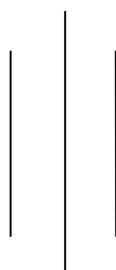
ANTIOXIDANT, ANTIBACTERIAL AND CELL TOXICITY ASSAY OF SOME LOCAL HERBAL PLANTS

M.Sc. Thesis
(2014)



Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

For Partial Fullfillment of Requirement for the
Master of Science in Biotechnology

Submitted By
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Roll No: BT 42-2067
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GLOSSARY ACRONYMS

ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
ACT	Artemisinin Combination Therapy
AGEs	Advanced Glycation End-products
ATCC	American Type Culture collection
BHA	Butylated Hydroxy Anisole
BHT	Butylated hydroxyl Toluene
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNA	Deoxyribo Nucleic Acid
DPPH	2,2-diphenyl-1 picrylhydrazyl
DPR	Department of Plant Resource
FIV	Feline Immunodeficency Virus
GAE	Gallic Acid Equivalent
HIV	Human Immunodeficency Virus
IUCN	International Union for Conservation of Nature
MAPDON	Medicinal and Aromatic plant database of Nepal
MAPs	Medicinal and Aromatic Plants
MHA	Muller and Hilton Agar
NAST	National Acadamy of Science and Technology
NK cells	Natural Killer cells
NO	Nitric Oxide
PEP	Prolyl endopeptidase
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Reverse transcriptase
WHO	World health organization

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ABSTRACT

Plants are used as traditional medicine for thousands of years, even long before modern medicine was introduced. More than half of world population still uses traditional medicine as primary health care system. Even developed countries have now acknowledged traditional medicine as alternate drugs system due to theirs advantage over modern drugs. From the last decade study on medicinal properties of phytochemicals are growing rapidly and this is due to plant being cheaper and harmless source of drugs. Since Nepal is rich in plant biodiversity, such study can be key for development of new drugs along with socioeconomic development of Nepal.

Therefore, in this research total of six plants were chosen to study their chemical and medicinal properties. Plants taken for this study were *Achyranthes aspera, Artemisia vulgaris, Citrullus colocynthis, Duranta repens, Euphorbia hirta, Urtica dioica* and *Woodfordia fruticosa*. These plants were subjected to phytochemical screening, estimation of total polyphenol and flavonoid content, then antioxidant, antibacterial and cell toxicity. *D. repens* and *E. hirta* showed the highest amount of total polyphenol content with 389±14.8 mg GAE/g dry wt and 383±7.42 mg GAE/g dry wt respectively whereas *A. vulgaris* and *U. dioica* showed high flavonoid content with168.65±1.98gm QE/g dry wt and 147±0.56gm QE/g dry wt respectively. Antioxidant assay was performed by DPPH and ABTS radical scavenging activities. In DPPH radical scavenging activities *D. repens* and *E. hirta* showed the highest scavenging activities with lowest EC_{50} value which is 60µg/ml and 70µg/ml respectively. Similarly in ABTS radical scavenging assay, *D. repens* and *C. colocynthis* showed high activities. In antibacterial assay, E. hirta showed high antibacterial activity inhibiting both Gram negative and Gram positive bacteria under study namely, *Escherichia coli, Klebsheilla pneumonae, Enterobacter sp, Salmonella typhimurium* and *Streptococcus aureus*. *D. repens* and *U. dioica* both showed effect on *Enterobacter* sp, *Salmonella typhimurium* and *Streptococcus aureus*. In cell cytotoxic assay, every plant's extract showed higher cell cytotoxicity (CC_{50}) value than reference market drug stating that these plant extracts are less cytotoxic than chemical drugs. In conclusion, this study supports the use of these six local plant as potential medicine in context of its polyphenol, flavonoid content, antioxidant, antibacterial and cell toxicity properties.

Keywords: Medicinal plant, Phytochemical screening, Antibacterial, Antioxidant, cell toxicity

CHAPTER 1: INTRODUCTION

1.1 Background

World Health Organization (WHO) document, described the traditional medicine as the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness. As Plants had been used for medicinal purpose from ancient period of time, knowledge about how to use plants accumulates thus leading to wisdom of present traditional medicinal practitioner or healers (Gautam, 2011). The use of plants for curing various human diseases were estimated to be started from very beginning of human civilization which are even engraved in an ancient manuscripts such as The Bible, The Rig- Vedas (2000 B.C.), The Atharvaveda (2000-100 B.C.), Ayurveda (1000-600 B.C.) etc (Taylor, 1992; Uprety et al., 2012). Traditional medicine covers a wide variety of therapies and practices which vary from country to country and region to region. Each culture has developed traditional herbal treatments based on their local flora. Unfortunately in developing countries, much of it is now being lost with the introduction of 'more scientific' Western health care, the destruction of the forests to feed and shelter the growing population, exploitation of the land by foreign multinationals, and the loss of culture through assimilation by colonists, governments and missionaries" (WHO, 2008). In many cases, theory and application of plants are quite different from those of conventional medicine. Long historical use of many practices of traditional medicine, including experience passed on from generation to generation, has demonstrated the safety and efficacy of traditional medicine (Tastai et al., 2002). However, scientific research is needed to provide additional evidence of its safety, efficacy, inexpensive medicine as more people are concern with adverse effect of synthetic chemical drugs (LI et al., 2008; Shukla et al., 2012). In conducting research and evaluating traditional medicine, knowledge and experience obtained through the long history of established practices should be respected (WHO, 2000).

Ethnobotany is scientific study of the relationships that exist between people and plants, especially the use of plants in different societies which are considered a rather sentimental discipline, a form of socially accepted or scientifically approved past tradition for a simpler way of life. Each and every culture has developed their own unique traditional treatment based on local flora and many of them has now being lost with uprising of modern and scientific western medicine and therapy, invasion of habitat of those flora, and exploitation of land (Taylor, 1992). Traditional medicine practices and ethnobotanical information play an important role in the scientific research which

can be seen in literature and field work data. The documentation of indigenous knowledge on the utilization of local plant resources by different ethnic groups or communities is one of the main objectives of ethnobotanical research. In general, ethnobotanical studies focus on the indigenous people and the minorities (Malla and Chhetri, 2009). Human have always depended on nature, plant or animal for their survival, welfare. Similar case in the medicinal field, as plant has been proved to be repository for organic molecules that are sometime virtually impossible to synthesize chemically. As plants are being extinct rapidly, the analysis of such plant and their product has become mandatory to continue its usefulness for the benefit of mankind (Schultes, 1994).

Traditional medicines are vaguely categories into Scholarly medical system, Folk medicine and Shamanistic medicine (Hamilton, 2004; Gewali, 2008). Scholarly medical systems are based on theories principles, formulation and experimentation. Ayurveda, traditional Chinese medicine, Japanese Kampo, Tibetan medicine (Unani) all belong to this class. They provide their health service from their own hospitals or dispensaries. Practitioners are qualified by mastering theoretical as well as clinical aspect which is totally based on research and recent development on experimental parameters and subjects (Gewali, 2008). Folk medicine refers to the society's indigenous medical wisdom that is handed down generation to generation usually through oral tradition. The folk medicine may have been generated from the contribution from a particular ethnic group or from a specific locality or may have coevolved with age-old culture. Worldwide, folk medicine practices are widespread especially on the traditional societies. Richard Evans Schultes, a world renowned ethnobotanist said that traditional healer, "is usually an accomplished botanist, represents probably the oldest professional man in the evolution of human culture" (Lewington, 1990). Lastly, Shamanistic medicine is a spiritual form of medicine where a person's illness is believed to be caused by the spirit possession. The shaman in a trance state will communicate with spirit healers and seek remedy of the sickness. The shamanism is still practiced with gusto in different parts of the world (Hamilton, 2004; Gewali, 2008).

By the middle of the 19th century at least 80 % of all medicines were derived from herbs while 25% of commercial drugs in the 21st century are still plant derived drugs and the number is increasing day by day (Gilani and Rahman, 2005). Medicinal plants or plant derived product have played a lead role in world health including them maintenance of health as well as treatment. The world health organization (WHO) has estimated that about 80% of the population in developing countries depend on traditional medicine for their primary health care needs (WHO, 2008; Wikispace, 2009). Many plants used in

traditional medicine also have other domestic uses. Therefore it is also very important to study ethnobotany and ethnopharmacology of that traditional medicinal plant so as to preserve traditional knowledge (Acharya, 2012)

In the world until now about 350,000 plant species have been identified and about 70,000 species are used worldwide for medicinal purposes and about 100 plant species are directly involved in 25% of all the drugs prescribed in modern medicine (Comer, 1996). In 1990s, 120 plant derived drugs were introduced and surprisingly those were discovered from only 95 species of flowering plants. Less than 0.5% of all flowering plant species have ever been studied for potential pharmaceutical activity (Balick and Cox, 1996). The demand and trade of medicinal plants is increasing day by day. According to a survey done in 2003, 61% of the 877 small-molecule introduced as drugs during 1981-2002 were natural products of plant origin. In last few decades, the annual global sale of herbal products over US$ 100 billion and which is due to revival of natural product sells in their crude form (Newman et al., 2003). The world market of the herbal remedies was worth 19.4 million US$ in 1999. In Canada the annual market sales of medicinal plants has reached 400 million US$ in 2001 and has been increasing by 15 % annually (Uprety et al., 2012). In world market, Asia and European countries are leading countries to import and use plant sources derived drugs, followed by North America, Japan.

Medicinal analysis of the plant species includes the test of the plant extract for its antimicrobial property against the pathogenic microorganisms. Furthermore, analysis of the total alkaloid, total phenol, total flavonoid content along with saponin, terpenes, acids, essential oils in the plant broadens the medicinal value of plants (Lee et al., 2003). Traditional medicinal plants have attracted the attention of pharmaceutical and scientific communities, and evidence has demonstrated the promising potential of antimicrobial plant derived substances and the antimicrobial effect of plant oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Savoia, 2012).

1.1 Plant as source of Antioxidant

Antioxidant is simply defined as compounds which quench the imbalance occur by generation of free radicals and oxidative stress. Oxygen Stress can be defined as physiological condition resulting from imbalance in ration of oxidants, pro-oxidants and antioxidants in our body. Oxidative stress generates Reactive Oxygen Species (ROS) like superoxide anion, hydroxyl radical, hydrogen peroxide, hypochlorite, singlet oxygen etc. or Reactive Nitrogen Species (RNS) like nitric oxide, peroxy nitrate, nitrogen dioxide etc. Classically antioxidant is defined as substance which can significantly delay or reduces

oxidation of substrate like reactive oxygen species and other free radicals even when present in very low concentration. In plants, defense of oxidants like Reactive Oxygen Species (ROS) or free radicals are done by antioxidant system includes Enzymatic and Non-Enzymatic system. Enzymatic systems include enzymes like Superoxide dismutase, catalase, peroxidase, glutathione reductase, polyphenol oxidase etc. whereas Non-enzymatic system includes compound with antioxidant properties like Vitamin A, Vitamin E, ascorbic acid, polyphenols, flavonoids, traces metals (selenium, magnesium, zinc) etc. Among these many are present in human body too as response to ROS in lungs, stomach or cellular fluid (Krishnaiah et al., 2011; Yordi et al., 2012). ROS are considered as causative element of many diseases like cardiovascular, cancer, neurodegenerative, Alzheimer's disease, Parkinson's disease (Engelhart et al., 2002; Halliwell, 2009; Pereira et al., 2009; Krishnaiah et al., 2011; Mishra et al., 2012). Due to side effect shown by many chemical drugs, more and more people, even from developed countries, are focusing on natural and herbal medicine as alternative source of treatment (Murugan and Parimelazhagan, 2013). Many herbal medicines have been proved to be excellent sources of antioxidant. This is due to presence of biologically active substance found in plants including high phenolic compound (Shukla et al., 2012). Plant derived phenolic compound, flavonoids are now documented as potent antioxidant and its activity as pro-oxidant activity are often being documented (Ren et al., 2003; Pourmorad et al., 2006).

Nowadays, western world are focusing in natural medicinal product for treatment of disease cause by oxygen stress (Engelhart et al., 2002; Chen et al., 2013). This is also due to side effects, toxicity and carcinogenicity of synthetic antioxidants like Butylated hydroxytoluene (BHT), Butylated hydroxyl anisole (BHA), tertiary butylated hydoxyquinone (TBHQ), propyl gallate (PG) and other Gallic acid esters which are also used as food additives (Emen et al., 2009; Ciesla et al., 2012; Mishra et al., 2012). Mechanisms of antioxidant activity of plants are not perfectly known but several mechanisms have been identified or postulate to explain this mechanism. Some of them are: donating hydrogen and neutralizing radicals, reducing hydroperoxides, stabilizing free radicals into complex with metal radicals, π electron containing antioxidant stabilize radical n form more stable radicals, inhibition of enzyme that generate radicals (lipoxygenases, cyclooxygenase, xanthine oxidase etc.), acts as prooxidant to intensify the metal chelators, biosynthesis of defense enzyme etc (Shahidi and Wanasundara, 1992; Pereira et al., 2009). Phenols and Flavonoids are recently proved as most effective plant antioxidants then synthetic and other body antioxidants like Vitamine C, Vitamin E, carotenoids etc (Shahidi and Wanasundara, 1992; Ren et al., 2003; Pereira et al., 2009; Dai and Mumper, 2010; Chen et al., 2013).

1.2 Plant as source of Antimicrobial agent

Antimicrobial activity of plant is being practically used in traditional medicine from very early era. Based on them, modern day drugs are formulated and focused from natural sources (Ripa, 2009; Savoia, 2012). Extensive research have been carried out over last 40 years to discover antibacterial and antifungal drugs form plant (Devi et al., 2007). Garlic, ginger, neem, turmeric are commonly used antimicrobial plant sources as a diet even in present time. 61% of the 877 small-molecule introduced as drugs during 1981-2002 were natural products of plant origin (Newman et al., 2003). Secondary metabolites are the constituent of plant which exert antimicrobial activity in first place. Secondary metabolites like alkaloids, steroids, polyphenols, flavonoids, quinones, coumarins, resins, tannins, fatty acids, gums and essential oils etc. produces by plants are responsible for antimicrobial activities including enzymes (Joshi et al., 2009). Antimicrobials from plant source are now considered as alternative to antibiotics, due to its side effects like allergies, hypersensitivity, immunosuppression depletion of beneficial microorganism (Parmar and Rawat, 2012). The antimicrobial compound of plants, either “Phytoalexins” which are produce de novo in plants or “Phytoanticipins” which are produce by induction of microbial infection, are low molecular weight compound produced by plant due to biotic or abiotic stress (Gonzalez-Lamothe et al., 2009). They may be flavonoids, alkaloids, tannins, terpenoids or other essential oils even some peptides as well (Cowan, 1999; Lewis and Ausubel, 2006). Plant derived Antimicrobial compounds disrupt microbial growth by efflux pump inhibition, inhibition of quorum sensing, inhibition of biofilms, membrane disruption, inactivation of enzymes, binding to proteins, forming metal ion complications, forming disulphide bridge, substrate deprivation etc. (Cowan, 1999; Savoia, 2012).

1.2 Hypothesis

- Local plants used for treatment of the disease in traditional medicinal methods have some significant phytochemicals which leads to their properties though not listed as medicinal plants
- The medicinal values of those plants are due to its antimicrobial, antioxidant and phytochemical constituent.

1.3 Research Objectives

1.3.1 General Objectives

Evaluation of phytochemical values of some local plants which are being used in traditional treatment systems.

1.3.2 Specific Objectives

The specific objectives are:

- To estimate the total polyphenolic content, total flavonoid content present in crude methanolic extracts of different plant extract.
- To assay antibacterial activity and cell toxicity activity of crude methanolic extracts of different plant extract.
- To evaluate the antioxidant activity of crude methanolic extracts of different plant extract.

1.4 Rationale

Nepal has wide range of the geoclimatic condition it has been considered as paradise for medicinal plants. Very low number of researches had been conducted in medicinal plants found here. Furthermore, the traditional healers like Dhami, jhakri, or kabiraj knows many herbs which are still to be categorized into the medicinal plants. Thus, study of local plants for the medicinal value or other ethnobotanical properties is very important. The validation of plant as ethnobotanical significance can be held as priority for the financial and commercial advantage for developing countries like Nepal. In industrial context, these types of study can be beneficial for the development of new kinds of drugs or any chemicals (bioactive products).

Nepal constitutes about 0.03% of total global land. It represent the 2.2% of the global plants of which 245 are endemic (CBS, 2011). Number of plants considered as medicinally significant has been increased from 571 to >2300 (DPR, 1984; Manandhar, 2002; Sharma, 2004; Luitel and Pathak, 2013). Thus if we study more about ethnobotanical plants, number of medicinal and aromatic plants may increase more which might leads to identification of new bioactive chemicals which cure diseases more efficiently than synthetic chemical drugs currently in use. Only 4-5 MAPs that are exported from Nepal to third countries (Sharma and Shrestha, 2011). If study on ethnobotanical plant with high medicinal values could be done and domesticated, then cultivating and exporting of those plants can be beneficial economically and health beneficial too.

CHAPTER 2: LITERATURE REVIEW

World Health Organization has made an attempt to identify all medicinal plants used globally and listed more than 20,000 species (Srivastav et al., 2011). 80% of the world population in Asia and Africa still depends on the traditional medicine despite of emergence of modern medicine and dispersion of its availability worldwide, and about 74% of pharmaceutical products introduced in the market are based on plant product originally used in indigenous cultures (WHO, 2008; Wikispace, 2009). Plant derived product used in traditional medicine can be its chemical, its oil or just fragrance, but it is recognized for having low or no toxic side effects, thus pharmaceutical companies are searching for more traditional or indigenous knowledge which can be help to develop new drugs (WHO, 2008; Shukla et al., 2012). 61% of the 877 small-molecule introduced as drugs during 1981-2002 were natural products of plant origin (Newman et al., 2003). So importance of plants as a therapeutic value can be predicted. These therapeutic uses can be as medicine as curative, antiseptic, preservative or antipyretic etc. it is believed that plant based medicinal herb or drugs are toxic and side effect free or with minimal ration as compared to modern allopathic drugs, thus million dollars have been invested in the medicinal plant research in many western countries as National product research. Even in our neighbor countries China and India have established artificial cultivation of many medicinal significant plants and are very forward in research in such field.

2.1 Medicinal Plants in Nepal: A prospective

Nepal is a multiethnic and multilingual country. There are more than sixty different ethnic groups speaking about seventy-five languages (Malla and Chhetri, 2009). Nepal is known as a rich in biological resources along with Medicinal and Aromatic Plants (MAPs). Physiographic diversity causes altitudinal variation thus creating different ecological belt which gave rise to diversity of topography, climate and other ecological factors. Due to which, plants we can found here have wide variation from tropical to subtropical, temperate, alpine to sub-alpine vegetation. Due to such variation, huge numbers and variety of medicinal plants can be found in a wild and natural condition (Acharya, 2012). Uses of medicinal plants from Higher Himalayan region are increasing at rate of 20-25% per annum (Ghimire, 2008). Medicinal value of those medicinal and aromatic plants (MAPs) are not well investigated for their tendency to influence human biochemistry though many of those species being used as traditional herbal medicine (Bhattarai, 1998; Singh, 2006). Number of medicinal and aromatic plants reported in Nepal varies according to various sources from 571 to 1950. In 2008, compilation of Medicinal and Aromatic plant database of Nepal (MAPDON) was published, which revealed 1950 species of medicinal in Nepal. Out of which 1906 species are identified under vascular

groups comprising 1614 native, 192 introduced and cultivated and 100 naturalized taxa. Whereas recent research showed that there are more than 2300 medicinal plant which are already had been identified till date (DPR, 1984; Manandhar, 2002; Sharma, 2004; Gewali, 2008; Ghimire, 2008; Luitel and Pathak, 2013). These reports suggest for the further exploration of valuable species having phytochemical and pharmacological properties.

Medicinal plants play vital role on the Nepalese livelihood and use of medicinal plant is frequent in several regions of Nepal. Nepal is considered as a natural store house for medicinal plants and several tons of plant raw materials have been exported to Asian countries (including India), Europe and America. However, the contribution of this sector to national economy is still nominal (Manandhar, 2002; Kunwar and Bussmann, 2008). The world market of the herbal remedies was worth 19.4 million US$ in 1999 (Uprety et al., 2012). The demand and trade of medicinal plants is increasing day by day. In Canada the annual market sales of medicinal plants has reached 400 million US$ in 2001 and has been increasing by 15 % annually (Uprety et al., 2012). In 2008 Food and Agriculture Organization (FAO) reported that Nepal is in fourth position for exporting the medicinal plants along with Madagascar with 4% of globally traded medicinal plants. Nepal is only behind of Sudan, Afghanistan and Tanzania which contributes 66%, 9% and 10% of globally traded high value medicinal plants. Annually 9,794,000 US $ equivalent plant material with ethnobotanical significance are exported from Nepal to Hong Kong, Singapore, India, China as major destination among Asian countries (Kunwar and Bussmann, 2008; Sharma and Shrestha, 2011).

About 400 plant that are found in Nepal and which are regarded as medicinal plant were even mention in Vedas (Atharvaveda, Ayurveda, Yajurveda) with their composition and applications (Joy et al., 1998). In 19th century, “Nepali Nighantu”, an herbal pharmacopoeia of medicinal plants had good information on 750 Nepelse medicinal plants its ethnobotanical and medicinal uses (Malla and Shakya, 1984; Kunwar and Bussmann, 2008). First recorded scientific study of Nepalese useful and medicinal plant was conducted by Francis Buchanan in 1802-1803, followed by Nathaniel Wallich 1820-1821. Since that time many plants has been isolated as ethnomedical plants. Total number of medicinal plants in Nepal has reaches to 2300 in 2013 reported by Luitel and pathak (2013). Still many traditional medicinal valued plants are not well investigated for their properties and phytochemicals (Bhattarai, 1998; Singh, 2006).

2.2 *Achyranthes aspera*

Achyranthes aspera belongs to Order – Caryophyllales and Family Amaranthaceae. They are also known as Aghata (Sanskrit), Latjira, chirchira, apamarga (Nepali). They are an erect, stiff, annual or perennial herb, which is usually found in waste places. The plant is about 1-3 feet to 1 meter high often with woody base. Steam is angular ribbed, simple or branched from the base, often with tinged purple color with simple finely softly pubescent on both side, entire, petiolate, petiole 6-20mm long, elliptic leaves, de-doublement of stamens, embryology, seed dormancy imposed by covering structures, anomocytic stomata and introse type of anther (Dey, 2011). Flowers are greenish white, numerous in axillary or terminal spikes up to 75 cm long, seeds subcylindric, truncate at the apex, rounded at the base, reddish brown (Srivastav et al., 2011).

A. aspera is distributed throughout the tropical world. It can be found in many places growing as an introduced species and a common weed. It is an invasive species in some areas, including many Pacific Islands environments. It is found on road sides, field boundaries and waste places as a weed throughout India up to an altitude of 2100 m and in South Andaman Islands. The plant is also widespread in Baluchistan, Ceylon, Tropical Asia, Africa, Australia and America.

2.2.1 Phytochemical constituents

Seven compounds are found in oil produced form leaves of *A. aspera* viz., p-benzoquinone, hydroquinone, spathulenol, nerol, α-ionone, asarone and eugenol which constitutie about 63.05% of the oil among which Hydroquinone (57.7%) was found to be the chief constituent (Dey, 2011; Srivastav et al., 2011).

From aerial parts, dihydroxy ketones like 36, 37- dihydroxyhenpentacontan-4-one and Triacontanol has been isolated. A long chain aliphatic alcohol are found like 27-Cyclohexylheptacosan-7-ol and 16- hydroxy-26- methylheptacosan-2-one, and 17-pentatriacontanol, β-sitosterol, spinasterol (Dey, 2011). Ecdysterone, 20-hydroxyecdysone, Pentatriaontane, 6-pentatriacontanone, Hexatriacontane and Tritriacontane are also found.

Achyranthine, a water soluble alkaloid, is also been isolated which possess pharmacological actions like dilation of the blood vessels, lowering of the blood pressure, depression of the heart and increase the rate and amplitude of respiration. Other alkaloids like betaine are also found in *A. aspera* (Dey, 2011; Priya et al., 2012). Beside them, three bisdesmosidic saponins (I-III) [indicated by α-L-rhamnopyranosyl-(1->4)-(β-Dglucopyranosyluronic acid)-(1->3)-oleanolic acid, α-L-rhamnopyranosyl-(1->4)-(β-Dglucopyranosyluronic acid)-(1->3)-oleanolic acid-28-O- β -D-glucopyranoside and

α-Lrhamnopyranosyl-(1->4)-(β-D- glucopyranosyluronic acid)-(1->3)-oleanolic acid-28-O- β -Dglucopyranosyl-(1->4)-β-D- glucopyranoside] 20-hydroxyecdysone] and quercetin-3-O-β-D-galactoside, were also isolated from the methanol extract of the aerial parts of *Achyranthes aspera*. From the seeds two saponin, Saponin A and B was identified in which saponins A was identified as D-Glucuronic Acid and saponins B was identified as β-Dgalactopyranosyl ester of D-Glucuronic Acid. Beside this other constituents were also isolated like sapogenin, oleanolic acid, cyclic aliphatic fatty acids, amino acids and hentriacontane. The seeds also contain chemical constituents like 10-tricosanone, 10-octacosanone & 4-tritriacontanone (Dey, 2011; Narayan and Kumar, 2012; Rani et al., 2012)

From methanolic extracts of root, ecdysterone, ecdysone was isolated and oleanolic acid from glycosidic fraction of the roots. From the ethanolic extracts of the roots, a new aliphatic acid was isolated and identified as n-hexacos-14-enoic acid. This compound was reported for the first time from any natural source. Certain other were also isolated and identified as strigmasta-5, 22-dien-3-β-ol, trans-13-docasenoic acid, n-hexacosanyl n-decaniate, n-hexacos-17-enoic acid and n-hexacos-11-enoic acid. Strigmasta-5, 22-dien-3-β-ol is a phytosterol which was obtained as a colorless crystalline mass from petroleum ether: benzene 75:25 elute. It responded positively to Liebermann Burchard test for sterols (Srivastav et al., 2011).

2.2.2 Medicinal uses

The traditional uses is described in “Nighantus” as purgative, pungent, digestive, a remedy for inflammation of the internal organs, piles, itch, abdominal enlargements and enlarged cervical glands (Krishnaveni and Thaakur, 2006).

The aqueous solution of plant of *Achyranthes aspera* showed antibacterial activity against *staphylococcus aureus, Streptococcus heamolyticus* and *Bacillus typhosus, Pseudomonas aeruginosa* (Tullanithi et al., 2010; Parmar and Rawat, 2012; Patel et al., 2013). While both alcoholic and aqueous extract of leaves showed antibacterial activity against *Staphylococcus aureu*s and *E. coli* (Tullanithi et al., 2010).

A. aspera has long been used in different systems of medicine in the treatment of cancer, leprosy, asthma, fistula, piles, arthritis, wound, insect and snake bite, dandruff, hepatitis, renal disorders, dermatological disorders, gynecological disorders, gonorrhea, malaria, fever, cough, diabetes, pyorrhea, dysentery, ophthalmic, rabies, hysteria, toothache etc (Narayan and Kumar, 2012; Parmar and Rawat, 2012; Rama et al., 2013). This plant has been used as antimicrobial, larvicidal, antifertility, immunostimulant, hypoglycemic, hypolipidemic, anti-inflammatory, antioxidant, diuretic, cardiac stimulant,

antihypertensive, anti-anasacra, analgesic, antipyretic, antinoiceptive, prothyrodic, antispasmodic and hepatoprotective (Khan, 2006; Krishnaveni and Thaakur, 2006; Dey, 2011; Rama et al., 2013). A decoction of the plant is used as diuretic in renal dropsy and general anasarca. In Philippines the plant is used to relieve toothache, dysentery and bowel complaint (Krishnaveni and Thaakur, 2006). *Achyranthes aspera* seeds are reported to contain of oleanene-type triterpenoid saponin thus seed are believed to prevent obesity by reducing the excess accumulation of body fat and changing the serum lipid profile which showed strong anti-obesity activity by inhibiting pancreatic lipase and suppressing the increase of body weight induced by a high-fat diet (Rani et al., 2012).

2.3 *Artemisia vulgaris*

Artemisia vulgaris belong to Class- Magnoliopsida and Family-Asteraceae. It is commonly known as Tetipaati (Nepali). It is a perennial plant which grows to 0.5 m to 1.5m. It is found throughout most temperate regions of northern hemisphere especially westward towards the Pacific Coast (Barney and DiTommaso, 2003). It is common in meadow, hedgebanks and waysides, uncultivated, waste land. It prefers dry or moist soil and can tolerate drought too. It flowers from July to September. It can grow in semi-shade (light woodland) or no shade. The flowers are hermaphrodite (have both male and female organs) and are pollinated by Wind. They have also been used as flavoring in beer though fell into virtual disuse once hops came into favor.

2.3.1 Phytochemical constituents

Previous studies on *Artemisia vulgaris* reported the isolation of 1,8-cineole, a-thujone, camphor and isoborneol, elemol, limonene, a-eudesmol and spathulenol, artemisia lactone and vulgaris lactone, 3,5-di-O-caffeoylquinic acid and 1,5-di-O-caffeoylquinic acid, α-phellandrene, *D*3-carene, camphene, trans-isoelemicin, c-elemene and lyratol, estrogenic flavanoids, b-caryophyllene and b-cubebene, eudesmane acids, a and b-pinenes, thujone, d-limonene, 1,8-cineole, linalool, citral, 8-methoxy-7,8-methylenedioxycoumarin, monoterpenes containing either artemisyl or santolinyl skeletons, Artimesinin, tauremisin, inulin vulgarole, vulgarin polyacetylenes, fernenol, a-amyrin and its acetate, sesquiterpene. The leaves of the plant also afforded caryophyllene oxide, phytyl fatty acid esters, squalene, stigmasterol and sitosterol (Ragasa et al., 2008; Khan and Gilani, 2009; Mannan et al., 2010). 1,2,3,4-diepoxy-11(13)eudesmen-12,8-olide and Yomogin has also been reported to be present which is known for its anti-carcinogenic properties (Natividada et al., 2011).

Twenty known flavonoids were isolated and identified as tricine, jaceosidine, eupafolin, chrysoeriol, diosmetin, homoeriodictyol, isorhamnetin, apigenin, eriodictyol, luteolin, luteolin 7-glucoside, kaempferol 3-glucoside, kaempferol 7-glucoside, kaempferol 3-rhamnoside, kaempferol 3-rutinoside, quercetin 3-glucoside, quercetin 3-galactoside, quercetrin, rutin, and vitexin. The most abundant flavonoid compounds are eriodictyol and luteolin (Lee et al., 1998).

2.3.2 Medicinal Uses

A. vulgaris has a long history of being used in herbal medicine especially in matters connected to the digestive system, menstrual complaints and the treatment of worms. All parts of the plant are anthelmintic, antiseptic, antispasmodic, carminative, cholagogue, diaphoretic, digestive, emmenagogue, expectorant, nervine, purgative, stimulant, slightly tonic and used in the treatment of women's complaints (Ashok and Kumud, 2010). They can be used internally or externally. The leaves are also said to be appetizer, diuretic, haemostatic and stomachic. An infusion of the leaves and flowering tops is used in the treatment of nervous and spasmodic affections, sterility, functional bleeding of the uterus, dysmenorrhoea, asthma and diseases of the brain. The leaves have an antibacterial action, inhibiting the growth of *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Proteus vulgaris, Bacillus dysenteriae*, streptococci, *E. coli, B. subtilis*, *Pseudomonas, Shigella flexneri* etc (Ahameethunisa and Hopper, 2010; Juvatkar et al., 2012). The stem is also said to be antirheumatic, antispasmodic, and stomachic ulcer. It can relief asthma symptoms which is believed to be mediated through the blockade of both cholinergic and histamine H1 receptors and through non-specific smooth muscle relaxation. Artemisinin found in this plant are effective against malaria caused by *Plasmodium falciparum*, and WHO recommended Artemisinin Combination Therapy (ACT) as first line treatment for Uncomplicated malaria (Khan and Gilani, 2009; Mannan et al., 2010; Ramazani et al., 2010). Artemisinin also demonstrated to be effective against *Leishamia, Schistosoma, Toxoplasm, Trypanosoma* etc. (Mannan et al., 2010).

The roots are antispasmodic and bronchodilator activities which were mediated through the dual blockade of muscarinic receptors and Ca^{2+} influx (Khan and Gilani, 2009; Natividada et al., 2011). They are said to be one of the best stomachics. The leaves, placed inside the shoes, are said to be soothing for sore feet. The compressed dried leaves and stems are used in moxibustion (plant or part of plant which is use to burn on the surface of skin in order to treat diseases, or pain) (IUCN Nepal, 2000). Yomogin has also been known for its anti-carcinogenic properties (Natividada et al., 2011). However, it is slightly toxic, should never be used by pregnant women, especially in their first

trimester, since it can cause a miscarriage. Large, prolonged dosage can damage the nervous system.

2.4. *Citrullus colocynthis*

Citrullus colocynthis is a member of family cucurbitaceae. It was originally named as *Colocynthis citrullus*, but is now classified as *Citrullus colocynthis*. It is commonly known as bitter apple or bitter cucumber. It is tropical plants that grow abundantly in whole tropical world, and found as indigenous in Arabia, West Asia, and Tropical Africa and in the Mediterranean region (Smet, 1997; Borhade et al., 2013). It is native to dry areas of North Africa, being common throughout Sahara, areas of Morocco, Egypt, and Sudan, eastward through Iran to India and other parts of tropical Asia. The physical appearances differ with the location and environmental condition of places it grow like altitude, soil type, rainfall, salinity, temperature (Al-Zahrani and Al-Amer, 2006; Zahra, 2006).

Leaves are alternate with long petioles, triangular shaped with many cleft. Each leaf is almost 5 to 10 centimeters in length and has around 3 to 7 lobes. Flowers are yellow, appearing singly at axils of leaves; fruit are globular, yellow and smooth, while ripe contains a hard coriaceous rind, a white spongy pulp enclosing numerous ovate compressed white or brownish seeds. Each bitter apple plant produces around 15 to 30 globular fruits having a diameter of almost 7 to 10 centimeters. The outer portion of the fruit is covered with a green skin having yellow stripes (Borhade et al., 2013). The fruits may also be yellow in color. The ripe fruits are characterized by a thin but hard rind. The seeds are around 6 mm in size, smooth, compressed and ovoid-shaped. They are located on the parietal placenta. The seeds are light yellowish-orange to dark brown in color. The Bitter Apple plant has a large perennial root that sends out long and slender, angular, tough, rough vine-like stems.

2.4.1 Phytochemical constituents

Fruits has 17 compound identified which is believed to be derivative from fatty acid pathway and are broadly divided into five classes viz. alcohol, ketones, epoxy compound, hydrocarbon and acids. Alcohols: 4-(1-methyl) ethoxy 1-Butanol, 5-methooxy 2-pentanol, 1-cyclopentyl 2-propene-1-ol and 2-Furanmethanol, tetrahydro-5-methyl- cis and trans isomers; Ketones: 3,4-Dimethyl 2-hexanone, 2-methyl 4-heptanone and 3-methyl 2heptanone; Epoxy compounds: 1-propoxy pentane and 2,3-epoxy methyl propionate. palmitic acid, Hydrocarbons: tridecane, tetradecane, pentadecane and

hexadecane. Lastly 2 compound have residual Trimethysilylmethol and 2-bensenedicarboxylic acid di-isocotylester (Gurudeeban et al., 2010).

Glycosides like curcurbitacins, Estrols classified to groups A, B, E , I, J, K, L, and T are also found (Shafaei et al., 2012; Ali et al., 2013). Flavonoid, quercetine is also found in almost all part of plant body, whereas flavone c-glucosides are found in fruits and aerial parts. Fruits also contain iso-vitexin, iso-orientin and iso-orientin 3'-methylether, while aerial parts contain 8-C-p-hydroxybenzylisovitexin, 6-C-p-hydroxybenzylvitexin and 8-C-p hydroxybenzylisovitexin 4'-O-glucoside. Plant also contains Lipase and phospholipase. Plants extract also seems to content 18 different amino acids in 20 essential amino acids. Many metals including heavy metals are also found in plants like S, Ca, K, Mg, Sb, Sn, Si, Ag, Sr, Mb, Se, P, Fe, Zn, Cd, Cu, Ar, Co, Cr, Pb, Hg, Ni etc.

2.4.2 Medicinal uses

Though regarded as a toxic plant, the Citrullus colocynthis has much medicinal significance. The principal medicinal part of plant is fruit pulp (Smet, 1997). The fruits are used as anthelmintic, antipyretic, carminative. They are believed to cures tumors, ascites, leucoderma, ulcers, asthma, bronchitis, jaundice, enlargement of spleen, tuberculosis glands of the neck, dyspepsia, constipation, anemia, throat diseases, elephantiasis, joints pain (Shafaei et al., 2012; Borhade et al., 2013). They are also used for relieving of tension by producing drastic hydragogue and catharsis (Huseini et al., 2009).

Similarly, Roots are also used for treatment of jaundice, ascites, urinary diseases, rheumatism, abdominal enlargements, cough and asthmatic attacks of children. A poultice of root useful in inflammation of the breast (Kirtikar and Basu, 2006; Borhade et al., 2013). Fruits or roots with or without stimulant is mixed with water and applied to boils and pimples.

Pharmacological testing have shown that immature fruits and seeds extract have proved to have best anti-inflammatory activities on induced paw edema assay in rats. Similarly its extract has high antibacterial and anticandidal activities too. Gram negative bacteria are more susceptible than gram positive bacteria (Marzouk et al., 2012). The crude extracts have high mosquito larvicidal activities. *C. colocynthis* has been clinically trialed for Type II diabetes and found to have significant result in reduction on blood glucose level with no gastrointestinal side effect (Huseini et al., 2009). Plant had also been tested for anticancerous activities. Cucurbitacin glucosides extracted from leaves seems to inhibit the growth of Human breast cancer cell growth by rapid reduction of key protein for G2/M phase transition in cell cycle and cause induced apoptosis (Tannin-Spitz et al.,

2007; Tannin-Spitz et al., 2007; Ali et al., 2013). Thus plants are being evaluated for possible therapeutic value against breast cancer cells.

2.5 *Duranta repens*

Duranta repens is a tree-type ornamental plant grown in tropical countries. It belongs to family Verbenaceae (Bircher, 1960). It is believed to be native to scrub and open woodland in West indies, Northen part of Pakistan and central and southern America (Abou-Setta et al., 2007). The plant is too vigorus and tall enough to uses against the foundation land mark of the small areas; multistemmed shrub is popular as a screen or background planting. It is commonly grown as a hedge plant and when trimmed, forms a strong compact hedge almost impenetrable to cattle (Ahmad et al., 2009).

This plant is known as Nil Khada (Nepali) due to its blue flowers and throne in the stem. The plant is also known as Sky flower as it have full clusters of fragrant, pale blue flowers which attract butterflies strongly in summer, Golden dewdrop, as blue flower followed by bunches of golden-orange berries, popular with birds, pigeon berry, angels-whisper. Flowers and fruit are often found on the plant simultaneously and make an attractive show. Yellow fruit often hangs on the plant into the winter if the birds don't eat them. Sometimes takes the form of a scrambling shrub or rarely a small tree (Sharma et al., 2012). It usually matures with a height 1 to 3 meters. The species develops tap and lateral root systems with abundant fine roots. There are normally several stems arising from below and just above the ground line. The bark is light gray, becomes rough and fissured when old. Light green, opposite leaves are elliptic to ovate and 1.5 to 8 cm long. Flowers are light-blue, lavender, or white; tubular, five-lobed which are borne on terminal or axillary racemes. The yellow or yellow-orange fleshy fruits are ellipsoidal with five lobes and grow in hanging clusters. These fruits may contain up to eight nutlets.

2.5.1 Phytochemical constituents

There are reports of finding a iridoid glycosides (Durantoside 1-5) that inhibits growth of plant which are lamiid and their esters with cinnamic acid (durantosid I), 4-methoxycinnamic acid (durantosid II), 3,4- dimethoxycinnamic acid (durantosid III). 4-hydroxy-cinnamic acid and 4-acetoxycinnamic acid have been reported in this plant (Hiradate et al., 1999).

Five triterpenoidal saponins has been reported along with oleanolic acid, the triterpene saponin 3-[(O-β-4C1- glucuronopyranosyl)-oxy] olean-12-en-28-oic acid O-β-D-4C1-

glucopyranosyl ester. Three phenylethanoids, namely campenoside I, cistanoside E and acteoside, and with pectolinaringenin and scutellareinb was also found in leaves of plant (Subramanian and Nair, 1972). Five flavonoids, identified as acacetin, diosmetin, apigenin, luteolin and quercetin, long with two Flavonoid glycoside 7-O-α-d-glucopyranosyl-3,4′-dihydroxy-3′-(4-hydroxy- 3-methylbutyl)-5,6-dimethoxyflavone and 7-O-α-dglucopyranosyl(6′′′-p-hydroxcinnamoyl)-3,4′-dihydroxy-3′-(4-hydroxy-3-methylbutyl)-5,6-dimethoxyflavone were also found (Hiradate et al., 1999; Ahmed et al., 2009; Ijaz et al., 2010).

Other chemicals isolated from this plants are; coumarinolignoids (Repenins A-D), Cleomiscosin A, (+)-Hardwickiic acid, (+)-3,12-Clerodadien-12,15-olid18-oic acid, Trimethoxycoumarin, Dihydroxy trimethoxyflavone, Betuline, stigmasterol, sitosterol glucopyranoside etc. (Iqbal, 2004).

2.5.2 Medicinal uses

The pharmacological significance was noted due to the presence of various bioactive compounds in the *Duranta repens* such as C- alkylated flavonoids ,terpenoids, steroids, and flavonoids, saponin pseudo-ginsenoside, acetosides , triterpene saponins, steroidal glycosides, iridiod glycosides (durantosides I, II, III, IV, V and lamiide). Furthermore, coumarinolignoids, Repenins A–D, cleomiscosin A and durantin A which was isolated recently has added the pharmacological significance (Anis et al., 2002; Ahmad et al., 2009; Stuart, 2010; Shankaraiah et al., 2013)

Extracts of the fruits showed in vivo antimalarial activity against *Plasmodium berghei* whereas glycosidic flavonoid along with five flavonoid isolated from leaves shows anti-plasmodial activities against *Plasmodium falciparum* (Shahat et al., 2005; Ijaz et al., 2010; Bahekar and Kale, 2013). Fruits has been used also used as febrifuge, malaria and intestinal worms, stimulant, and along with infusion of leaves as diuretic in traditional medicine in Philippines (Stuart, 2010). Prolyl endopeptidase (PEP) Thrombin inhibitory coumarins were isolated from fruits (Anis et al., 2002; Ahmed et al., 2009). Specific inhibitors of PEP are expected to have anti-amnesic effects. Many PEP inhibitors have been synthesized as candidates for the treatment of neuropathological disorders (Toide et al., 1997; Anis et al., 2002).

Crude extract of *D. repens* showed antiviral activities with 76% inhibition of viral titre of Hepatitis A virus (Abou-Setta et al., 2007; Ahmed et al., 2009). Fruits are also reported to treatment of Malaria and intestinal worms, Leaves are used to treat abscess in chinese traditional medicine (Nikkon et al., 2008; Shankaraiah et al., 2013).

Coumarinolignoids as phytoalexins inhibit growth and sporulation of fungal. They acts as repellent against beetle and terrestrial invertebrate. Its bioactivities are anticoagulants, estrogenic, antimicrobial, vasodilator, molluscacidal, antihelmintic, sedative, hypnotic analgesic. Some Caumarins have inhibitory effects on DNA gyrase so Anti-HIV activities are also being studied on this compound (Ahmad, 1999).

Diabetic patient have accelerated accumulation of advanced glycation end-products (AGEs) during biochemical process in body, and generation of AGEs is further amplified with oxidative stress. Phytochemicals like Glycosidic flavonoids, steroids, flavonoids, terpenoids, triterpenes, diterpenoids, cinnamic acids, irridoid glycoside, lignan and phenyl propanoids acts as strong antioxidant, free radical scavengers and AGEs inhibitory, reducing AGEs and oxygen stress (Shankaraiah et al., 2013).

2.6. *Euphorbia hirta*

Euphorbia hirta is a pantropical weed i.e. a wild herbaceous plant is very common in all tropical countries. It belongs to Euphorbiaceae family and commonly called as Dudhe jhar in Nepal (Hashemia et al., 2012). It is a hairy herb that grows in open grasslands, roadsides and pathways.

It is annual, branched herb, prostrate to ascending, with branches up to 50 cm long. Stem is solid, hairy that produced abundant white latex. There are stipules present. The leaves are simple, elliptical, hairy (on both upper and lower surfaces but particularly on the veins on the lower leaf surface), with a finely dentate margin. Leaves occur in opposite pairs on the stem. The flowers are unisexual and found in terminal or axillary leaf node. Several cyathium densely clustered into a cyme. They lack petals and are generally on a stalk. The fruits are a capsules with three valves and produces tiny, oblong, four-sided red seeds. Seeds are oblong-conical, slightly wrinkled, pinkish brown, without caruncle. It has a white or brown taproot (Mamun-Or-Rashid et al., 2013).

2.6.1 Phytochemical constituents

Phytochemicals identified from this plant were scopoletin, scopar-one, isoscopoletin, quercetin, isorhamnetin, pinocembrin, kaempferol, luteolin and gallic acid. Important constituents of the aerial parts are terpenoids, including triterpenes: α-amyrin, β-amyrin, friedelin, taraxerol, and esters of it: taraxerone, 11α, 12α-oxidotaraxerol, cycloartenol, 24-methylene-cycloartenol, and euphorbol hexacosoate (Upadhyay et al., 2010; Mamun-Or-Rashid et al., 2013).

The aerial parts and roots also contain diterpene esters of the phorbol type and ingenol type, including 12-deoxyphorbol-13-dodecanoate-20-acetate, 12-deoxyphorbol-13-phenylacetate-20-acetate, ingenol triacetate, as well as the highly toxic tinyatoxin, a resiniferonol derivative. Other terpenoids isolated are sterols including β-sitosterol, campesterol, cholesterol and stigmasterol. Tannins isolated include the dimeric hydrolysable dehydroellagitannins euphorbins A, B, C, E and terchebin, the monomeric hydrolysable tannins geraniin, 2,4,6-tri-O-galloyl-β-D-glucose and 1,2,3,4,6-penta-O-galloyl-β-D-glucose and the esters 5-O-caffeoylquinic acid (neochlorogenic acid) and 3,4-di-O-galloylquinic acid, and benzyl gallate (Ping et al., 2013). Acids isolated include shikimic acid, protocatechuic acid, ellagic acid, gallic acid, tannic acid, maleic acid and tartaric acid. Flavonoids isolated are quercetin, quercitrin, quercitol and derivatives containing rhamnose, quercetin-rhamoside, a chlorophenolic acid, rutin, leucocyanidin, leucocyanidol, myricitrin, cyanidin 3,5-diglucoside, pelargonium 3,5-diglucoside and camphol (Rathnakumar et al., 2013). The flavonol glycoside, xanthorhamnin, was also isolated. The stems contain the hydrocarbon hentriacontane and myricyl alcohol. The latex contains inositol, taraxerol, friedelin, β-sitosterol, ellagic acid, kaempferol, quercitol and quercitrin. Fresh leaves from Euphorbia hirta plants of Nigerian origin were found to contain high levels of Mn (189 ppm), Cu (30.5 ppm), Zn (152 ppm), and NO_3 (4600 ppm).

2.6.2 Medicinal uses

Euphorbia hirta is an important medicinal herb used throughout its distribution area, including tropical Africa. It is held in high esteem, as a decoction or infusion, to treat gastrointestinal disorders, including intestinal parasites, diarrhea, peptic ulcers, heartburn, vomiting and amoebic dysentery (Hore et al., 2006). *Euphorbia hirta* is included in the African pharmacopoeia of the Organization of African Unity as a dysentery medication.

It is also regarded as an outstanding medication to treat respiratory system disorders, including asthma, bronchitis, hay fever, laryngeal spasms, emphysema, coughs and colds (Ogbulie et al., 2007; Gyuris et al., 2009). The leaves of this plant are mixed with those of *Datura metel* L. in preparing 'asthma cigarettes'. Other principal uses are as a diuretic to treat uro-genital diseases, such as kidney stones, menstrual problems, sterility and venereal diseases. The plants are also used to treat affections of the skin and mucous membranes, including warts, scabies, tinea, thrush, aphthae, fungal afflictions, measles, Guinea-worm and as an antiseptic to treat wounds, sores and conjunctivitis (Yi et al., 2012). Paste of leaves are applied externally (twice daily) on the place of scorpion bite (Chitra et al., 2011).

The plant has a reputation as an analgesic to treat severe headache, toothache, rheumatism, colic and pains during pregnancy. It is used as an antidote and pain relief of scorpion stings and snakebites. It is antipyretic and anti-inflammatory (Joshi and Joshi, 2000; Upadhyay et al., 2010). The use of the latex to facilitate removal of thorns from the skin is common. Its use in the treatment of jaundice, hypertension, oedema, anaemia and malaria, as an aphrodisiac, and to facilitate childbirth has also been reported. Whole plants are chewed to induce labor during childbirth. Besides these, other properties have also been recorded for *E. hirta*, e.g. hypotensive, tonic, antipyretic, anti-inflammatory and sedative effects (Gyuris et al., 2009).

Methanolic extract of this plant was found to exert a higher antiretroviral. A dose-dependent inhibition of Reverse transcriptase (RT) activity was observed for all three viruses HIV-1, HIV-2 and SIV_{mac251}. The HIV-1 inhibitory potency of *E. hirta* is being further studied (Gyuris et al., 2009)

Antibacterial (against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtili, Klebsiella pneumonia*), antimalarial, antifungal (against *Colletotrichum capsici, Fusariumpallidoroseum, Botryodiplodia theobromae, Phomopsis caricae-papayae*, *Aspergillus niger)* and antiamoebic (against *Entamoeba histolytica*) activity are also shown by this plant (Sudhakar et al., 2006; Kumar et al., 2010; Yi et al., 2012; Mamun-Or-Rashid et al., 2013)

2.7 *Urtica dioica*

Urtica dioica, commonly called Sisno or Stringing Nettle, is belonged to Family Urticaceae. The plant is used as local vegetable as well as the local medicinal plant. It is indigenous occasional vegetable that is found abundantly in Nepal (Joshi et al., 2007). It is regarded as vegetables for poor people too in some locality. But the nutritional value of this plant is being extensively studied in eastern society like Japan, Korean. But there is no such studied carried out in Nepal even if had been not known to all (Chahardehi et al., 2009; Upton, 2013). *Urtica dioica* L. are broadly distributed all over the world. Naturally, they are found in mild climate areas, bottom of barriers, ruins and grassy places, between cultivated plants, street, and water cannels, or field, sidewalk passage. Plant prefers nutrient riches soils and lighted places, hot and mild climate (Upton, 2013).

This plant has dark green leaves which are have 2–4 cm long, oval, and core shape leaves, serrated and contain nettle. These nettle gave stinking rashes when injected to a human body, hence gave the name Stinging nettle. The nettle flowers are small and green. It gives flowers from May to September. The fruits of nettle are arid and single germ. It is used as drug, food, fibrous, dye, and cosmetic from centuries. Numbers of

medical and pharmacologic researches about nettle are increased day by day. On the other hand, nettle has valuable fibrous content which is light, elegant, long, and resistant (Otles and Yalcin, 2012; Upton, 2013).

2.7.1 Phytochemical constituents

Polar extract of the *U. dioica* contains lignans (+)-neoolivil, (-)-secoisolariciresinol, dehydrodiconiferyl alcohol, isolariciresinol, pinoresinol, and 3,4-divanillyltetrahydrofuran (Kanter et al., 2005). Beside those antioxidant present are: sitosterol, sitosterol glucoside, sopoletine, vitamin C. Seven flavonol glycosides (kaempferol-3-Oglycoside, quercetin-3-O-glycoside, isorhamnetin-3-O glycoside, quercetin-3-O-rutinoside, isorhamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside and isorhamnetin-3-Oneohesperidoside) have been isolated (Chaurasia and Wichtl, 1987; Akbay et al., 2003; Otles and Yalcin, 2012)

Nettle has pharmacologically significant phytochemicals like agglutinin, acetophenone, alkaloids, acetylcholine, chlorogenic acid, butyric acid, chlorophyll, caffeic acid, carbonic acid, choline, histamine, coumaric acid, formic acid, pantothenic acid, kaempferol, coproporphyrin, lectin, lecithin, lignan, linoleic and linolenic acids, palmitic acid, xanthophyll, quercetin, quinic acid, serotonin, stigmasterol, terpenes, violaxanthin, and succinic acid in its chemical content. Seeds of nettle contain 8–10% fixed oil, ascorbic acid, carotene, and oxalate. Linoleic acid, palmitic acid, oleic acid, palmitoleic acid, stearic acid, gadoleic acid, and erucic acid were fattly acides found in roots of this plant (Guil-Guerreroa et al., 2003). Stinging hair of nettle contains formic acid, histamine, hydroxytryptamine and acetylcholine (Chahardehi et al., 2009; Upton, 2013).

Leaves of nettle contain provitamin A, vitamin B1, K, xanthophylls, and sistosterin and ashes of nettle contain 6,3% ferric oxide, potassium, calcium, and silicium vanillic acid, homovanillic acid, 2-hydroxycinnamic acid, 4-hydroxycinnamic acid, and ferulic acid were found. Methanolic extract of nettle leaves syringic acid, gallic acid, and ferulic acid were found (Otles and Yalcin, 2012)

2.7.2 Medicinal uses

People use nettle as vegetables as well as local medicine. Nettles are a very nutritious food that are easily digested and are high in minerals (especially iron), vitamin C and pro-vitamin A (Guil-Guerreroa et al., 2003). People believe that it has benefits on various condition like coughs, high blood pressure, tuberculosis, arthritis, n in some extent stimulant for hair growth and antidandruff agent (Manganelli et al., 2005; Golalipour et al., 2007; Upton, 2013). It has been found that it has anti-inflammatory action, in which preventing prostaglandin formation. Stinging nettle roots also found to have effects on

the hyperplasia by affecting hormones and proteins that carry sex hormones in the body. It can be used as Diuretic, expectorant, blood purifier as well as in gastrointestinal diseases, rheumatism pains, diarrhea (Tahri et al., 2000; Otles and Yalcin, 2012). *U. dioica* traditionally used as antidiabetic hypoglycemic and hypotensive medicine in Morocco. Bnouham et al. (2003) reported antidiabetic, hypoglycemic properties of this plant is due to reduction of glucose absorption in intestinal tract.

Some of significant effects of the Stinging nettle are- reduction of allergy in people with hay fever, help in prostatic hyperplasia, prevention of free radical accumulation in central nervous system or towards iron-promoted oxidation of phospholipids, linoleic acid and deoxyribose (Kamkar et al., 2010). It is used as antiallergenic anti-inflammatory agent, by inhibiting NF-kappa B activation involve in chronic inflammatory disease (Kamkar et al., 2010). Methanolic extract of stinging nettle are being exploited as anticancer activity, as it was found that they have concentration dependent and significant antiproliferative effect on LNCaP cells. They are also found to be beneficial in Benign prostatic hyperplasia (BHP) (Ganzera et al., 2005). Carotenoid and flavonoids present in this plant is being studied for anti-carcinogenic, anti-ulcer or anti-ageing properties. Researchers in Germany had found that it has immunomodulating effect on joint pain and arthritis. It inhibits lippopolysachharide but stimulates monocyte cytokine expression. It inhibits inflammatory cascade in autoimmune disease like rheumatoid arthritis (Riehemann et al., 1999). Lectin isolated from this plant has shown some antiHIV and anti-FIV properties too (Manganelli et al., 2005).

This plant also shows antimicrobial activities, anti-ulcer activity against ethanol-induced ulcergenesis and analgesic effect on acetic acid induced stretching. Extract of *U. dioica* shows antibacterial effect on *Salmonella typhi, Aeromonas hydrophila, Salmonella typhi, Staphylococcus aureus, Bacillus cereus, Escherichia coli* etc (AlShuwayeb and Al-Khatib, 2013).

2.8 *Woodfordia fruticosa*

Woodfordia fruticosa also called as Agnijwala (Nepali) or Tamra-pushpi (Sanskrit), belongs to family Lythraceae (Baravalia et al., 2011). The plant is found in altitude up to 1500m, found throughout Nepal and India, also in a majority of the countries of South East and Far East Asia like Malaysia, Indonesia, Sri Lanka, China, Japan and Pakistan as well as Tropical Africa (Das et al., 2007).

Full-grown plants are leafy shrubs, with about 3.5m high, and have long spreading branches with fluted stems. The bark is similar to cinnamon bark i.e. has cinnamon

brown colored, smooth, peels off as fibers and the young shoots are tapering, often clothed with fine white pubescence. The leaves are opposite or sub-opposite in nature. Flowers are brilliant red, innumerable, arranged in dense axillary paniculate-cymose clusters, with short glandular pubescent pedicels. The seeds are brown, numerous, very minute, smooth, shining, angular and obovated (Baravalia et al., 2011).

2.8.1 Phytochemical constituents

The compounds identified are predominantly phenolics, particularly hydrolysable tannins and flavonoids (Das et al., 2007). Besides those, octacosanol and beta-sitosterol are also reported to found in the stems apart from the flowers. Other non-phenolic constituents which are reported to be found include the bergenin (a C-glycoside of gallic acid), steroid, sapogenin, hecogenin, meso-inositol, chrysophanol-8-O-dglucopyranoside from the flowers, triterpenoids lupeol, betulin, betulinic acid, oleanolic acid, ursolic acid and naphthaquinone pigment from the leaves, and norbergenin in stems. Gallic acid is found in both leaves and stems similarly ellagic acid in leaves and flowers (Choi et al., 2010).

Flavonoid constituents found in leaves and flowers include six Quercetin glycosides which are 3-rhamnoside from flowers, 3-l-arabinoside (polystachoside) from flowers and leaves, and 3-O-α-l-arabinopyranoside, 3-O-β-D-xylopyranoside, 3-O-(6”-galloyl)-β-d-glucopyranoside, and 3-O-(6”-galloyl)- β -d-galactopyranoside from leaves. Three myricetin glycosides are found they are: [3-O- β -d-galactoside in flowers and leaves , 3-O- α -l-arabinopyranoside, 3- O-(6”-galloyl)- β -d-galactopyranoside in leaves. Naringenin 7-glucoside and kaempferol 3-O-glucoside are also found in flowers (Chandan et al., 2008; Finose and Devaki, 2011). The brilliant orange-red pigment of the flower was found to be due to pelargonidin 3,5-diglucoside and anthocyanidin pigment cyanidin 3,5-diglucoside. Japanese group “Yoshida” have isolated and characterized large number of hydrolysable tannins from the flowers. The known tannins reported so far are: 1,2,3,6-tetra-O-galloyl- β -d-glucose, 1,2,4,6-tetra-O-galloyl- β -d-glucose, 1,2,3,4,6-penta-O-galloyl- β -d-glucose, tellimagrandin, gemin D, heterophylliin A, oenothein A-B, Isoschimacoalin-A, woodfruticosin and woodfordins A–I (Yoshida et al., 1990; Chandan et al., 2008).

2.8.2 Medicinal uses

Woodfordia fruticosa is a very much-used medicinal plant in Ayurvedic, and Unani systems of medicines (Das et al., 2007; Chandan et al., 2008). In Nepal, the flower and leaf of *W. fruticosa* have exceptionally wide diversity of traditional uses to treat various illnesses. It has been found to treat various ailments that may be of bacterial origin, for

example boils, diarrhea, dysentery, fever, cough, menstrual disorders, urinary disorders, wounds, swellings, cuts, skin diseases (Bhattarai and Bhuju, 2011).

It has also been found to be used in treatment of rheumatism, leucorrhea, menorrhagia, hemorrhoids, asthma, liver disorder, and inflammatory conditions, burning sensation in stomach, weakness and other rheumatic diseases as well as considered as safe stimulant in pregnancy (Das et al., 2007; Baravalia and Chanda, 2011; Mihira et al., 2011). It has also proved to be effective in dysfunctional uterine bleeding (Das et al., 2007). In a controlled clinical trial, *Woodfordia fruticosa*-based plant formulation was found to be as efficacious in the treatment of reversible asthma as salbutamol alone or in combination with theophylline (Murali et al., 2006).

Woodfordin C, a macrocyclic dimeric hydrolysable tannin isolated from *W. fruticosa*, has been reported to show remarkable growth inhibition of Human tumor cells i.e. it shows anti-tumor activity in vivo and it was found to be related to be inhibition of DNA topoisomerase II in vitro condition (Yoshida et al., 1990; Kuramochimotegi et al., 1992; Das et al., 2007). Cytotoxic effect in cell culture was demonstrated to be selective, and is perhaps expressed through potentiation of host-immune defense system, like activation of NK cells and augmentation of interleukin secretion (Miyamoto et al., 1993; Wang et al., 1999).

Oenothein B, dimeric ellagitannin, is considered as one of the medicinally important constituents of *Woodfordia fruticosa* flowers because it has been demonstrated to inhibit 5-alpha reductase and aromatase, two enzymes which are involved in the causing of benign prostatic hyperplasia, and also been found to suppress tumor gene expression via inhibition of poly(ADP-ribose) glycohydrolase (Lesuisse et al., 1996)(Aoki et al., 1995). Further, this macrocyclic dimeric ellagitannin has recently been shown to induce neutral peptidase activity in prostate cancer cells implying its possible beneficial effect in managing prostate cancer (Yang et al., 1999). Oenothein B can also be used in treating of hyperandrogenism in warm-blooded animals, it acts via inhibition of 5-alpha reductase, a key enzyme in the biosynthesis of certain androgens, has been patented by Gourvest et al. (1996).

Several naturally occurring polyphenolic compounds isolated from this plants like flavonoids, anthraquinones, macrocyclic ellagitannins etc shows putative role in management of inflammation inhibited LPS-induced iNOS and COX-2 gene expression (Chen et al., 2000). The tannins are has equal probability of having anti-oxidant properties or capacity that is equivalent to vitamin C. Other traditional and modern uses are currently being used are:- the prevention and treatment of dental plaque formation, flowers are used for making cooling drinks in Central and Eastern India, as ointment

form it is used skin whitening in cosmetic product and were claimed to be capable of suppressing the activity of hyaluronidase, elastase, tyrosinase, etc (Yoshida et al., 1990; Vermaa et al., 2013), and are thus give softness to rough skin, decoction of flower effectively quenches excess thirst especially in diabetic patient (Sengupta, 2013). Plant extract seems to have antimicrobial activities against many enteric and pathogenic microbes like *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus subflava, Micrococcus flavus, Alcaligenes fecalis, Enterobacter aerogenes, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas pseudoalcaligenes, Pseudomonas testosterone, Salmonella typhimurium, Salmonella typhi* etc (Parekh and Chanda, 2007).

CHAPTER 3: MATERIALS AND METHODS

3.1 Collection of sample

Plant selection was based on convenience, availability on the nearby localities. The plants selected for the study were *Achyranthes asperal* (Apamarga), *Artemisia vulgaris* (titehpaati), *Citrullus colocynthis* (bitter apple), *Duranta repens* (Nilkadha), *Euphorbia hirta* (Dudhejhar), *Urtica dioica* (Sissnu) and *Woodfordia fruticosa* (Agni jwala).

Artemisia vulgaris, *Citrullus colocynthis*, *Duranta repens* and *Woodfordia fruticosa* were collected from near vicinity of Central Departments of Tribhuvan University, Kirtipur. *Urtica dioica* and *Euphorbia hirta* was collected from Phulchowki. Plants were verified by Dr. Deepak Pant and in Central Department of Botany. They were also authenticated with Annoted Checklist of Flowering Plants in Nepal (Press et al., 2000). Stem and leaves were taken as samples for *Achyranthes aspera, Artemisia vulgaris, Euphorbia hirta, Urtica dioica*; whereas - flower and leaves were taken for *Duranta repens, Woodfordia fruticosa* finally fruits were taken for *Citrullus colocynthis*.

3.2 Sample preparation

Sample was collected from different places were cleaned for dust or mud. Then it was subjected to dry which was done by shade drying for few days or up to a week for complete removal of moisture. Then it was crushed into fine powder by use of electric grinder. The powder was seal in a polyethylene bag for further use.

3.3 Extraction preparation

About 10 gram fine powder of each species was taken separately and filled in the thimble and was subjected for the methanol extraction on the Soxhlet apparatus on 250 ml of the methanol at 45^0C. The continuous heat was provided by the heating mantle in order to evaporate the alcohol. The process was carried out up to nearly 25 fluxes till the clear solution of alcohol was obtained. The extract was filtered and the ethanol was evaporated on the rotatory evaporator under the vacuum at the room temperature till the solid mass was obtained. The solid mass was weighed carefully to express the gram of extract extracted per 10 gram of the plant powder. For each sample, extract was prepared individually. The extracts were kept at 4°C until further drying was done.
The sample was subjected to evaporation under reduced pressure by Rotavapour in Laboratory of National Acadamy of Science and Technology (NAST), Khumaltar and Central Department of Chemistry, Kirtipur. Dried products obtained were stored in stored at 4^0C for further use.

Extraction efficiency was calculated as Follows.

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

3.4 Phytochemical screening

Phytochemical screening of major phytochemicals or secondary metabolites was performed as given by Raman (2006) and other research articles (Njoku and Obi, 2009; Sasidharan et al., 2011; Yadav and Agarwala, 2011). The summary of the protocol which was followed during this work are described below:

3.4.1 Detection of Alkaloids

About 50mg of the solvent free extracts were extracted with few ml of dil. HCL and were filtered and divided into two parts.

Mayer's test

Two drops of Mayer's reagent was added to the filtrate obtained above by the side of test tube. Formation of white or creamy precipitate indicates the presence of alkaloids.

Wagner test

To 2ml of filtrate 1% HCl was added, and then it was treated with six drop of Wagner's Reagent. Formation of Brownish-red precipitation conform presence of alkaloids

3.4.2 Detection of Carbohydrates

About 50-100mg of the crude extracts was dissolved in 5ml of distilled water and filtered. This aqueous fraction was subjected to these tests.

Molisch's test

With few ml of extract, 2 drops of alcoholic solution of α-napthol was added and shaken well, and then 1ml of Conc. Sulfuric acid was added along with wall of the test tube without any disturbance. Formation of violet ring is positive result for presence of carbohydrates.

Fehling's test

For 1ml of extract solution, 1ml of each of Fehling's solutions A and B were added and then the test tube was placed over boiling water bath for few minutes. Formation of red precipitate shows presence of reducing sugars.

Benedict 's test

To 0.2ml of extract solution, 2ml of Benedict solution was added and heated. Formation of brick red to greenish blue precipitate shows presence of carbohydrates.

3.4.3 Detection of Saponins

Foam test

About 50 mg of the extract was taken in a test tube containing about 20ml of distilled water. The solution was then vigorously shaken. Formation of stable foams in the upper layer of the test tube after it was stand for few minutes shows presence of saponins.

3.4.4 Detection of Cardiac glycosides

Kellar-killiani test

To 2ml of glacial acetic acid, 0.5ml of the methanolic extract was added. Then 1ml of ferric chloride solution was added and lightly shaken. The solution was then overlaid with 1ml of conc. sulfuric acid. Formation of greenish-blue coloration shows presence of cardiac glycosides. Sometimes a brown ring or a violet ring at the interphase might appear which is also a positive result.

Salkowski Test

To 5ml of methanolic extract, 2ml of chloroform and 3ml of concentrated sulfuric acid was added slowly along the wall of test tubes. The yellow ring at the interference which turn reddish brown in few minutes shows presence of steroidal ring i.e. glycine portion of glycoside

3.4.5 Detection of Phytosterols and Terpenoids

Libermann-Burchard's test

Few milligrams of extract were dissolved in methanol. 1ml of that methanolic extract was treated with 1ml of Chloroform, 1-2ml of acetic anhydride and few drops of concentrated sulfuric acid along the wall of the test tube. Dark green coloration of solution shows the presence of steroids or phytosterols.

3.4.6 Detection of Proteins and Amino acids

Aqueous fraction of the extract was obtained by dissolving 100mg crude extract in 10ml distilled water and filtered.

Xanthoproteic Test

To 1ml of extract solution, 2 drops of concentrated nitric acid was added. Deep blue coloration confirmed the presence of proteins.

Biuret test

To 2ml of the filtrate 2 drops of 2% copper sulfate solution and 1ml of 95% ethanol was added which was followed by addition of KOH pellets in excess. Appearance of pink color in the ethanolic layer confirms presence of proteins.

3.4.7 Detection of Phenolic compounds and tannins

Ferric chloride Test

To the aqueous fraction of the extract, few drops of alcoholic ferric chloride were added. Dark green coloration of solution shows presence of phenolics.

Alkaline Reagent Test

To the aqueous fraction of the extract, 10% ammonium hydroxide solution was added. Development of yellow fluorescence on addition of aqueous fraction of the extract confirms presence of flavonoids.

3.4.8 Detection of Tannins

Braemer's test

An equal volume of 10% ferric chloride solution when added to methanolic extract results in dark blue or greenish coloration of the solution if the extract contains tannins.

Gelatin test

About 1% gelatin was mixed with 10mg/ml aqueous extract with 10% of Sodium Chloride, shaken and left for few minutes. White precipitate shows presences of tannin

3.4.9 Detection of Flavonoids

Shinoda test

Extract was mixed with little Zinc and treated with concentrated hydrochloric acid. Coloration of solution shows presence of flavonoids. Red-orange color shows presence of flavonoid where as white-violet shows flavonone presences

Lead Acetate test

About 2-3ml of extract was treated with 10% Lead acetate solution and shaken. The formation of yellow precipitate shows the presence of Flavonoids.

3.4.10 Detection of Resin

Acetone water test

Dry extract was treated with 5ml acetone then diluted with equal volume of water. Formation of turbidity will show presence of resin.

3.4.11. Detection of Fixed oil and Fats.

Spot test

Extract was placed between two layers of filter paper and pressed for few minute. Formation of oil stain in the filter paper shows presence of fixed oil and fats.

Saponification test

Few drops of 0.5N alcoholic potassium hydroxide was added to extract and phenolphthalein was added. Then solution was heated for 1 hour in water bath. Formation of soap or partial neutralization of phenolphthalein's color will show presence of oils and fat.

3.5 Total Polyphenol content determination

The total Polyphenol content of the different species was determined using the Folin–Ciocalteu phenol reagent as described by Gülçin et al. (2004) and Otles and Yalcin (2012) with slight modification. To 0.5 ml of each extract (25 mg/ml), 0.5 ml of Folin–Ciocalteu phenol reagent (Merck Specialities pvt Ltd, India) diluted to 1:10 with distilled water was mixed. After 3 minutes, 1.5ml of aqueous 2% Na_2CO_3 solution was added. The reaction mixture was allowed to stand for about 2 hours and the absorbance of the reactants was measured at 760 nm using the UV- visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained using the solution of gallic acid (Merck Specialities pvt Ltd, India) as standard in methanol and water (50:50 v/v) using the concentration ranging from 25-250μg/ml. Based on this standard graph, the concentration of the individual samples were calculated. The total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE g^{-1}). For each extract, three replicates were performed for the reproducibility of results.

3.6 Total Flavonoid content determination

Total flavonoid estimation was performed as described by Pourmorad et al. (2006). Total flavonoid content of extract was determined by estimating ability to form flavonoid-aluminum complex by aluminum chloride with flavonoid rings of compound present in extract (Chang et al., 2002). The total flavonoid content in the plant extract was estimated using the Aluminum chloride ($AlCl_3$) colorimetric method. 0.5 ml of extract (5 mg/ml) was separately mixed with the 1.5 ml of 80% methanol, 0.1 ml of the 10% aluminum chloride, 0.1 ml of the 1 M potassium acetate (CH_3COOK) and 2.8 ml of the distilled water. The reaction mixture was allowed to stand for about 30 minutes in room temperature. The absorbance of the mixture was measured at 415 nm using the UV – visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained with the help of the quercetin (Sigma) standard solutions in ethanol with the concentration ranging from the 10-100µg /ml. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass (mg QE/g). For each sample, triplicate test was done for the accuracy and reproducibility of results.

3.7 Antioxidant activity

The antioxidant activities of the extracts were determined by two different methods, DPPH Free Radical Scavenging Activity assay as described by Otles and Yalcin (2012) and ABTS radical scavenging assay as described by Thaipong et al. (2006).

3.7.1 DPPH Free Radical Scavenging Activity assay

The antioxidant activity of extract of different plant sample, with Gallic acid as standard antioxidant, was subjected to antioxidant activity assay by use of free and stable radical 1, 1- diphenyl-2 picryhydrazyl (DPPH). Different concentration of plant extract (50-1000 µg/ml) and Gallic acid (10-100µg /ml) were prepared in methanol on the clean and clear test tubes. To 10 µL of sample of each concentration, 1 ml of the 0.2mM DPPH solution was added. The tubes were shaken vigorously for the uniform mixing. These tubes were incubated for half an hour in the dark. The control was prepared as above but without the plant extract or Gallic acid. Methanol was taken to collect the baseline on the spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The absorbance was taken on spectrophotometer at 517 nm.

Now the radical scavenging activity was calculated using the following formula.

$$\% \text{ Radical scavenging activity } = [\frac{(\text{Control abs} - \text{sample abs})}{\text{Control abs}}] \text{x}100\%$$

3.7.2 ABTS Antioxidant Assay

To the 1ml of diluted ABTS solution, 10µl of different concentration of sample solution was added and the absorbance of the resulting solution was measured at 734nm after 6 minute. Gallic acid was used as positive control. The solvent (methanol) and ABTS cation solution without extract was taken as Sample blank and control respectively.

Radical scangavanging activity (RSA), which is equivalent to decolorization of ABTS solution, was calculated as:

$$\% \text{ Radical scavenging activity } = [\frac{(\text{Control abs} - \text{sample abs})}{\text{Control abs}}] \text{x}100\%$$

In both Antioxidant assay, Standard graph was plotted taking the concentration on the X-axis and percentage scavenging activity on the Y-axis. Based on % radical scavenging data, EC_{50} value of each sample was calculated based on the formula:-

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

Where, exp: exponential function, ln: is natural log function, Conc >50% =concentration of signal >50% and conc <50%= concentration of signal <50%. All the tests were performed in triplicates (n=3) and calculated as described by Maes and Cos (2010).

The EC_{50} value of the different species was compared. The species having the lowest EC_{50} is considered to have the best antioxidant property.

3.8 Antimicrobial activity Assay

The antimicrobial activity of different extract was assayed by the agar well diffusion technique (Devi et al., 2007; Sudha et al., 2011). It was done by fixed volume per milligram of extract solution placed on the equally sized well bored on the agar media with bacterial culture along with positive control and negative control i.e. antibiotic disc and solvent (methanol) respectively. The halo zone or clear zone formed around the well which is known as Zone of Inhibition (ZOI) was measured to screen the antimicrobial activities. This method is qualitative method rather than quantitative. For the assay, five strains of Gram negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853), *Salmonella typhimurium* ATCC 14028 and *Serratia marcescens* ATCC 13880 and a single strain of Gram positive bacterium (*Staphylococcus aureus* ATCC 25923) were used. The strains were obtained

from National Public Health Laboratory, Teku, and Nepal Academy of Science and Technology (NAST), Lalitpur Nepal. All tests were carried out in Triplicate.

3.8.1 Preparation of Culture media necessary

Nutrient agar (NA)

About 28 gram of nutrient agar powder (Hi Media Laboratories Pvt. Ltd, India) was carefully weighed and poured in distilled water. Then the media was sterilized on an autoclave at 15 lbs pressure at 121°C for 15 minutes. The autoclave tape was used as an indicator for the completeness of sterilization. After that the media was taken out of the autoclave and cooled to about 45-50°C and poured on sterilized and properly labeled petri-dishes. About 20 ml of the media was poured on each petri-dishes of 9 cm diameter. After that plates were left for the solidification. The pouring process was carried out on the laminar airflow cabinet.

Luria Bertani (LB) media

The Luria Bertani (LB) broth is a liquid media. About 6.25 gram (Hi Media Laboratories Pvt. Ltd, India) of LB media powder was carefully weighed and transferred on a conical flask. The content was dissolved in distilled water and final volume was maintained to 250 ml. Then the media was transferred to the screw bottles and sterilized on autoclave at 15 lbs pressure and 121°C for 15 minutes. Autoclave tape was used for the indication of the completeness of the sterilization. Finally that media was cooled in laminar airflow and was used for the suspension bacterial culture.

Preparation of Mueller Hinton Agar (MHA)

About 38 grams of Mueller Hinton agar (MHA) powder (Hi Media Laboratories Pvt. Ltd, India) was weighed and suspended in distilled water. The final volume was maintained 1000 ml. The content was heated to boiling to dissolve the medium completely. The media was sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes. The media was mixed carefully with gentle swirling before pouring. The media was poured on sterile petridishes under aseptic conditions for further proposes.

Preparation of the standard culture Inoculums

The individual pure cultures of bacteria were streaked on the different nutrient agar plates. Those plates were incubated on the incubator at 37°C for about 24 hours and pure and isolated colonies were obtained. Each distant colony was aseptically transferred to the Luria Bertani Broth (LB broth) for the suspension culture with the help of the sterilized inoculating loop. The inoculated bottles were kept on the shaking incubator at 37°C and 120 rpm for overnight. The turbidity of the bacterial suspension

was adjusted at the 0.5 McFarland standards (1.0-1.5 X 10^8 CFU per mL) for the antibacterial test. These inoculums were used for the swapping of the plates to test the antimicrobial effects of the plant extracts.

Transfer of the bacteria on the petriplates

The test plates for the antimicrobial activity were first labeled with date, name of bacteria, and name of the plant species and the concentration of the plant extract to be added. The MHA plates were inoculated with the appropriate bacterial culture by a sterile cotton swab. Different swabs were used for different bacterium. The culture plates were allowed to dry for about five minutes.

3.8.2 Antibacterial test

The antimicrobial test was performed by modified agar diffusion method as described by Sudha et al. (2011) with slight modification. On the above prepared MHA plates five wells were prepared on the solid MHA media with the help of the sterile borer of 0.4 mm diameter. The Extract samples were prepared on the methanol. With the help of the sterile pipette the 40 µl of the each individual plant extract were poured in the above prepared well. The methanol was taken as negative control while the Antibiotic disc Amikacin and Nitrofuranioin (10 mcg/d) was taken as the positive The plates were incubated on the microbial incubator overnight at 37°C and the zone of inhibition was observed and noted for individual bacteria with respect to individual plant extract.

3.9 Assay for cytotoxicity activity

Cytotoxicity assay was conducted by MTT Assay. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. Then absorbance of that colored solution was quantified by measuring at a 540nm wavelength by a spectrophotometer. The reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent was compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells could be deduced, through the production of a dose-response curve (Garcez, 2002).

3.9.1 Macrophage isolation and culture

Peritoneal macrophages of a healthy BALB/C mouse were used to study the cell toxicity of the different concentration of the Plant extract. The peritoneal macrophage from BALB/C mice was isolated and cultured according to the protocol of Lusis (1990) with slight modification.

Briefly, a BALB/C mouse was injected with 2% starch in its peritoneal cavity 2 days prior to macrophage extraction. The mouse was then anesthetized by chloroform and it was eutanize and with sterile forceps and scissors laparotomy incision, short, horizontal incision at the abdominal region, was done to expose its peritoneal cavity. About 10ml harvest medium (i.e. cold incomplete Roswell Park memorial Institute iRPMI) was injected in peritoneal cavity with syringe and using the same syringe, the peritoneal fluid was aspirated along with the harvest medium. The fluid was then centrifuged at 4°C at 1000 rpm for 10 minutes. The pellet was washed with iRMPI for two times and finally re-suspended in 1ml complete RPMI (cRPMI). The macrophage was counted using Neubauer's chamber and the volume was made upto 4ml adding cRPMI and the cell suspension was then transferred to the T25 culture flask and incubated for 1 hour at 37°C in a humidified CO_2 incubator containing 5% CO_2. Non adherent cells were removed by gently washing three times with warm phosphate buffer saline. For execution of cytotoxicity assay, the cells were detached from flasks using 0.25 % trypsin/ EDTA solution and seeded in 96-well tissue culture plates.

3.9.2 MTT Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as described by Garcez (2002) with slight modicication as needed. Cytotoxicity assay was performed on culture of primary mouse peritoneal macrophages by determining the percentage reduction in cell viability by the reference drugs and extracts.

Briefly, macrophages was well seeded on 96 well culture plate with a concentration of approx. $2x10^4$ cells/well in a complete RPMI-1640 medium. The plates were incubated in a humidified CO_2 incubator containing 5% CO_2 at 37°C for 4 hours. After incubation, cells were washed with PBS to remove non-adherent cells and 100 µl of fresh complete RPMI medium was dispensed. Then 100µl reference drugs were serially diluted from concentrations 200 µg/mL to 6.25 µg/mL and extracts were also serially diluted from 4mg/mL to 0.03125 mg/mL. Each dilution was dispensed in each well in a series of four fold dilution and plates were incubated for 72 hours at 5% CO_2 at 37°C. After completion of incubation, 50 µl of 0.5% MTT was added to each well and plates were further

incubated for 4 hours. Then 100 µl of DMSO was added in each well to dissolve blue formazan formed by reduction of yellow tetrazolium salt within the cells. The absorbance of the plate was read at 540 nm. The experiments were performed in triplicate and cells without extract were taken as control. Results were expressed as percentage reduction in cell proliferation. Wells with

3.10 Statistical analysis

All the experiments were performed in triplicates and the data were represented as mean ± standard deviation from three independent assays. The Effective Inhibitory concentration or EC_{50} value and correlation between total polyphenol, flavonoid and antioxidant were computed using Microsoft Excel 2007 (Microsoft Corporation, USA) and GraphPad Prism version 5 (Graphpad Software Inc.). An unpaired t-test (two-tailed) was computed to assess the statistical significance. P value <0.001 was considered significant.

CHAPTER 4: RESULTS

4.1 Yield of Plant extracts

Seven plant samples collected were subjected for the methanolic extraction. Maximum yield was obtained in *Euphorbia hirta* with 42% and lowest yield was seen in *Duranta repens* with 9.3%. The consistencies of extracts were thick and sticky except for *Woodfordia fruticosa* which was brownish and crystalline extract (Table 4.1).

Table 1 Percentage Yield and Physical characteristics of the crude methanolic extracts

Plant	Characteristics Of Extracts		Dry Wt. taken (gm)	Wt. of Extract (gm)	Percentage yield (%)
	Color	Consistency			
Achyranthus aspera	Dirty green	Semi solid	10	2.02	20.2
Artemisia vulgaris	Dark brown	Liquid	10	4.05	40.5
Citrullus colocynthis	Brown	Sticky	10	1.1	11
Duranta repens	Blackish	Very Sticky	10	0.93	9.3
Euphorbia hirta	Dark brown	Slight Sticky	10	4.2	42
Urtica dioica	Greenish Brownish	Very Sticky	20	2.02	10.1
Woodfordia fruticosa	Brown	crystalline	10	3.2	32

4.2 Phytochemical screening

Preliminary Phytochemical screening of crude methanolic extract of samples were performed. Total alkaloid, Carbohydrate, saponin, Cardiac Glycoside, Phytosteroid, terpenoid, Protein and Amino acid, Phenolic compound, Tanin, Flavonoid, Resin and Fixed oil and Fats were performed.

Total Alkoloid, Carbohydrate, Phytosterol, fixed oil and fats were found to be present in all seven plants taken in this study. Saponin was detected in all plant extract but not found in *A. vulgaris*. Cardiac Glycoside was not detected in any plants but only in *D. repens*. Tanin was detected by Bramer’s test in all but *W. fruticosa* and *A. aspera* (Table 4.2).

Table 2 Phytochemical Screening test

Test performed	A. aspera	A. vulgaris	C. colocynth	E. hirta	D. repens	U. dioica	W. fruticosa
Alkaloid test							
Mayer's test	-	+	+	+	+	-	+
Wagner test	+	+	+	+	+	+	+
Carbohydrate test							
Molish test	+	-	+	+	+	+	-
Fehling's test	+	-	+	+	+	+	-
Benedict test	-	-	-	-	+	-	-
Saponin test	+	-	+	+	+	+	+
Cardiac Glycoside test							
Kellar killani test	-	-	-	-	+	-	-
Phytosteroid and terpenoid test							
Liebermann-Burchard's test	+	-	+	-	+	+	+
Salkawski Test	+	+	+	+	+	+	+
Protein and Amino acid detection							
Xanthoproteic test	-	-	+	+	-	-	-
Biuret test	+	-	+	+	+	-	-
Phenolic compound test							
Ferric chloride test	+	+		+	+	+	-
Alkaline reagent test	-	+	+	+	+	+	-
Tannin test							
Bramer's test		+	+	+	+	+	-
Gelatin test	-	-	-	-	-	-	-
Flavonoid test							
Shioda test	+	+	+	+	-	-	+
Lead acetate test	-	-	+	+	-	+	+
Resin test							
Acetone water test	-	-	-	-	-	-	-
Fixed oil and fats test							
Spot test	+	+	+	+	+	+	+

= Positive(Detected) - = Negative (Not detected)

4.3 Total Polyphenol content determination

Total Polyphenolics content were calculated and results were expressed as mg Gallic Acid Equivalent (GAE)/gram dry weight of extract. For this, Standard Calibration Curve which was drawn in Microsoft Office 2010 (appendix) using various concentration of Gallic acid. The equation of standard curve was $y = 0.0035x + 0.0269$ ($R^2 = 0.9701$).

Among the seven plant, the highest phenol content (Fig4.3) was of *Euphorbia hirta* with 389±14.8 mg GAE/g dry wt followed by *Duranta repe*ns 383±7.42 mg GAE/g dry wt. Polyphenol content of *Urtica Dioica, Citrulus colocynthis, Artemisia vulgaris* and *Woodfordia fruticosa* was found to be 195.18±4.95, 165.07±1.84, 164.12 ±2.31 and 100.12 ±1.84 mg GAE/g dry wt respectively. *Achyranthus aspera* showed lowest polyphenol content of all with 55.46±1.41 gm GAE/g dry wt.

Graphical Representation of Total polyphenol content of different plant extracts had been given below:

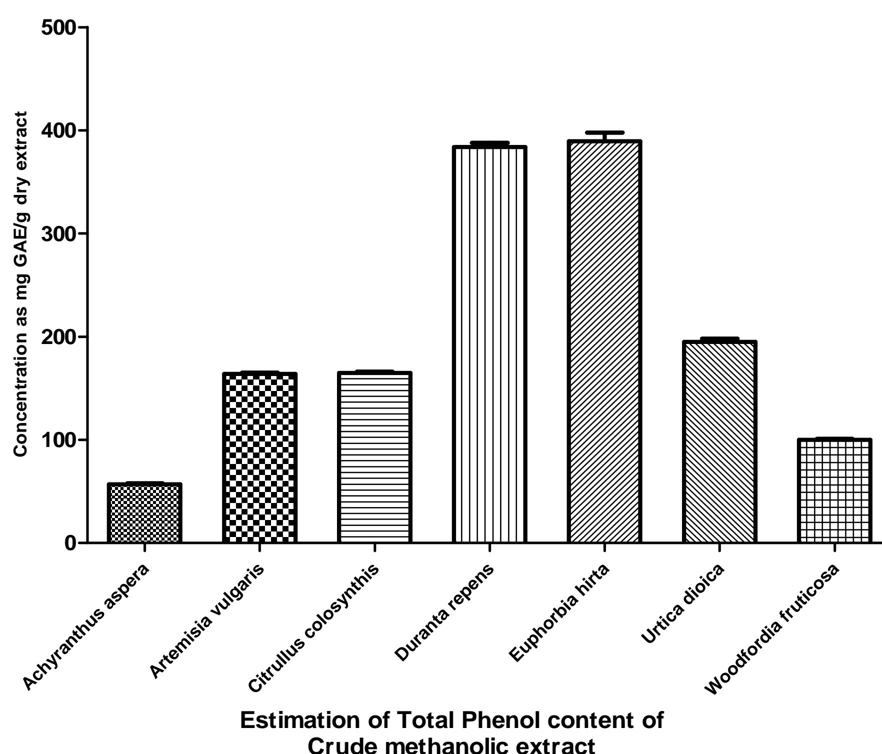

Figure 1 Estimation of Total Polyphenol content of Crude methanolic extract

4.4 Total Flavonoid content determination

Total flavonoid content in plants was estimation using standard curve of Quercetin as standard flavonoid (appendix). The line of equation was generated by Microsoft Excel 2010 which was Y = 0.0101x - 0.0134 (R^2 = 0.9974). Estimated Flavonoids content in the crude extracts were expressed in mg Quercetin/g dry weight of plant material. *Artemisia vulgaris* showed highest flavonoid content of 168.65±1.98gm QE/g dry wt, followed by *Urtica dioica* with 147±0.56gm QE/g dry wt of extact. Similarly *Citrulus colocynthis, Duranta repens, Euohorbia hirta* and *Woodfordia frutica* have flavonoid content of 70.40±0.35 gmQE/g dry wt, 75.81±2.37 gm QE/g dry wt, 96.88 gm QE/g dry wt, 68.22±0.06 gmQE dry wt respectively. *Achyrantus aspera* showed the lowest amount of flavonoid with 17.89±0.6 gm QE/g dry wt. Graphical representation of the Flavonoid content are shown below:

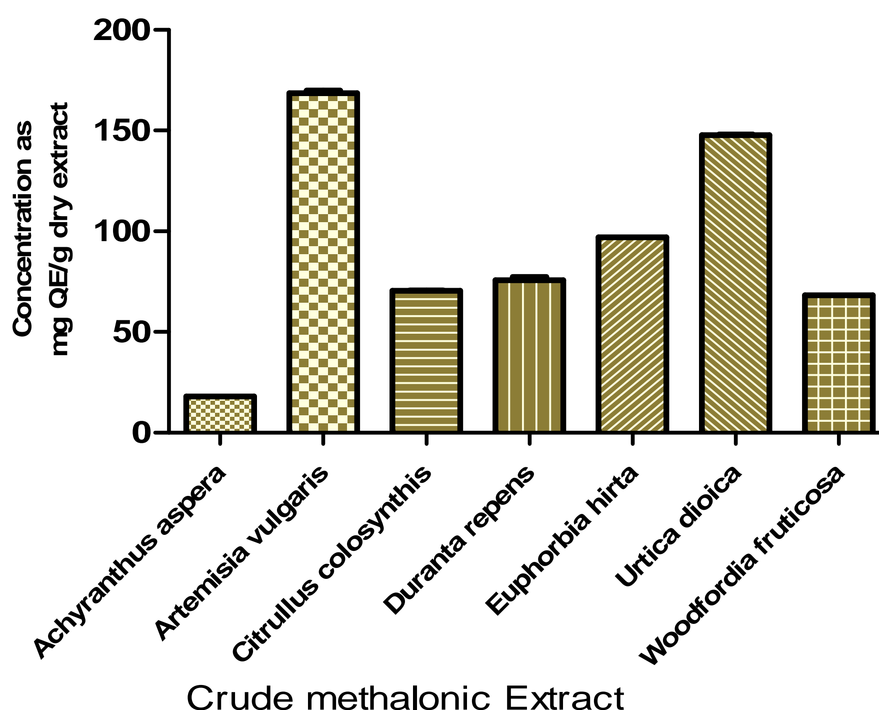

Figure 2 Estimation of Total Flavonoid content of crude methanolic extracts.

4.5 Antioxidant Activity

4.5.1 DPPH Free Radical Scavenging Activity assay

DPPH was used as source of free radical which would react with plant extract and change in coloration of solution from Violet to yellow is due to the radical scavenging activity of plant extract. The absorbance was measured at 517nm with Gallic acid as standard solution. EC_{50} value of standard compound Gallic acid was 0.04mg/ml. Lowest EC_{50} value was of *Duranta repens* of about 0.06mg/ml whereas *Woodfordia fruticosa* showed highest Ec_{50} value of 0.3mg/ml followed by *Achyranthus aspera* and *Artemisia vulgaris* with 0.21mg/ml and 0.13mg/ml respectively. Others plant extract had relatively similar EC_{50} value which is shown in table 4.5.1

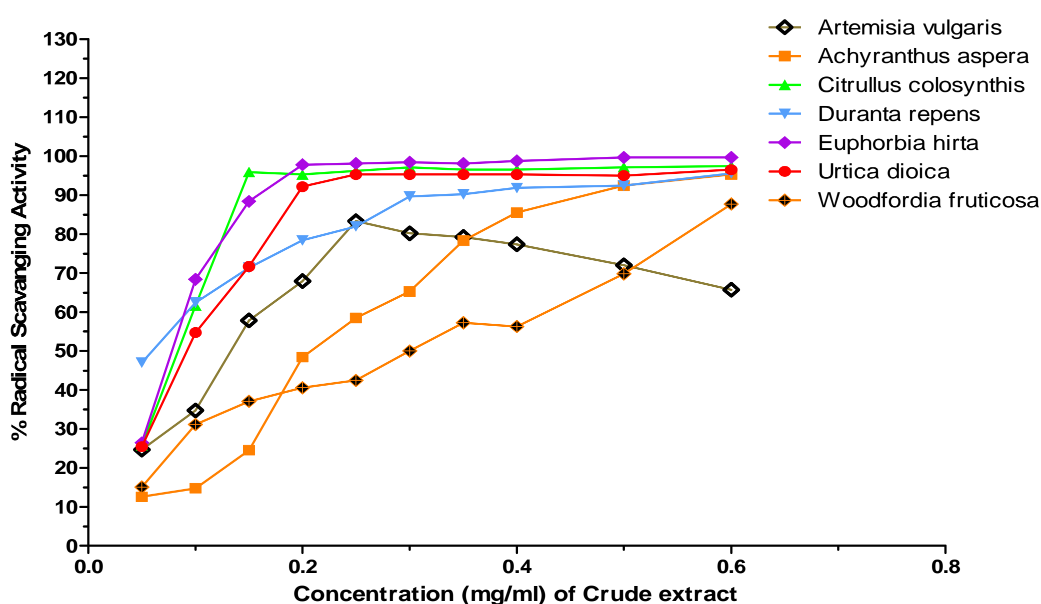

Figure 3 DPPH Free radical Scavenging Activity Assay

Table 3 EC_{50} values of Crude methanolic extracts in DPPH Assay

Sample Methanolic extract	EC_{50} value Mg/ml
Artemisia vulgaris	0.13
Achyranthus aspera	0.21
Citrullus colocynthis	0.08
Duranta repens	0.06
Euphorbia hirta	0.07
Urtica dioica	0.09
Woodfordia fruticosa	0.3

Gallic acid	0.04

4.5.2 ABTS Antioxidant Assay

ABTS Radical Scavenging assay was performed to determine total antioxidant activity of the sample and the efficacy of antioxidant capacity was compare with Gallic acid as a standard antioxidant compound. ABTS was reacted over night to get the ABTS•+ which is blue in color and reduction of ABTS was indicated by decolorization of solution upon addition of extract which was measured by taking absorbance 734nm. All the seven extracts showed regular scavenging activity with each other. Similarly, *Euphorbia hirta* sample was not studied due to formation precipitate upon addition of ABTS+ radical solution. Highest EC_{50} was displayed by *Achyranthus aspera* with 5.98 µg/ml followed by *Artemisia vulgaris, Urtica dioica* with 5.68 and 4.12 ug/ml repectively. *Duranta repens* shows lowest EC_{50} value of 1.48 ug/ml (Table 4.5.2). Percentage Radical scavenging curve is shown below

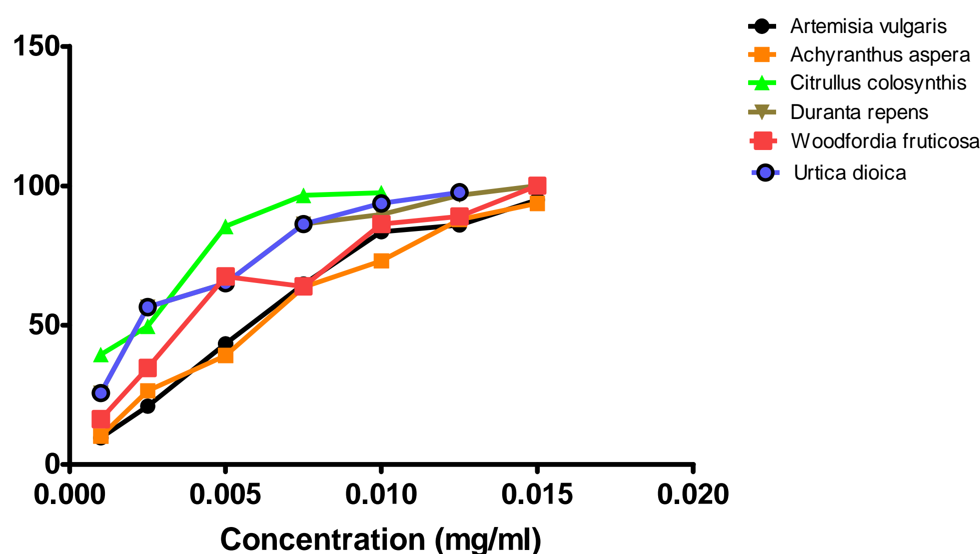

Figure 4 ABTS Antioxidant Activity Assay

Table 4 EC_{50} values of Crude methanolic extracts in ABTS Antioxidant Assay

SN	Sample Methanolic extract	EC_{50} value ug/ml
1	*Artemisia vulgaris*	5.68
2	Achyranthus aspera	5.98
3	Citrullus colocynthis	2.52
4	Duranta repens	1.48
5	Euphorbia hirta	-
6	Urtica dioica	4.12
7	Woodfordia fruticosa	3.45

4.6 Antimicrobial Screening Assay

Antimicrobial activity of plant extracts were studies by Agar Well diffusion method. Six bacterial strain were used for antimicrobial screening which were *E. Coli* (ATCC 25922), *K. Pneumonia* (ATCC 700603), *Enterobacter sp*(ATCC 23373), *S. Typhimurim* (ATCC 14028), *S. marcesens* (ATCC 13880) and *S. aureus*(ATCC 25525). Broad spectrum Antibiotic Nalidixic acid was used for reference drug. *E. hirta* seems to inhibit all bacteria except *S. marcesens. A. vulgaris* and *W. fruticosa* inhibit two bacterial strain whereas *D. repens* and *Urtica dioica* inhibit 3 strains. The Respective inhibition of bacterial strain is given below in table 4.6

Table 5 Antimicrobial activity of methanolic extracts of plants.

Plants	Zone of Inhibition (cm) Diameter of well= 0.8cm					
	E. Coli 25922	*K. Pneumonia* 700603	Enterobacter sp 23373	*S. Typhimurim* 14028	*S. marcesens* 13880	*S. aureus* 25525
A. vulgaris	0	0	0	1.2	0	1.5
A. aspera	0	0	0	0	0	0
C. colocynthis	0	0	0	0	0	0
D. repens	0	0	2	1.4	0	1.4
E. hirta	1.7	2.1	2.4	2	0	1.4
U. dioica	0	0	1.2	0.8	0	1.1
W. fruticosa	0	1.3	0	1.3	0	0
Methanol	0	0	0	0	0	0
Nalidixic acid	1.9	3.4	2.9	1.9	1.7	3

4.7 Assay for Cell toxicity activity

Cell cytotoxicity was tested with mouse peritoneal macrophage. The reference drug, Fluorouracil was taken for comparative study of cell toxicity of extract. Cell toxicity CC_{50} value of reference drug was found to be 31µg/m. *D. repens* had highest CC_{50} value with 445.45µg/ml, followed by *A. vulgaris* with 224.373 µg/ml. *U. dioica, A. aspera, C. colocynthis and E. hirta* have CC_{50} value of 220.894 µg/ml, 189.273 µg/ml, 147.301 µg/ml and 132.031 µg/ml respectively. *W. fruticosa* have lowest CC_{50} value with 65.181 µg/ml (Table 4.7)

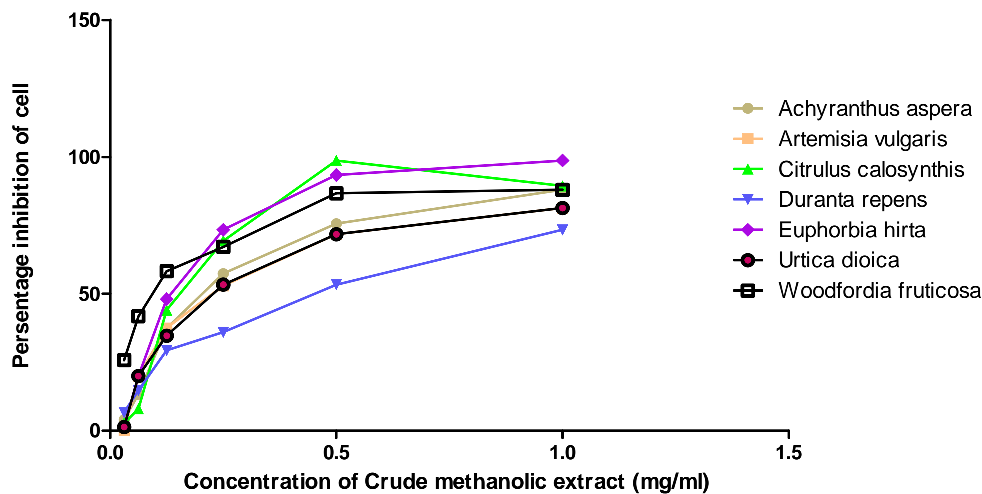

Figure 5 Percentage inhibition of mice peritoneal Macrophage by crude methanolic extract

Table 6 CC_{50} value of crude methanolic extract on mice peritoneal macrophage.

Plants	CC_{50} µg/ml
Duranta repens	445.449
Artemisia vulgaris	224.373
Urtica dioica	220.894
Achyranthus aspera	189.273
Citrulus colocynthis	147.301
Euphorbia hirta	132.031
Woodfordia fruticosa	65.181
Fluorouracil	31

4.8 Correlation between Total Polyphenol and Flavonoid content with Antioxidant activities

Correlation coefficient (r) of polyphenol to DPPH and ABTS was found to be 0.79 and 0.62 whereas correlation coefficient of Flavonoid with DPPH and ABTS was found to be 0.8 and 0.6 respectively. Correlation between Total polyphenol content with antioxidants were found to be satisfactory and result in acceptable level of 0.6 – 08.

CHAPTER 5: DISCUSSION

Each year new important phytochemicals are being identified and assayed for their uses for medicinal purpose (Handa et al., 2008). Several bioreactive molecules can and have been identified by use of traditional folk medicinal plants (Tastai et al., 2002). Environmental variation cause stress on plant due to which its composition in terms of antioxidants and other phytochemicals also varies thus those environmental change in turn enhance or retard the medicinal properties of plant which may not be detected in molecular analysis but can be determined by biochemical tests (Saleh and Plieth, 2009). This research was also done to compare the result of some plants extract's activity with the result with other research so far carried out. All the plants above have ethnobotanical significance and are still being used in traditional medicine as direct or indirect route (Riehemann et al., 1999; Shahat et al., 2005; Khan, 2006; Khan and Gilani, 2009; Kumar et al., 2010; Borhade et al., 2013; Vermaa et al., 2013).

Plants were shade dried for this study. It was done so as to prevent bioreactive compounds present in it (Doughari, 2012). Methanol was used as solvent because it is capable to dissolve polar solute more efficiently then ethanol and after extraction, it is more convenient for evaporation as it is more easily evaporate at low temperature as 40^0C under pressure (Cowan, 1999). Methanol can extract high number of compounds than other alcohol. It is because most of plant matrix contain medium sized bioreactive compound with π electron which is highly polarizable by polar solvent (Kumoro et al., 2009). Though, quantities of extraction depend on other factors like polarity, temperature, solvent concentration, metabolite composition.

Phytochemical screening was done to detect the presence of various components like alkaloid, phytosterol, saponin, tannin, cardio glycosides, carbohydrate etc. in the obtained extract by use of methanol. Resin was not found in any of the extract. It may be because methanol is not much selective to resin extraction (Cowan, 1999). *A. aspera* showed similar result to phytochemical screening as Reddy et al. (2011) and Rani et al. (2012). Similarly results was found in *C colocynthis* by Jayaraman and Christina (2013), *D. repens* by Sharma et al. (2012).

Total Polyphenolic content was found from 390 mg to 56 mg GAE/ gm of dry weight. Since phenolic components of the plant are important class of compounds that acts as good antioxidant and free radical scavengers, the plant said to be more potent as medicinal used if it have high phenolic content (Pourmorad et al., 2006). But having high content of phenol and antioxidant doesn't always mean it is safe and good for health so further analysis have to be done (Halliwell, 2009). *Euphorbia hirta* showed highest phenolic content. Basma et al. (2011) reported to found similar result like our findings.

In case of *Urtica dioica,* Otles and Yalcin (2012) reported that there was twice as much as total phenol present in their experiment than this study. Total phenol content was estimated by Folin-Ciocalteu method which is based on transfer of electron form phenolic compound of extract to Phosphomolybdic/ phosphotungstic acid complex present in F-C reagent giving bluish chromophore which is then determined spectrometrically at 765nm (Ainsworth and Gillespie, 2007; Blainski et al., 2013). Phenol content in the food is regarded to have significant effect on animal species, like it reduces the inflammation in several cardiac heart disease by oxidative down regulation of Low Density Lippoprrotein LDL (Serafini et al., 2000). They also have acts as antioxidant and prevent form cancer. Limited research have suggested that polyphenolic diet may decrease the risk of chronic disease like diabetes, cataracts, macular degeneration, rheumatoid arthritis, neurodegenerative diseases and hypertension (Yordi et al., 2012). Furthermore, interest of phenolics are increasing in food industry due to its ability to reduce auto oxidation or rancidity of dietary lipids in food thereby improving quality and nutritional value of food (Shukla et al., 2012).

Flavonoid is subgroup of polyphenol group found in the plant. *Artimemisia vulgaris* and *Urtica dioica* shows highest amount of flavonoid content whereas *A. aspera* showed lowest flavonoid content. Temraz and El-Tantawy (2008) and Kumar (2010) had found very low flavonoid content in *E. hirta* sample in compare to this study. High flavonoid content in our sample do not coincide with other’s report which may be due to environmental variation, time of plant condition and its maturity, soil and other factors (Bhattarai, 1998). Contrary, *U. dioica* had showed high flavonoid content in this study and results are back by study done by Chaurasia and Wichtl (1987) and Akbay et al. (2003), which was pretty close to our result. About 8-9 antioxidant flavonoids so far have been identified from *U. dioica*. Similarly, Rama et al. (2013) also reported that *A. aspera* have very low amount of flavonoid content as this study. During experiment, the color decolorization occurs which is due to Aluminum chloride froming a complex with functional group of flavonoid ring thus forming stable acid labile compound whose absorbance is maximum at 415nm (Chang et al., 2002). As flavonoid is as part of polyphenolic group, it has too multiple biological effects as important antioxidant, anti-inflammatory, anticancerous, anti-ischemic, antiviral and antibacterial and vasodilators (Prochazkova et al., 2011). Flavonoid has also shown neuromodulatory effects on age related dementia and Alzheimer’s disease as well as neuroprotective agent in Perkinson’s disease (Nijveldt et al., 2001; Spencer, 2008)

Antioxidant are considered as important nutraceuticals due to its health benefits in food industries (Deng et al., 2011). In food, antioxidant helps to inhibit lipid peroxidation, and

other degradation cause by free radicals n reactive oxygen species, and human body it prevent activities of reactive free radicals which facilitates development of numerous disease like cardiovascular disease, cancer, neurodegenerative disease, Alzheimer and other inflammatory disease (Deng et al., 2011; Krishnaiah et al., 2011; Shukla et al., 2012). Synthetic antioxidant like Butylated hydroxyanisol BHA, Butylated hydroxytoluene BHT etc shows some side effects due to which search of Natural antioxidant is considered as prior to other research in plant phytochemistry nowadays (Ito et al., 1985; Shukla et al., 2012). Antioxidant properties of plant may be differs from location to location of same species or due to change in experimental parameters. A single assay cannot give full quantity of any chemicals even (Chu et al., 2000). In this study, two antioxidant assays was implied namely DPPH and ABTS radical scavenging assay as they are must common, recently modified, rapid and reliable method than any other (Krishnaiah et al., 2011) . DPPH (1,1-diphenyl-2-picryl-hydrazyl) is stable free radical due to delocalize electron due to which it give deep violet color with high absorbance at 515-517nm. When DPPH react with hydrogen donor or free radicals, it become reduces and violet color become faint yellow due to picryl group (Molyneux, 2004). ABTS (2,2 Azino,bis-3-ethylbenzothiazioline-6-sulfonic Acid) is also single free radicle assay in which blue chormophor $ABTS^{+\bullet}$ radicle is developed by reaction between ABTS and potassioum persulfate 16-20 hours prior to experiment. The absorbance is taken at 735nm (Thaipong et al., 2006; Walker and Everette, 2009). ABTS assay seems to have much advantages on DPPH as the radical are priory generated, no cross reaction between free radical an radical generating steps could occur and $ABTS^{+}$ radical react with both hydrophilic and lipophilic system, whereas DPPH is much more stable and is significant in hydrophobic system only (Re et al., 1999; Floegel et al., 2011).

In DPPH free radical scavenging assay, *W. Fruticosa* and *A. aspera* showed highest EC_{50} value whereas *D. repens* showed lowest. EC_{50} represent effective concentrations of extract which scavenge 50% of Radical, so higher the EC_{50} value is lower the antioxidant activities. So, *D. repens* can be considered to have high antioxidant properties from this study. This result coincided with Total Polyphenolic content of *D. repens* as it has highest Polyphenol content after *E. hirta*. Shahat et al. (2005) and Abou-Setta et al. (2007) also reported that *D. repens* has high antioxidant properties and reasoned this high value is due to various types of glycosides and saponins along with alkaloids and flavonoids present in plant. Furthermore, this test have justified the ongoing theory of having high antioxidant value of plant is due to Total Polyphenolic content of Plant (Halliwell, 2009; Pandey and Rizvi, 2009; Tsao, 2010; Yordi et al., 2012). Similarly in ABTS scavenging assay too showed similar result. *D. repens* shows lowest EC_{50} value showing its high antioxidant properties. EC_{50} value of *E. hirta* in ABTS scavenging assay was not shown

here because even with very low concentration of about 1 µg/ml the $ABTS^+$ radical solution was decolorized completely, this may be due to high total phenolic content of the plant sample. The correlation between total phenol and flavonoid content with antioxidant properties are between 0.6 to -0.8. This justify our study because as the concentration of phenol or flavonoid increase in the sample, more antioxidant properties it would show which inversely give lower effective concentration i.e. EC_{50} value. Similarly, value of r ranges for 0 to +1 and since our value lies between 0.6 to 0.8 so data is statistically acceptable as valid data (Kufs, 2010).

Even though both assays are based on single proton/electron transfer mechanism, EC_{50} value of DPPH was about 10 to 100 times higher than EC_{50} value generated from ABTS. Such large difference in the EC_{50} value may be because DPPH react with hydrophobic free radicals only whereas ABTS radical interact with both hydrophilic and lipophilic radical. Secondly, in DPPH itself being oxidant when added with plant sample containing free radicals, they react with DP PH before DPPH• radical formation giving low quantity of discoloration whereas in ABTS assay radicals are prepared before reaction thus whole radicals are involved in reaction giving high number of reacting molecules that results in low absorbance. Lastly, most antioxidant that reacts with peryoxyl radical may react slowly or doesn't react with DPPH giving lower result than actual concentration (Re et al., 1999; Prior et al., 2005; Shalaby and Shanab, 2013).

Plant contain vast array of phytochemicals among them the secondary metabolite may also present which is produced against microorganism. Phenolics, terpenoids, essesential oils, Alkaloids, Lectins, and Polyacetylenes are major group of antimicrobial present in plant (Cowan, 1999). Thus, assay for antimicrobial activity is also one of important aspect of phytochemical study. Antimicrobial screening was done on the basis of well diffusion method. Well diffusion (or disc diffusion) method is based on the diffusion of antibiotic or antimicrobial compound from point of dispense in Bacterial agar medium. Muller Hilton agar was used for this purpose due to low or no interference of Agar itself to antimicrobial agents like antibiotics or phytochemicals. Minimum Inhibitory Concentration is the use to screening the antimicrobial effect whereas Minimum Bacteriocidal Concentration is used to find concentration at which bacteria are killed completely. 1mg/ml concentration of crude extract solution was used for this study. Six microorganism was used for screening purpose. They are Gram positive *Staphylococcus aureus* (ATCC25525*)*, Gram negative bacteria like *Escherichia Coli* (ATCC259220*), Kelbshiella pneumonia* (ATCC 700603*), Enterobacter sp* (ATCC23373*), Salmonella typhimurim* (ATCC14028*), Serratia marcesens* (ATCC13880*)*. Nalidixic acid a broad spectrum antibiotics, was used as reference drugs. Among sample, *E. hirta* shows

high antimicrobial activities and shows its efficacy against all but *S. marcesens*, whereas *U. dioica* and *D. repens* shows its antibacterial activities against three microorganisms. All three sample showed high Polyphenol content and *E. hirta* even shows highest antioxidant properties which may be reason of high antimicrobial activities (Cowan, 1999). *A. vulgaris* inhibit two orgamism and *W. fruticosa* one. Among sample, *A. aspera* and *C. colocynthis* shows no any antimicrobial activities. There was no any halo zone or Zone of Inhibition (ZOI) was seen in the methanol as control. ZOI is halozone produce by diffusion and inhibition of microorganisms by antimicrobial substance. ZOI is quantitative assay for detection of antimicrobial assay but it can also be uses as quantitative by use of dose variation standard curve with respect to sample (Korgenski and Daly, 1998; Reller et al., 2009). Well diffusion technique depend on diffusion properties of chemicals, its solubility in extract thus non responsiveness of *A. aspera* and *C. colocynthis* may also be due to this reason or due to extraction and storing incompatibility with antimicrobial component of the extract.

Drugs must be clinically trial for assurance for their safety toward human subject. Pharmacological active compound may shows toxic nature to our cells so cell toxicity assay was perform with peritoneal macrophage cells via MTT assay taking synthetic cancer drug 5-Fluorouracil as reference. Fluorouracil has lowest CC_{50} value of 31 µg/ml. Lower the CC_{50} value higher the toxicity of a chemical. *W. fruticosa* have highest cell toxicity with CC_{50} value 65.181 µg/ml, followed by *E. hirta* and *C. colocynthis* with 132.031 µg/ml and 147.3 µg/ml. *A. aspera, U. dioica, A. vulgaris* and *D. repens* had CC_{50} value of 189.273 µg/ml, 220.89 µg/ml, 224.373 µg/ml and 445.449 µg/ml respectively. *D. repens* showed lowest cell toxicity and highest CC_{50} value.

CHAPTER 6: CONCLUSION

The uses of plants in traditional medicine have deeply rooted among human. Recently, the scientific communities have also shown interest in plant derived products used in traditional medicine. However, in Nepal about 20% of medicinal plants have been studied (Bhattarai and Bhuju, 2011) and due to lack of scientific data on proclaimed benefits, their usage have been limited to traditional medicine only. So, to assist in modern medicinal fields, all the possible plants with their applications have to be studied. Therefore, this research is a step to generate valid data on applications of seven medicinal plants of Nepal which have been used in traditional medicine.

The extracted crudes from seven medicinal plants used under this study showed the promising effect on antioxidant assay and antibacterial effect with respect to their phenolic and flavonoid contents. Similar results reported previously along with the correlation coefficient calculated in this study suggest that the results are reliable and the validation of this data is acceptable in terms of statistical parameters. Thus, it can be stated that the traditional uses of these seven plants are justified by this study. Besides, the results of this study have opened up avenues for promotion and preservation of ethno medicine. For pharmacologists, this kind of study forms a basis of drug discovery and development.

Nepal is ecologically diverse. Thus the possibility of commercial cultivation of Medicinal and Aromatic Plants are very high which will ultimately develop the socioeconomic condition of country. Furthermore, instead of exporting medicinal plants to other countries if extraction and purification of phytochemicals and pharmaceutical bioactive compounds could be done in our country, the development of our country is inevitable.

However, polyphenols, flavonoids and other compounds like alkaloids, saponin, terpenes, tannin etc. may be present in plants which have significant effects on antioxidant and cell toxicity. These compounds are not tested separately on this study. Thus further research on isolation, identification and characterization of specific bioactive constituents of plants must be studied before full medicinal significance profiling of any plants.

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APPENDIX

1. Preparation of 1 M Na_2CO_3 -100 ml

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

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

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

3. Preparation of aluminium chloride (10%) -100 ml

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

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

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

5. Preparation of 0.2 mM DPPH solution - 100 ml

DPPH (1, 1- diphenyl-2 picryhydrazyl) has the molecular weight of 394.32 gm/mol. Thus, 100 ml of 0.2 mM 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.

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

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

7. Mayer's reagent:

To 1.358 gm of mercuric chloride, 60 ml of water and 5.0 gm of potassium iodide was added and dissolved in 10ml water. The two solutions were mixed up and the final volume was made 100 ml.

8. Wager's Reagnet

To 1.27 gm of iodine and 2 gm of potassium iodide 5 ml water was added and dissolved, then made the volume upto 100ml with distilled water.

9. Molisch's Reagent

α –napthol or thymole (5 gm) is dissolved in methanol (50ml).

10. Fehling's solution A:

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

11. Fehling's solution B:

Potassium tartrate (173 gm) and sodium hydroxide (50 gm) were dissolved in water and the final volume was made 500 ml.

12. Preparation of ABTS reaction solution

ABTS solution was prepared in a 20 mM Sodium Acetate buffer. Monocation radical of ABTS•+ was generated in a solution of (7.4 mM) ABTS•+ salt solution in 20 mM Sodium Acetate buffer of pH 6.5. Then ABTS solution was oxidized by reacting with 2.45 mM potassium persulphate ($K_2S_20_8$) (R & M Chemicals, UK). The reaction mixture was allowed to stand for 16 hours in the dark at room temperature (24 to 26°C) before use. After incubation of 16-18 hours mixture was diluted about 4-6 fold with Sodium Acetate buffer at pH 6.5, in order to adjust the absorbance of the reaction solution to 0.70 ± 0.02 at ambient temperature at a wavelength of 734 nm.

13. 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

14. Composition of Luria Bertani media

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

Components	gram/L
Casein enzyme hydrolysate	10
Yeast extract	5.0
Sodium chloride	10.0

Final PH 7.5± 0.2

15. 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

16. 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.

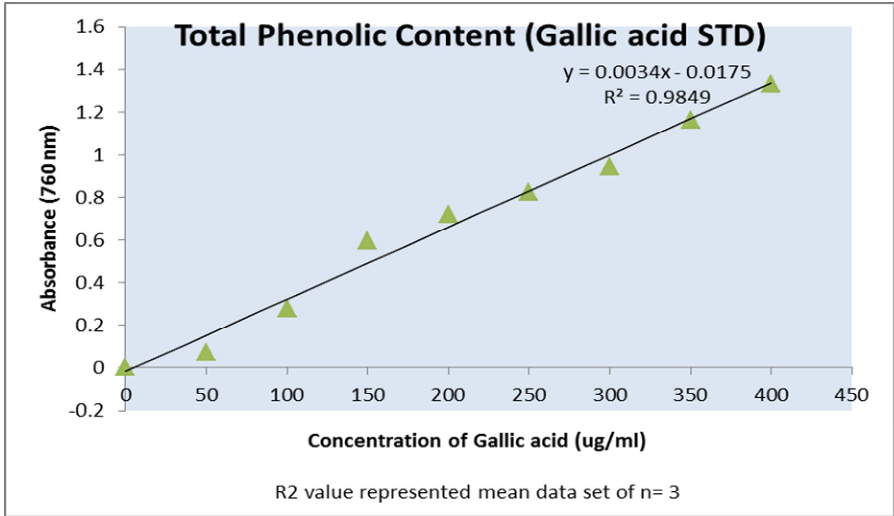
Components	Amount in ml
Sulfuric acid,	0.18 M 99.5

17. RPMI (Roswell Park Memorial Institute) complete medium

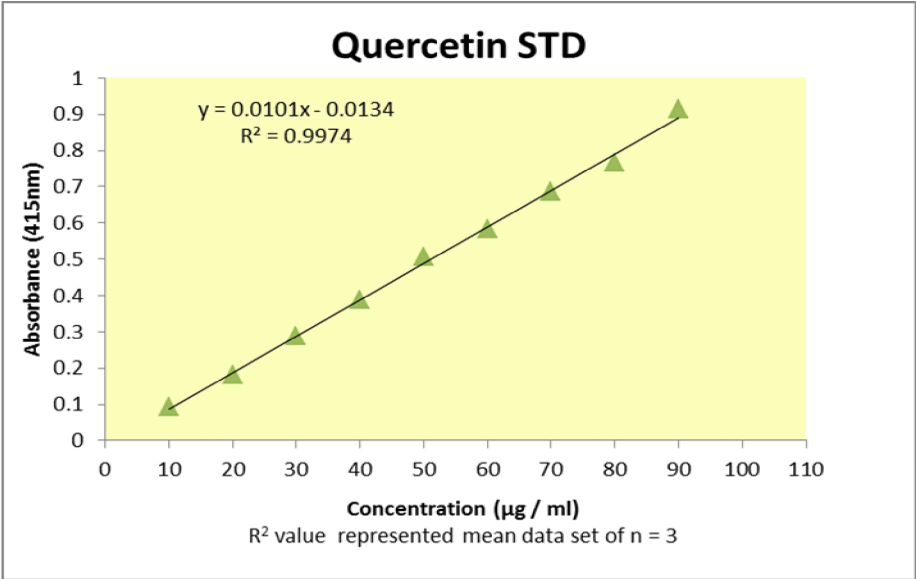
$NaHCO_3$	2.00 gm
HEPES:	1.40 gm
L-Glutamine:	2 mM
TDW:	1 L
Gentamycin:	20 µg/ml
Streptomycin:	100 µg/ml
Penicillin:	100 U/ml
pH:	7.4

18. PBS (Phosphate Buffer Saline)

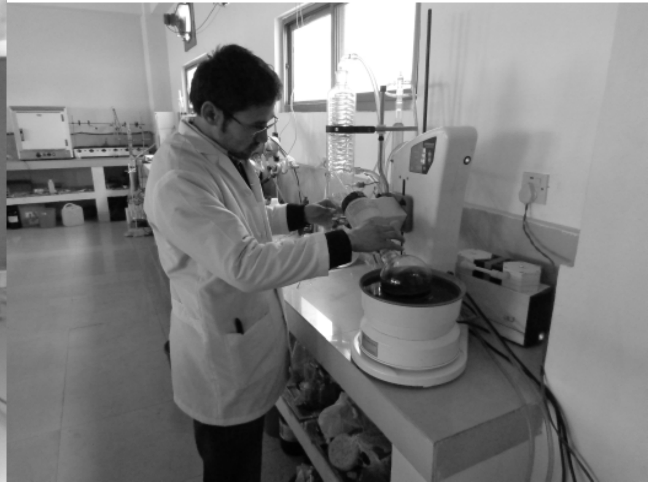
NaCl	: 8 gm
$Na_2HPO_4.2H_2O$	: 1.44 gm
KCl	: 0.2 gm
KH_2PO_4	: 0.2 gm
pH	: 7.3 to 7.4
DDW	: 1 L



19. Standard curve for estimation of Total Polyphenol content.



20. Standard Curve of Quercetin for estimation of Total flavonoid Content.



Soxhlet Extraction

Vacuum Evaporation

Durenta repens

Citrullus colocynthis

Urtica dioica

Woodfordia fruticosa

Extraction of peritoneal Macrophage from Balb/C Mouse

Antibacterial assay