PHYTOCHEMICAL ANALYSIS AND INVESTIGATION OF ANTIOXIDANT, ANTIBACTERIAL AND ANTICANCEROUS PROPERTIES OF THREE MEDICINAL PLANTS OF NEPAL

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LIST OF ABBREVIATIONS

ABTS 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)

ATCC American Type Culture collection

BHA Butylated Hydroxy Anisole

BHT Butylated hydroxyl Toluene

CAM Complementary and Alternative Medicine

CFU Colony Forming Unit

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleic acid

DPPH 2,2-diphenyl-1 picrylhydrazyl

ELISA Enzyme Linked immuno sorbent Assay

FRAP Ferric reducing antioxidant power

G6PD Glucose 6 phosphate dehydrogenase

GAE Gallic aid equivalent

GCMS Gas Chromatography-Mass spectrometry

GSH Glutathione

GSSG Glutathione disulfide

HL60 Human promyelocytic leukemia cells

HPLC High Performance liquid Chromatography

HPTLC High Performance Thin layer Chromatography

IC50 50% Inhibitory concentration

IR Infrared Spectroscopy

MBC Minimum bactericidal concentration

MCF7 Michigan Cancer Foundation 7

MDR Multi Drug Resistant

MHA Muller and Hilton Agar

MIC Minimum Inhibitory Concentration

MRSA Methicillin resistant Staphylococcus aureus

MTT 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide

NADPH Nicotinamide phosphate dinucleotide

CLSI Clinical and Laboratory standard Institute

NMR Nuclear Magnetic Resonance

NO Nitric Oxide

ORAC Oxygen radical absorption capacity

PUFA Polyunsaturated Fattyacid

ROS Reactive Oxygen Species

RPMI Rosewell Park Memorial Institute

RSA Radical Scavenging Activity

SOD Super Oxide Dismutase

TBHQ Tert- Butyl Hydroxylated Quinone

TPC Total Polyphenol Content

WHO World Health Organization

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ABSTRACT

The use of plant as medicine started from the prehistoric days and its use is still continuing in different forms whether as synthetic drugs or as complementary medicine. Whole plant was taken as sample in *Primula rotundifolia* and *Potentilla fulgens* and bark was used in *Rhododendron arboreum*. Aqueous, Methanolic and Chloroform extract of three plants *Potentilla fulgens, Rhododendron arboreum* and *Primula rotundifolia* were analysed for their total flavonoid, polyphenol content, antioxidant activity, antibacterial and anticancerous activity. Chloroform extract of *Primula rotundifolia* was found to be highest in flavonoid content i.e. 23±0.71 mg quercetin eqv/gm plant extract. Methanolic extract was also found to be slightly less in flavonoid content than chloroform extract ie. 19±707 mg quercetin eqv/gm plant extract. Polyphenolic content was found to be high in methanolic extract of *Potentilla fulgens* (448±6 mg gallic acid/gm equivalent) and *Rhododendron* (449.605±4mg gallic acid/gm equivalent). On contrary chloroform fraction of *Primula rotundifolia* was rich in phenol content than aqueous and methanolic extract. Antioxidant activity was found to be in good correlation to the total polyphenol content. Methanolic extract of *Potentilla fulgens* and *Rhododendron arboreum* showed high scavenging potential with IC_{50} value at 477.32±5.46 µg/ml and 602±3.11 µg/ml respectively in (2,2-azinobis 3-ethyl-benzothiazoline-6-sulfonic acid) ABTS assay and 340.93±4.09 µg/ml and 517.69±6.33 µg/ml respectively in (2,2-diphenyl-1 picrylhydrazyl) DPPH assay. On contrary in both assays chloroform extract of *Primula rotundifolia* showed higher free radical scavenging potential with IC_{50} value at 807.08±3.78 µg/ml in (2,2-diphenyl-1 picrylhydrazyl) DPPH assay and 1420±5.66 µg/ml in (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) ABTS assay. Antibacterial activity was performed in all extract and chloroform extract of *Primula rotundifolia* exhibited very impressive result with (Minimum Bactericidal concentration) MBC of 1 mg/ml for *Pseudomonas aerugenosa* and 800µg/ml for *Staphylococcus aureus*. Antiproliferative and cytoxicity study was also performed in the extract and Chloroform extract of *Primula rotundifolia* showed lowest IC_{50} value at 107.096 µg/ml and low cytotoxicity as well. Methanolic extract of *Potentilla fulgens* and *Rhododendron arboreum* showed moderate type of antiproliferative and less cytotoxic property. These findings indicate the possible potential use of these plants as anticancer agent but needs further identification, isolation and purification of the compound responsible.

Keywords: Reactive oxygen species (ROS), Flavonoids, antioxidants, Polyphenols, antibacterial, cytotoxicity, Medicinal plants.

CHAPTER I: INTRODUCTION

1.1 Background

Plants have been used as a medicine from the very ancient time in crude form even the main constituent in the extract was unknown. Present study is mainly focused on the screening of the medically important chemical compound by studying possible effects in special molecular targets or studying its biological effects followed by the identification of putative lead compound. Once the lead compound is identified it can be used in preclinical studies. In addition the elucidation of the chemical structure of many natural products allows chemists to synthesize them, rather than isolating them from natural sources, which markedly reduce the cost of drug production. Subsequently, a large number of well-known natural compounds were identified, analyzed and synthesized e.g. salicin from *Salix alba* (white willow), emetine from *Cephaelis ipecacuanha* (ipecacuanha), strychnine and brucine from *Strychnos nuxvomica* (strychnos), quinine from *Cinchona ledgeriana* (cinchona bark), colchicine from *Colchicum autumale* (colch icum), caffeine from *Coffea arabica*, nicotine from *Nicotiana tobaccum*, atropine from *Atropa belladonna* and cocaine from *Erythroxylum coca*. Many of these compounds are still widely used as drugs (HongFang, 2009).

Secondary metabolites of plants are important class of natural products known to have medicinal importance. Polyphenolic compounds, alkaloids, Saponins and terpenoids being major class of secondary metabolites having clinical relevance.

1.2 Medicinal plants in Nepal: A brief overview

Nepal is a country sandwiched between two Asian giants, India on the south and China on the north, Nepal (147,181 Km^2) and lies on the central region of the great Himalayan range. Altitudinal variation starts from almost sea level (~70 meter) to the top of the world (8,848 meter). Climatic differences, varied topography and abundant ecological habitats of our country offer rich flora and fauna life. The prosperous biodiversity of the living organisms has thrived inside the country.

The use of medicinal plants for treating various ailments started long back .The first written record of using medicinal plants in Nepal is Saushrut Nighantu, the oldest Nepali medicinal plant book which was produced during the rule of the Great King Man Dev in the 5th century. It describes 278 plants in Sanskrit as well as 282 medicines for curing the illness (Gewali, 2008) In 1970, Nepal Government's Department of Plant Resources (then known as Department of Medicinal Plants) published the first detailed survey of the medicinal plants found in Nepal and the number of the medicinal and aromatic plant species was estimated to be 483 (Jiang and Liu, 2007). A more recent publication of Plant Department Resource recorded the number of medicinal plants of Nepal as 701 species and out of which only 30 species are prioritized for research and development (DPR, 2012). In 2002,

Manandhar reported ethnobotanical importance of 1500 plant spp, of which majority of them have medicinal value.

Traditional medicine or folk medicine in Nepal refers to the use of those natural things based on beliefs that were in existence often hundreds to thousands of years before the development of modern medicine. This method of traditional medicine is handed down from generation to generation by the oral method or by apprenticeship rather than through documentation. Recently there are 33 Nepalese Ayurvedic and Unani medicine manufacturing industry in operation reported in the drug bulletin of Nepal 2005 published by Department of Drug Administration (Drug Bulletin of Nepal, 2005). This shows that the interest of people towards complementary and ayurvedic medicine is growing and on other aspect a lot of Nepalese rely on the trade of these valuable products for livelihood.

1.3 Cancer and plant derived drugs

In a scenario where conventional medicine has failed to develop techniques that could reduce the incidence of death due to cancer, complementary and alternative medicine (CAM) is slowly emerging as an option. According to the National Institute of Health's National Centre for Alternative and Complementary medicine, around 36% of people in the US use alternative medicine in some form or the other. Several studies indicate that a majority of cancer patients use CAM extensively as a mode of treatment or as a means to reduce the side effects of conventional treatment methods (Shafi et al., 2009).

From the past few decades, there has been an upsurge in the search for new plant derived drugs. This process has facilitated to produce remarkably a diverse array of over 1,39,000 natural products, containing medicinally useful terpenoid derivatives, alkaloids, glycosides, polyphenolics, steroids, and so forth. The National Cancer Institute (NCI) of the United States of America (USA) has screened about 1,14,000 extracts from an estimated 35,000 plant samples against a number of tumor systems (Cragg and Boyd, 1996). Of the 92 anti cancer drugs commercially available prior to 1983 in the USA and approved world-wide between 1983 and 1994, approximately 62% can be related to natural origin (Newman and Sna 1997). Some examples include vinblastine and vincristine (*Catharanthus roseus*), epipodophyllotoxin, anisomer of podophyllotoxin (*Podophyllum peltatum* roots) paclitaxel (*Taxus baccata*, *T.brevifolia*, *T.canadensis*), camptothecin (*Camptotheca acuminate*), homoharringtonin (*Cephalotaxusharringtonia var.drupacea*).The two plant derived natural products paclitaxel and camptothecin were estimated to account for nearly one third of the global anticancer market, respectively to the tune of about $ 3 and $ 9 billion, in the year 2002 (Boopathy and Kathiresan, 2010).

Apart from this the other global issues on drugs are the antibiotics which are facing the problem of resistance. Now this has led to understand that the lifespan of antibiotics is

limited and has created pressure to switch on for the second or third line drugs and ultimately a need to develop alternative approaches (Ciocan and Bara, 2007).

Three plants having ethnomedicinal value were used in studying their flavonoid, polyphenol content, antioxidant, anticancer and cytotoxicity activity. Primula rotundifolia, *Potentilla fulgens* and *Rhododendron arboreum* are well known for their medicinal value.

Primula rotundifolia belongs to the family Primulaceae. It is a hardy perennial plant with high medicinal value. Various spp of *Primula* are well known for their use in fever, food poisoning, asthamas etc. This plant is found to be rich in saponins and flavonoids (Callis et al., 1992).

Potentilla fulgens belongs to family Rosaceae and it is an erect perennial herb. Various ethnomedicinal use of this plant includes its use in diarrhea, diabetes, cancer, stomatitis cough, tooth infection etc. Potentilla spp are known to contain high amount of tannins and triterpenoids while flavonoids, Phenol carboxylic acid, organic acids are present in less amount (Kaul et al., 2011).

Rhododendron arboreum also known as laligurans belongs to the family Ericaceae. Various parts of this plant are well known for their medicinal value. Flowers are used for the treatment of hill diarrohoea and dysentery. Leaves are used in headache and fever (Bhattacharya, 2011).Various secondary metabolites like alkaloids, steroids, flavonoids, terpenoids anthraquinones, saponins, tannins and reducing sugars (Nisar et al., 2011).

1.4 Research plan

1.4.1 Research hypothesis

Plants that are used ethnomedically are reported to have medicinal value. Their medicinal value in terms of scientific basis needs to be explored. People consuming the food containing high flavonoid and polyphenols are known to have low risk for cancer but the actual mechanism underlying the process is ellusive. Plants rich in polyphenol and flavonoid content are capable of inhibiting the proliferation of cancer cell line without affecting the normal cell line. Also these plants extracts containing high amount of flavonoid and polyphenols are capable of scavenging free radicals which are known to promote dreadful disease like cancer.

1.4.2 Objectives

a) To explore the amount of polyphenols and flavonoid in these ethnomedically important plants.

b) To screen their antimicrobial, free radical scavenging and antiproliferative activities of plant extracts.

c) To determine correlation between total phenol content to antioxidant capacity of the extracts.

1.4.3 Rationale

From the very ancient time recorded in the different Vedas, plants in different forms are used for the remedy of various ailments. Plants being a pool of chemical are believed to contain potent bioactive compounds. Being better compatibility with human body plants are taken as an alternative to modern medicine because of their complication emerging in a greater rate. Polyphenolic and flavonoids are found to inhibit the prognosis of cancer by various mechanisms like scavenging of free radicals or by indirect inhibition of cancer promoting cellular metabolic product or abiological chemicals. Plants under study are reported to have high polyphenolic content and are potent scavenger of free radicals generated in vivo which suggests us that these plants have greater possibility of inhibiting cancer prognosis. Hence there is a need in screening of their anticancer property and further identification and isolation of the lead compound.

1.4.4 Scope

Plants that are used by the indigenous population are known to have medicinal value. This study initiates the way to explore the true value of the prolonged used herbs through detail investigation. Also it creates an interest among the researcher for exploration of other medicinally used plants and if found to be effective, opens a new arena for further research and finally if approved can go for medical prescription. This research will give a scientific validation on the use of selected plants as medicine by local people. Apart from this study would be a preliminary step towards isolation of active compounds and further process in drug discovery. Also the most important thing is that it will create awareness among the people to use these medicinal plants in better way, exploiting it for their own benefit or say benefit sharing.

CHAPTER II: LITERATURE REVIEW

2.1 Plant secondary metabolites and their importance

From the very ancient times, plants have been used as a medicine. Palaeoanthropological studies at the cave site of Shanidar, located in the zagros Mountains of Kurdistan in Iraq, have suggested that more than 60,000 years ago Neanderthals might have been aware of the medicinal properties of various plants, as evidenced by pollen deposits in one of the graves at the site (Solecki, 1975). Throughout our evolution, the importance of natural products for medicine and health has been enormous. Since our earliest ancestors chewed on certain herbs to relieve pain, or wrapped leaves around wounds to improve healing, natural products have often been the sole means to treat diseases and injuries (Hong-Fang et al., 2009) Despite the use of these plants as medicine people were unknown about the specific constituent in the extract which is targeted to treat the disease. Modern chemistry of natural products has now opened a new arena to study and use the plant derived natural products. Furthermore structural and analytical chemistry has helped to elucidate the structure and purify the chemical compounds and finally studying its therapeutic value in the mankind. In fact, it has only been during the past decades that natural products have taken a secondary role in drug discovery and drug development, after the advent of molecular biology and combinatorial chemistry made possible the rational design of specific molecules (Ji et al., 2009).

Plant during their lifetime produce two types of metabolites named as primary metabolites and secondary metabolites. Primary metabolites are those which have low molecular weight that are essential to maintain life or to survive such as sugars, organic acids and those amino acids which are used to synthesize proteins (Hadacek F, 2002). Secondary metabolites are not required for survival of the cell but are necessary as a whole to protect the plant from external invaders. These are produced at certain stage of lifecycle or during the pathogen invasion in special celltypes so that the other cells remain unaffected by the toxic nature of the metabolites. Later on these toxic metabolites are fed into the primary metabolic pathway thereby conserving the metabolic compounds. Certain secondary metabolites are restricted to a few plant species, where they fulfill specific functions, such as attracting insects to transfer pollen, disperse seeds, act as natural pesticides (Heldt 2004). Plants secondary metabolites can be grouped into three parts: alkaloids, phenylpropanoids and isoprenoids and will be described below.

2.1.1 Alkaloids

Alkaloids belong to a group of secondary metabolites that are synthesized from amino acids and contain one or several N atoms as constituents of heterocycles. Many of these alkaloids act as defense substances against animals and microorganisms. Since alkaloids

usually are bases, they are stored in the protonated form, mostly in the vacuole, which is acidic. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden and Fessenden,1982): the name morphine comes from the Greek Morpheus, god of dreams. Codeine and heroin are both derivatives of morphine.

Vincristine and vinblastin so called vinca alkaloids isolated from Madagascar Periwinkle are used as anticancer agent. More recently the semisynthetic analog of these alkaloids are vinorelbine and Vindacene in combination with other drugs are used to treat various types of cancer. Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma (Cragg and Newman, 2005). The mechanism of action of Vinca alkaloids is that they inhibit the cell proliferation by affecting the microtubular dynamics during mitosis, and this causes a characteristic block during mitosis leading to apoptosis (Nirmala et al., 2011). Another important addition to the anticancer drug armamentarium is the class of clinically active agents derived from camptothecin, which is isolated from the Chinese ornamental tree, Camptothecin acuminate Decne (Nyssaceae), known in China as the tree of joy (Cragg and Newman, 2005). Similarly, two novel alkaloids, schischkinnin and montamine have been isolated from the seeds of *Centaurea schischkinii* and *Centaurea montana* (Shoeb et al., 2006).

Besides the anticancer potency of alkaloids, some alkaloids have other medicinal uses including the antimalarial drug quinine extracted from the Cinchona plants. Furthermore two new semisynthetic derivatives of age-old drugs, morphine and atropine, have been developed and are being used clinically for Parkinson's disease and for chronic obstructive pulmonary disease respectively.

2.1.2 Phenylpropanoids

These are the next important secondary metabolites of plants and are also present in microorganism. Phenylpropanoid pathway is linked to the primary metabolic pathway, glycolysis through the shikimate pathway. Precursors for shikimate pathway are Erythrose 4 phosphate and Phosphoenolpyruvate that are derived from glycolysis. Shikimate pathway provides limited set of core structure i.e. aromatic amino acids phenylalanine and tyrosine which undergoes efficient modification as well as amplification to give the diversified structures of phenylpropanoid (Herrmann, 1995). Diversification of the phenyl propanoid pathway leads to the production of various secondary metabolites like flavonoids, flavanols, coumarins, isoflavonoids, stillbenes, cutins, suberins, proanthocyanidins, lignin phenyl propanoid esters etc.

Phenylpropanoids contribute to all aspect of plant responses towards biotic and abiotic stimuli. They are not only indicators of plant stress responses upon variation of light or mineral treatment, but are also key mediators of plants resistance towards pests (Camera et al., 2004) and provide the biochemical resources for successful reproduction (Dudareva et al., 2004). Phenyl propanoid based polymers, like lignin, suberin or condensed tannins, contribute substantially to the stability and robustness of gymnosperms and angiosperms towards mechanical or environmental damage, like drought or wounding (Vogt, 2009). In case of mammals it has been found to be antitoxic, hepatoprotective, radical scavenger and cytoprotective. The effect of flavonoids on CCl_4-induced toxicity in isolated rat hepatocytes was studied by Perrissoud and Testa (1986). The ability to interfere with CCl_4-induced release of aspartate aminotransferase was tested with 55 flavonoid compounds. The more hydrophilic compounds were observed to inhibit the CCl_4-induced toxicity, whereas the more lipophilic derivatives actually potentiated the toxicity.

The flavonoids quercetin, kaempferol, catechin, and taxifolin suppressed the cytotoxicity of O_2 and H_2O_2 on Chinese hamster V79 cells, as assessed with a colony formation assay (Nakayama et. al, 1993). Quercetin and kaempferol showed protective effects at 5 to 10 mM concentrations, whereas much higher concentrations of catechin and taxifolin were necessary for the prevention of cytotoxicity. The mechanism of antiradical action of quercetin and its glycoside, rutin was evaluated by Afanas'ev et al. (1989) using NADPH and carbon tetrachloride (CCl_4)- dependent lipid peroxidation of rat liver microsomes and iron ion-induced peroxidation of lecithin liposomes. Both flavonoids were significantly more effective inhibitors of the iron ion-dependent lipid peroxidation system due to their chelation of iron ions. The chelating mechanism of inhibition was more important for rutin than for quercetin. The flavonoids were reported to chelate iron ions and to form inert complexes unable to initiate lipid peroxidation, yet they retained their free radical-scavenging properties more pronounced on NADPH-dependent than on CCl_4- dependent lipid peroxidation in rat liver microsomes.

2.1.3 Isoprenoids

Isoprenoids or terpenoids are metabolites produced by plants and miroorganisms constisting of isoprene units as building blocks. Based on the number of building blocks, terpenoids are classified into several classes, such as monoterpenes (e.g., carvone, geraniol, d-limonene, and perillyl alcohol), diterpenes (e.g. retinol and trans-retinoic acid), triterpenes [e.g., betulinic acid (BA), lupeol, oleanic acid, and ursolic acid (UA)], and tetraterpenes eg. α-carotene, β-carotene, lutein, and lycopene (Rabi and Bishayee, 2009). Several terpenoids are found to be used in pharmaceutical application like artemesin and taxol as antimalarial and cancer drugs respectively.

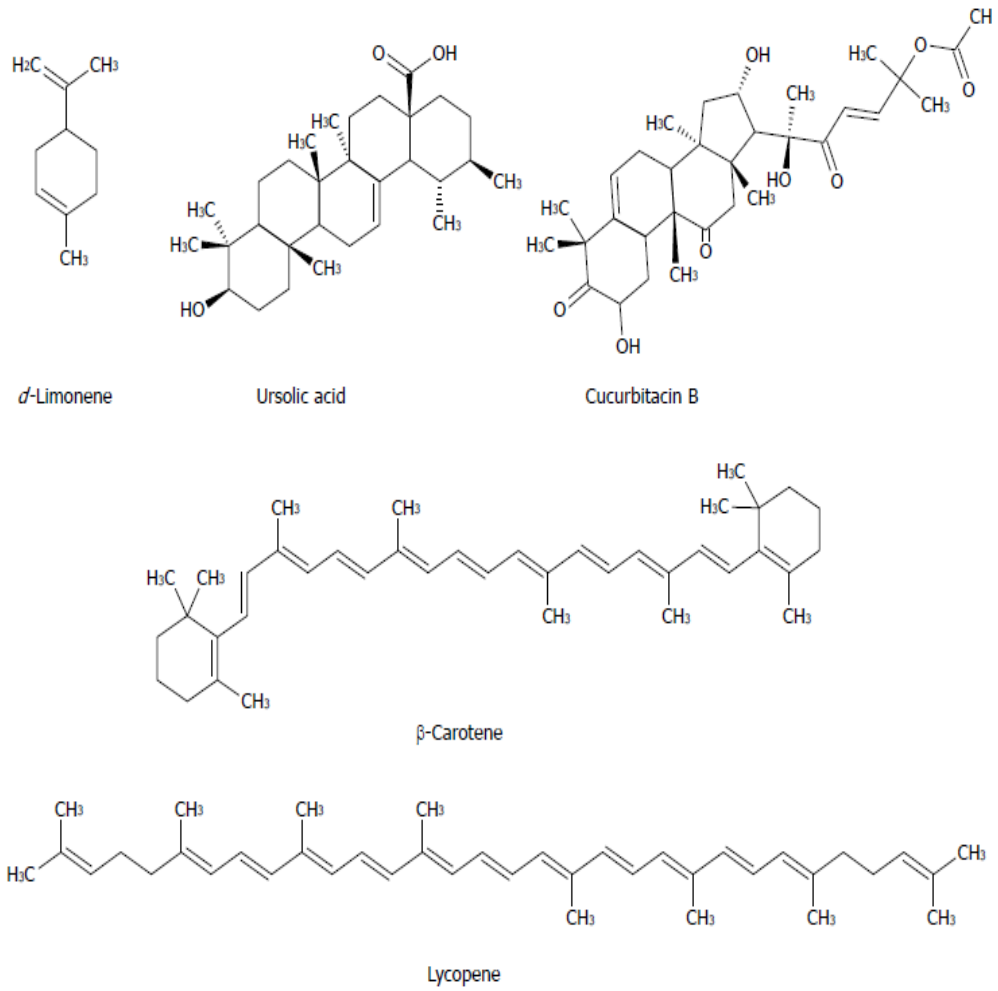


Figure 1 : Structure of some isoprenoids

2.2 Reactive oxygen species and antioxidants

A chemical compound that is capable of accepting an electron is called oxidant or oxidizing agent while on the contrary the chemical compound that is capable of donating an electron is said to be reductant or reducing agent. This process of oxidation and reduction forms a redox reaction i.e. oxidation process is always followed by reduction and this redox reaction is the basis of various types of biochemical reaction taking place in the living beings. The process of accepting or donating electron takes place in the form of hydrogen donation or removal of oxygen. In biological terms we can say that a reducing agent or reductant is an antioxidant and an oxidizing agent is pro oxidant (Prior and Cao, 1999; Baud and Ardaillou, 1986). Even though these redox reactions are necessary for life process they also are found to be detrimental to the cell. In the biological system these oxidants are generally reactive oxygen species (ROS) or reactive nitrogen species (RNS).

These reactive species are produced inside the cell of the living system either as a strategy of cell to combat its pathogens or as an incomplete or little inefficient process that takes

place in the cell or as a signaling molecule endogenously or exogenously. For example Reactive oxygen species (ROS), i.e. substances like hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) or the highly reactive hydroxyl ion (HO^-) as well as reactive nitrogen species (RNS) with nitric oxide (NO) as its most important member are ideally suited to serve as signaling molecules since they are locally generated, highly and rapidly diffusible and can be neutralized by a bulk of anti-oxidative agents organized in the cellular anti-oxidative defense system. On the other way, During the process of respiration and photosynthesis there is flow of electron in the cyclic electron transport chain from which the electrons are leaked and this electrons convert the molecular oxygen to the superoxide form thereby acting as a prooxidant (Turrens, 2003) which damages proteins, DNA etc. Similarly in case of pathogen invasion, several phagocytic cells like neutrophils, eosinophils, basophils, mononuclear cells (monocytes), and lymphocytes are major producers of endogenous ROS (Ginsburg, 1998; Ginsburg, 1995). Following stimulation, these cells undergo a respiratory burst characterized by a 20-fold increase in oxygen consumption, which is accompanied by an increase in glucose utilization and production of reduced nicotinamide phosphatedinucleotide (NADPH) by the pentosephosphate pathway which donates electron to NADPH oxidase (Babior et al., 2002). In animals the NADPH-oxidase is found in phagocytes and B lymphocytes. It catalyzes the production of superoxide by the one electron reduction of oxygen using NADPH as the electron donor. The O_2^- generated by this enzyme serves as a starting material for the production of large variety of reactive oxidants, including oxidized halogens, free radicals and singlet oxygen. These oxidants are used by phagocytes to kill invading microorganisms, but at the same time they may also damage surrounding cells of the host (Apel and Hirt, 2004). While most enzymes produce ROS as a by-product of their activity, exemplified by the formation of superoxide radicals by xanthine oxidase, there are some enzymes designed to produce ROS, such as nitric oxide synthase that yields NO radicals (Canas, 1999; Lewen et al., 2000).

Exogenous source of these reactive oxygen species includes UV radiation, ϒ radiation, ultrasound, food, drugs, pollutants, xenobiotics and toxins. Thus we can conclude that reactive species or free radical produced in the body have physiological significance and a detrimental effect depending upon the cellular condition. In order to combat the harm done by these reactive species our body has developed a defense mechanism to overcome the stress i.e. by enzymatically or non-enzymatically called as endogenous antioxidant. Likewise the antioxidants that we get from our dietary sources are known as exogenous antioxidants.

2.2.1 Endogenous antioxidants

Some of the important endogenous antioxidants are described below

a) **Superoxide dismutase**

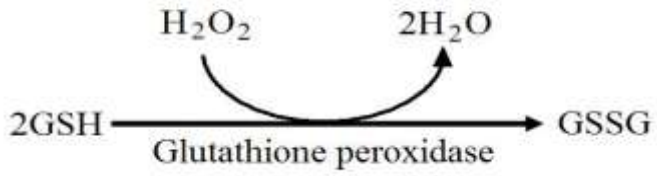
These are the enzymes that convert superoxide radicals into oxygen and hydrogenperoxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen. Structure analysis shows that these are cofactored with metals like Cu , Zn, Mn etc. Mammalian tissues and cells have three types of SOD isozymes: the first (Cu/Zn-SOD), the homodimer, localizes in cytosol and contains 1 mol of Cu and Zn per mole of monomeric subunit; the second (Mn-SOD), homotetramer localizes in mitochondrial matrix and contains 1 mol of Mn per subunit; the third (extracellular SOD, EC- SOD), homotetramer, contains 1 mol of Cu and Zn per mole of monomeric subunit and localizes bound to cell surface matrix via its heparin-binding domain (Inoue, 2001). The genes are located on chromosomes 21, 6, and 4, respectively.

b) Glutathione reductase

Glutathione (ϒ-glutamyl cysteinyl glycine, GSH) is a sulfhydryl (-SH) an antioxidant, antitoxin and cofactor. Glutathione is ubiquitous in animals, plants and microorganisms, and being water soluble is found mainly in the cell cytosol and other aqueous phases of the solvent system. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants (Kosower 1978). Glutathione exists in two forms: The antioxidant “reduced glutathione” tripeptide is conventionally called glutathione and abbreviated GSH; the oxidized form is a sulphur–sulphur linked compound, known as glutathione disulfide or GSSG. Once oxidized glutathione can be reduced by glutathione reductase using NADPH as electron donor. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress. High the ratio greater is the oxidative stress (Kidd 2001).

Various functions of Glutathione in the cells are:

- It is the cofactor of glutathione transferase enzyme that is involved in the detoxification pathways.
- It also acts as substrate for the gamma-glutamyl transpeptidase, enzymes which are located in the outer cell surface and transfers the glutamine moiety from GSH to other amino acids for subsequent uptake into the cell.
- Direct free radical scavenging eg glutathione peroxidase which breaks down H_2O_2 into water. Also acts as an antioxidant enzyme cofactor viz enzymes known as GSH transhydrogenase use GSH as a cofactor to reconvert dehydroascorbate to ascorbate.



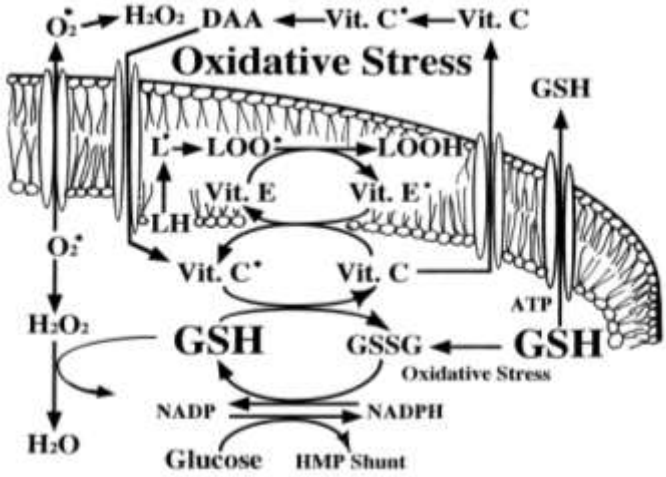


Figure 2 Radical chain reaction and antioxidant network in membranes and aqueous compartments (Inoue, 2001).

$O^{2\bullet}$: superoxide radical, LH: lipids, $L^{\bullet}$: lipid radicals, LOO•: peroxy radical, Vit.E: vitamin E, Vit.E•: vitamin E radical, Vit.C: vitamin C, Vit.C•: vitamin C radical, DAA: dehydroascorbic acid.

c) Catalase

Catalase is an important enzyme found in nearly all aerobic organisms, located in the peroxisomes. It catalyzes the conversion of hydrogen peroxide to water and oxygen. Catalase is a tetrameric protein of 250 kDa, which consists of four similar subunits, each containing a heme group. It is encoded by a single gene in mammalian species. Catalase is highly expressed in some tissues, protecting cells against an excess formation of ROS (Glorieuxa, 2011). In mammalian tissues the highest levels of catalase are found in the liver, kidney, and erythrocytes while the lowest levels are found in connective tissues. In tissues such as the liver, catalase is found predominantly in peroxisomes. However in mature human erythrocytes catalase is freely found in the cytosol (Friel et al., 2002).

d) Glucose 6 phosphate dehydrogenase:

Glucose-6-phosphate dehydrogenase (G6PD), a vital enzyme in all cells, catalyzes the first reaction of the pentose phosphate pathway allowing the conversion of glucose-6-phosphate to 6-phosphogluconolactone. In this reaction, nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH. NADPH is a key hydrogen donor for

the reduction of oxidized glutathione (GSSG) to a tripeptide known as reduced glutathione (GSH). This tripeptide is used as a reducing agent by glutathione peroxidase, which is involved in the detoxification of hydrogen peroxide. In this process, GSH is converted to GSSG leading to a decrease in GSH. The regeneration of GSH occurs by the action of glutathione reductase, which catalyzes the reduction of GSSG to GSH in the presence of NADPH (Njålsson and Norgren, 2005). Since there are no other sources of NADPH in red blood cells, G6PD is essential in protecting hemoglobin sulfhydryl groups and preventing red blood cell membrane oxidation (Pandolf et al., 1995).

2.2.2 Exogenous antioxidants

These antioxidants are found in the plant products and upon consumption they protect body against free radicals directly or enhance the endogenous antioxidants. Exogenous antioxidants include vitamin C, vitamin E, polyphenols, carotenoids etc. Plants rich in antioxidants include fruits and vegetables like apple, broccolis, banana, plums, onions, potatoes, leafy vegetables, soybeans, peanuts, bell pepper etc. In addition to their natural occurrence in foods, fortification, supplementation with isolated components and intake of synthetic antioxidant additives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butyl hydroquinone (TBHQ) and propyl, octyl and dodecylgallates (used initially to protect and to preserve the nutritional quality and to increase shelf-life of processed foods) constitute further sources of antioxidants (Bouayed and Bohn 2010)

a) Vitamin E

Vitamin E is a chain breaking antioxidant having the ability to repair oxidizing radicals directly, preventing the chain breaking propagation step during lipid autooxidation. (Serbinova and Packer 1994). It reacts with alkoxy radicals (LO°), lipid peroxyl radicals (LOO°) and with alkoxy radicals (L°), derived from PUFA (Polyunsaturated Fatty Acid) oxidation. The reaction between Vitamin E and lipid radical occurs in the membrane –water interphase where vitamin E donates a hydrogen ion to lipid radical with consequent tocopheroxyl radical formation. Regeneration of the reduced form of tocopheroxyl radical can be achieved by the vitamin C (Buettner 1993).

b) Ascorbic acid

VitaminC (ascorbic acid) is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not by humans, non-human primates and guinea pigs. These species do not have the enzyme gulonolactone oxidase, which is essential for synthesis of the ascorbic acid immediate precursor 2-keto-l-gulonolactone (Padayatty et al., 2003) Because of its high electron donating capacity it is considered as a potent antioxidant, and it is converted to a stable free radical semi dehydroascorbic acid or

ascorbyl radical when it loses one electron and after the loss of second electron it is converted to dehydroascorbate. Thus formed oxidized ascorbic acid can be reduced back to ascorbic acid via different enzymes as well as by reducing compounds like glutathione in humans. Ascorbic acid are capable of reducing oxygen related radicals like superoxide radicals, hydroxyl radicals and peroxyl radicals, nonradicals like hypochlorous acid, nitrosamines and other nitrosating compounds, nitrous acid related compounds and ozone. It also regenerates vitamin E or alpha Tocopherol from its radical form (Buettner, 1993).

c) Polyphenols

These ranges from simple phenolic acids to highly complex structure like tannins. Phenolic acids are derivates of cinamic acids or benzoic acids like Gallic acid, Ferulic acids, Coumaric acid, Caffeic acid etc. Likewise slightly moderate polyphenols includes flavonoids, flavonols, flavones, stillbenes, lignans etc. Tanins are the complex ones having high molecular weight.

2.2.3 Antioxidant activity determination

Antioxidant activity of a tested substance indicates its capacity to inhibit the oxidation reaction in a definite set of condition. Different plants differ in their polyphenolic content as well as other small molecules like glutathione, vitamin E content etc. and thus this makes a difference in the antioxidant activity of the extracts obtained from those plants. Apart from this the solvent system used for extraction, also affects the antioxidant activity. There are a number of antioxidant activity determining method viz ABTS asay 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid), DPPH assay (2,2-diphenyl-1 picrylhydrazyl), FRAP (ferric reducing antioxidant power), ORAC assay (Oxygen radical absorption capacity) etc.

ABTS assay

2,2- azinobis(3- ethyl-benzothiazoline - 6 - sulfonic acid) is a molecule that is converted to a free radical in presence of oxidizing agent like Potassium persulphate by undergoing one electron oxidation and is reported to be stable in p^H 6.5–3.5 range (Ozgen et al., 2006) . Above or below this P^H there is rapid degradation of the radical into its metabolites. Once the oxidation process proceeds the colorless ABTS is converted to a deep blue green colored radical which is not stable until 6 hrs. The absorbance maxima reach only after 12 to 14 hrs. This free radical has maximal absorbance at wavelengths 645 nm, 734 nm and 815 nm, as well as the more commonly used maximum at 415 nm. The antioxidants present in the sample quench this free radical to a colorless non radical product. A standard curve of antioxidant is plotted and the amount of ABTS scavenged by the sample or extracts is determined with the help of standard curve spectrophotometrically.

DPPH assay

This method was first developed by Blois in 1958 and is followed to determine the antioxidant activity. DPPH is a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, like most other free radicals. The delocalization also gives rise to the deep violet colour, with an absorption in ethanol solution at around 520nm (Singh, 2011).

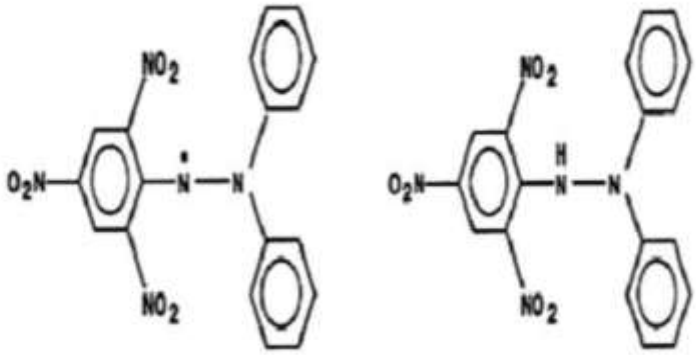


Figure3: (1)Diphenylpicrylhydrazyl (radical form) (2)Diphenylpicrylhydrazyl (nonradical)

This free radical in the solution accepts electron or the hydrogen radical and is converted to a nonradical form thereby the color changes from deep violet to colorless form. Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

Z• + AH = ZH + A•

where ZH is the reduced form and A• is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry,i.e. the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant (Molyneux, 2003). Since it is a rapid, simple, widely used and inexpensive method, it can be used to measure the antioxidant capacity of fruits and vegetables juices. It is a convenient method for the antioxidant assay of cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds (Masahiro et al., 2005) for olive oil, fruits, juices and wines (Sanchez-Moreno, 2002).

2.3 Consequences of oxidative stress

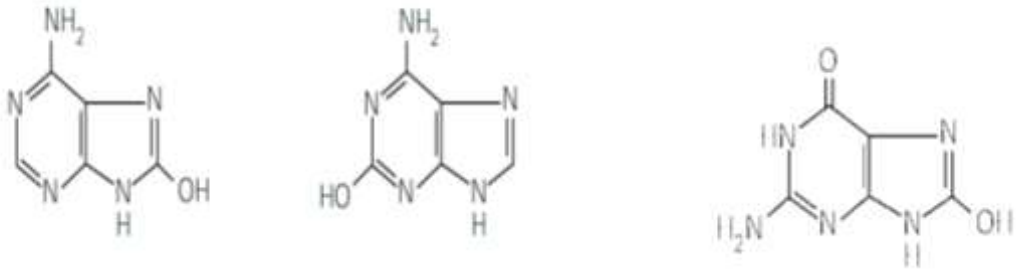
Oxidative stress in the cell can damage three main components of the cell biology viz. Lipids, Proteins and DNA.

Cell membrane is made up of phospholipid bilayer consisting mainly of polyunsaturated fatty acid. These polyunsaturated fatty acids are vulnerable to attack of reactive oxygen species thereby causing lipid peroxidation. This process of lipid peroxidation occurs is a three step process, first step involving the attack of the methylene group by a free radical

(Smirnoff, 1995). This leads to the weakening of the bond between hydrogen and carbon and thus there is formation of conjugated diene. In presence of oxygen in the vicinity this conjugated dimer is converted to peroxyl radical. Unstable peroxyl radical now to maintain its stability abstracts hydrogen from the nearby lipid molecule thus starting chain reaction. A single initiation can lead to a chain reaction resulting in peroxidation of all the unsaturated fatty acids in the membrane. Probably these peroxyl radicals are converted to cyclic peroxides. Termination of this reaction can occur via α- Tocopherol which is a chain breaking lipophilic molecule (Baud and Ardaillou, 1986).

Proteins are the next possible target of ROS. Proteins can undergo direct and indirect damage following interaction with ROS, including peroxidation, damage to specifc amino acid residues, changes in their tertiary structure, degradation and fragmentation (Davis 1987). The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins. Protein oxidation products are usually aldehydes, keto compound, and carbonyls.

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage: modification of DNA bases, single and double DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage, and damage to the DNA repair system. Not all ROS can cause damage; most is attributable to hydroxyl radicals. For example, following exposure of DNA to the hydroxyl radical leads to the modification of the DNA bases like guanine which is oxidized to 8-hydroxyguanine, adenine to 8- hydroxyadenine , 2-hydroxyadenine etc. (Kohen and Nyska,2002). Nitric oxide and its derivatives like $NO_2^{\bullet}$, $ONOO^-$, N_2O_3, and HNO_2 are mutagenic agents with the potential to produce nitration, nitrosation and deamination reaction on DNA bases.



8-Hydroxyadenine 2-Hydroxyadenine 8-Hydroxyguanine

Figure 4 : Structure of some modified DNA bases

As we know that oxidative stress in a cell causes various types of damages to the macromolecules that makes cells basic unit. Studies have shown that there is correlation between the oxidative stress and the deteriorating health condition. One of the diseases is

Alzheimer's disease which is a neurodegenerative disease. Brain cells especially the neurons are more susceptible to the oxidative stress because their glutathione content is low, their membrane consists of large amount of polyunsaturated fatty acids and brain metabolism requires a substantial amount of oxygen which can lead to the excessive damage of the neuron cells (Christen, 2000). Similarly oxidative stress leads to other various pathophysiological condition, and its implication in the dreadful disease like cancer, atherosclerosis and diabetes has been revealed (Halliwell and Whiteman 2004).

2.4 Antibiotics

Antibiotics are the natural compounds obtained from microorganism and are capable of inhibiting or killing the selective organism. The first known antibiotic is Penicillin. Nowadays because of the emergence of drug resistance the need for development of still newer, potent antimicrobial is felt.

The problem of antibiotic resistance, which has limited the use of cheap and old antibiotics, has necessitated the need for a continued search for new antimicrobial compounds. Understanding the mechanisms of resistance is important in the development of strategies to solving the problem. Active efflux of drugs, alteration of target sites and enzymatic degradations are the strategies by which pathogenic bacteria acquire or develop intrinsic resistance to antibiotics (Cowan, 1999; Kumoro, 2012). Multi-drug resistance (MDR) pumps, capable of recognizing and expelling a variety of structurally unrelated compounds from the bacterial cell and conferring resistance to a wide range of antibiotics have since been characterized in many gram positive and gram negative pathogens like *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and more recently, in mycobacteria (Sibanda and Okoh, 2007). Worldwide spending on finding new antiinfective agents (including vaccines) is expected to increase 60% from the spending levels in 1993. New sources, especially plant sources, are also being investigated (Ciocan, 2007).

2.4.1 Plant as a source of antimicrobial agent

The driving factor for the renewed interest in plant antimicrobials in the past 20 years has been the rapid rate of plant species extinction (Lewis et al., 1995). The multitude of potentially useful phytochemical structures is at risk of being lost irretrievably (Borris, 1996). Natural products research perspectives form a major field in pharmaceutical company (Cowan, 1999).

Caffeic acid, Pyrogallol, Catechol are found to be inhibitory possibly because of their power to alter the enzyme function either through direct oxidation of sulfhydral and other susceptible groups in protein or through nonspecific interaction with protein. Quinones are aromatic ring with two ketone substitution. In addition to providing a source of stable free

radical quinones are known to complex irreversibly with nucleophilic aminoacids in proteins (Kubo, 1995), often leading to inactivation of the protein and loss of function.

Flavonoids are capable of inhibiting not only the bacterial population but also are known to inhibit viral replication. The possible mechanism underlying the effect is conflicting. Flavonoids lacking hydroxyl groups on their ϐ-rings are more active against microorganisms than are those with the 2 OH groups (Chabot et al., 1992). This finding supports the idea that their microbial target is the membrane. Lipophilic compounds would be more disruptive by this structure. However, several authors have also found the opposite effect i.e., the more hydroxylation, the greater the antimicrobial activity (Sato et al. , 1996). These finding reflects similar results for simple phenolics.

Tannins are group of polymeric phenolic substances found in almost all plant parts and their molecular weight ranges from 500 to 3000. Tannins are also found to be antimicrobial and this property attributed is due to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, and forming complex with polysaccharides (Ya et al., 1988). Coumarins are well known for their antithrombotic, anti-inflammatory and vasodilatory activity. Their antimicrobial activity is found to be not much effective.

2.5 Cancer a brief overview

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Cancers (carcinomas) are characterized by their unregulated growth and spread of cells to other parts of the body (LeMarbre and Greon-wald, 2000). All cancer are initiated by a small alteration in gene and further involve multistep genetic alteration. They are categorized into 6 types and involves selfsufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000). Cancer is caused by both external factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations). These causal factors may act together or in sequence to initiate or promote carcinogenesis. The development of most cancers requires multiple steps that occur over many years. When countries are grouped according to economic development, cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries (following heart diseases). According to recent World Health Organization projections, cancer will have replaced ischemic heart disease as the overall leading cause of death (World Health Organization, 2007).

Even though the biomedical research has been done from very long time for cancer diagnosis and treatment, proper medicine without side effects and remedy for cancer remains unsolved. When solid tumor enters the metastatic phase, the chance for curing the

disease drops dramatically. These tumors should be eradicated by surgery or by using noninvasive therapies. Many clinical protocols rely on the high dose of irradiation or chemotherapy (Tosseti et al., 2002).

2.5.1 MTT assay

MTT assay is a quantitative colorimetric assay used in the cell biology to determine the cell survival and cell proliferation (Mosmann 1983). The colorless and cell soluble MTT dye is reduced to form a colored formazan crystal inside the cell and this forms the basis of cellular cytotoxicity assays, histochemical procedures and enzyme assay. MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced to insoluble formazan crystal which is further solubilised by the addition of various solvents like DMSO, isopropanol, mineral oil etc.

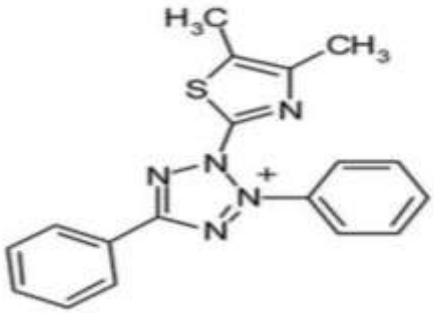


Figure 5 : Structure of MTT

The positive charge present in the tetrazole ring facilitates it to cross the plasma membrane potential (ΨPM -30 to -60Mv) and if not reduced in the cytoplasm, crosses the mitochondrial membrane potential (ΨPM-150 to-170mV). The view that MTT readily enters viable cells via the plasmamembrane potential and is reduced intracellularly is supported by imaging studies with HepG2 cells (Bernas and Dobrucki, 2002; Bernas and Dobrucki, 2000). Various oxidoreductase enzymes present in the cell are involved in the reduction of tetrazolium salts. Although many cofactors and metabolites are potential donors of reducing electrons: NADH, NADPH, succinate and pyruvate have been the main focus of attention. The most commonly studied systems are the oxidoreductases of the mitochondrial electron transport chain, but numerous other cellular dehydrogenases, oxidases and peroxidases have been shown to reduce tetrazolium dyes biochemically. Confocal imaging study has shown that most formazan crystals are formed not in the mitochondria but in the cytoplasm and the peripheral areas of plasma membrane (Bernas and Dobrucki, 2000; Bernas and Dobrucki, 2002). Till to date the actual site and mechanism of MTT reduction is not revealed. Since 1990 MTT assay has been used for various purposes

mostly in determining the viability of cell lines in drug screening program by National Cancer Institute.

Cell viability assay using MTT is based upon the principle of treating the growing cells with drug and after incubating for certain period of time the cells are treated with MTT. Those cells which are alive are capable of taking the dye and the other dead cells are unable to uptake the dye. Here only the living cells are able to reduce the dye because of the reducing agents present inside the cell and hence the formazan crystals formed after dissolving by suitable solvent can be measured spectrophotometrically at 500 to 600nm.

2.5.2 Hela cell line

HeLa was the first human cell line established in culture (Gey et al., 1952) and has since become the most widely used human cell line in biological research. Its application as a model organism has contributed to the characterization of important biological processes and over 60,000 publications. The cell line originates from a cervical cancer tumor of a patient named Henrietta Lacks, who later died of her cancer in 1951 (Lucey et al., 2009). This robust immortal cell line easily propagates in culture and this aberrant beheviour of Hela cell line is attributed to its extensive genomic rearrangements called chromothripsis and remarkably high level of aneuploidy (Landry et al., 2013). Recent investigation on Hela Kyoto cell line expression profile revealed that several pathways, including cell cycle and DNA repair exhibit significantly different expression patterns from those normal cell lines. Scientists used Hela cells in order to make advances in all of the following: virology, polio, scientific standards, live cell transport, genetic medicine, clones, space biology, genetic hybrids, ethics, salmonella, HPV, HIV, telomerase, tuberculosis, and nanotech (Biba, 2010).

Cell count in Neubauer's chamber and determining the viability

Throughout the experiment Naubauer's chamber was used to count the cells; Hela cells as well as macrophages. 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.Ten microlitre of the sample containing cells was taken and mixed with 1:1 Trypan blue solution (0.8mM) subsequently loaded in the counting chamber covered with coverslip and observed in the phase contrast microscope. The viable cells exclude trypan blue while the dead ones uptake the dye.

2.6 *Potentilla fulgens*

2.6.1 Introduction

Classification
Kingdom –Plantae
Phylum – Tracheophyta

Class- Magnoliopsida
Order-Rosales
Family-Rosaceae
Genus-*Potentilla*
Species-*Fulgens*

Habitat

This species finds habitat in open meadows and grassy slopes of oak and rhododendron campanulatum in Temperate and alpine Himalaya.

Taxonomic description

The plant is an erect perennial herb, 15-75cm high, with a thick root stick, pinnate leaves and yellow flowers. It possesses both radical and cauline leaves. The plant reproduces both by seed and underground parts.

2.6.2 Recorded ethnomedicinal importance of *Potentilla fulgens*

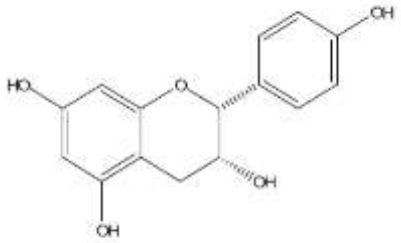
Table 2.6 Recorded Ethnomedicinal importance of *Potentilla fulgens*

Himalayan	Parts used	Ethnomedicinal use	References
Assam India	Whole herb	Gum and tooth ailments (pyorrhea,toothache,and caries),diarrhea, stomach problems ,coμgh and cold, diabetes mellitus, cancer	Kumar (1998) Syiem (2003)
Nepal and Bhutan	Plant juice	Stomach problems , coμgh and cold	
	Root powder	Toothache, stomach disorders, antihelmintic	Kaul et al. (2011)
	Fresh root	Cough	
	Root juice	Strenthening gums, Tooth infection	
	Root paste	Peptic ulcer and disusia masticated for pyorrhea	
	Leaves	Respiratory complaints	
Uttarakhand india	Twigs ,	Used as toothbrush	
	whole plant	Stomatitis and aphthae	Pala et al. (2010)
	Root	Wounds and tiger bites, mouth ulcer	

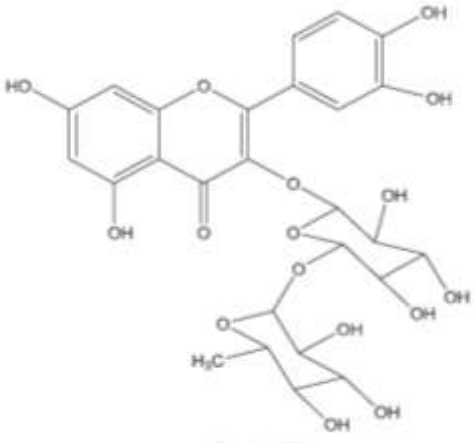
2.6.3 Phytochemical constitutents of *Potentilla fulgens*

Different Phytochemical constituents in the roots and rhizome of this genus include tannins (hydrolysable and condensed) and triterpenoids in large amount while flavonoids, organic acids, phenol carboxylic acids in lesser amount. Due to the high amount 17–22% of tannins in the rhizomes of *Potentilla spp* (i.e. 15–20% condensed tannins, ca. 3.5% hydrolysable

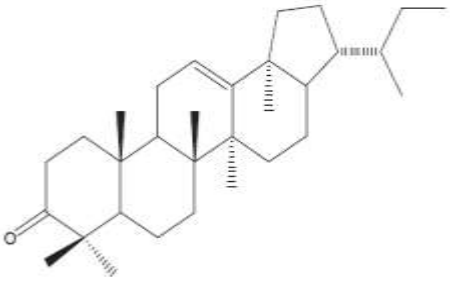
tannins, this group of natural compounds has been in the focus of many phytochemical studies. Predominant in aerial parts of plants are generally flavonoids which were found in cinquefoils (more than 20 species) in a large number (57 compounds) and in a wide structural variety. A common feature is the mono-, di- and also tri-hydroxy substitution of ring B in the instances isolated. Other important constituents in the aerial part include triterpenoid compounds similar to those found in the roots and rhizomes (Kaul et al., 2011).



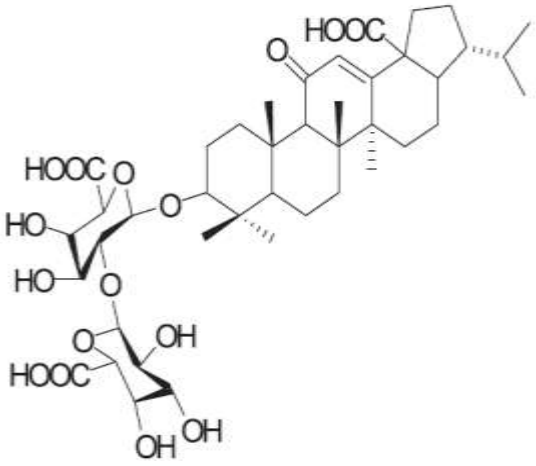
Epiefzelchin



Rutin



Potentene –A



Potentene -B

Figure 6 : Structure of some compounds isolated from Potentilla fulgens

Phytochemical investigation of the root parts of *Potentilla fulgens* led to the isolation of a novel bioflavonoid potifulgene (Epiafzelchin-6-o-8'' epiafzelchin) along with epicatechin (Jaitak et al., 2010). Investigation of its aerial parts yielded two new triterpenes, Potentene-A(3-O-β-D-gluconopyranosyl-(1,2)-β-D-glucuronopyranosyl hopan-12-eno-oxo-28oic acid)

along with three known compounds, afzelchin-4 α→8'' catechin, epiafzelchin and rutin (Jaitak et al., 2010).

2.6.4 Recorded antioxidant, TPC, flavonoid content, anticancerous and antibacterial activity

The study on the total phenolic content in aqueous extract of various species of *Potentilla* ranged from 49.9 ± 1.5 mg GAE/g dry weight for the aerial parts of *P*. *pensylvanica* to 116.3 ± 3.9 mg GAE/g dry weight for P. fruticosa. Flavonoid content also varied from 1.9±0.7 mg quercetin /gm dry wt for *Potentilla argentea* L. to7±1.1 mg quercetin /gm dry wt for *Potentilla fruticose* (Tomczyk, 2010).

Antioxidant Potential of *P. fulgens* was found to be very high with an IC_{50} value of 40.5 against H_2O_2 while the antioxidant activity with other radicals like ABTS, DPPH, $OH^{\cdot}$ was moderate type (Syiem, 2002). *Potentilla fulgens* is used in the preparation of herbal pastes because of its inhibitory activity against the bacteria tha causes dental caries. Good anticariogenic activity of this spp is reported as it has been found to inhibit the gram positive bacteria *Streptococcus spp*. This high anticariogenic effect is due to the high amount of polyphenols extracted in the ethylacetate fraction (Tomczyk et al., 2011). Antibacterial study of the other different spp of *Potentilla* showed inhibiton of gram positive bacteria, *Pseudomonas aerugenosa* and *Candida albicans* with MIC value ranging from 0.78 to 6.25mg/ml (Wang et al., 2013). Methanolic root extract of *P. fulgens* was active against certain tumors like Dalton's Lymphoma cell transplanted intraperitoneally in Swiss albino mice (Syiem, 2003).

2.7 *Primula rotundifolia*

2.7.1 Introduction

Classification
Kingdom- Plantae
Subkingdom – Viridaeplantae
Division- Tracheophyta
Subdivision- Spermatophytina
Class- Magnoliopsida
Order – Ericales
Family- Primulaceae
Genus- *Primula*
Species- *Primual rotundifolia*

Habitat

Primula spp. are a hardy perennial alpine plant which can withstand cold conditions, but it is ideal setting is woodland which contains soil that is rich in humus and remains moist throughout the year but is also well drained. The *Primula* plant grows close to the ground.

Taxonomic description

Primula is a genus of 400–500 species of mainly flowering plants in the family Primulaceae. Primulaceae are herbaceous plants, usually perennial but some are annual. The family has leaves that are basal, opposite or alternate, but usually entire and always without stipules. Glandular hairs, simple or compound are common. The flowers grow in clusters of different shapes. They are radially symmetric and bisexual. The fruit is a 5 valved capsule with many seeds.

2.7.2 Recorded Ethnomedicinal value of *Primula*

Primula spp have been used from the very ancient time and it has been reported to be used by the inhabitants of Greece as antidote to the snake poison and their juice administered through nostrils to remove pituitary matter from the head to relieve toothache.

Table 2.7 Ethnomedicinal importance of *Primula spp*.

***Primula* spp**	**Part used**	**Medicinal value**	**References**
Primula denticulata	Flower	Used in the eye for improvement of eyesight and during opthalmia	Lama (2001)
Primula veris Huds.	Root,leaf	Expectorant	Lama (2001)
Primula macrophylla	Whole plant	Antidote to food poisoning	Lama (2001)
Primula macrophylla	Flowers	Fever,indigestion,dysentery,ulcer	Lama (2001)
Primula sikkimensis	Flowers	Channel disorders and diarrohea	Lama (2001)
Primula acaulis	Flower, leaf	Relieves asthamas	Fico et al. (2007)

2.7.3 Reported Phytochemical constituents

It has been reported that the rhizome and roots of this *Primula* species is rich in saponins which has attributed the medicinal property in this plant (Calis et al., 1992; Ahmad and Shah 1993)

Similarly the aerial part of this genus is rich in flavonoid was revealed by the study done by Nestler 1904 while his investigation on the allergic reactions caused by irritant compounds in glandular trichomes of *P. obconica*. Among flavonols, mono- and diglycosilated derivatives characterize this genus. The most common sugars are glucose, rhamnose, xylose and galactose, while the main aglycons are quercetin, kaempferol and isorhamnetin; on average, sugar moiety linkage favours the 3-position (Vitalini et al., 2011). Isolation and identification of two new flavonol glycosides, isorhamnetin 3-O-(2,6-di-O-β-D-glucopyranosyl-b-D-glucopyranoside) and kaempferol 3-O-(2-O-a-L-rhamnopyranosyl-6-O-

β-D-xylopyranosyl-b-D-glucopyranoside) along with eight known flavonoids quercetin 3-O-(glucosyl(1→2)gentiobioside), kaempferol 3-O-neohesperidoside, isorhamnetin 3-O-neohesperidoside ,tamarixin , isorhamnetin 3-Oglucopyranoside, isorhamnetin 3-O-(2-O-a-L-rhamnopyranosyl-6-O-β-Dxylopyranosyl- β-D-glucopyranoside), quercetin 3-Oneohesperidoside, 7,2'- dihydroxyflavone 7-O-β-Dglucopyranoside in three italian *Primula* species ; *Primula auricular, Primula daonensis* and *Primula hirsute* was performed in 2007 by Gelsomina Fico and his coworkers (Fico et al., 2007). In another species *Primula spectabilis Tratt*, detail study of volatile compounds and flavonoids present in the leaves and flowers was done. From a Methanolic extract of the leaves two flavone glycosides, 8-C-β-glucopyranosylluteolin 7-O-α-arabinofuranoside and 6-C-α-arabinofuranosylapigenin were isolated, in addition to a flavone and three flavonols already known from species of *Primula* From an EtOH extract of leaf exudates, 7,3',4'-tri-O-methylquercetin was obtained. Investigation on the volatiles in flower revealed the presence of sesquiterpene hydrocarbons in large amount followed by non-terpene hydrocarbons, monoterpenes, diterpenes and salicylate. The volatiles emitted by the leaves were mainly constituted by non-terpene derivatives, followed by comparable proportions of hemiterpens, oxygenated monoterpenes and sesquiterpene hydrocarbons. In flowers, monoterpene hydrocarbons were the most represented chemical class followed by non-terpene derivatives (Vitalini et al., 2011).

2.7.4 Recorded antioxidant, TPC, flavonoid content, anticancerous and antibacterial activity

Investigation on the *Primula rotundifolia* has not been recorded so far. The radical scavenging activity of leaf exudates of *Primula denticulata* using DPPH showed less antioxidant potential (25µg/ml) as compared to the standard quercetin (0.75µg/ml) (Sergey et al., 2004). The biological effects of epicuticular substances in farinose exudates accumulated on inflorescence shafts and calyces of *Primula denticulata* on human acute myeloid leukemia cells (HL-60) were analyzed. The crude material possessed little antioxidative capacity but strong cytostatic properties. Compared to quercetin the flavones induce little apoptosis (up to 40µM), but despite their low toxicity, the *Primula* flavonoids possessed strong cytostatic properties even at low concentrations (Sergey et al., 2004). Methanolic extract of *Primula auriculata* showed significant cytotoxic activity against breast, liver and colon cancer cell lines with IC_{50} values ranging from 25.79 to 43.34 µg/ml (Behzad et al., 2014). Antibacterial activity of *Primula* is also found to be high against *Escherichia coli* and *Pseudomonas aerugenosa* with zone of inhibition of 18mm and 14mm respectively with methanolic extract (Majid et al., 2014).

2.8 *Rhododendron arboreum*

2.8.1 Introduction

Classification
Kingdom- Plantae
Subkingdom- Tracheobionta
Superdivision- Spermatophyta
Division- Magnoliophyta
Order- Ericales
Family- Ericaceae
Genus- *Rhododendron*
species- *Rhododendron arboreum*

Habitat-The plant prefers light (sandy) to medium (loamy) soil and requires fairly moist and acidic soil. It can grow in semi shade (light woodland) or no shade, requires protection from hot afternoon sun thus requires a place in the green house or conservatory.

Taxonomic description:

A medium sized tree. Leaves evergreen, oblong-lanceolate, leathery, glabrous, crowded at the end of the branches. Inflorescence terminal with flowers in corymb. Calyx 5-8 lobed and corolla capanulate, tubular, 5-8 lobed. Stamens inserted at the base of corolla. Ovary superior,multilocular. Capsule cylindrical, scaly, curved with numerous winged seeds.

2.8.2 Recorded ethnomedicinal values of *Rhododendron arboreum*

Table 2.8 Recorded ethnomedicinal values of *Rhododendron arboreum*

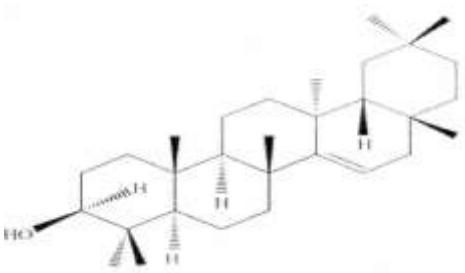
Medicinal uses	Species	Parts used	References
Treatment of hill diarrhea and dysentery	*R. arboreum*	Fresh flowers	Bhattacharyya (2011)
Taken with ghee after frying to check blood dysentery	*R. arboreum*	Dried flowers	Bhattacharyya (2011)
Taken when fish bones stuck in the gullet	*R. arboreum*	Fresh and dried corolla	Bhattacharyya (2011)
Used as poultice in high fever and headache	*R. arboreum*	Leaves	Bhattacharyya (2011)

Apart from the ethnomedicinal importance recent study in this plant has shown high medicinal value. Reports are there showing Anti-inflammatory, Anti-nociceptive, Hepatoprotective, Anti-diabetic and Anti-diarrhoeal activity (Srivastav, 2012).

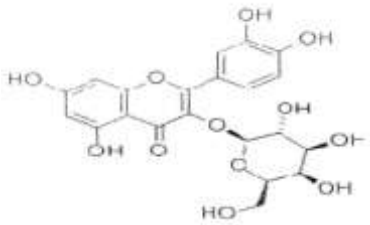
2.8.3 Recorded antioxidant, TPC, flavonoid content, anticancerous and antibacterial activity

Preliminary phytochemical screening of various parts viz flowers, leaves, bark, stem and roots of *Rhododendron arboreum* in methanol using conventional natural products identification tests indicated the presence of different classes of secondary metabolites such as alkaloids, steroids, flavonoids, terpenoids, anthraquinones, phlobatanins, saponins, glycosides, tannins and reducing sugars (Nisar et al., 2011).

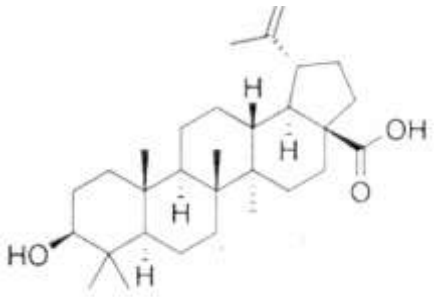
Bark indicated the presence of a single triterpenoid substance taraxerol ($C_{30}H_{50}O$) and ursolic acid acetate ($C_{32}H_{50}O_4$), betulinic acid ($C_{30}H_{48}O_3$) and leuco-pelargonidin ($C_{15}H_{14}O_6$) (Hariharan and Rangaswami, 1966). Green leaves are reported to contain glucoside, ericolin (arbutin) ($C_{12}H_{16}O_7$), ursolic acid ($C_{30}H_{48}O_4$), α-amyrin ($C_{30}H_{50}O$), epifriedelinol ($C_{30}H_{52}O$), a new triterpenoid named campanulin, quercetin and hyperoside ($C_{21}H_{20}O_{12}$) (Orwa et al., 2009). Chemical analysis of the leaves of R. *arboreum* var. nilagiricum revealed the presence of hyperoside (3-D -galactoside of quercetin), ursolic acid and epifriedelinol, a triterpenoid compound (Rangaswamy and Sambamurthy, 1959). The leaves are also reported to contain the flavone glycoside and dimethyl ester of terephthalic acid and certain flavonoids (Verma A et al., 2011). Quercetin-3-rhamnoside a crystalline chemical compound has been reported from the flowers of this species (Rangaswamy and Sambamurthy, 1960). Three biologically active phenolic compounds i.e. quercetin ($C_{15}H_{10}O_7$), rutin ($C_{27}H_{30}O_{16}$) and coumaric acid ($C_9H_8O_3$) have been reported in flowers of R. *arboreum* using high-performance thin-layer chromatography (HPTLC) (Swaroop et al., 2005).



Taraxerol



Hyperoside



Betulinic acid

Figure 7 : Chemical structure of compounds isolated from *Rhododendron arboreum*

2.8.4 Recorded antioxidant, TPC, flavonoid content, anticancerous and antibacterial activity

Antioxidant potential of the 21 species of *Rhododendron* along with total polyphenol content and flavonoid content was analysed by Prakash, 2007. In the study TPC varied from 37.3 to 208.9 mg/g, flavonoids from 11.5 to 137.1 mg/g and Antioxidant activity from 30.4 to 97.4 % depending upon the species. *Rhododendron arboreum* arb showed the IC_{50} value at 0.47 mg/ml and *R*. *arboreum* cinn .at 0.34 mg/ml in reference compound quercetin it was found to be 0.2 mg/ml. Similarly in case of total polyphenol, *Rhododendron arboreum* arb has total Polyphenol Content of 57.3 mg gallic acid equivalent/gm plant extract and R. *arboreum* cinn 78.5 mg gallic acid equivalent/g plant extract. R. *arboreum* cinn was found to contain higher flavonoid, 62.6 mg quercetin equivalent/g plant extract as compared to the *Rhododendron arboreum* arb, 43.8 mg quercetin equivalent/gm plant extract (Prakash et al., 2007).

Anti-proliferative activity of *Rhododendron lepidotum* against HeLa cell line was relatively high in comparison to other plants (Bhattarai, 2010). The growth of RL952 cells was inhibited by treatment with hyperin extracted from the Manchurian *Rhododendron* leaf. Following the treatment of hyperin after 24hrs OD (optical density) values of caspase-3 and caspase-9 were increased, expression of bcl-2 was increased and bax was decreased in protein levels in RL952 (endometrial cancer cell line) cell (Li et al., 2012). The antibacterial activity of the methanolic extract of various parts of *Rhododendron arboreum* such as flowers, leaves, bark, stem and roots were investigated against medically important pathogens by determining the zone inhibition. Methanolic extract of *Rhododendron arboreum* had good antimicrobial activity against the tested bacterial strains such as *Escherichia coli, Staphylococcus aureus, Bacillus subtilus* and *Salmonella typhi* (Nisar, 2013).

CHAPTER III: MATERIALS AND METHODS

3.1 Sample collection and identification

Rhododendron arboreum was collected from the Phulchoki of Kathmandu (3050m) and other plants *Primula rotundifolia* and *Potentilla fulgens* were collected from the Rasuwa district at the height of 4000 m. Whole plant was taken as sample in *Primula rotundifolia* and *Potentilla fulgens* whereas bark was taken in *Rhododendron arboreum*. All the samples were collected in the month of June/July 2013. Collected plants were identified and verified by the Central Department of Botany.

3.2 Extraction

Plant samples were shade dried and crushed to fine powder with the help of electric mixer. For methanolic extract, 20gm of the powder was taken filled in thimble and soxhalation was performed until the color of the solvent became colorless. Thus obtained solvent was subjected to rotavapour and the concentrated plant extract was obtained. In case of aqueous and chloroform extract simple maceration method was adopted with slight modification (Bandar et al., 2013). Ten grams of each powdered plant was taken in conical flask and 80 ml of the distilled water and chloroform was poured for each extraction .The flask was cotton plugged and incubated in shaker incubator at 40° C for 24 hrs. After 12 hrs the solvent was filtered using whatman filter and again water was added, incubated in same condition for next 12 hrs. Thus filtrate was then dried in waterbath at 40°C. Thus dried extracts was then weighed in amber colored bottle, dissolved in (Dimethyl sulfoxide) DMSO and stored in freeze. All the test performed throughout the experiment was performed using the stored sample. Percentage yield of each extract was calculated using the formula:

$$\text{Percentage yield} = \frac{\textbf{Dry wt of plant extract}}{\textbf{Dry wt of plant material}} \times 100$$

3.3 Research Outline

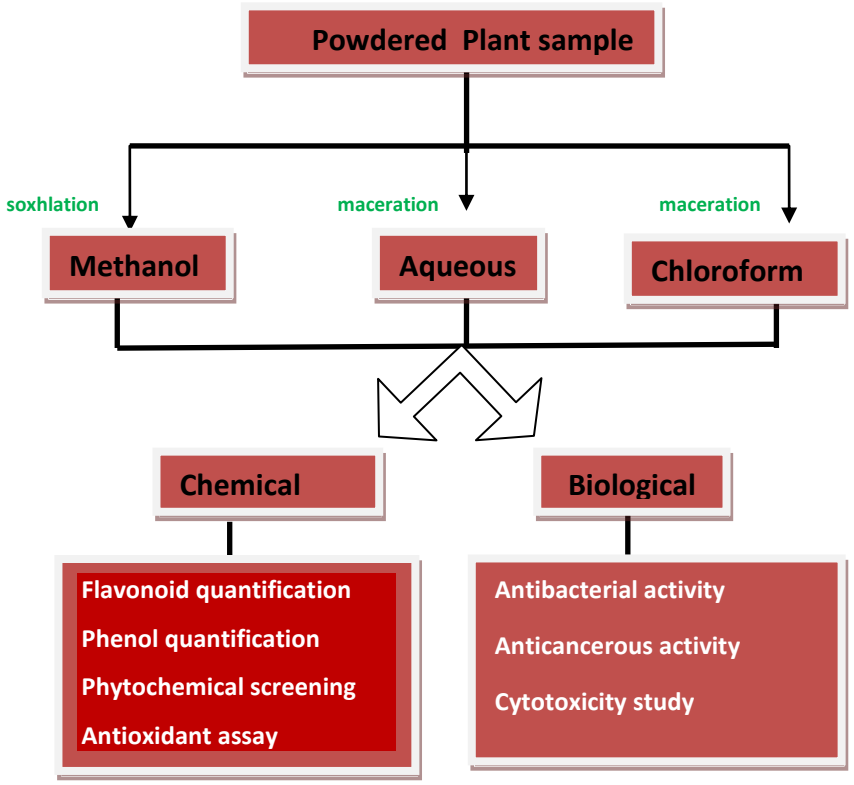


Fig 8 : Outline of research procedure

3.4 Phytochemical screening

The crude extracts (aqueous, methanolic and chloroform) 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 and Todkar *et al.*, 2010. The following tests were carried out on extracts to detect various phytoconstituents present in them.

3.4.1 Detection of Alkaloids

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

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.

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.

3.4.2 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.

3.4.3 Detection of Terepenoids and Steroids

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.

b. Salkowski test

Five ml 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.

3.4.4 Detection of Tannins

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.

3.4.5 Detection of Flavonoids

a Shinoda test

Two ml 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.

b Alkaline reagent test

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

3.5 Method of flavonoid estimation

Amount of flavonoid present in the sample was determined using Alumunium chloride colorimetric method (Chang et al., 2002).

At first 1 mg/ml of Quercetin was prepared and different concentration ranging from 10 µg/ml to 100 µg/ml was prepared. Similarly for sample 5 mg/ml concentration was prepared .The diluted standard solutions and sample (0.5 ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% alumunium chloride, 0.1 ml of 1Mpotassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 430 nm in spectrophotometer. The amount of flavonoid in the sample was calculated using standard curve of quercetin prepared in MS word excel 2007. All the determination was performed in triplicate and the results were interpreted as n±3 standard deviation

3.6 Total phenol estimation

Method for total phenol estimation was done using Phenol-Ciocalteu method as described by Gao (2000). Different concentration of gallic acid was prepared ranging from 60µg/ml to 300 µg/ml. Similarly sample of conc 5mg/ml was prepared. Hundred microlitre of the plant sample and standard was mixed with 0.2ml of Folin –Ciocalteu reagent and 2ml of $H_2O$was added. After the incubation of 3min, 1ml of 20% Na_2CO_3 was added and the resulting mixture was further incubated for 1 hr. Finally the absorbance of the resulting blue color was taken at 765nm. Results are expressed as gallic acid equivalent GAE per gram plant extract. The amount of phenol in the sample was calculated using standard curve of gallic acid prepared in MS word excel 2007. All the determination was performed in triplicate and the results were interpreted as n±3 standard deviation.

3.7 ABTS assay

The method for ABTS assay was used according to the method of Re et al. (1999).

ABTS radical preparation

The stock solutions included 7.4mM ABTS solution and 2.6mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml sodium acetate buffer of P^H 6.5 to make an absorbance of about 0.75nm. Reaction was carried out using the protocol of Re et al. (1999) with slight modification.

Gallic acid concentration 10-100 µg/ml was prepared. For samples at first varying concentration over large range was taken to know the surrounding IC_{50} value of each sample and then in next phase the required concentration was prepared having shorter range.Ten microlitre of the sample or standard was taken and 1 ml ABTS solution was added followed by dark incubation for 30min. Then absorbance was taken at 734nm. All the readings were taken in triplicate an expressed n±3.

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

$$\%RSA = [A_o\text{-}A/A_o\}*100$$

Where, A_o= Absorbance of control (ABTS solution + methanol)

A= Absorbance of plants sample/ standard (gallic acid).

3.8 DPPH Assay

For DPPH assay the procedure followed the method of Brand- Williams et al. (1995). Stock solution of DPPH was prepared by dissolving 24 mg DPPH in 100ml methanol. Working solution was prepared by mixing 10ml stock solution with 45ml methanol to obtain an absorbance of 0.75 units at 515nm. Gallic acid concentration ranging from 0 to 200µg/ml was prepared as standard and for the plant extract at first varying concentration over large range was taken to know the surrounding IC_{50} value of each sample and then in next phase the required concentration was prepared having shorter range. Ten microlitre of plant extract was taken and mixed with 1000µl of the DPPH solution. The resulting solution is allowed to stand for 1 hr and the absorbance of the solution was taken at 515nm.

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

$$\text{Percentage radical scavenging activity} = [A_o\text{-}A/A_o\}*100$$

Where, A_o= Absorbance of control (DPPH solution in methanol)

A= Absorbance of plants sample/ standard (gallic acid).

In both the cases, the IC_{50} value 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)

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

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

All the tests were performed in triplicates.

3.9 Antimicrobial activity

The screening of the antimicrobial activity of crude extract were 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 four strains of gram negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603,

Pseudomonas aeruginosa ATCC 27853, *Salmonella typhimurim* ATCC 14028). These microorganisms were provided by Nepal Academy of Science and Technology (NAST), Lalitpur Nepal.

The antimicrobial activity was determined according to the CLSI guidelines and standard (CLSI, 1999).

3.9.1 Disc diffusion method

Bacterial strains were revived by inoculating in Nutrient broth and incubated at 37°C for 24hrs. Next day the turbidity of the actively growing broth culture was adjusted with sterile saline solution to achieve a turbidity equivalent to 0.5 Mc Farland standard which results in a suspension containing approximately 1 X 10^8 cfu/ml. The microbial suspension prepared was used to make a lawn culture in the Mueller and Hilton agar plates and 5mg/ml of plant extract was prepared. Plant extract impregnated filter paper disc was placed on the surface of agar plates. Plates were then incubated at 37°C for 24 hrs. On the other day the zone of inhibition produced was measured. Those plant extract which showed zone of inhibition were further used for determining their MIC and MBC. Antibiotics Gentamicin was taken as positive control.

3.9.2 Determination of Minimum inhibitory concentration

Different concentration of the plant extract was prepared in 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 18-24 hours at 37°C and all the tubes were further taken for MBC.

3.9.3 Determination of Minimum bactericidal concentration

Nutrient agar plates were prepared .With sterile loop the extract from each tube of MIC performed was taken and streaked in the Nutrient agar plates. Plates were then incubated at 37°C for 24 hrs and further more for 48 hrs. Growth of the bacteria was checked and the minimum bactericidal concentration was calculated. Antibiotic Gentamicin (50 mcg) was taken as positive control and the solvent DMSO was used as negative control. To avoid the suspicion that the traces amount of methanol present in the extract could inhibit the cell, methanol was also taken as negative control.

3.10 Anticancer activity

3.10.1 Culture of Hela cell line

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 Unit/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^H 7.4) and harvested from 25cm^3 flasks using 0.25 % trypsin/ EDTA solution. Then cells were sub-cultured in 96 well plates (Merck, Germany).

Proliferating cell lines were trypsinised using 0.05% trypsin. Thus trypsinised cells were then centrifuged to get the pellet and the pellet was redissolved in the fresh RPMI media .Cells were counted by using trypan blue staining method ,in which the dead cells are stained blue while the live ones remains unstained. Further unstained cells were counted using neuber's haemocytometer. About 25,000 cells were seeded in each well of microtitre plate following the incubation of about 3 days. Cell growth was checked every day and media was added to the plates in which the color of media has been changed to yellow because of the production of acids after the media utilization. When the cells reached confluent growth then the plant extracts at different concentration 400µg/ml to 50µg/ml was treated in the well. Also the anticancerous drug fluorouracil was taken as positive control, DMSO as vehicle control and the well without cells as negative control. Plates were then incubated at temperature 37°C in 5% CO_2 incubator for 24 hrs.

3.10.2 MTT reagent preparation and treatment

Yellow powder of MTT was dissolved in the PBS to prepare the concentration of 5mg/ml 50ul of MTT was added in each well and the plates were incubated at 37°C for 4 hrs. After incubation each well was treated with 100 µl DMSO to dissolve the formazan crystals formed. Then absorbance of the plates were taken using ELISA plate reader at 560nm.

3.11 Cytotoxicity study

The study done to identify whether the compound or substance is toxic to the cells or not is cytotoxicity study. Even though the compound or extract is very potent to inhibit the cancerous cells but if its cytotoxicity is high then it can't be a selective drugs. So it is very important to study the cytotoxicity study in normal cell line in parallel with the drug screening in diseased cell line. Here mouse peritoneal macrophages are taken as a normal cell line.

3.11.1 Mouse Peritoneal Macrophage extraction and the cytotoxicity study by MTT

Healthy mice were taken and injected with 3 ml of 2% starch solution. After 2 days mouse were sacrifised to obtain the macrophages from peritoneal cavity. Then the cells were centrifuged at 12000 rpm for 10 min. Pellets thus obtained was slowly mixed with fresh media, stained with trypan blue and counted in haemocytometer. Forty thousand cells were seeded in each well and the plates were incubated at 37°C in 5% CO_2. Next day the cells were treated with the same concentration as used in the anticancer study. Plates after

24 hrs of incubation was again treated with MTT reagent and incubated at 37°C for 4 hrs followed by addition of DMSO to dissolve the crystals formed. Finally the absorbance was taken at 450nm in ELISA plate reader.

Determination of IC_{50} value

At first results were expressed as percentage reduction in cell proliferation, compared with controls (cells without extracts).

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

Percentage Inhibition (PI) =100 -

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 $(IC_{50})^H$ and 50% of mouse peritoneal macrophages proliferation $(IC_{50})^M$ were calculated by using formulae as described by Prof. Dr. Louis Maes and Prof. Dr. Paul Cos (Louis and Paul, 2010).

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

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)**

3.12 Determination of selectivity index

The cytotoxicity of crude extracts and drugs on the mice peritoneal 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 $(IC_{50})^M$ to inhibitory concentration of the cancer cell $(IC_{50})^H$. A value greater than 1 indicates the treatment is more selective towards the cancer cell.

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

CHAPTER IV : RESULTS

4.1 Sample preparation

Three different solvent were taken to prepare the crude extract of each plant. Percentage yield of each extract along with their vernacular name is given below.

Table 4.1 Percentage yield of different extracts with parts used and vernacular name

			% yield of extract		
Plant sample	**Vernacular name**	**Parts used**	**Methanolic**	**Aqueous**	**Chloroform**
Potentilla fulgens	Bajradanti	Whole plant	23.44	17.5	0.87
Rhododendron arboreum	Laligurans	Bark	17	5.4	2.1
Primula rotundifolia	Medosero	Whole plant	22.12	21.8	3.3

4.2 Phytochemical screening

Methanolic and aqueous extract of all the plant showed positive test for Alkaloid, Saponin,Tanin, Flavonoid and Phenol. Chloroform extract of most plant were found to be devoid of most of the phytochemicals

Table 3.14 Phytochemical screening of aqueous and methanolic extracts of the plants

Tests	***P. fulgens***			***R.arboreum***			***P. rotundifolia***		
Alkaloid Test	M	A	C	M	A	C	M	A	C
Mayer's Test	+	+	-	+	+	-	+	+	+
Wagner's Test	+	+	-	+	+	-	+	+	+
Saponin test	+	+	-	+	+	-	+	+	+
Steroid test									
Libermann- Buchard test	+	+	-	+	+	-	+	+	-
Salkowski test	+	+	-	+	+	-	+	+	-
Tannin test	+	+	+	+	+	-	+	+	+
Flavonoid test									
Shinoda test	+	+	+	+	+	-	+	+	+
Alkaline reagent test	+	+	+	+	+	-	+	+	+
Phenol test	+	+	+	+	+	+	+	+	+

+: positive, -: negative; M: methanolic extract, A: Aqueous extract, C: Chloroform extract

4.3 Flavonoid and Polyphenol content

Among all the plants taken under study methanolic and chloroform extract of *Primula rotundifolia* exhibited the highest amount of flavonoid as compared to other plants viz 19 ± 0.70 mg quercetin eqv/gm plant extract and 23± 0.71 mg quercetin eqv/gm plant extract respectively. In case of *Potentilla fulgens* and *Rhododendron arboreum* methanolic and aqueous extract exhibited the moderate amount of flavonoid whereas the chloroform extract of these two plants were found to contain very few amount of flavonoid. Methanolic extract of *Potentilla fulgens* and *R. arboreum* contained 6.25±0.35 and 7.65±0.15 mg quercetin eqv/gm plant respectively. Chloroform extract of these two plants showed very low amount of flavonoids.

Methanolic extract of Potentillla *fulgens* and *Rhododendron arboreum* showed very high amount of Polyphenols 448±6 and 449±4 mg gallic acid eqv/gm plant extract in comparision to *Primula rotundifolia* which exhibited low phenolic content in all three extract. Aqueous extract of *Potentilla fulgens* and *Rhododendron arboreum* showed 123±3.2 and 210.83±5.4 mg gallic acid eqv/gm plant extract respectively.

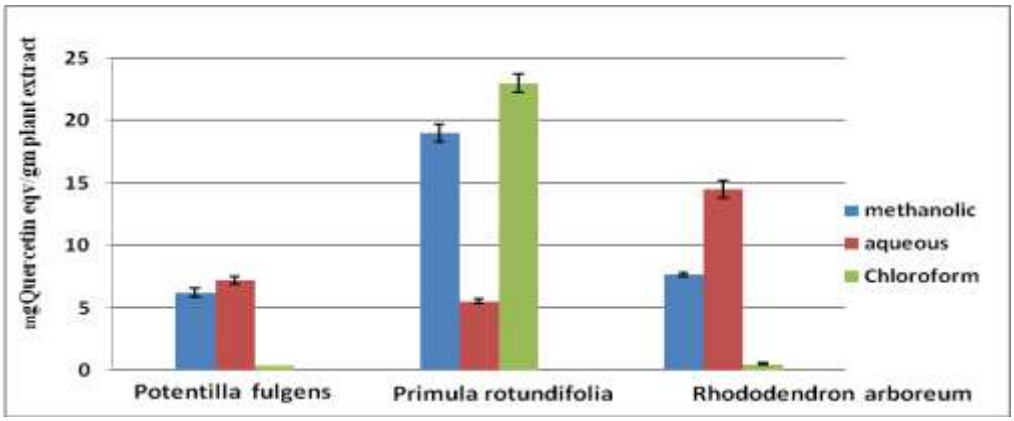


Figure 8 : Estimation of flavonoid content in different plant extracts by Aluminium chloride colorimetric method

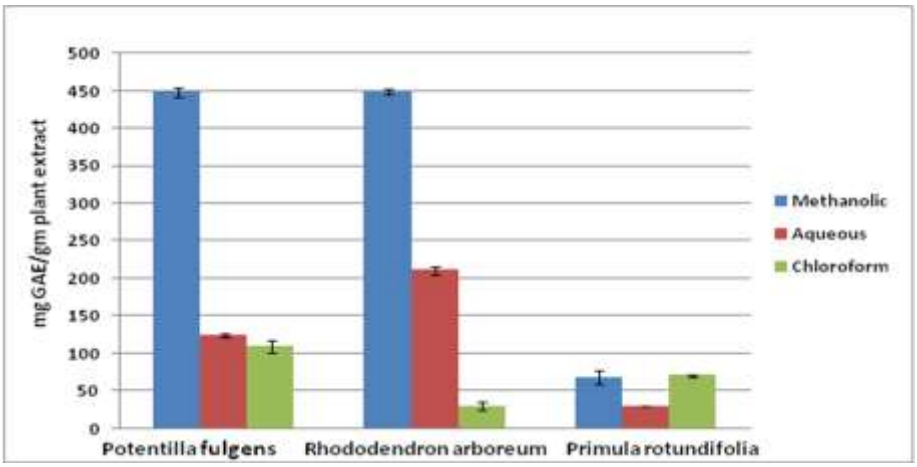


Figure 9 : Estimation of total phenolic content in various plant extract by Phenol- Ciocalteu method

4.4 Antioxidant activity

Two methods were used to investigate the antioxidant activity of the plant sample viz DPPH and ABTS method. Two plants *Rhododendron arboreum* and *Potentilla fulgens* showed high radical scavenging capability as compared to *Primula rotundifolia*. Among the three extract methanolic extract has been found to posses better radical scavenging potential in *Potentilla* while in *Rhododendron* both Aqueous and methanolic extract showed almost similar readings.

4.4.1 ABTS Assay

Table 4.4 (a) IC_{50} values of different plant extracts and standard gallic acid in ABTS cation decolorization assay

Plant sample	IC_{50} values (µg/ml) of different extracts in ABTS assay		
	Methanol	Aqueous	Chloroform
P.fulgens	477.2	734.82	11996.58
R. arboreum	600	560.83	1189.47
P. rotundifolia	8000	6508.86	1420
Gallic acid	36		

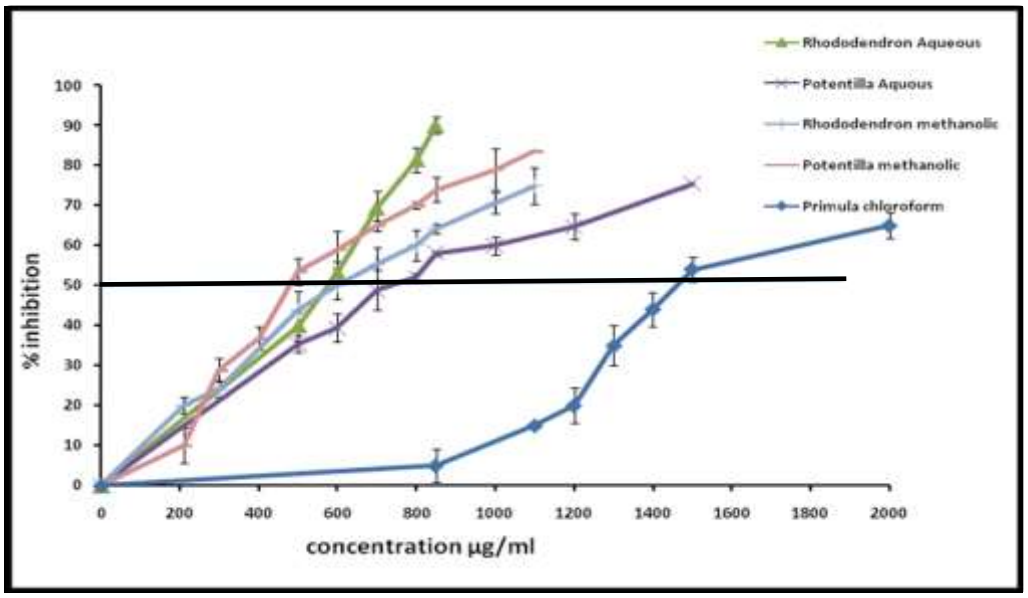


Figure 10 : ABTS radical scavenging activity of different plant extracts. Extracts showing IC_{50} value >1500 µg/ml is not included in graph.

4.4.2 DPPH Assay

Table 4.4 (b): IC_{50} Values of different extracts and the standard gallic acid in DPPH assay

Plant sample	IC_{50} values (µg/ml) of different extracts in DPPH assay		
	Methanol	**Aqueous**	**Chloroform**
P. fulgens	340.93	2550.20	2611.38
R. arboreum	517.69	702.43	2500
P. rotundifolia	13000	12000.39	807.08
Gallic acid	86		

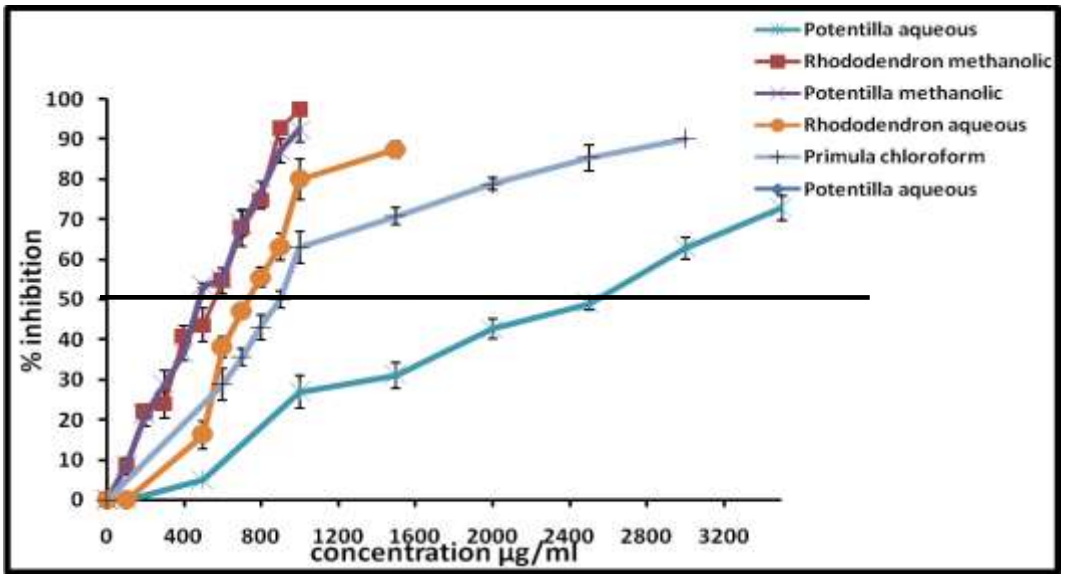


Figure 11 : DPPH radical scavenging activity of different plant extracts. Extracts showing IC_{50} value >2600 µg/ml is not included in graph.

4.5 Antibacterial activity

Methanolic extract of *P. fulgens* and Chloroform extract of *P. rotundifolia* exhibited good antimicrobial activity while the all other didn't show the antibacterial activity. Even the same plant extracted in different solvent showed the difference in antimicrobial activity. Only the two bacterial species; *Staphylococcus aureus* and *Pseudomonas aerugenosa* were found to be susceptible. DMSO was taken as negative control and Gentamicin was taken as positive control.

Table 4.5 (a): Antimicrobial activity of Aqueous, Methanolic and Chlorform extracts

Zone of Inhibition (mm) Diameter of well= 6mm							
Plant	**Bacterial strains**	*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. typhimurim*	*S. Marcesens*
	ATCC no.	25525	*25922*	*700603*	27853	14028	13880
P.*fulgens*	Methanolic	11	0	0	10	0	0
	Aqueous	0	0	0	0	0	0
	Chloroform	0	0	0	0	0	0
R.arboreum	Methanolic	10	0	0	0	0	0
	Aqueous	0	0	0	0	0	0
	chloroform	0	0	0	0	0	0
P.rotundifola	Methanolic	10	0	0	11	0	0
	Aqueous	0	0	0	0	0	0
	Chloroform	12	0	0	12	0	0
Gentamycin		31	19	10	30	21	30
DMSO		0	0	0	0	0	0

Table 4.5 (b): Minimum Bactericidal concentration of plant extracts and Gentamicin

Plant species	Bacteria	Extract	MBC	MBC (gentamicin)
P. rotundifolia		Methanolic	16 mg/ml	250 µg/ml
P. rotundifolia	*S. aureus*	Chloroform	800 µg/ml	250 µg/ml
P. fulgens		Methanolic	16 mg/ml	250 µg/ml
P. rotundifolia	*P. aerugenosa*	Chloroform	1 mg/ml	200 µg/ml

4.6 Anticancer and cytotoxicity study

Chloroform extract of *Primula rotundifolia* exhibited the best antiproliferative activity with IC_{50} value of 107.09 µg/ml. Methanolic extract of *Rhododendron arboreum* and *Potentilla fulgens* showed a moderate type of activity while the antiproliferative activity shown by all other extracts was very low. Further the plant extracts showing better antiproliferative activity were further studied for their cytotoxic activity in mouse peritoneal macrophages and the result was found to be very selective. Those extracts which showed better

antiproliferative activity showed less cytotoxic activity which suggests a better medically beneficial compound.

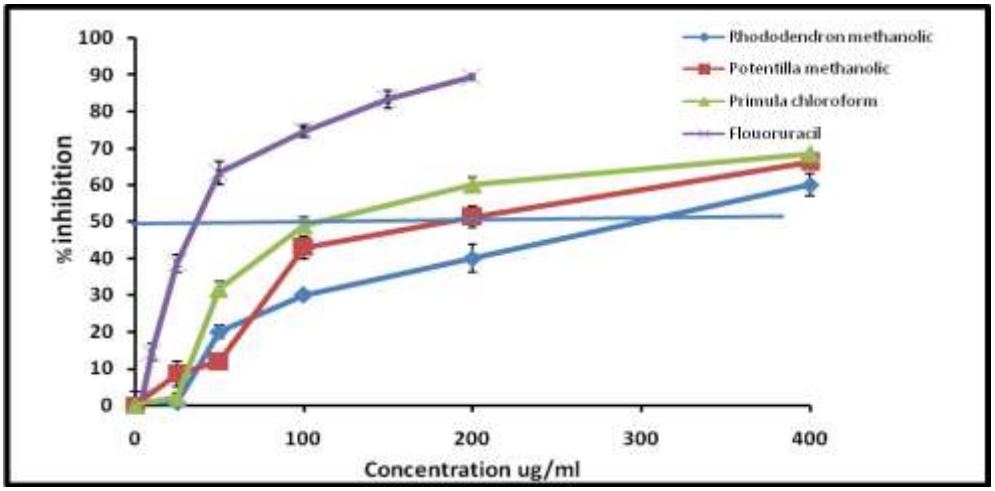


Figure 12 : Dose dependent percentage inhibiton of Hela cell line by different plant etracts and 5-Fluorouracil

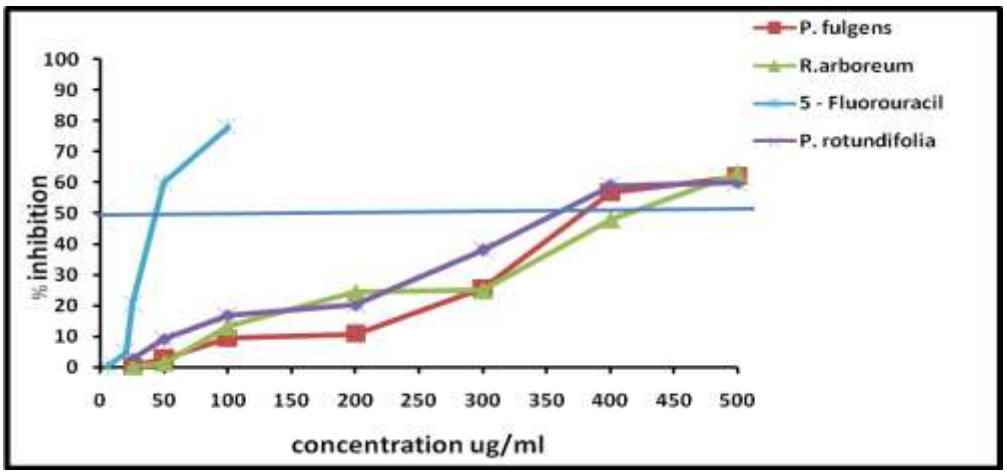


Figure 13 : Cytotoxicity study of mouse peritoneal macrophages by diferent plant extract and standard drug 5-fluorouracil.

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

Table 3.19: In virto cytotoxic activity of extracts against HeLa cell $(IC_{50})^H$ and Mice peritoneal macrophage $(IC_{50})^M$ and their respective selective indices

Extracts/Drug	Cytotoxicity on HeLa Cell IC_{50} (µg/ml)	Cytotoxicity on Macrophages IC_{50} (µg/ml)	Selectivity index (SI)
Fluorouracil	34.42±1.3	48.01±0.81	1.39
R. arboreum Methanolic	312.35 ± 2.8	421.94±3.11	1.35
P. fulgens Methanolic	203.34 ± 1.4	376.102±1.94	1.84
P. rotundifolia Chloroform	107.09 ± 0.9	351.09±0.84	3.27

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 were found to be higher than 1 indicating their selectivity towards cancer cell than that of normal cell.

4.8 Correlation between Total Phenol content and Antioxidant Activity

A good correlation was observed between Total Phenol Content and the antioxidant activity of the plant extracts in both of the assay with correlation coefficient value, r = 0.80343 for ABTS and r = 0.8927 for DPPH assay. The value was calculated using Excel 2007 with $1/IC_{50}$ values taken to indicate antioxidant activity.

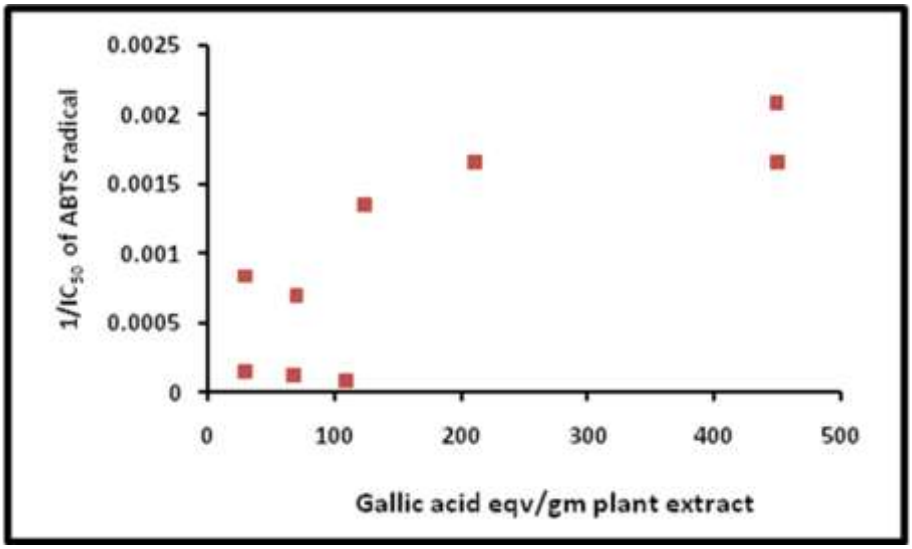


Figure 14 : Correlation between total phenol content and ABTS scavenging activity

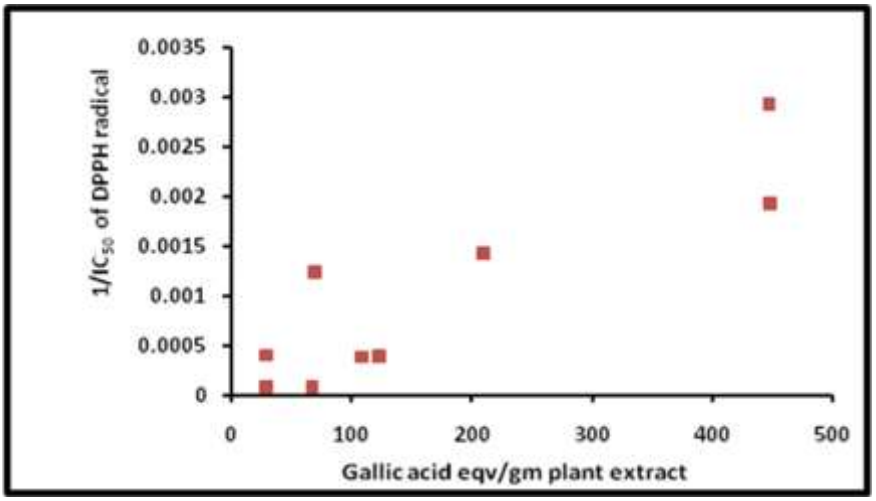


Figure 15 : Correlation between total polyphenol content and DPPH scavenging activity

CHAPTER V : DISCUSSION

In our study we took different parts of the plants and this was choosen on the basis of traditional use of these plant parts. Three different solvent were taken water, methanol and Chloroform. Water being universal solvent is used by many traditional healers because of its intoxicity. Similarly another polar solvent methanol is believed to extract nearly all active plant constituents. Chloroform being nonpolar extracts most of the nonpolar phytochemical constituents. The percentage yield of extract is seen highest in methanol and lowest in chloroform. This variation seen is due to the number of reasons for e.g. Amount of phytochemicals present, plant life stage, extraction method used, nature of solvent etc.

5.1 Flavonoid and Phenolic content

Among all plants *Primula rotundifolia* was found to be rich in flavonoid content .Our finding of very high content of flavonoid in *Primula rotundifolia* is similar in findings of other researcher (Fico et. al, 2007). In case of *Rhododendron* moderate amount of flavonoid is reported in our study. Similarly flavonoid quantification done by Prakash et al. (2007) in 21 *Rhododendron* species reported a large variation in the flavonoid content among the species. *Rhododendron arboreum* arb was found to contain 57.3mg quercetin equivalent/gm sample while *Rhododendron arboreum* cinn. contained 78.5mg quercetin equivalent/gm sample of flavonoid (Prakash et al., 2007). This deviation in data may be because sample taken in our study was bark while they took leaves as sample and leaves are reported to contain high flavonoid and bark are almost devoid of flavonoids (Mohammad, 2011**).** Extract of *Potentilla fulgens* exhibited very low amount of flavonoid content in all extract and the result is similar to the work of Anzielczyk et al. (2010).

Total phenolic estimation in different plant extract was carried out and two plants P. fulgens and *R. arboreum* were rich in polyphenols. They contained 448±6mg and 449±4mg GAE/gm plant extract of phenol in methanolic extract. Similar work performed by Syiem (2002) showed high amount of polyphenol compound in methanolic extract and aqueous extract i.e. 529.3±18.5 and 412±1.6 mg GAE/gm plant extract. Aqueous extract of *P. fulgens* showed low phenolic content in our study and this variation in data may be due to the different extraction method used in our study. Our data of polyphenol content in *R. arboreum* varies from the study of Prakash (2007) where they found polyphenol content of only 78.5mg GAE/gm plant extract. This variation may be due to the use of absolute methanol in our study rather than 50% MeOH: H_2O. In our *s*tudy *P. rotundifolia* exhibited low amount of polyphenols in all three extracts.

Very important parameter is solvent polarity higher the polarity, better the solubility of phenolic compounds (Naczk and Shahidi, 2006). The highest extract yields (up to 22.8%)

were obtained with polar alcohol based solvents (Grigonisa, 2005) which is similar to our research where we found high polyphenol content in methanolic extract.

5.2 Antioxidant activity

There is a large variation in the free radical scavenging potential of plants studied. Because different plants taken were of different families and genus so it is obvious that they have different antioxidant potential. Apart from this the physiological state of plant, genetic variation and even the environmental factors determines the amount of polyphenol accumulation and other antioxidants present in the plant cell. There was a dose dependent decrement in the absorbance for all samples tested. The high antioxidant activity was shown by the methanolic extract. This may be due to the high extraction of polyphenols in methanol, (up to 30% variation) in fruits and vegetables (Michiels et al., 2012). Also the other reason may be the difference in the extraction method used for different solvent system.

Among three plants taken under study, *P. fulgens* showed the best antioxidant activity in both ABTS and DPPH assay. Secondly, *R. arboreum* showed slightly less antioxidant activity as compared to *P. Fulgens.* On other hand very low antioxidant activity was seen in *P. rotundifolia.* A good correlation between the total polyphenol content and antioxidant potential of the extract was seen in the study and this suggests that high antioxidant potential shown by the plants are due to their high polyphenolic content.

Methanolic extract of *P. fulgens* showed low IC_{50} at 477.2µg/ml and 340.93µg/ml in ABTS and DPPH assay. Our result of high antioxidant activity of *Potentilla fulgens* is similar to work done by Syiem (2002) in which they found the antioxidant activity of this plant three times less than the standard used. Similarly, another study done on *Potentilla fulgens* showed that it is active in reducing the DPPH molecule and this high activity is attributed to the compounds like afzelchin- 4α 8''-catechin, epiafzelchin, rutin and new bioflavonoid potifulgene (Kaul,2011).

We found the IC_{50} of methanolic extract of *R. arboreum* at 600µg/ml and 517.6µg/ml in ABTS and DPPH assay. Similarly our finding of high antioxidant capacity is in accordance to the work done by Prakash et al. (2007). They reported the IC_{50} value of *R. arboreum* at 470µg/ml using DPPH assay and suggest the reason behind high antioxidant activity of *Rhododendron spp* is flavonoids, rutin, caffeic acid, gallic acid, ellagic acid and chlorogenic acids (Prakash, 2007).

Low antioxidant activity shown by *Primula* in our research is similar to the DPPH scavenging power of *Primula spp*, studied by Tokalov (2004). Their study includes weak DPPH radical scavenging potential shown by *Primula spp* as compared to the standard

quercetin. Main antioxidant constituent in *Primula* are known to be tannins and saponin which are found highly in their roots and rhizome (Calis, 1992).

In all cases we found that the DPPH scavenging potential is quite lower than ABTS radical. These both assays are electron transfer based assays and as per said by authors and are not much comparable. The results obtained are hardly comparable because of the different mechanisms, redox potentials, P^H and solvent dependencies, etc. of various assays (Thaipong et al., 2006). Reactions of phenols with ABTS radical cations are usually rapid, but the reactions with DPPH radical differ from compound to compound. Three types of kinetics, rapid, intermediate and slow, have been distinguished for the reactions between DPPH radicals and phenols (Campos and Lissi, 1996; Moreno, 2002). Another reason may be that reaction of antioxidants is influenced so strongly by, and in some cases is controlled by, steric effects rather than innate chemical characteristics makes this DPPH assay unacceptable for screening antioxidant activity of extracts, comparing antioxidants of different structural classes, or comparing extracts of unknown composition and concentration because preference is given to small reactive compounds and large molecule antioxidants are underestimated (Thaipong et al., 2006). Beside this the DPPH is insoluble in water making it unsuitable to measure the antioxidant activity of hydrophilic antioxidants. ABTS being soluble in both aqueous and organic solvents and is not affected by ionic strength, so can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluid (Huang, 2005).

The advantages of ABTS/TEAC were reported to be operational simplicity, reproducibility, diversity, and the most important of all, flexible usage in multiple media to determine both hydrophilic and lipophilic antioxidant capacity of food extracts and physiological fluids, since the reagent is soluble in both aqueous and organic solvent.

5.3 Invitro antiproliferative and cytotoxicity study

In our study the high antiproliferative capacity is in concordance with the investigation done by other researcher. Methanolic extract of *Potentilla fulgens* was found to be very active against certain tumours, Daltons lymphoma cells in a dose dependent manner. Also recent investigation on the methanolic extract of *Potentilla fulgens* showed a very impressive result with depletion of significant amount of glutathione after the treatment of methanolic extract on MCF-7 cell lines .This high level of suppression is mainly attributed to the inhibition of antiapoptotic protein survivin and reduced level of glutathione in the treated cell line (Radhika, 2012). But in our study the effect seen can't be said or assigned to a particular compound unless detail investigation is done and the result obtained may also be due to the synergestic effect of compound present.

In case of *Primula rotundifolia* the chloroform extract showed a very impressing result with an IC_{50} value at 107.0965 µg/ml and the result obtained is in concordance with the others

work. Strong cytostatic property of human acute myeloid leukemia cell was seen after the treatment of cell with extract of *Primula spp* even at low concentration (Tokalov, 2004).

In case of *Rhododendron arboreum* the result obtained was not much significant.This may be due to the use of bark as sample. Study done by others in the leaf extract of *Rhododendron arboreum* obtained a very good result. Flavonoid hyperin (quercetin -3-O-beta-d-galactose) extracted from the leaf of *Rhododendron* was able to inhibit many cancer cell lines as well as RL952 (endometrial cancer cell). Optical density values of caspase -3 and caspase-9 was increased and expression of bcl-2 was also increased while the antiapoptotic protein bax was decreased in RL952 cells after 24 hrs of hyperin treatment (Li et al., 2012). Since our extract was of bark it couldn't show the inhibition of Hela cell line and this may be due to the absence of flavonoid hyperin in the extract. Also Preliminay phytochemical screening of different parts of this plant showed that bark is devoid of flavonoids, anthraquinones, and glycosides (Nisar et al., 2011).

5.4 Cytotoxicity study

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, invitro test, invivo test and then clinical trials in order to render them for human treatment. 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 good anti proliferative activity against HeLa cells.

Cytotoxicity study was carried out using mouse peritoneal macrophage as normal cell and those plant extract which showed good anticancerous activity in Hela cell line. 5-Fluorouracil was taken as positive control which showed IC_{50} value at 48.01µg/ml while all the other plant extracts showed IC_{50} value >300µg/ml. This data shows that these plants have selective cytotoxicity similar to the standard drug suggesting the possibility of using it in the clinical field of cancer. Good selectivity index of all the extracts indicates their potential use as therapeutics in treating the dreadful disease like cancer.

Potentilla fulgens has been used from the very long time in the treatment of various ailments. There is no recorded toxicity of this plant.

There is no recorded toxicity of *P. rotundifolia* but other species of this genus may be toxic or nontoxic. In the United States, flowers of *Primula veris* and *Primula elatior* are classified as Class 1 botanicals, which means they can be safely consumed when used appropriately

(McGuffin et al., 1997). Chibanguza et al. (1984) performed in vivo studies on rabbits which contain some information concerning toxicological considerations. Except for the red blood cell count, none of the parameters tested (respiration rate, pulse rate, prothrombin time, electrolyte concentrations of calcium, potassium and sodium) was influenced by the intragastral application of the extract from *Primula*e *flos* in the 50-fold therapeutic concentration.

Ethnopharmacological data point to the therapeutic potential of the genus *Rhododendron* for the treatment of inflammatory conditions and pain and thus, research should focus on identification of active compounds and related mechanistic studies. On the other hand researcher suggest prolonged and high dose intake of traditional formulations containing *Rhododendron*s should be avoided until more in depth toxicity studies become available (Popescu and Kopp, 2013). Potent active compound with appropriate dosage should be found out along with its cytotoxic study before using it in medical formulation.

5.5 Antibacterial activity

Methanolic extract of *Potentilla fulgens* and both methanolic and chloroform extract of *Primula rotundifolia* were found to be significant as antibacterial agent.

Methanolic and chloroform extract of the *Primula rotundifolia* was found to be potent in inhibiting the growth of *Staphylococcus aureus* whereas only the chloroform extract inhibited the growth of *Pseudomonas aureus*. Very high antibacterial activity of extract of *Primula rotundifolia* may be due to the presence of some active compound in it. Since there are no recorded study of antibacterial activity of *P. rotundifolia*, other spp of this genus has shown good antimicrobial activity. Crude extract, benzene and ethylacetate fraction of *Primula macrophylla* showed an excellent antifungal activity with different MIC value. Wolters (1966) has compared the antifungal and antibacterial effects of 30 herbal drugs containing saponins. Among these drugs, *Primula* root extracts showed antibacterial as well as good antifungal activity. Also in our study high flavonoid content seen in the chloroform extract might be responsible for the antibacterial activity. Similarly chloroform fraction of *Primula* spp contains lipophilic flavones (Budzianowski, 2005) and these lipophilic flavones are capable of disrupting the microbial cell membranes (Cowan, 1999; Mishra et al., 2009).

Methanolic extract of *Potentilla fulgens* contains alkaloid and phenols as shown in phytochemical screening and these secondary metabolites may have attributed the good antibacterial property in this plant. Researcher suggests that most of the pharmacological effects of this plant are due to the presence of high tannins (Tomczyk and Latte, 2009).

CHAPTER V: CONCLUSION

In this study some of traditionally used medicinal plants were taken and study on their flavonoid, polyphenol, antioxidant, antibacterial and anticancerous activity was done or say tried to establish a scientific basis for using it ethnomedically. Documentation of these plants as medicine in different ailments was found in different literature. Some plants were found to be potent in antioxidant activity while others showed very impressive antibacterial activity even in crude form. Methicillin resistant *Staphylococcus aureus* and *Pseudomonas aerugenosa* were inhibited by different extracts of *Primula* and *Potentilla* even at low concentration. In chloroform extract of *Primula rotundifolia* better selectivity towards cancer cell line as compared to normal cell line was found. Among the three plants taken in our study chloroform extract of *Primula rotundifolia* was found to be most potent plant in terms of antibacterial and anticancer activity. Very low number of literature regarding the investigation of *Primula rotundifolia* is found.There is not much study done in this species of *Primula*. So further study on this plant is recommended.

We used crude extracts in our study and it is very necessary to find out the responsible lead compound in that extract. For that it is necessary to fractionate the extract by using various range of solvent based on polarity. In the next step further separation can be done using chromatography, crystallization. Finally various techniques of modern analytical chemistry like HPLC, GCMS, NMR, IR spectroscopy can be used to find the compound present.

This research recommends the study of more medicinal plants with extensive study using modern equipments and thus find out the scientific basis of using it. Thus it can be used by the local people to raise their economy as well as encourage to develop the rational use of local resources. On one hand if found as a candidate molecule or drugs after detailed investigation it can save life against various chronic disorders like atherosclerosis, Diabetes, Alzheimer's disease, cancer etc. which has threatened the modern medicine and on other it can help for the country's economic development.

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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
TDW:	1 L
Gentamycin:	20 µg/ml
Streptomycin:	100 µg/ml
Penicillin:	100 U/ml
pH:	7.4

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

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

3. Preparation of 1M potassium acetate (CH3COOK) – 100 ml

Weigh 9.814 gram of the potassium acetate Merk Specialities Pvt. Ltd, Mumbai, India) and dissolve on water. Finally maintain the volume to 100 ml by the addition of water.

4. Preparation of 0.2mM DPPH solution - 100 ml

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

5. Composition of Nutrient agar media

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

Components	gram/L
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Agar	15.0
PH 7.4 ± 0.2	

6. Composition of Mueller Hinton Agar (MHA)

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

Components	gram/L
Beef infusion form	300
Casein hydrolysate	17.5
Starch	1.56
Agar	17
Final PH 7.3 ± 0.2	

7. Preparation of 0.5 McFarland standards- 100 ml

The components that were used for the preparation of the 0.5 McFarland standard is as follows.

Components	Amount in ml
Sulfuric acid,	0.18 M 99.5

PHOTOGRAPHS

Figure : Rhododendron arboreum

Figure : Primula rotundifolia

Figure: Potentilla fulgens

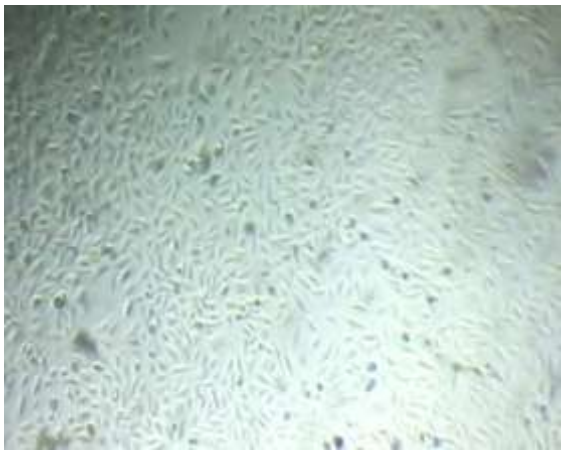

Figure: Hela cell

Fig: soxhalation of plant extract

Figure : Rotary evaporator