Abdel-Wahab YH (2010) Terminalia bellirica stimulates the secretion and action of insulin and inhibits starch digestion and protein glycation in vitro. *Br J Nutr*. **103**: 212-217

Kaur P, Sharma M and Mathur S (2003) Effect of 1-oxo-5beta, 6beta epoxy-witha-2-ene-27-ethoxy-olide isolated from the roots of Withania somnifera on stress indices in Wistar rats. *J Altern Complement Med.* **9**: 897–907

Kaur R, Kapoor K and Kaur H (2011) Plants as a source of anticancer agents. *J Nat Prod Plant Resour.* **1**(1): 119-124

Kaur S, Michael H, Arora S, Harkonen PL and Kumar S (2005)The in vitro cytotoxic and apoptotic activity of Triphala-an Indian herbal drug*. J Ethnopharmacol* **97**: 15–20

Khan AQ and Malik A (1989) A steroid from Calotropis procera. *Phytochemistry*. **28**(10): 2859-2861

Khan AQ, Ahmed Z, Kazmi SNH and Malik A (1988) A New Pentacyclic Triterpene from Calotropis procera*. J Nat Prod.* **51**: 925-928

Khan AU and Gilani AH (2009) Antispasmodic and bronchodilator activities of Artemisia vulgaris are mediated through dual blockade of muscarinic receptors and calcium influx. *J Ethnopharmacol.* **126**: 480-486

Khan KH and Jain SK (2009) Regular intake of Terminalia chebula can reduce the risk of getting typhoid fever. *Adv Biotech.* **8**(9): 10-15

Khanzada SK, Shaikh W, Kazi TG, Sofia S, Kabir A, Usmanghani K and Kandhro AA (2008) Analysis of fatty acid, elemental and total protein of Calotropis procera medicinal plant from Sindh, Pakistan. *Pak J Bot*. **40**(5): 1913-1921

Khare CP (1982) *Encyclopedia of Indian medicinal plants,* 419-421pp.

Khare CP (2004) *Indian herbal remedies: Rational western therapy, Ayurvedic and other traditional usage,* Botany, Pub Springer, Berlin: 451-452 pp.

Kim DO, Lee KW, Lee HJ and Lee CY (2002) Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. J Agri & Food Chem. **50:** 3713–3717

Kim HG, Cho JH, Jeong EY, Lim JH, Lee SH and Lee HS (2006) Growth-inhibiting activity of active component isolated from Terminalia chebula fruits against intestinal bacteria. *J Food Prod.* **69**(9): 2205-2209

Kim DO, Chun OK, Kim YJ, Moon HY and Lee CY(2003) Quantification of polyphenolics and their antioxidant capacity in fresh plums. *J Agri & Food Chem*. **51**: 6509–6515

Kirson and Glotter E (1980) 4α-hydroxy steroids from Withania somnifera (L) Dunal*. J Chemistry Res Synop*, **10**:338-339

Kohen R and Nyska A (2002) Invited Review: Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification. *Toxicol Pathol*. **30**(6): 620–650

Kotzekidou P, Giannakidis P and Boulamatsis A (2008) Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens in vitro and on the fate of inoculated pathogens in chocolate. *LWT.* **41**: 119–127

Kumar KJ (2006) Effect of geographical variation on contents of tannic acid, gallic acid, chebulinic acid and ethyl gallate in Terminalia chebula. *Nat products.* **2**(3): 170-175

Kumar A, Raut A, Sawant NS, Badre AS, Amonkar AJ, Ashok D and Vaidya B (2007) Bhallatak (Semecarpus anacardium Linn.)-A review. *Ind. J Trad Knowledge*. **6**(4): 653-659

Kumar VL and Shivkar YM (2004) In vivo and in vitro effect of latex of Calotropis procera on gastrointestinal smooth muscles. *J Ethnopharmacol*. **93**: 377-379

Kunwar RM and Bussmann RW (2008) Ethnobotany in the Nepal Himalaya. *J Ethnobiol and Ethnomed.* **4**: 2-11

Kunwar RM, Nepal BK, Kshhetri HB, Rai SK and Bussmann RW (2006) Ethnomedicine in Himalaya: a case study from Dolpa, Humla, Jumla and Mustang districts of Nepal. *J Ethnobiomed.* **2**(27): 1-12

Lamture JB, Gondgaon NM, Nayak UR, Patwardhan BK and Ghooi RB (1982) Semecarpus anacardium- Separation of the Bhilawanol A and Bhilawanol B and A Comparative Study of Their Growth Inhibitory Effect on Clostridium tetani and General Pharmacology, *Bulletin of Haffkine Institute*. **10**: 87-92

Larhsini M, Bousaid M, Lazrek HB and Jana M (1997) Evaluation of antifungal and molluscicidal properties of extracts of Calotropis procera. *Fitotrapia.* **68**: 371-373

Lavi D, Glotter E and Shro Y (1965) Constituents of Withania somnifera Dun, The structure of Withaferin A. *Journal Chem Soc*. **30**: 7517-7531

Lee HS, Won NH, Kim KH, Lee H, Jun W, Lee KW (2005) Antioxidant effects of aqueous extract of Terminalia chebula in vivo and in vitro. *Biol Pharm Bull*. **28**(9): 1639-1644

Lee SH, Ryu SY, Sang U, Lee CO, No ZK, Kim SK, Ahn JW (1995) Hydrolyzable tannins and related compound having cyotoxic activity from the fruits of Terminalia chebula. *Archives of Pharmacol Res*. **18**: 118-120

Li Y, Wang SF, Zhao YL, Liu KC, Wang XM, Yang YP and Li XL (2009) Chemical constituents from Clematis delavayi var. spinescens. *Molecules*. **14(**11**)**: 4433–4439

Lozano I (2001) The therapeutic use of Cannabis sativa (L.) in Arabic medicine. *J Cannabis Ther*. **1**: 63-67

Louis M and Paul C. (2010) Kaladrug-R: Laboratory SOP#18 [Online] <http://www.leishrisk.net/Leishrisk/UserFiles/File/Kaladrug-R/SOPs/lab/labSOP18_IC50tool.pdf>.

Mahesh R Bhuvana S Begum VM (2009) Effect of Terminalia chebula aqueous extract on oxidative stress and antioxidant status in the liver and kidney of young and aged rats. *Cell Biochem Funct.* **27**(6): 358-363

Majumdar SH, Chakraborthy GS and Kulkarni KS (2008) Medicinal potentials of Semecarpus anacardium nut- A review. *J Herbal Med &Toxicol*. **2**(2): 9-13

Malckzadeh F, Ehsanifar H, Shahamat N, Levin M and Colwell RR (2001) Antibacterial activity of black myrobalan (Terminalia chebula Retz.) against Helicobactor pyroli. *Int J Antimicrob Agent*. **18**(1): 85-88

Malik CP, Garg p, Singh Y and Grover S (2012) Medicinal uses, chemical constituents and micro propagation of three potential medicinal plants. *Int J of life science*. **2**(3):57-76

Mammen D, Bapat S, Sane R (2012) An investigation to variation in constituents in the fruits of Terminalia chebula Retz. at differ maturity stages. *Int J Pharm Bio Sci*. **3**(1): 416-419

Mandal N, Chaudhuri D, Ghate NB and Sarkar R (2012) Phytochemical analysis and evaluation of antioxidant and free radical scavenging activity of Withamnia somnifera root. *Asian J Pharm Clin Res.* **5**(4): 193-199

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

Martindale JL and Holbrook NJ (2002) Cellular Response to Oxidative Stress: Signaling for Suicide and Survival. *J cellular physiol.* **192**: 1–15

Mathur R, Gupta SK, Mathur SR and Velpandian T (2009) Anti-tumor studies with extracts of Calotropis procera (Ait.) R.Br. root employing Hep2 cells and their possible mechanism of action. *Indian J Exp Biol.* **47**(5): 343-348

Matsuda H, Murakami T, Kishi A and Yoshikawa M (2001) Structures of withanosides I, II, III, IV, V, VI and VII new withanolide glycosides from the roots of Indian Withania somnifera D and inhibitory activity fortachyphylaxis to clonidine in isolated guinea pig ileum. *Bioorg Med Chemistry.* **96**: 1499-1507

Masters JRW (2000) *Animal Cell Culture: A practical approach*, Oxford University Press. 20-75pp.

Meena AK, Yadav A and Rao MM (2011) Ayurvedic uses and pharmacological activities of Calotropis procera Linn. *Asian J Traditional Med.* **6**(2): 45-52

Menon LG, Kuttan R and Kuttan G (1997) Effect of rasayanas in the inhibition of lung metastasis induced by B16F-10 melanoma cells. *J Exp Clinical Cancer Res*. **16**(4): 365-368

Mikage M and Mouri C (2000) Altitudinal variations of Berberine contentin the bank of Berberis plants from West Nepal. *Newsletter of Himalayan Botany*. **26**: 16-19

Mirjalili HJ Moyano E, Bonfill M, Cusido RM and Palazón J (2009) Steroidal Lactones from Withania somnifera, an Ancient Plant for Novel Medicine. *Molecules*. **14**: 2373-2393

Mohsin A, Shah AH, Alaha MA, Tariqi MO and Ageel AM (1989) Analytic anti-pyretic activity and phytochemical screening of some plants used in traditional Arab systems of medicine. *Fitoterapai.* **60**(3): 174-177

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* **65**(1-2): 55-63

Mossa JS, Tariq M, Mohin A, Ageel AM, Al-Yahya MA and Al-said MS (1991) Pharmacological studies on aerial parts of Calotropis procera. *Am J Chin Med*. **19**: 223-231

Muhammad S, Khan BA, Akhtar N, Mahmood T, Rasul A and Hussain I (2012) The morphology, extractions, chemical constituents and uses of Terminalia chebula: A review. *J Med Plants Res.* **6**(33): 4772-4775

Murthy SS (1985) A biflavonoid from Semecarpus anacardium. *Phytochem*. **24**(5): 1065–1070

Murty D, Rajesh E, Raghava D, Raghavan TV and Surulivel MK (2010) Hypolipidemic effect of arborium plus in experimentally induced hypercholestermic rabbits. *Yakugaku Zasshi.* **130**: 841-846

Mwitari PG, Ayeka PA, OndichoJ, Matu EN and Bii CC (2013) Antimicrobial Activity and Probable Mechanisms of Action of Medicinal Plants of Kenya: Withania somnifera, Warbugia ugandensis, Prunus africana and Plectrunthus barbatus. *PLoS ONE.* **8**(6): e65619

Nadkarni KM (2005) *The Indian Materia Medica*, Vol I, 486 pp.

Naik GH, Priyadarsini KI, Naik DB, Gangabhagirathi R, Mohan H (2004) Studies on the aqueous extract of Terminalia chebula as a potent antioxidant and a probable radioprotector. *Phytomed*. **11**: 530-538

Nammi S, Boini KM, Koppula S, Sreemantula S (2005) Reserpine-induced central effects: pharmacological evidence for the lack of central effects of reserpine methiodide. *Can J Physiol Pharmacol*. **83**: 509-515

NCCLS (1999). Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline.NCCLS document M26-A [ISBN 1-56238-384-1]. NCCLS, 940 West Valley Road, Suite 1400, Wayne,Pennsylvania 19087 USA.

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

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

Nobori T, Miurak K, Wu DJ, Takabayashik LA and Carson DA (1994) Deletion of cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*. **46**: 753-756

Oberlines NH, Kroll DJ (2004) Camptothecins and taxol: historic achievement in natural products research. *J Nat Prod.* **67**: 129-135

Ojala T (2001) *Biological Screening of Plant Coumarins*. Academic Dissertation, Department Of Pharmacy, Faculty of Science, University of Helsinki, Finland.

Okwu DE and Josiah C (2006) Evaluation of the chemical composition of two Nigerian medicinal plants. *Afri J Biotech*. **5**: 357-361

Okwu DE and Okwu ME (2004) Chemical composition of Spondias mombin linn. plant parts. *J Sustain Agric Environ*. **6**(2): 140-147

Panda S and Kar A (1997) Evidence for free radical scavenging activity of Ashwagandha root. *Ind J Physiol &Pharmacol.* **41**(4): 424-426

Pant R and Chaturvedi K (1989) Chemical analysis of Calotropis procera latex. *Curr Sci*. **58**(13):740-724

Panunto W, Jaijoy K, Lerdvuthisopon N, Lertprasertsuke N, Jiruntanat N, Soonthornchareonnon N (2011) Acute and chronic toxicity studies of the water extract from dried fruits of Terminalia chebula Rezt. in rats. *Int J Appl Res Nat Prod*. **3**(4): 36-43s

Paras J and Sharma HP (2013) A potential Ethnomedicinal palnt: Semecarpus anacardium Linn- A review. *Int J Res in Pharmacy & Chemistry.* **3**(3): 564-572

Parekh J and Chanda S (2006) Antibacterial and phytochemical studies on twelve species of Indian medicinal Plants. *African J Biomed Res*. **10**: 175-181

Parveen Z, Siddique S, Shafique M, Khan JS and Khanum R (2011) Volatile constituents, Antibacterial and Antioxidant activities of essential oil from Nardostachys jatamansi DC. Roots. *Pharmacology online* **3**: 329-337

Patel SR, Suthar AP and Patel RM (2009) In Vitro Cytotoxicity Activity of Semecarpus anacardium Extract Against Hep 2 Cell Line and Vero Cell Line. *Int J PharmTech Res.* **1**(4): 1429-1433

Pe ´ rez-Jime ´nez J and Saura CF (2006) Effect of solvent and certain food constituents on different antioxidant capacity assays. *Food Res Int.* **39**: 791–800

Ponnusankar S, Pandit S, Venkatesh M, Bandyopadhyay A and Mukherjee PK (2011) Cytochrome P450 inhibition assay for standardized extract of Terminalia chebula Retz. *Phytother Res*. **25**(1): 151-154

Potmeisel M and Pinedo H (1995) *Camptothecins: new anticancer agents*. Boca Raton, Florida, CRC Press, 149-150 pp.

Powell RG, Weisleder D, Smith CRJr and Rohwedder WK (1970) Structures of harringtonine, isoharringtonine, and homoharringtonine. *Tetrahedron Lett.* **11**: 815-818.

Premalatha B, Muthulakshmi V and Sachdanandam P (1999) Anticancer potency of the milk extract of Semecarpus anacardium Linn. Nuts against aflatoxin B1 mediated hepatocellular carcinoma bearing Wistar rats with reference to tumour marker enzymes. Phytother Res. **13**:183–187.

Premalatha B, Muthulakshmi V, Vijayalakshmi T and Sachdanandam P (1997) Semecarpus anacardium nut extract induced changes in enzymic antioxidants studied in a flatoxinB1 caused hepatocellular carcinoma bearing Wistar rats. Int J Pharmacol. **35**: 1–6

Premalatha B and Sachdanandam P (1998) Immunomodulatory activity of Semecarpus anacardium Linn. Nut milk extract in Aflatoxin B1 induced hepatocellular carcinoma in rats. *Pharmacol Commun.* **4**: 507–510

Premalatha B and Sachdanandam P (1998) Regulation of mineral status by Semecarpus anacardium Linn. Nut milk extract in aflatoxin B1 induced hepatocellular carcinoma. *J Clin Biochem Nutr* **25**: 63-70

Premalatha B and Sachdanandam P (1999) Effect of Semecarpus anacardium nut extract against aflatoxin B1 induced hepatocellular carcinoma. *Fitoterapia.* **70**: 484–492

Premalatha B and Sachdanandam P (2000) Potency of Semecarpusan acardium Linn. Nut milk extract against aflatoxinB1 induced hepatocarcinogenesis: reflection on microsomal biotransformation enzymes. *Pharmacol Res*. **42**: 161–166.

Premalatha B and Sachdanandam P (2000) Stabilization of lyzosomal membrane and cell membrane glycoprotein profile by Semecarpus anacardium Linn. Nut milk extract in experimental hepatocellular carcinoma. *Phytother Res.* **14**: 352–355

Premalatha B, Sujatha V and Sachdanandam P (1997) Modulating effect of Semecarpus anacardium Linn. Nut extract on glucose metabolizing enzymes in aflatoxinB1 induced experimental hepatocellular carcinoma. *Pharmacol Res*. **36**: 187–192

Press JR, Shrestha KK and Sutton DA (2000) *Annoted Checklist of the Flowering Plants of Nepal,* J. R. Press, National History Meseum, London) pp

Rahman AU, Abbas S, Shawar DE, Jamal AS and Choudhary MI (1993) New withanolides from Withania spp. *J Nat Prod*. **56:** 1000-1006

Rahman AU, Jamal AS, Choudary MI and Asif I (1991) Two withanolides from Withania somnifera. *Phytochemistry.* **30**: 3824-3825

Rahman H, Shaik HA, Madhavi P and Eswaraiah MC (2011) A review: pharmacognostics and pharmacological profiles of nardastachys jatamansi dc. *Elixir Pharmacy.* **39**(6): 5017-5020

Rajagopalan S, Tamm Ch and Reichstein T (1995) Die Glykoside der Samen von Calotropis procera R .Br. *Helv Chim Acta.* **38**: 1809-1824

RajaSankar S, Manivasagam T, Sankar V, Prakash S, Muthusamy R, Krishnamurti A and Surendran S (2009) Withania somnifera root extract improves catecholamines and physiological abnormalities seen in a Parkinson's disease model mouse. *J Ethnopharmacol*. **125**(3): 369-373

Rajbhandari KR (2001) Ethnobotany *of Nepal Ethnobotanical Society of Nepal*, Kathmandu.

Rajkumar V, Guha G and Kumar RA (2011) Antioxidant and anti-neoplastic activities of Picrorhiza kurroa extracts. *Food Chem Toxicol.* **49**: 363-369

Ramaan N (2006) *Phytochemical Techniques,* New India Publishing Agency, 19-24pp.

Ramad MF, Kinni SG, Seshagiri M and Mörsel JT (2010) Fat-Soluble Bioactives, Fatty Acid Profile and Radical Scavenging Activity of Semecarpus anacardium Seed Oil. *J Am Oil Chemists' Society.* **87**(8): 885-894

Rangsriwong P, Rangkadilok N, Satayavivad J, Goto M and Shotipruk A (2009) Subcritical water extraction of polyphenolic compounds from Terminalia chebula Retz. fruits. *Sep Puri Tech*. **66**: 51-56.

Ranjith A, Kumar SK, Venugopalan VV, Arumughan C, Sawhney RC and Singh V (2006) Fatty acids, tocols and carotenoids in pulp oil of three Sea Buckthron species (Hippophae rhamnoides, H. salicifolia, and H. tibetana) grown in the Indian Himalayas. *JAOCS*. **83**:359-364

Ray DK and Thokchom IS (2006) Antipyretic, antidiarrhoeal, hypoglycaemic and hepato-protective activities of ethyl acetate extract of Acacia catechu in albino rats. *Ind J Pharmacol*. **38**: 408-413

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol & Med.* **26**: 1231-1237

Reddy KP, Bid HK, Nayak VL, Chaudhary P, Chaturvedi JP, Arya KR, Konwar R and Narender T (2009) In vitro and in vivo anticancer activity of 2-deacetoxytaxinine J and synthesis of novel taxoids and their in vitro anticancer activity. *Eur J Med Chem*. **44**: 3947-3953

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.

Rokaya MB, Münzbergová and Timsina B (2010) Ethnobotanical study of medicinal plants from the Humla district of western Nepal. *J Ethnopharmacol*. **130**(3)**:** 485-504

Rowinsky EK, Onetto N, Canetta RM and Arbuck SG (1992)Taxol-the 1st of the texanes, an important new class of anti tumor agents. *Semin Oncol*. **1**: 646-662

Roy S, Sehgal R, Padhy BM and Kumar VL (2005) Antioxidant and protective effect of latex of Calotropis procera against alloxan-induced diabetes in rats. *J Ethnopharmacol*. **102**(3): 470-473

Roy N, Laskar RA, Ismail Sk, Kumari D, Ghosh T, Begum NA (2011) A detailed study on the antioxidant activity of the stem bark of Dalbergia sissoo Roxb., an Indian medicinal plant. *Food Chem***. 126**: 1115–1121

Rucker G, Paknikar SK, Mayer R, Breitmaier E, Will G and Wiehl L (1993) Revised structure and stereochemistry of jatamansic acid. *Phytochem.* **33**(1): 141-143

Rucker G, Tautges J. Sleck A, Wenzl H and Graf E (1978) Isolation and pharmacological activity of the sesquiterpene valeranone from Nardostachys jatamansi DC (in German). *Arzneimittel forschung.* **28**(1): 7-13

Sabir M and Bhide MK (1971) Study of some pharmacological activities of berberine. *Indian J Physiol Pharmacol*.**15**:111-132

Sabu MC, Kuttan R (2002) Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *J Ethnopharmacol*. **81**:155-160

Sakaguchi K, Kitajima J and Iwashina T (2012) Kaempferol tri- and tetraglycosides from the flowers of Clematis cultivar. *Nat Prod Commun.* **7**(2): 173–174

Salah N, Miller NJ, Pagange G, Tijburg L, Bolwell GP and Rice- Evans C (1995) Polyphenolic flavonoids as scavenger of aqueous phase radicals as chain breaking antioxidant. *Arc Biochem Broph.* **2**: 339-346

Saleem A, Husheem m, Harkonen P and Pihlaja k (2002) Inhibition of cancer cell growth by crude extract and the phenolics of Terminalia chebula retz. Fruit. *J Ethnopharmacol*. **81**(3): 327-336

Samvatsar S and Diwanji VB (2000) Plant sources for the treatment of jaundice in the tribals of Western Madhya Pradesh of India*. J Ethnopharmacol*. **73**: 313-316

Santhi R, Lakshmi G, Priyadharshini AM and Anandaraj L (2011) Phytochemical screening of Nerium oleander leaves and Momordica chrantia leaves. *Int Res J Pharm.* **2:** 131-135

Sarbhoy AK, Varshney JL, Maheshwari ML and Saxena DB (1978) Efficacy of some essential oils and their constituents on few ubiquitous molds*. Zentralbl Bakteriol Naturwiss*. **133**(7-8): 723-725

Sastry SD, Maheswari ML, Chakravarti KK and Bhatacharya SC (1967) Terpenoids-CV1: the structure of calarenol. *Tetrahedron.* **23**: 1997-2000

Sastry SD, Maheswari ML, Chakravarti KK and Bhattacharyya SC (1967) Terpenoids, CXV, Chemical Constituent Of Nardostachys Jatamansi. *Perfum Essent Oil.* **58:** 154-158

Sato Y, Oketani H, Singyouchi K, Ohtsubo T, Kihara M and Shibata H (1997) Extraction and purification of effective antimicrobial constituents of Terminalia chebula Retz. against methicillin- resistant Staphylococcus aureus. *Biol Pharm Bull*. **20**(4): 401-404

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

Sebastian MK and Bhandari MM (1984) Medico-ethno botany of mount Abu, Rajasthan. *Ind J Ethnopharmacol.* **12**(2): 223-230

Seiber JN, Nelson CJ and Lee SM (1982) Cardenolides in the Latex and Leaves of 7 Asclepias-Spp and Calotropis-Procera. *Phytochemistry.* **21**: 2343-2348

Sharma AK and Singh RH (1981) Clinical and biological studies on Amrita Bhallatak in management of Rheumatoid Arthritis. *J Res in Ayurveda & Siddha.* **2.**

Sharma DK, Varshneya C and Mehta M (2012) Total Phenolic Content and Antioxidant Activity of Triphala (an Ayurvedic formulation) and Its Constituents. *Am J Pharm Tech Res.* **2**(1): 459-465

Sharma SK and Singh AP (2011) Standarization and preliminary phytochemical screening of Nardostachys jatamansi DC. Rhizome. *Int J Res In Ayurveda and Pharm.* **2**(3): 978-982

Sharma V, Sharma S and Paliwal R (2011) Withania somnifera: A Rejuvenating Ayurvedic Medicinal Herb for the Treatment of various Human ailments. *Int.J. PharmTech Res*. **3**(1): 187-192

Shengji P (2001) Ethnobotanical approaches of traditional medicine studies: some experiences from Asia. *Pharmacoceutical Biol*. **39**(1): 74-79

Shivkar YM and Kumar VL (2003) Antihelmintic Activity of Latex of Calotropis procera. *Pharm Biol*. **41**(4): 263-265

Shivkar YM and Kumar VL (2004) Effect of anti-inflammatory drugs on pleurisy induced by latex of Calotropis procera in rats. *Pharmacol Res*. **50**: 335-340.

Sing AK, Raghubanshi AS and Sing JS (2002) Medical ethnobotany of the tribals of Sonaghati of Sonbhadra district, Uttar Pradesh. *Ind J Ethnopharmacol.* **81**(1): 31-41

Singh G and Kumar P (2011) Antibacterial potential of Alkaloids of Withania somnifera L. & Euphorbia Hirtal L. *Int J Pharm Pharm Sci*. **4(**1): 78-81

Singh G, Sharma PK, Dudhe R, and Singh S (2010) Biological activities of Withania somnifera. *Annals of Biol Res.* **1**(3): 56-63

Singh RM (2002) An assessment of ayurvedic concept of cancer and a new paradigm of anticancer treatment in Ayurveda. *J Altern Complement Med*. **8**: 609–614

Singh VP, Sharma SK and Khare VS (1980) Medicinal plants from Ujjain district, madhya pradesh Part II. *Ind Drugs Pharmacol*. **15**(5): 7-12

Smit HF, Woerdenbag HJ, Singh RH, Meulenbeld GJ, Labadie RP and Zwaving JH (1995) Ayurvedic herbal drugs with possible cytostatic activity. *J Ethnopharmacol*. **47**: 75-84

Sodipo OA, Akiniyi JA and Ogunbamosu JU (2000) Studies on certain on certain characteristics of extracts of bark of Pansinystalia macruceras (K schemp) picrre Exbeille. *Global J Pure Appl Sci*. **6**: 83-87

Stahelin H (1973) Activity of a new glycosidic lignan derivative (VP 16-213) related to podophyllotoxin in experimental tumors. *Eur J Cancer*. **9**: 215-221

Stern JL, Hagerman AE, Steinberg PD and Mason PK (1996) Phlorotannin- protein interactions. *J Chem Ecol*. **22**:1887–1899

Subashini R, Gnanapragasam A, Senthilkumar S, Yogeeta S, Devaki T (2007) Protective effect of Nardostachys jatamansi (Rhizomes) on mitochondrial respiration and lysosomal hydrolases during doxorubicin induced myocardial injury in rats. *J Health Sci.* **53**(1): 67-76

Subashini R, Yogeeta S, Gnanapragasam A and Devaki T (2006) Protective effect of Nardostachys jatamansi on oxidative injury and cellular abnormalities during doxorubicin-induced cardiac damage in rats. *J Pharm Pharmacol.* **58**(2): 257-262

Sun F and Yang D (2009) Advance in chemical constituents of genus Clematis. *Zhongguo Zhong Yao Za Zhi.* **34**(20): 2660-2668

Talwar GP, Dar SA, Rai MK, Reddy KV, Mitra D, Kulkarni SV, Doncel GF, Buck CB, Schiller JT, Muralidhar S, Bala M, Agrawal SS, Bansal K and Verma JK (2008) A novel polyherbal microbicide with inhibitory effect on bacterial, fungal and viral genital pathogens. *Int J Antimicrob Agents*. **32**: 180-185

Thaipong K, Boonprakob U, Crosby K, Zevallos LC and Byrne DH (2006) Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Composition & Analysis*. **19**: 669–675

Thakur M, Rana RC and Thakur S (2008) Physiochemical evaluation of Terminalia chebula fruits. *J Non Timber Forest Prod*. **15**: 37-42

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

Trivedi PC (2006) *Herbal medicine: traditional practices*, Pub Aavishkar, Jaipur, 322 pp

Tsuchiya H, Sato M, Miyazaki T Fujiwara S, Tanigaki S, Ohyama M, Tanaka T and Iinuma M (1996) Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant Staphylococcus aureus. *J Ethnopharmacol.* **50**: 27–34

Tubtimdee C and Shotipruk A (2011) Extraction of phenolics from Terminalia chebula Retz. with water-ethanol and water-propylene glycol and sugaring-out concentration of extracts. *Sep Puri Tech.* **77**(3): 339-346

Usha C, Satyanarayanan R and Velmurugan A (2007) Use of an aqueous extract of Terminalia chebula as an anticaries agent. A clinical study. *Ind J Dent Res*. **18**(4): 152-156

Vaishnav R, Sankaranarayanan A, Chaudhury RR, Mathur VS and Charavarthi RN (1983) Toxicity studies on proprietary preparation of Semecarpus anacardium. *Ind J Med Res.* **77**: 907–908

Valko M, Leibfritz D, Moncola J and Cronin MD (2007) Free radicals and antioxidants in normal physiological functions and human disease. Review. *Int J Biochem Cell Biol*. **39**(1):44-84.

Verma N and Vinayak M (2009) Semecarpus anacardium nut extract promotes the antioxidant defence system and inhibits anaerobic metabolism during development of lymphoma. *Biosci Rep.* **29**(3): 151-164

Wang H, Cao G and Prior RL (1996) Total antioxidant capacity of fruits. *J Agri & Food Chem.* **44:** 701–705

Wang XP, Qian HB and Huang J (2009) Comparative study on Clematis chinensis and Clematis uncinata by HPLC in vitro and in vivo. Lishizhen. *Med Mater Med Res.* **20**: 2224–2225

Wang YC and Huang TL (2005) Screening of anti-Helicobacter pylori herbs deriving from Taiwanese folk medicinal plants. *FEMS Immunol & Med Microbiol*. **43** :295-300

Wani MC, Taylor HL, Wall M E, Coggon P and McPhail AT (1971) Plant anti-tumor agents. VI. The isolation and structure of taxol, a novel anti-leukemic and anti-tumor agent from Taxus brevifolia. *J Am Chem Soc*. **93**: 2325-2327.

Weyermann J, Lochmann D and Zimmer A (2005) A practical note on the use of cytotoxicity assays. *Int J Pharm*. **288**(2): 369-376

Williumson EN (2002*) Major herbs of Ayurveda*, Churchill Livingstone, London: 1-299pp.

Wu JY, Zhang QX and Leung PH (2007) Inhibitory effects of ethyl acetate extract of Cordyceps sinensis mycelium on various cancer cells in culture and B16 melanoma in C57BL/6 mice. *Phytomed*. **14**: 43-49

Yan H, Ma Y, Liu M and Zhou L (2008) The dual actions of Paederia scandens extract as a hypouricemic agent: xanthine oxidase inhibitory activity and uricosuric effect. Planta Med. **74**: 1345-1350

Yan LH, Xu LZ, Wang ZM,Zhang QW, Yang SL (2010) Two new phenolic glycosides from the stems of Clematis parviloba. *Yao Xue Xue Bao*. **45**(12): 1527–1532

Yang AM, Du J, Miao ZH and Yuan HJ (2009) Study on the chemical constituents from Clematis brevicaudata. *Zhong Yao Cai.* **32**(10): 1534–1537

Yesmin MN, Uddin SN, Mubassara S and Akond MA (2008) Antioxidant and Antibacterial Activities of Calotropis procera Linn. *American-Eurasian J Agri & Environ Sci.* **4**(5): 550-553

Yildirim A, Oktay M and Bulaloulu V (2001) The antioxidant activity of the leaves of Cydonia vulgaris *Turkish J Med Sci*. **31**: 23-27.

Yonzon M, Lee DJ, Yokochi T, Kawano Y and Nakahara T (2005) Antimicrobial activities of essential oils of Nepal*. J Essent Oil Res*. **17**: 107-111

Young I and Woodside J (2001) Antioxidants in health and disease. *J Clin Pathol*. **54**(3): 176–186

Zavery M, Khandhar A, Patel S and Patel A (2010) Chemistry and pharmacology of Piper longum L. *Intl J. Pharmaceutical Sci Review and Res.* **5**(1): 67-76

Zhang X, Chen C, He S and Ge F (1997) Supercritical-CO2 fluid extraction of the fatty oil in Terminalia chebula and GC-MS analysis. *Zhong Yao Cai*. **20**(9): 463-464

Zhang X, Goncalves R and Mosser DM (2010) *The Isolation and Characterization of Murine Macrophages,* Curr Protoc Immunol*.* Author manuscript.

Appendices

RPMI (Roswell Park Memorial Institute) complete medium

RPMI powder	: 10.40 gm
NaHCO3	: 2.00 gm
HEPES	: 1.40 gm
L-Glutamine	: 2 mM
DDW	: 1 L
Gentamycin	: 20 µg/ml
Streptomycin	: 100 µg/ml
Penicillin	:100 U/ml
pH	:7.2

PBS (Phosphate Buffer Saline)

NaCl	: 8 gm
Na2HPO4.2H2O	: 1.44 gm
KCl	: 0.2 gm
KH2PO4	: 0.2 gm
pH	: 7.3 to 7.4
DDW	: 1 L

Preparation of Reagents: (Ramaan, 2006)

1. Mayer's reagent: 1.358g mercuric chloride was dissolved in 60ml of water and 5.0 g of potassium iodide was dissolved in 10ml water. Two solutions were mixed and the final volume was made 100ml.

2. Fehling's solution A: 34.66g of copper sulfate was dissolved in distilled water and the final volume was made 500ml and the resulting solution was Fehling's solution A.

3. Fehling's solution B: Potassium tartarate (173g) and sodium hydroxide (50g) were dissolved in water and the final volume was made 500ml.

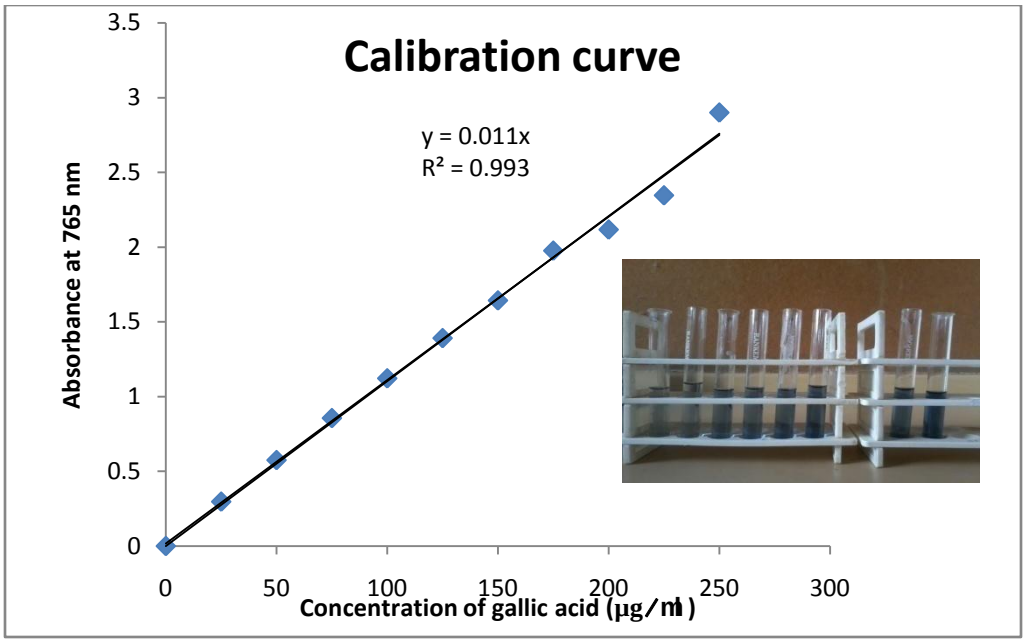


Figure Standard curve for estimation of total phenol content

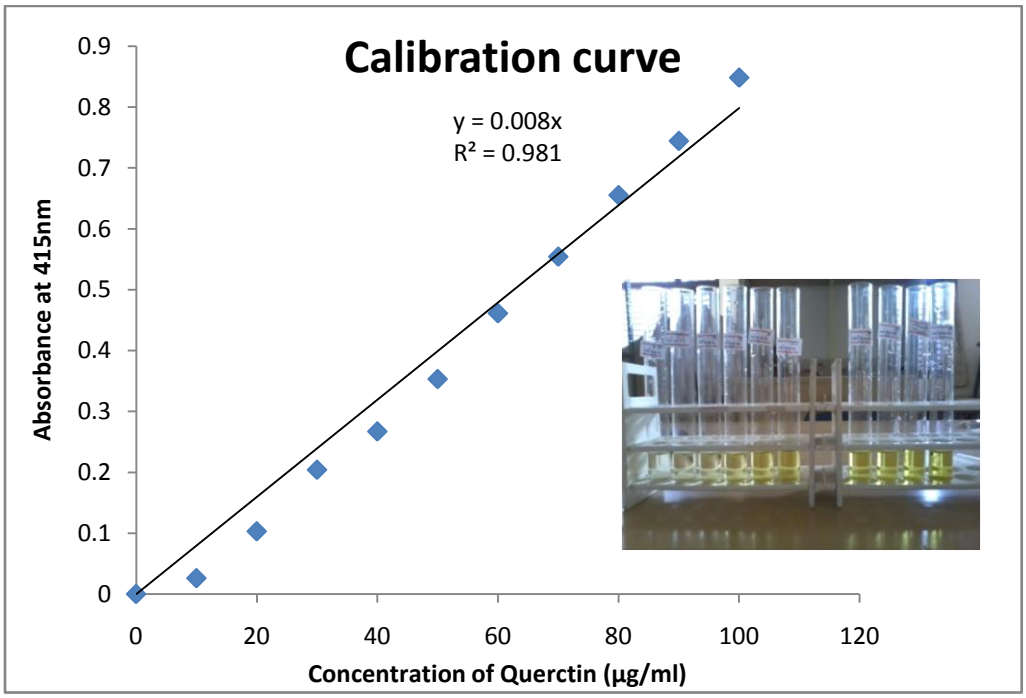


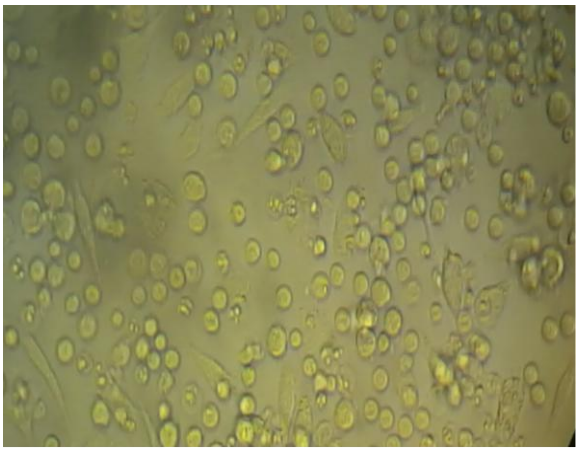
Figure Standard curve for calibration of total Flavanoid content

Table 4.2 Phytochemical screening of aqueous and methanolic extracts of the plants

Tests	***T.chebula***		***S.anacardium***		***C.montana***		***C.procera***		***N.jatamansi***		***W.somnifera***	
	M	A	M	A	M	A	M	A	M	A	M	A
Alkaloid test												
Mayer's Test	++	+	++	++	+	++	–	–	++	–	+++	–
Wagner's Test	++	++	++	+	+	+	–	–	++	–	+++	+
Saponin test	+++	++++	+++	+	+	++	–	+	++	+	+	+
Steriod test												
Libermann- Buchard test	++	++	+	+	+	+	–	–	++	–	++	–
Salkowski test	+	++	+	–	–	–	–	–	++	+	+	+
Tannin test	++++	++++	+++	++	++	++	+	+	+	–	+	–
Flavonoids test												
Shinoda test	++	+	++	–	+	+	+	–	–	–	+	–
Alkaline reagent test	+	+	+	–	–	+	+	–	–	–	+	–
Phenol test	++++	+++	+++	++	++	++	+	+	–	–	–	–
Carbohydrates and Glycosides test												
Molisch's test	+++	+++	+++	++	+	+	++	++	+	+	++	–
Fehling's test	++	+	++	+	–	–	+	+	++	–	++	–
proteins and amino acids test												
Biuret test	+	+	–	–	+	+	++	+	–	–	+	–
Ninhydrin test	–	–	–	–	++	++	++++	+++	–	–	+	–

++++: extremely positive, +++: highly positive, ++: moderately positive, +: positive, -: negative; M: methanolic extract, A: Aqueous extract

Plate 1. (a) Soxhlation with methanol, (b) in vacuo concentration using Rotatory evaporator



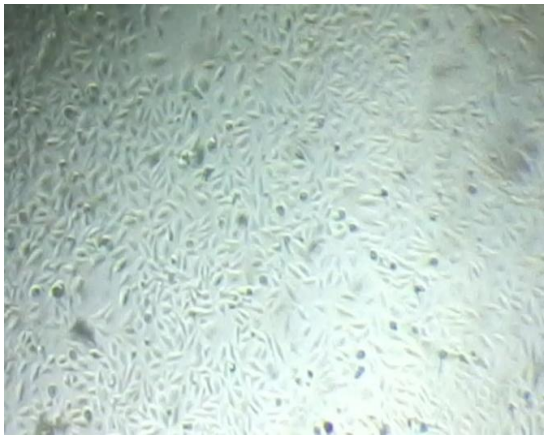


Plate 2. (a) Mice peritonial macrophages, (b) Hela cell

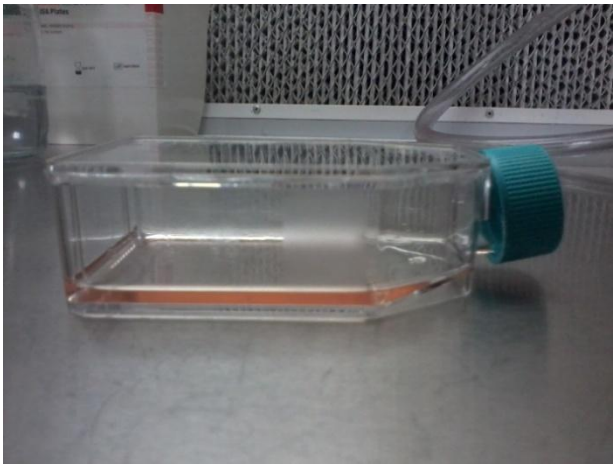


Plate 3. Hela cell in culture flask, (b) working on laminar hood