BIOPROSPECTING OF SOME MEDICINAL PLANTS OF NEPAL BASED ON ETHNOBOTANICAL KNOWLEDGE OF THE TAMANG COMMUNITY

M.Sc Thesis 2018

Submitted to:

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For the partial fulfillment of the requirements for the Master of Science in Biotechnology

Pratikshya Mainali

Roll no: BT 210/071

TU Registration: 5-2-37-449-2010

Recommendation

Certificate of Evaluation

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GLOSSARY ACRONYMS

Ul	Microlitre
Ug	Microgram
ATCC	American Type Culture Collection
DMSO	Dimethyl Sulfoxide
DPPH	1, 1-diphenyl-2 picryhydrazyl
DW	Dry Weight
ELISA	Enzyme Linked Immunosorbent Assay
Gm	Gram
GAE	Gallic Acid Equivalent
GC-MS	Gas chromatography-Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
IC50	Inhibitory Concentration 50
MHA	Muller Hinton Agar
Mg	Milligram
Ml	Milliliter
NB	Nutrient Broth
NOD	Non Obese diabetes
OD	Optical Density
PBS	PhosophateBuffer System
QE	Quercetin Equivalent
RSA	Radical Scavenging Activity
RT	Room Temperature
SD	Standard Deviation
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
UV	Ultra Violet
Wt	Weight
ZOI	Zone of Inhibition

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ABSTRACT

Nepal has always been the center of herbal richness with more than ten thousand species of herbs in its alpine belt. The medical herbs databases listing for Nepal shows 1,624 species of medical and aromatic species. This study investigates the medicinal property of some popularly used medicinal plants of Nepal by quantitative and qualitative evaluation of antioxidant, antibacterial and cytotoxicity properties. Eight plants namely, *Cinamomum camphora, Schima wallichii, Reinwardtia indica, Woodfordia fruticosa, Castanopsis indica, Measa chisia, Mimulus nepalensis and Mahonia nepalensis were* selected on the basis of their reported traditional uses in the treatment of various ailments. Preliminary phytochemical screening carried out for detection of alkaloid, steroids, tannins, saponins, polyphenols and terpenoids. Total phenolic and flavonoids content were determined spectrophotometricaly and in vitro DPPH radical scavenging assay was measured. Agar well diffusion method was employed for the antibacterial assays. Fluorescent study was done through administrating different organic solvents and observed under UV light and visible light. Nanoparticles were prepared by the use of $AgNo_3$ and the change in color confirms the nanoparticles synthesis. It is also to assess the cytotoxicity of the extract.

Many extracts showed the considerable value of phenolics and flavonoids. Similarly the antioxidant property was variable as according to the extracting solvent which were comparable to the standard compound querctin.In among all the extract the highest phenol content was observed in *Cinamomum camphora* ethyl acetate extract (381 mg GAE/G dry weight) and flavonoid content highest in ethyl acetate extract of *Castanopsis indica* (160 mg GAE/G dry weight). In case of antioxidant the aqeous extract of *Mahonia* with 138 ug/ml.During the treatment with silver nitrate every sample after colour change was with the peak at 421 nm except *Castanopsis* which was of 447 nm. For fluorescence slight colour change was observed in every solvent in visible and UV light. Antibacterial test was in-effective for hexane extracts whereas for ethyl acetate extracts, *Staphylocoocus aureus* showed highest zone of inhibition of 18 mm by Schima. *Castanopsis indica* showed high zone of inhibition in considering to methanolic while aqeous extracts were ineffective in the entire organism. During the GC-MS of methanolic extracts *Mimulus* was with 29 peaks and *Maesa chisia* with 17 peaks. The Rf value during TLC was different for each extract. For the cytotoxicity ethyl acetate extract was found to be more toxic for *Cinamomum camphora* with 30% while for methanolic extract Woodfordia was with high toxic with same percentage. Various bioassays and quantification along with analysis indicated that various medicinal plant species might be a good candidate against infectious and chronic diseases.

Keywords: Phytochemical, Antioxidant, Cytotoxicity

CHAPTER 1

INTRODUCTION

1.1 Background

Medicinal plants have been used by all civilizations as a source of medicines since ancient times. Interest in medicinal plants as a re-emerging health aid in the maintenance of personal health and well being has been fuelled by rising costs of prescription drugs, and the bio prospecting of new plant derived drugs (Sharma, Ethanobotanical claims, 2012). The plant kingdom can be considered as a treasure house of potential drugs and in recent years. And also there has been an increasing awareness about the importance of medicinal plants. Using the plant for the potential drug development is being increased also due to the increasing awareness of the people as well as other. Drugs from the plants are easily available renewable resource, less expensive, safe, and efficient and rarely have side effects. The plants which have been selected for medicinal use over thousands of years constitute the most obvious choice for examining the current search for therapeutically effective new drugs such as anticancer drugs, antimicrobial drugs (Wright, 1996) and anti-hepatotoxic compounds (Evans, 1996).

The World Health Organization (WHO) has reported that around 21,000 plants had been used for medicinal purpose in the world. About 500 higher species had been thoroughly investigated as potential source of new drugs. Nearly 119 pure chemicals were extracted from 90 plant species. In the recent times, there has been growing interest in exploiting the biological activities of different ayurvedic medicinal herbs (Naik, 2003). In developing countries, a large proportion of the rural population depends on biodiversity for livelihood, nutrition, and health. Higher plants are the major sources of natural products and are used as pharmaceuticals, agrochemicals, flavors and fragrance, ingredients, food additives and pesticides (Clocke, 1988).

Historically pharmacological screening of compounds of natural or synthetic origin has been thesource of innumerable therapeutic agents. Random screening as a tool in discovering biologically active molecules have been productive in the area of antibiotics. Medicinal plants are valuable antimicrobial agents and are a source of many potent and powerful drugs (Cathrine, 2011). The medicinal value of the plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body (Akinmoladum, 2007). These natural compounds formed the foundations of modern prescription drugs as we know today (Goh, 1995). Phytochemical is a natural bioactive compound found in plants, such as vegetables, fruits, medicinal plants, flowers, leaves and roots that work with nutrients and fibers to act as a defense system against disease or to protect against diseases.

Primary constituents comprise common sugars, amino acids, proteins and chlorophyll while secondary constituents consist of alkaloids, terpenoids andphenol compounds and many more such as flavonoids, tannins and so on (Krishnaih et.al, 2004).Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today plant materials continue to play a major role as therapeutic remedies in many developing countries (ody, 1997).

Forests are the principal source of raw material forplant based medicine. Medicinal plants have been used as a major source of therapeutic agents by man for thousands of years. The herbal drugs obtained from the plants are believed to be more effective in the treatment of different ailment (Shiddqui, 2013). More than 90% of raw matter required in the field of pharmaceutical is obtained from the wild resources (Kehimker, 2000). Collection of information and documentation of traditional knowledge plays an important role in scientific research on drug development (S.Ragupathy et al., 2008).

Usually local names of plants vary from place to place, therefore, the correct determination of the plants is only based on the botanical names, and subsequently the information is considered as authentic. Thus, the primary aim of the study is to tap the information in systematic manner. For the proper scientific transformation of traditional knowledge a sound rapport to local communities, understanding of their language and ethanobotanical knowledge of plant species and pattern of utilization system is essential. Throughout the world there has been a great concern to restore the available old traditional practices among different ethnic societies. But unfortunately much of this prosperity of knowledge is today becoming lost as traditional culture becomes ended. So there is a great need of ethno botanical operations for the documentation of this knowledge an important component of traditional wisdom as soon as possible. Various ways have been applied for the drugs formulation from the plants extracts and it also depends upon the ethanobotanical idea of the people how they use it.

In this research work, 8 different plants of different spacies having medicinally important features are choosen and their general background is mentioned below:

1.1.1 Cinamomum camphora

Systematic Classification

Kingdom: Plantae
Division: Angiosperm
Class: Magnoliids
Order: Laurales
Family: Lauraceae
Genus: *Cinamomum*

Species: *camphora*

Figure 1.1 *Cinamomum camphora*

It is commonly called as Camphor tree, camphorwood or camphor laurel. It is native to China south of the Yangtze River, Taiwan, southern Japan, Korea and Vietnam, and has been introduced to many other countries. (Xi-en et.al, 2013)

Botanical Description

It is a large evergreen tree that grows up to 20–30 m (66–98 ft) tall. The leaves have a glossy, waxy appearance and smell of camphor when crushed. In spring, it produces bright green foliage with masses of small white flowers. It produces clusters of black, berry-like fruit around 1 cm (0.39 in) in diameter. Its pale bark is very rough and fissured vertically.

1.1.2 *Schima wallichii*

Systematic classification

Kingdom: Plantae
Clade: Angiosperm
Division: Eudicots
Class: Asterids
Order: Ericales
Family: Theaceae

Genus: *Schima*
Species: wallichii

Figure 1. 2 Schima wallichii

It is commonly called as needlewood tree, Khiang, Makria, Makrisaal, Kanak; Kapaiwon. It is originated in South Western China.

Botanical Description

Schima is a medium evergreen tree up to 35 m tall. However, in most places it may be seen only 40 ft high. The stem is cylindrical, branchless for up to 25 m, diameter up to 1 m, with a steep buttresses rarely up to 1.8 m high; bark surface ruggedly cracked into small, thick, angular pieces, red-brown to dark grey; inner bark with skin-irritating fibres, bright red in colour. Leathery leaves are elliptic-oblong in shape and look somewhat like Champa (*Michelia*) leaves. Leaf margins are entire or slightly toothed. Flowers are white, fragrant, 3-4 cm across. Sepals rounded. Five white petals are broadly ovate and rounded. There is a dense bunch of orange-yellow stamens in the center. The genus name is derived from the Greek word *skiasma* (shadow), probably referring to the dense crown. *Schima* grows in moist and dry evergreen as well as in mixed deciduous forests. Schima has a fast growth even under infertile soil conditions. Flowering is in April-May and fruiting is in February-March. The main value of *Schima* is its hard and durable timber. *Schima* timber is used for fence posts and beams and boards for house construction. *Schima* wood is little used for fuel because the bark causes irritation.

1.1.3 *Reinwardtia indica*

Systematic classification
Kingdom: Plantae
Clade: Angiosperm
Division: Eudicots
Sub class: Rosids
Order: Malpighiales
Family: Linaceae
Genus: *Reinwardtia*
Species*: indica*

Figure 1.3 Reinwardtia indica

It is commonly called as yellow flax or pyoli and is originated from Northern India and China.

Botanical Description

Glabrous subshrub, to 3 feet; leaves elliptic-obovate or oblong-obovate, mucronate, entire to serrulate; flowers 1-2 inches across, petals united into a tube at base, styles 3-5. It is alao sometimes erroneously grown as *Linum flavum*. Native to mountains of northern India and China.

1.1.4 Woodfordia fruticosa

Systematic Classification
Kingdom: Plantae
Clade: Angiosperm
Division: Eudicots
Class: Rosids
Order: Myrtales
Family: Lythraceae
Genus: *Woodfordia*
Species*: fruticosa*

It is also known as fire flame Bush, Dabai, Dhai, Dhawai and originated from India.

Figure 1.4 Woodfordia fruticosa

Botanical Distribution

Perennialdeciduous shrubs have a numerous fluted branches. This shrub has spreading branches reaching height up to 5-12 ft. Branches of this shrub are long and multi spreading and stem is fluted and long. Bark of the shrub is smooth and reddish brown in color with very thin and small fibrous stripes. Leaves are about 5-9cm long, oblong or ovate and lanceolate. This shrub bears numerous flowers that are bright red in color. Small flowers of this herb grow singly or in groups along the twigs and branches. Every flower of this herb, borne a tiny stem, slender tube, curved and greenish base. Fruits are capsules about 1 cm long, ellipsoid and membranous and it

contains very minute, brown colored smooth seeds. Shrub is overloaded with bright red flowers in the month of February to April and its leaves shed off and new leaves appear. Its fruits appear from April to June.

Fire-flame bush, shinajitea and dhataki all are common names of *Woodfordia.* In Ayurveda this herb is used for various medicinal properties mentioned in Sanskrit scripture. Acharya Charka considers this herb in Asava yoni. Flowers are astringent and analgesics and used for preparing various medicines in ayurvedic medicine system. In ayurvedic scriptures this herb possesses various properties like fertility agent (Garbhasthapaka), anti-diarrheal (controls Atisara and Pravahikakara), Erysipelas (Visarpa), poisoning (Visha) and menstrual disorders (Pradara).

1.1.5 *Castanopsis indica*

Systematic classification

Kingdom: Plantae
Clade: Angiosperm
Division: Eudicots
Class: Rosids
Order: Fagales
Family: Fagaceae
Genus: *Castanopsis*
Species: *indica*

Figure 1.5 Castanopsis indica

It is commonly called as chinquapin or chinkapin. It is originated from Southwestern China.

Botanical Distribution

It is the genus of evergreen trees belonging to the beech family, Fagaceaea. The genus contains about 120 species, which are today restricted to tropical and subtropical eastern Asia. A total of 58 species are native to China, with 30 endemic; the other species occur further south, through Indochina to Indonesia, and also in Japan. The English name chinkapin is shared with other related plants, including the golden chinkapins of the Pacific United States, which are sometimes included within *Castanopsis* but are more often considered a separate but very closely related genus, *Chrysolepsis.*

Figure 1.0.2 *Maesa chisia*

1.1.6 *Maesa chisia*

Scientific classification

Kingdom: Plantae
Phylum: Angiosperm
Class: Dicotyledoneae
Order: Primulales
Family: Myrsinaceae
Genus: *Maesa*
Species*: chisia*

The common name is Bilaune and origin is China.

Botanical Description

Shrubs have 1-2(-6) m tall. Branchlets angular, sparsely brown scaly, glabrescent, lenticellate; pith solid. Petiole slightly canaliculate, 0.7-1.5 cm; leaf blade oblong, elliptic-lanceolate, to oblanceolate, 8.5-14 × 2.5-5.5 cm, papery, glabrous, base cuneate, margin serrate, densely and minutely orange punctate-lineate, apex caudate-acuminate, acumen 0.8-2 cm; midrib and lateral veins prominently raised abaxially, lateral veins ca. 10 on each side of midrib, ending in teeth, submarginal vein absent. Inflorescences axillary on biennial branches, paniculate, 1.5-4 cm in fruit, many branched, glabrous; bracts lanceolate, ca. 0.5 mm; bracteoles ovate, glabrous. Flowers are unknown. Pedicel 1-2 mm in fruit, glabrous.Fruit globose, 3-4 mm in diam., somewhat fleshy, glabrous, not punctate; persistent calyx lobes ovate-triangular, glabrous, punctate at tip, ciliate.

1.1.7 *Mimulus nepalensis*

Systematic classification

Kingdom: Plantae
Clade: Angiosperm
Division: Eudicots
Class: Asterids
Order: Lamiales
Family: Frymaceaea
Genus: *Mimulus*
Species: *nepalensis*

Figure 1.3 *Mimulus nepalensis*

It is commonly called as Nepal Monkey flower*/ Mimulus assamicus and about its origin* the genus now contains only seven species, two native to eastern North America and the other five native to Asia, Australia, Africa, or Madagascar (Barker.et.al, 2012)

Botanical Description

Nepal Monkey Flower is a perennial spreading herb, rooting at nodes, or erect leafy stems 15-25 cm tall. Oppositely arranged elliptic leaves are coarsely toothed and stalked, mostly 2-2.5 cm. Flowers are yellow, tubular, weakly 2-lipped, the upper lip erect, 2-lobed, the lower one 3-lobed, spreading. Flowers are born singly in leaf axils, on stalks which are generally longer than the leaves. Flowers are 1.5-2.5 cm long. Sepal cup is 1 cm long, tubular, distinctly 5-angled, with very short sepals. Sepal cup is inflated in fruit. Nepal Monkey Flower is found in forests and damp place of the Eastern part of Himalayas, from C. Nepal to SW China and Burma, at altitudes of 1200-300 m. Flowering: April-September.

1.1.8 *Mahonia nepalensis*

Systematic classification
Kingdom: Plantae
Clade: Angiosperm
Division: Eudicots
Order: Ranunculales
Family: Berberidaceae
Genus: *Mahonia*
Species: *nepalensis*

Figure 1.4 *Mahonia nepalensis*

It is commonly called as Darruhaldi or Jamanemaandro. It is originated from native to eastern Asia.

Botanical Description

Mahonia is a genus of about 70 species of evergreen shrubsin the family Berberidaceae, native to eastern Asia, the Himalaya North America, and Central America. They are closely related to the genus *Berberis*. Botanists disagree on the acceptability of the genus name *Mahonia*. Several authorities argue plants in this genus should be included in the genus *Berberis* because several species in both genera are able to hybridize, and when the two genera are looked at as a whole, there is no consistent morphological separation except simple vs compound leaves. *Mahonia* typically have large, pinnate leaves 10–50 cm (3.9–19.7 in) long with five to fifteen leaflets, and flowers in racemes which are 5–20 cm (2.0–7.9 in) long.

The genus name *Mahonia* honours the Philadelphia horticulturist Bernard Mcmohan.The type species of the genus is *Mahonia aquifolium*, (Oregon grape) from the Pacific Coast of North America.

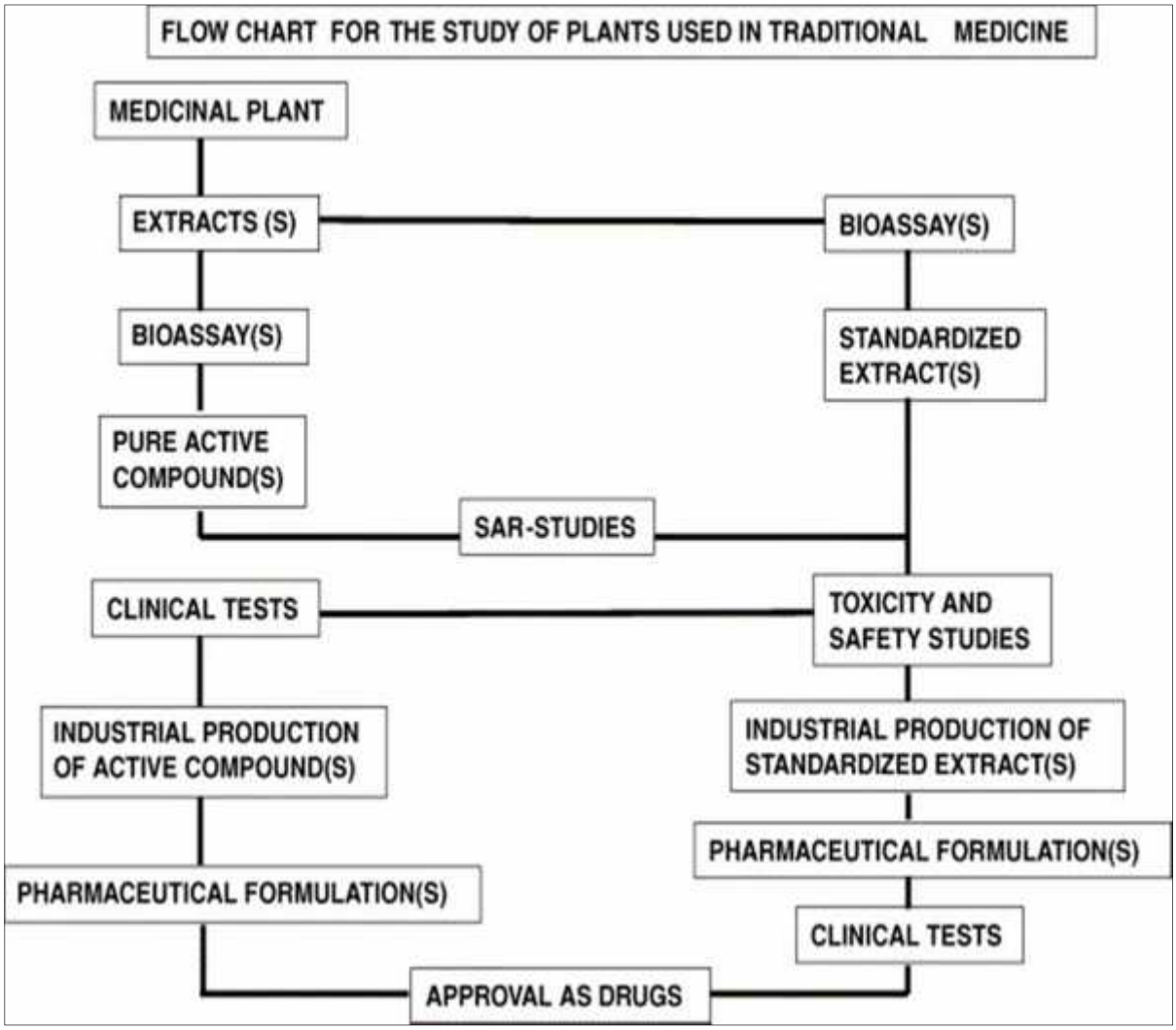

Figure 1.5 Flow chart for the study of plants as a traditional medicine to drug approval (Source: Science direct.com)

1.2 Ethnobotany

Harshberger in 1895 coined the term ethnobotany to indicate plants used by the aboriginals. It included the study and evaluation of plant-human relations in all phases and the effect of plant environment on human society. Subsequently Schulte's (1962) defined ethnobotany as "the study of the relationship which exists between people of primitive societies and their plant environment."

Ethnobotany is the study of regions plants and their practical uses through the traditional knowledge of a local culture and people (USDA Definition). An ethnobotanist thus strives to document the local customs involving the practical use of local flora for many aspects of life, such as plants as medicines, foods and clothing. (Bio cyclopedia; Ethnobotany)

Richard Evans Schultes, often referred to as “Father of Ethnobotany” explained the discipline in this way: Ethnobotany simply means investigating plants used by primitive societies in various parts of the world (Kochchar, 2016). Since the time of Schultes, the field of ethnobotany has grown from simply acquiring ethno botanical knowledge to that of applying it to a modern society, primarily in the form of pharmaceuticals.

Beginning in the 20th century, the field of ethnobotany experienced a shift from the raw compilation of data to a greater methodological and conceptual reorientation. This is also the beginning of academic ethno botany. Today the field of ethnobotany requires a variety of skills: botanical training for the identification and preservation of plant specimens; anthropological training to understand the cultural concepts around the perception of plants; linguistic training, at least enough to transcribe local terms and understand native morphology, syntax, and semantics. "Ethnobotany is a term used to refer to the academic discipline that deals with people’s interactions with plants. As an academic discipline, the definition of Ethnobotany is varied but there are some common elements in the concepts. It is broadly defined as the study of the total relationships between plants and people (Cox and Balick 1996).

1.3 Phytochemical Analysis

Phytochemicals are the chemicals produced by the plants, generally to help them thrive or thwart competitors, predators, or pathogens. The name comes from the Greek word *phyton*, meaning plant. Some phytochemicals have been used as poisons and others as traditional medicine.

Plants consist of different kind of chemical compounds as well as other components that may be valuable from various points of aspects. They consist of different secondary metabolites and other components that have the found to be playing role in the different biological activity of the plant, plant growth and defense against predators, pathogens and predators (Molyneux et.al, 2007).

Phytochemicals are generally regarded as the research compounds rather than the essential nutrients and other because proof of their health effects till now is not been established. Most of the developing countries have adopted the traditional medicinal plants as their part of culture. People used to use the plant materials in the form of raw materials and other forms but now been used in the more processed form.In today world the medicinal plants are gaining the attention in owing the fact that it is cost effective and fewer side effects.

1.4 Research Plan

1.4.1 Hypothesis

The selected medicinal plants for the research have been frequently used by the ethnic community and have been obtained as according to the ethanobotanical knowledge. People use these medicinal plants against various diseases and the search lead to the findings of various useful bioactive compounds.

1.4.2 Work Plan

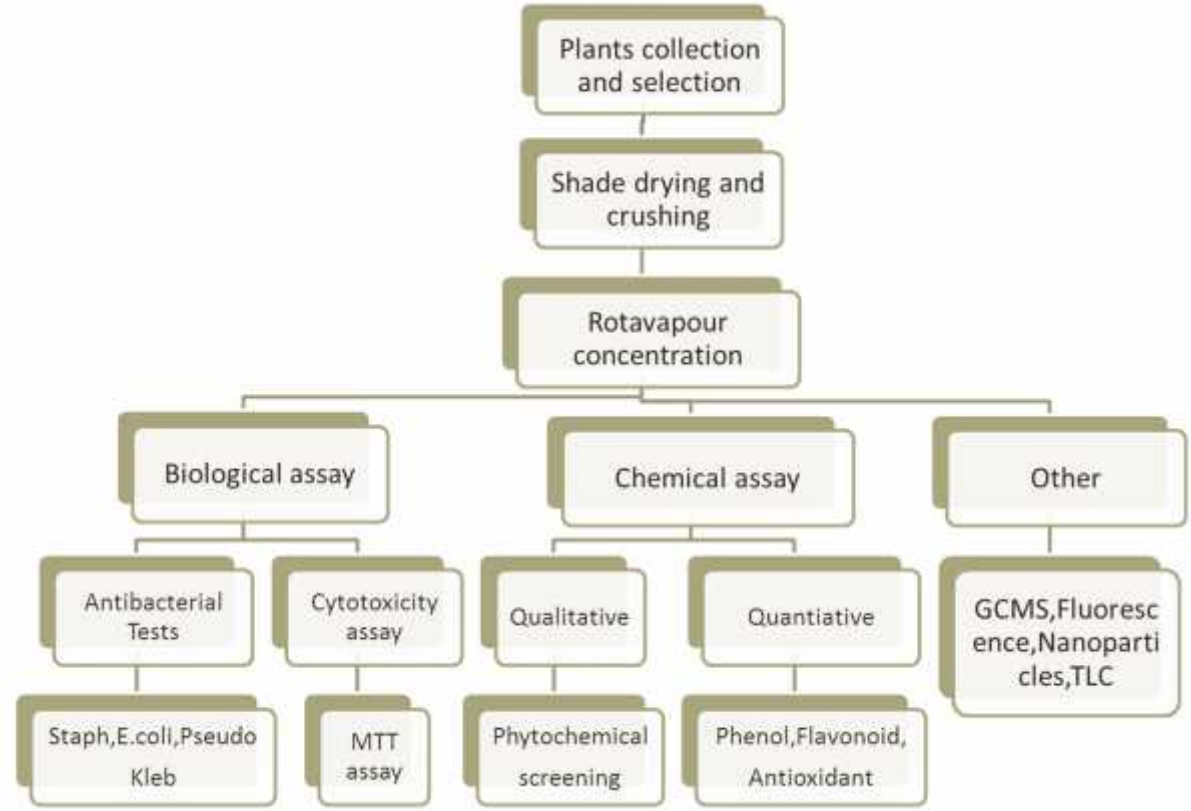

Figure 1.6 Flowchart showing the work plan of the research

1.5 Aims and Objectives

1.5.1 General objectives

To estimate the major bioactive compounds of 8 species of plants that are considered as medicinally important from the ethanobotanical aspects based on community knowledge through the phytochemical analysis.

1.5.2 Specific Objectives

I. Enrich the knowledge of medicinal plants through the documentation ethanobotanical knowledge of the ethnic community.

II. Bioactive compounds extraction from plants using n-hexane, ethyl acetate, methanol and water

III. Phytochemical screening of plants samples including phenolic, flavonoid, antioxidant, antibacterial, fluorescence.

V. Identification of the major phytoconstituents by GC-MS.

VI. Study of plant species in order to find the cytotoxicity of plant extracts that have been used for medicinal purposes.

1.6 Rationale of the study

The medicinal plants are the reservoir of the different kind of drugs for many diseases. Medicinal plants are the different potential sources of antioxidant, antibacterial and other bioactive compounds that are the major components of traditional healing systems in rural communities. The detailed phytochemical analysis and bioactivity of the medicinal plants have been extensively used.

Nepal for being located at the central part of the Himalaya has always remained a place of great interest to the botanists and phytochemists involved in researching medicinal herbs. So, the present study aims to compare the major phytochemicals in different medicinal plants. The qualitative grading of the different medicinal plant species can be done in accordance to the need and presence of important compounds.

1.7 Scope of the study

Nepal houses different medicinal plants of different genera and species which provides an ample traditional and pharmacognostic importance. Medicinal plants always play the role in the health of mankind. In developing countries, 80% of people are totally dependent upon the plants for their primary health care (Tabassum et al, 2012)

Medicinal plants have the great importance in the field of medicine.Various plants extracts have been extensively used by the community as the medicine and their active bioconstituents have been highy acknowledged though unknowingly. Screening the medicinal plants with its natural properties like phyto constituents and other bioactive compounds has been a very essential part to combat against various diseases. Plants act as a rich source of all those potential compounds that are an important part in order to act as a medicine. The choice and availability of plants, their traditional uses or ethanopharmacology, chemotaxonomy, plant ecological observations are important in determining the plant therapeutic aspects, the usual strategy being the observation of its

biological assay or panel of assays. The plants may undergo bioassay guided fractionation of active constituents or rigorous phytochemical isolation can lead to the discovery of active cobstituents (Heinrich, 2008)

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Nepal is the country of different natural sources and various medicinal herbs. Their exploration from different parts of the country always focus on the result that we have many sources that we can go further for the research.

Plants are the source of medicines and produce an amazing diversity of low molecular weight compounds. Nepal has always been the center of herbal richness with more than ten thousand species of herbs in its alpine belt. These herbs have been integral part of traditional medicine practices of the indigenous community in Nepal. Seeing the same impact and long history of herbal use, these herbs of Nepal are now exported to many countries and companies for medicinal purposes. Many big companies import such herbs to make medical extract and derivatives which are used in treating various diseases and health deficiency (Herbal medicinal plants of Nepal).

2.2 Medicinal plants of Nepal

Nepal is rich in flora and fauna. It has been gifted by diverse flora from fodder to prime medicinal importance. Medicinal plants hold a supreme value from Ayurveda to modern drug discovery. They served as a common link between the traditional and modern science as they are the source of medicaments involved through the centuries (Akatar 1997). Even today about 80 % of the world's population especially in the rural area are dependent on traditional medicines for their primary health security (Vieira and Skorupa 1993).

Medicinal plants play a vital role in modern research and are the raw materials for the new type of medicine used in various purposes like in oncology, heart diseases and many more. Medicinal plants are sources of active pharmaceutical compounds so they are considered as one of the important natural resources for raising the economy of the country. Thus, research on the medicinal plants for the developing country like ours is highly essential. The medicinal plants need to be mobilized which is indeed a huge potential for country's progress and economic benefit of human kind. Several medicinal plants research has revealed that they contain alkaloids, saponins, flavonoids, phenolics, and triterpenoids. (Doughari, 2012)

2.2.1 Cinamomum camphora

2.2.1.1 Chemical Constituents

Camphor laurel contains volatile chemical compounds in all plant parts, and the wood and leaves are steam distilled compounds for the essential compounds. Camphor laurel has six different chemical variants called chemotypes which are camphor, linalool,1,8-

cineole, nerolidol, safrole and borneol. In China, field workers avoid mixing chemotypes when harvesting by their odour (Hirota et.al, 1967; Lawrence et.al).

2.2.1.2 Medicinal Importance

Cinamomum camphora has several chemical varieties which have different essential oil compositions (Hattori, 1981; Huergo et al., 1978; Lin et al., 1994; Akeng´a et al., 1994; Moellenbeck et al., 1997; Dung et al., 1993). Two varieties have been exploited commercially, Hon-Sho (*C. camphora* Nees & Eberm.), the most valuable for the presence of camphor, and Ho-Sho (*C. camphora* Nees & Eberm var. *linaloolifera* Fujita) for it high content of linalool. These varieties are morphologically similar, but they show different essential oil compositions and for this reason are considerated physiological varieties (Guenther, 1950).

Camphor is a natural product with many applications in traditional and modern medicines. Camphor has several chemical varieties, each with different essential oil compositions. Traditionally, camphor has been used as a cold remedy for the relief of chest congestion and the treatment of inflammation related diseases such as rheumatism, sprains, bronchitis, asthma and muscle pain (Salman et.al, 2012). The leaf of *Cinnamomum camphora* contains camphor, as the main component along with cineol, linalool, eugenol, limonene, safrole, α-pinene, ß-pinene, ß-myrecene, α-humulene, *p*-cymene, nerolidol, borneol, camphene and some other components. (Abiya et.al, 2008)

There are many reports which prove that the use of camphor, solely or in combination with other treatments can be very effective for treating and preventing some serious diseases. A cancer study says that the use of camphor odor as a conditioning agent for the cancer cells of YC8 lymphoma in mice could have a suppressive effect on the growth of YC8 tumor, when it is combined with immunotherapy treatment (Ghanta et.al 1990). Cinnamomin has shown to have inhibitory effect on the growth of solid melanoma in the skin of the nude mouse (Ling et.al 1996)

A camphor based drug called 714-X, was developed by a Canadian researcher more than forty years ago and it is reported by some institutions, to be effective on the treatment of some patients with cancer, especially breast and prostate cancer (Kaegi et.al, 1998). Padma 28 is another multi compound herbal preparation, based on camphor formula which has shown to be effective against chronic inflammatory diseases. The result of a study indicates that Padma 28 has the ability to suppress the development of autoimmune diabetes in female non-obese diabetic (NOD) mice which could be an experimental model for type 1 diabetes mellitus in humans. (Weiss et.al, 2015)

The essential oil of *Cinnamomum camphora* and some other aromatic camphor containing plants such as sage, rosemary and basil which are widely used in traditional medicines contain monoterpenes. The studies have shown that some essential oil components, especially monoterpenes have suppressive and anti-mutagenic effect in number of human cancer cells including colon cancer, gastric cancer, liver tumor, breast cancer, leukemia and others. (Edris et.al, 2007). Many studies have been done about the various applications and benefits of camphor in pharmaceutical, industrial and environmental fields. Camphor has been used traditionally for many years as a remedy for the relief of pain, inflammation and

irritation in the body and skin. Recent studies have focused on the role of camphor in preventing and curing serious and life threatening diseases, when it is used purely or combined with other treatments. The study on some species in the Lauraceae family, shows that a number of extracts have significant antioxidant, anti-inflammation and anti-tumor activities (Lung Ho.et.al, 2009). These studies indicate that Lauraceae tree species and other camphor containing plants could have very important potential nutraceutical and pharmaceutical applications in the future, taking medicine just another step forward (Lin et.al,2007)

Camphor is a botanical hydrocarbon which is very cheap and can be easily cultivated without fear of shortages unlike petroleum products. Therefore, camphor is an excellent carbon source for the production of a high yield, high purity and high efficiency carbon nanotubes in future (Mukul et.al, 2007)

2.2.2 Schima wallichii

2.2.2.1 Chemical Constituents

Four compounds were isolated and elucidated from the bark of *Schima wallichii* (DC) Korthals. On the basis of spectroscopic and chemical evidences, the structures of four compounds were established as α-spinasterol, α-spinasterol glycoside, epicatechin and a new tritepenoid saponin. The structure of D evidences as 22-O-angelic acid ester-A 1-barrigenol-3-O-[α-L-rhamnopyransyl (1→2)]-[β-D-glucopyranosyl (→2)-β-D-galacto-pyranosyl (1→4)]-β-D-glucuronopyranose.

2.2.2.2 Medicinal Importance

The barks are used as an antiseptic for cut and wound, vermicide, mechanical irritant and to cure gonorrhoea. The barks juice is given to animal infested with liver flukes. Decoction of barks is good for fever and said effective against head lice.

2.2.3 Reinwardtia indica

2.2.3.1 Chemical Constituents

Investigation on the whole plant of Reinwardtia trigyna (Roxb.) has led to the isolation of three new secondary metabolites, namely wardtiamide A 1 (1,3-Dihydroxy-2-(2′,3′-dihydroxy,11′-(E)-ene-heptadecanoylamino)-tetracosane], wardtiamide B 2 (1,3-Dihydroxy-2-(2′,3′-dihydroxy,11′-(E)-ene-heptadecanoyamino)-pentacosane), and 2-((2,6-dimethoxybenzyloxy)methyl)-6-((Z)-3-hydroxyprop-1-enyloxy)tetrahydro-2H-pyran-3,4,5-triol (Chemical_constituents _of_Schima_wallichii ,accessed Jan 09 2018].)

2.2.3.2 Medicinal Importance

Reinwardtia indica is used for the treatment of paralysis. Crushed leaves and stems are applied to wounds infested with maggots (Yusuf *et al.* 2009).

2.2.4 *Woodfordia fruticosa*

2.2.4.1 Chemical Constituents

Main chemical compound available in the flowers of dhataki are tannins and cyanidine, diglucoside, octacosanol and beta-sitosterol are isolated from leaves. Leaves are rich in ellagic acid, polystachoside, pelargonidin-3 and 5-diglucoside. Dhataki also contains woodfordins A, B, C, D, E and F, trimeric hydrolysable tannins and tetrameric hydrolysable tannins. Other chemical compounds available in this herb are lupeol, betulin, betulinic acid, urosolic acid sisterol and olealonic acid.

2.2.4.2 Medicinal Usage

- It is very beneficial herb to cure range of diseases; it is used to cure diarrhea, piles and dysentery because it is a powerful astringent.
- The flower applied externally to relieve burning sensation of skin.
- Flowers are sprinkled over wounds and ulcers for quick healing and to stop discharge and granulation. For the local burns on body dhataki flowers are applied along with coconut oil to relieve burns.
- Flowers of this herb are immune modulatory and is also used to boost up immunity and overall body health.
- Flower decoctionare used for treating vaginal prolapse and anal prolapse and it is also used as gargles to cure dental disorders.
- Flowers and roots are very effective in curing rheumatism, lumbar and rib fractures, and foot and mouth diseases.
- Fresh flowers juice is applied externally to reduce headache caused by pitta dosha.
- In diabetic patient decoction of flowers are used to reduce thirst and other mouth related disorders.
- Being a mutra-viranjniye herb it is used to restore normal color of the urine in diabetic patients.
- It is also used in the treatment of fever that is caused due to pitta dosha.
- Flowers are used for the victims who are addicted to opium and suffering with diarrhea.
- Dried flowers are very beneficial for overall heart health and used to treat various heart related disorders.

2.2.5 *Castanopsis indica*

2.2.5.1 Chemical Constituents

The bark is a rich source of tannins and can be used as a dye, to weatherproof fibres etc (Soepadmo et al, 2002)

2.2.5.2 Medicinal Importance

Bark paste is used to control the chest pain (Joshi and Joshi et .al, 2010).

2.2.6 *Maesa chisia*

2.2.6.1 Chemical constituents

Using the extract prepared with solvent ethanol and performing the initial level phytochemical screening of the root portion of *Maesa perlaria* showed the presence of major bioactive constituent like- 2, 4-di-tert-butylphenol, sigmasterol and campesterol (Yang et al., 2016). Several reports provide plant extract containing 2; 4-di-tert-butylphenol possesses significant antioxidant and neural protective effects (Chen & Dai, 2015; Choi et al., 2013; Yoon et al., 2006).

2.2.6.2 Medicinal importance

This plant species is used to treat scabies and mouth sores (Ethanobotanical knowledge))

2.2.7. *Mimulus nepalensis*

2.2.7.1 Medicinal Importance

This plant species are Used during Allergy and Inflammation (Ethnobotanical knowledge)

2.2.8 *Mahonia nepalensis*

2.2.8.1 Chemical constituents

In etiolated plants, when bark is ringed out, shoots are rich in alkaloid sprouts.

2.2.8.2 Medicinal Importance

Several species are popular garden shrubs, grown for their ornamental, often spiny, evergreen foliage, yellow flowers in autumn, winter and early spring, and blue-black berries. The flowers are borne in terminal clusters or spreading racemes, and may be among the earliest flowers to appear in the growing season. The berries are edible, and rich in Vitamin C, though with a very sharp flavor. Although edible, the plants contain berberine a compound found in many *Berberis* and *Mahonia* species which can cause vomiting, lower blood pressure, reduced heart rate, lethargy, and other ill-effects when consumed in large quantities.

2.3 Phytochemical Extraction, Identification and Quantification

2.3.1 Phytochemical Extraction

Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of

preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after *Galen*, the second century Greek physician. The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstruum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such astablets and capsules. These products all contain complex mixture of many medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans.

In order to be used as a modern drug, an extract maybe further processed through various techniques of fractionation to isolate individual chemical entities such as vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin and codeine. (Handa S.S et.al)

The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents).

For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed. Some of the latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, microdistillation, thermomicrodistillation and molecular distillation.

With the increasing demand for herbal medicinal products, nutraceuticals and natural products for health care all over the world, medicinal plant extract manufacturers and essential oil producers have started using the most appropriate extraction technologies in order to produce extracts and essential oils of defined quality with the least variations from batch to batch. Various parameters influences the extract quality such as parts of the plant used, solvent used for extraction and extraction protocol. The extracted plant materials phytochemical property depends on the nature of plant material, its origin, degree of processing, moisture content and particle size as well.

The variations in different extraction methods such as type and time of extraction, temperature, solvent nature, polarity and concentration affect quantity and secondary metabolite composition of an extract**.**

Plant based natural constituents can be derived from any part of the plant like bark,leaves, flowers, roots, fruits, seeds, etc. i.e. anypart of the plant may contain active components.For Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. A property of a good solvent in plant extractions includes low toxicity, ease of evaporation at low heat, preservative action, and promotion of rapid psychological absorption of the extract. The factors affecting the choice of solvent are quantity of phytochemical to be

extracted, rate of extraction, diversity of inhibitary compounds extracted, toxicity of solvent in bioassay process, ease of subsequent handling of the extracts.

The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted.

Variation in extraction methods usually depends upon length of extraction period, solvent used, pH of solvent, temperature, particle size of plant tissues, solvent and sample ratio. The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction. Earlier studies reported that solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used as ideal.Solvents used for active component extraction are: Water, Ethanol, Methanol, Chloroform, Ether, and Acetone.

Rotary Vaccum Evaporator or Rota Vapor

A rotary vaccum evaporator is a gentle and efficient device which was used in chemistry laboratories for removal of organic solvent by evaporation under pressure. It was invented by Lyman C. Craig. A vacuum system is also used in it harmonically in evaporators .Vacuum evaporators is a class function because lowering the pressure above a bulk liquid lowers the boiling points of the component liquids in it. Generally, the component liquids of interest in applications of rotary evaporation are research solvents that one desires to remove from a sample after an extraction, such as following natural product isolation or a step in an organic synthesis. Use of a "rotavap" therefore allows liquid solvents to be removed without excessive heating of what are often complex and sensitive solvent-solute combinations (Laurence MH et al, 1989).

Rotary evaporation is most often and conveniently applied to separate "low boiling" solvents such as n-hexane or ethyl acetate, ethanol, acetone etc. from compounds which are non-volatile solid at room temperature and pressure.

Distillations can be performed under vacuum. This increases performance and helps to protect products. Distillations can be performed under vacuum and atmospheric pressure.

Different kinds of chemical distillates and extracts are finally obtained in powdered form which can be used in testing and analysis whereas; solvent was re-collected so that can be re-used.

Solvents with higher boiling points such as water (100 °C at standard atmospheric pressure, 760 torr or 1 bar), dimethyl formamide (DMF, 153 °C at the same), or dimethyl sulfoxide (DMSO, 189 °C at the same), can also be evaporated if the unit's vacuum system is capable of sufficiently low pressure. (For instance, both DMF and DMSO will boil below 50 °C if the vacuum is reduced from 760 torr to 5 torr (from 1 bar to 6.6 mbar) However, more recent developments are often applied in these cases (e.g., evaporation while centrifuging or vortexing at high speed) (Craig, LC et al, 1950)

Evaporation under vacuum can also, in principle, be performed using standard organic distillation glassware i.e., without rotation of the sample. The key advantages in use of a rotary evaporator are: the centrifugal force and the frictional force between the wall of the rotating flask and the liquid sample result in the formation of a thin film of warm solvent being spread over a large surface and the forces created by the rotation suppress bumping. The combination of these characteristics and the conveniences built into modern rotary evaporators allow for quick, gentle evaporation of solvents from most samples, even in the hands of relatively inexperienced users. Solvent remaining after rotary evaporation can be removed by exposing the sample to even deeper vacuum, on a more tightly sealed vacuum system, at ambient or higher temperature (e.g., on a Schlenk line or in a vacuum oven)

The main components of a rotary evaporator are: A motor unit that rotates the evaporation flask or vial containing the user's sample. A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off of the sample, vacuum system to substantially reduce the pressure within the evaporator system, heated fluid bath (generally water) to heat the sample, condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed,condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses and mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

The solvent is heated over a heating bath. In the rotating evaporating flask, a thin solvent film forms on the inside of the flask, resulting in increased evaporation rate. Rotation also results in even mixing of the sample and thus, prevents stationary over heating in the flask.

The unit makes sure that the evaporating flask rotates evenly with the resulting advantages. The solvent vapour flows very quickly in the condenser. Here, the energy in the solvent vapour is transferred to the cooling medium (mostly water) and the solvent condenses. Receiving flask receive the condensing solvent. Vacuum reduces the boiling temperature and thus increases performance of distillation. Evaporating performance is influenced by distillation pressure (vacuum), heating bath temperature, rotation speed and size of evaporating flask.

2.4 Identification of Major Compounds

The impact of different types of solvents, such as methanol, hexane, and ethyl acetate, for the purpose of extraction from various plants parts, such as leaves and seeds. In order to extract different phenolic compounds from plants with a high degree ofaccuracy, various solvents of differing polarities must be used is analyzed (Wong et al 2004)

Solvents used for the extraction of biomolecules from plants are chosen based on the polarity of the solute of interest. A solvent of similar polarity to the solute will properly dissolve the solute. Multiple solvents can be used sequentially in order to limit the amount of analogous compounds in the desired yield. The polarity, from least polar to most polar, of a few common solvents is as follows:

Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water.
Antioxidants can be defined as bioactive compounds that inhibit or delay the oxidation of molecules (Halliwell et.al 1995). Many of these medicinal plants are indeed good sources of phytochemicals that possess antioxidant activities. Some typical examples of common ingredients that have been used in ethnic foods are tamarind, cardamom, lemon grass, and galangal basil. These spices or herbs have been shown to contain antioxidants (Javanmardi et.al 2003)

2.5 Quantification of Major compounds

Quantification of major compounds present in plant samples can be achieved by HPLC, HPTLC, and other type of commercial software programs. HPLC is a type of liquid chromatography technique mostly used to identify, separate and quantify the active compounds in a solution (Martin et.al, 2005). HPTLC is an alternative to the HPLC technique and enhanced form of HPLC technique and enhanced form of Thin Layer Chromatography. Various enhancements can be made to the TLC to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements. This may be done by using a UV scanner to identify various compounds that are present in the sample used (Morolock et.al, 2010)

2.6 Nanoparticles Synthesis

Silver nanoparticles (AgNPs) have been extensively studied for many decades due to their unique features and wide range of applications. Their uses include catalysis (Pradhan, Pal & Pal, 2002), biosensing (Anker et al., 2008), imaging (Lee & El-Sayed, 2006), and antibacterial activity (Morones et al., 2005; Rai, Yadav & Gade, 2009). Among these applications, antibacterial activities have gained much attention because they potentially offer a solution to the problem of antibiotic resistance (Cho et al., 2005). There are a variety of methods to synthesize AgNPs including physical and chemical methods (Chudasama et al., 2010).

Silver nanoparticles are widely used in pharmaceutical industry in the fabrication of ointments and creams to inhibit burns and wounds related infections (Satyavani et al.; 2011). The silver ion has strong inhibitory effect against a number of microorganisms (Mohanta and Behera, 2014). Biological synthesis or green synthesis of nanoparticles is an alternative and eco-friendly method for production of nanoparticles (Firdhouse and Lalitha, 2015; Chung et al; 2016; Nayak et.al 2016)

Silver nanoparticles can be synthesized using a variety of chemicals and physical methods, involving chemical reduction (Vorobyova et al., 1999; Tan et al., 2002; Yu, 2007), photochemical reduction (Kéki et al., 2000; Pileni, 2000; Sun et al., 2001; Mallick et al., 2005), electrochemical reduction (Sandmann et al., 2000; Liu and Lin, 2004), and heat vaporization (Bae et al., 2002; Smetana et al., 2005). These processes involve several toxic chemicals as reducing agents. Because of using noble metal nanoparticles in areas of human contact (Song and Kim, 2008), there is an emergent need to develop ecofriendly biosynthesis processes that hinders the use of toxic chemicals.

2.7 Fluorescent Study

Fluorescence is the phenomenon exhibited by various chemical constituents present in the plant material. Some show fluorescence in the visible range in daylight. The ultraviolet light produces fluorescence in many natural products (e.g. alkaloids like berberine) which do not visibly fluoresce in daylight. Some of the substances may be often converted into fluorescent derivatives by using different chemical reagents though they are not fluorescent, hence we can often assess qualitatively some crude drugs using fluorescence as it is the most important parameter of pharmacognostical evaluation (Gupta et al 2006, Ansari et.al 2006)

2.8 Antibacterial Test

The antimicrobial activity of plant extracts and phytochemicals was evaluated with antibiotic susceptible and resistant microorganisms.The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Almagboul et.al, 1985). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils (Jansen et.al, 1986), as well as in tannin (Saxena et.al,1997).

The use of plant compounds for pharmaceutical purposes has gradually increased in Brazil. According to World Health Organization (Santos.et.al, 1995) medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency (Elof et.al 1998).

The definition and practice of pharmacognosy have been evolving since the term was first introduced about 200 years ago (Kinghorn, 2001; Samuelsson, 2004), as drug use from medicinal plants has progressed from the formulation of crude drugs to the isolation of active compounds in drug discovery.

Plants are prospective source of antimicrobial agents in different countries (Alviano et.al, 2010). About 60 to 90% of populations in the developing countries use plant-derived medicine. Traditionally, crude plant extracts are used as herbal medicine for the treatment of human infectious diseases (Zhang et.al, 2010). Plants are rich in a variety of phytochemicals including tannins, terpenoids, alkaloids, and flavonoids which have been found *in vitro* to have antimicrobial properties (Dorman et.al, 2010). Although the mechanism of action and efficacy of these herbal extracts in most cases is still needed to be validated scientifically, these preparations mediate important host responses (Cruz et.al, 2007).

2.9 Gas Chromatography-Mass Spectrometry

Gas chromatography–mass spectrometry (GC-MS) is ananalytical method that combines the features of gas chromatography and mass spectrometry to identify different unknown samples. Its application includes drug detection, fire investigation, environmental analysis, explosive detection, and identification of test samples.

Instrumentation

The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio.

A mass spectrometer is typically utilized in one of two ways: full scan or selective ion monitoring (SIM). The typical GC-MS instrument is capable of performing both functions either individually or concomitantly, depending on the setup of the particular instrument.

The primary goal of instrument analysis is to quantify an amount of substance. This is done by comparing the relative concentrations among the atomic masses in the generated spectrum. Two kinds of analysis are possible, comparative and original. Comparative analysis essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library. This is best performed by a computer because there are a myriad of visual distortions that can take place due to variations in scale. Computers can also simultaneously correlate more data (such as the retention times identified by GC), to more accurately relate certain data.

Another method of analysis measures the peaks in relation to one another. In this method, the tallest peak is assigned 100% of the value, and the other peaks being assigned proportionate values. All values above 3% are assigned. The total mass of the unknown compound is normally indicated by the parent peak. The value of this parent peak can be used to fit with a chemical formula containing the various elements which are believed to be in the compound. The isotope pattern in the spectrum, which is unique for elements that have many natural isotopes, can also be used to identify the various elements present. Once a chemical formula has been matched to the spectrum, the molecular structure and bonding can be identified, and must be consistent with the characteristics recorded by GC-MS. Typically; this identification is done automatically by programs which come with the instrument, given a list of the elements which could be present in the sample.

A “full spectrum” analysis considers all the “peaks” within a spectrum. Conversely, selective ion monitoring (SIM) only monitors selected ions associated with a specific substance. This

is done on the assumption that at a given retention time, a set of ions is characteristic of a certain compound. This is a fast and efficient analysis, especially if the analyst has previous information about a sample or is only looking for a few specific substances. When the amount of information collected about the ions in a given gas chromatographic peak decreases, the sensitivity of the analysis increases. So, SIM analysis allows for a smaller quantity of a compound to be detected and measured, but the degree of certainty about the identity of that compound is reduced.

2.10 Thin Layer Chromatography

Thin Layer Chromatography is a technique which is used to separate the nonvolatile compounds or mixtures.Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. To quantify the results, the distance traveled by the substance being considered is divided by the total distance traveled by the mobile phase. (The mobile phase must not be allowed to reach the end of the stationary phase.) This ratio is called the retention factor (R_F). In general, a substance whose structure resembles the stationary phase will have low R_F, while one that has a similar structure to the mobile phase will have high retention factor. Retention factors are characteristic, but will change depending on the exact condition of the mobile and stationary phase.

Thin-layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance. Specific examples of these applications include: analyzing ceramides and fatty acids, detection of pesticides or insecticides in food and water, analyzing the dye composition of fibers ,in forensics, assaying the radiochemical purity of radiopharmaceuticals, or identification of medicinal plants and their constituents

Plant extracts can be prepared for TLC in many ways. Common methods include extracting plant material in alcohol-water mixtures such as 80% ethanol (Khurram et.al 2011) possibly with the addition of acid or base (Robinson et al 1963). Following an extraction in such solvents, which contain some water and are possibly acidic or basic, extracts must be concentrated so that they can be applied to TLC plates in a minimal volume. The concentration of alcohol water extracts can be achieved by partitioning with water-immiscible organic solvents (Khurram et.al, 2011)

Different plant metabolites are extracted into different organic solvents, depending on their polarities. To ensure that plant organic acids or bases are extracted into organic solvents at this stage, the pH of an alcohol-water extract can be raised or lowered with awater-soluble acid or base to convert dissociated analytes into their non -dissociated forms, which are then soluble in neutral organic solvents (Robinson et, al 1963).The organic phase can then be evaporated under reduced pressure or under nitrogen and adjusted to the desired

volume for TLC. The pH of the extract is unlikely to be lethal to bioassay microorganisms due to the partitioning of analytes into neutral solvents, small final volume, and evaporation of the extract on the TLC plate prior to separation. TLC is widely used because it is relatively rapid and can be performed on different adsorbents (*e.g,* silica, starch, alumina), as well as providing good resolution and sensitivity (Stahl 1969)

Separations by paper or thin-layer chromatography (PC or TLC) are convenient because many compounds can be separated on a planar surface. Separation is based on polarity, with some compounds binding tightly to the adsorbent (cellulose in the case of PC, and a variety of adsorbents in the case of TLC) and migrating less than others.

2.11 Cytotoxicity

2.11.1 Cell Culture

Cell culture is a very systematic technique that was performed under the controlled condition or culture laboratory. Simply It involves the dispersal of cells in an artificial environment composed of nutrient solutions, a suitable surface to support the growth of cells, and ideal conditions of temperature, humidity, and gaseous atmosphere. In general practices cell culture of multicellular eukaryotes (Animals), plants, fungi's, bacteria's, microbes including viruses is done by isolating cell from them. The historical development of cell culture was related with the development of organ culture and tissue culture technique ("Cell Culture" Retrieved 2006-04-19).

Primary culture refers to the stage of the culture after the cells are isolated from the tissue proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be subcultured (i.e.passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. After the first subculture, the primary culture known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

There are various applications of cell culture such as used in cellular and molecular biology, providing model systems for studying the normal physiology and biochemistry of cells (e.g . metabolic studies, aging), the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

2.11.2 MTT Assay

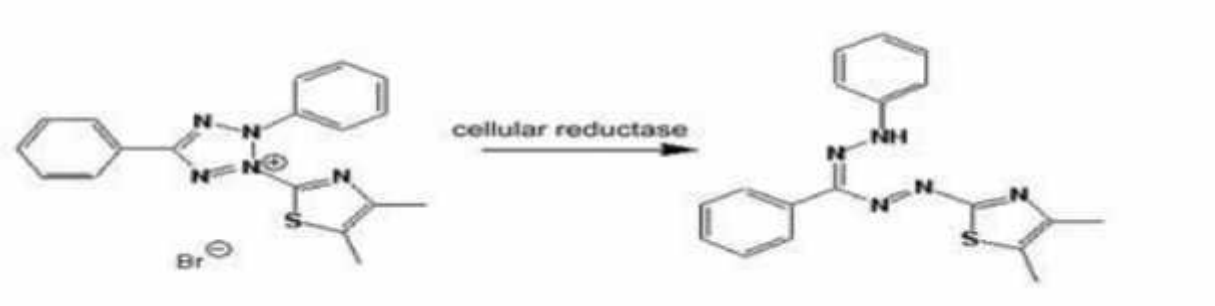

Figure 0.1 MTT reaction

MTT is (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. (Mosmann et al, 1983). Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. Isopropanol or DMSO; used DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically.

Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced, through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan

formed and an increase in absorbance. The use of the MTT method does have limitations influenced by physiological state of cells. Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in multiwell plates. For best results, cell numbers should be determined during log growth stage. Each test should include a blank containing complete culture.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

All the chemicals and reagents used during the research were of Analytical Grade. The total research work was done in the Central Department of Biotechnology, T.U except GC-MS which was carried out in forensic.

3.1.2 Instruments

All the instruments used during the research were in good working conditions.

3.1.3 Plant Samples

Plant sample was collected from the ethanobotanical survey of the community especially tamang community was selected from the study. Plant sample were as per the knowledge of the indigenous community knowledge, their traditional uses and other aspects too.They were collected from the knowledge of experties of National Herbarium and Plant Laboratories and were identified by them and also from Central Department of Botany.

3.1.4. Bacterial strains

Cultures of *Pseudomonas aeruginosa, E coli, Klebsiella pnemoniae, and Staphylococcus aureus* from CDBT laboratory collected from TUTH.

3.1.5 Selection of plants samples

The plant samples were choosen according to the higher ethno medicinal value among Nepalese folklore and potential drug formulation and less studies done.

3.2 Collection and identification of the plant samples

Whole plants were collected from different parts of Nepal during their flowering period. The selected samples collected photographed. Identification of plant was done by Mr.Hem Raj Poudel, Taxonomist of National Herbarium and Plant Laboratory (NHPL) Godawari Kathmandu and also some plants were identified from the Central Department of Botany, TU, and Kirtipur.

3.3 Preparation of plant materials

The collected plants were cleaned off mud, fungi and many other unwanted materials, and then air/shade dried at 30-35 degree centigrade for 5 days to remove all their moisture. The dried plant material were powdered with the help of grinder, passed through wire sieve (porosity 220 microns) and the fine powder was collected on sterile and dry polythene bag for extraction

3.4 Preparation of plant extracts and extract dilution

50 grams of powder of each plant samples was taken separately and dissolved in 325 ml of solvents n-hexane (HEX), ethyl acetate (EA), Methanol (MTH) and Distilled Water (AQ) and allowed to percolate for 24 hours. Solvents used were of analytical grade. After percolation these samples were subjected to ultrasonication for 1.15 hours for three days at 50 degree centigrade. The solvent was filtered through Whattman No 1 filter paper and the extract was concentrated in rota vapour at 37 degree centigrade at 60 to 80 rpm under the reduced pressure until the extract is concentrated enough. The extract was then poured in the petridish and left at 37 degree centigrade in the incubator for three days. The extract was then scratched from the petriplate with the help of spatula and transferred in cryovial. The cryovial is then sealed with the cello tape and stored at 4 degree centigrade for further tests.

Each 100 mg of plant extracts was weighed accurately and dissolved in 1ml solvent (HEX,EA, MTH and AQ) respectively on which the extract is prepared. This 100 mg/ml stock was used for the antimicrobial tests, antioxidant activity, quantification of total phenol and flavonoids. Other dilutions were also prepared from this stock solution.

3.5 Qualitative Preliminary Phytochemical Analysis

The hexane, ethyl acetate, methanol and aqeous extract of plant were used to screen for the presence of various secondary metabolites by using the protocol suggested by Harborne, 1973 and Trease and Evans, 1989.

Test for Flavonoids

Alkaline Reagent Test Crude extract was mixed with 2 ml of 2% solution of NaOH. An intense yellow colour was formed which turn colourless on addition of few drops of dilute acid which indicated the presence of flavonoids.

Test for glycosides

Libermann test

Crude extract was mixed with eachof 2 ml of chloroform and 2 ml of Acetic acid. The mixture was cooled on ice. Carefully concentrated sulphuric acid was added sidewise. A colour change from violet to blue to green indicated the presence of steroidal nucleus, i.e glycine portion of glycoside.

Test for steroid

Crude extract was mixed with2 ml of chloroform and concentrated sulphuric acid was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing the crude extract with the 2 ml of chloroform. Then 2 ml of each of concentrated sulphuric acid and acetic acid were poured in the solution mixture. The development of greenish colouration indicated the presence of steroids

Test for terpenoids

Crude extract was dissolved in 2 ml of Chloroform and evaporated to dryness.To this; 2 ml of conc suluric acid was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

Test for Alkaloids

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

Test for phenols and tannins

Crude extract was mixed with2 ml of 2% ferric chloride. A blue green or black colouration indicates the presence of phenols and tannins.

3.6 Quantitaive Phytochemical Analysis

3.6.1 Quantitative Phenolic Determination

In the procedure, different concentrations of the extracts were mixed with 0.4 ml FCR (diluted 1:10 v/v). After 5 min 4 ml of sodium carbonate solution was added. The final volume of the tubes were made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using gallic acid solutions as standard and total phenolic content of the extract was expressed in terms of milligrams of gallic acid per gram of dry weight and the standard graph.

3.6.2 Quantitative Flavonoid Determination

Total flavonoid content was determined by Aluminium chloride method using querctin as a standard. 1ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 min 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% aluminium chloride was added. After 6 min incubation at room temperature, 2 ml of 1 M sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as equivalents (mg querctin/g dried extract).

3.6.3 Total antioxidant activity via DPPH free radical scavenging assay

DPPH assay is used for the determination of antioxidant activity. The plant extracts were assessed on the basis of the radical scavenging activity of the stable 1, 1-diphenyl-2 picryhydrazyl (DPPH) –free radical activity following the protocol of (Singh et.al, 2002).Different concentration of plant extracts and quercetin (50-1000 ug/ml) were prepared in methanol on clean test tubes. 0.5 ml of samples of plant extracts as well as querctin of each concentration was taken separately in clean test tubes. To this sample 0.5 ml of 0.2mM DPPH solution was added. The tubes were then shaken uniformlyfor proper mixing and incubated in dark for half an hour. The control was prepared as above but

without the plant extracts or querctin and methanol was taken as blank.The absorbance was taken on the spectrophotometer at 517 nm.

The radical scanvenging activity was calculated using the formulae:

% Radical Scavenging Assay = [(Control-sample)/Control]*100%

Then a standard graph was plotted taking the concentration of Quercetin. Following Maes.et.al. 2010. The IC50 value of different species was compared. The species having the lowest IC50 was considered to have the best antioxidant property.

3.7 Nanoparticles synthesis

3.7.1 Aqeous Extract Preparation

10 gram of powdered form was mixed with 10 ml of sterile distilled water and was sonicated for 20 min. Centrifugation was done and supernatant was collected for further use at 5000 rpm for 15 min. Purified extracts was filtered through Whatman.1 filter paper and filtrate was store at 4 degree Celsius.

3.7.2 Biosynthesis of silver Nanoparticles using Extract

0.5 ml of aqueous extract was mixed with 4.5 ml of aqeous solution of 1 mM silver nitrate and similarly 0.5 ml aqeous extract was mixed with 4.5 ml of distilled water for control.Both the samples and control were incubated at dark overnight at room temperature.The colour change conform the nanoparticle synthesis. The solution along with the nanoparticle was centrifuged at 10000 rpm for 45 minute with successive washing with deionized water. Remaining pellet was lyophilized and stored for further characterization.

3.7.3 Characterization

Bioreduction of silver ions (Ag+) into silver nanoparticles (Ag0) was monitored in aqeous solution by U.V spectrophotometer at regular interval in wavelength ranges from 200 to 1000 nm.

3.8 Fluorescent study

0.5 gram of powder plant sample was taken in clean and dried test tubes. 5 ml of organic solvent was taken and shaken well and allowed to stand for 20-25 minute and was observed under visible light and U.V light for characteristic colour.

3.9 Determination of Antibacterial activity

3.9.1 Preparation of Nutrient Agar (NA)

NA plates and NA broth was prepared for the antibacterial tests. About 28 grams of NA powder (Hi Media Laboratories Pvt.Ltd, Mumbai, India) from the powder supplied was carefully weighed and transferred in a conical flask. The content was dissolved in water completely in a conical flask and the final volume was maintained to 1000 ml followed by boiling for uniform mixing. This media was sterilized by autoclaving at 15 lbs pressure at

121^{o}C for 15 minutes. The autoclave tape was used as the indicator for completeness of sterilization. This media was allowed to cool to 45-50^{o}C, the media was poured and sterilized and well labeled petridishes. About 25 ml of the media was poured on each petridish of 9 cm diameter in sterile condition under a laminar flow hood. All the plates were left for the media solidification. For the preparation of NA broth, screw tight bottles were filled with media and autoclaved.

3.9.2 Preparation of Luria Bertani Miller (LB) and Muller Hinton Agar (MHA)

The LB broth was required to culture and sub culture the microorganism prior to antimicrobial tests. About 25 gram media powder was weighed and transferred to the conical flask. The content was then dissolved in distilled water and final volume was maintained in distilled water to 1000 ml and was allowed to autoclave at standard condition. Autoclave tape was used for the indication of completeness of autoclave. Finally this media was cooled in laminar air flow and dispensed in sterile and dry culture tubes.

38 grams of the powder was weighed and the final volume was maintained upto 1000 ml. The media was sterilized by autoclaving at standard condition. The media was mixed carefully before pouring. The media was poured on sterile anddry petriplate under asceptic condition.

3.9.3 Preparation of the standard bacterial culture inoculums

The individual pure cultures of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumonia* was obtained from CDBT, TU and was streaked on LB broth with the help of sterilized inoculating loop.The inoculated culture bottles were kept on the incubator at 37^{o}C for 3 hours.The turbidity of the bacterial suspension was compared to McFarland standards.These bacterial standards were used for the swabbing on the MHA plates to test the antimicrobial effects of the plants extracts.

3.9.4 Antibacterial test

First all the sterile and dry plates were properly labeled with the name of the bacteria name of the plant species and different concentration of plants extract to be added. The MHA Plates were inoculated with the different bacterial culture along with proper concentration and was swabbed asceptically under sterile condition. The culture plates were allowed to dry for about 15 to 20 minutes under the laminar flow.

The antimicrobial test is performed by modified agar well diffusion method as per Lindequist et.al, 2006 with slight modifications. On the above prepared MHA plates five well were prepared on the solid MHA plates with the help of sterile cork borer (4 mm) diameter. Different concentration of the plant extracts samples were prepared in the respective solvents. With the help of sterile pipette 20 ul of each individual plant extracts were poured in the above prepared wells. The solvent was taken as the negative control and the antibiotics respective were taken as the positive control. The plates were incubated in the incubator overnight at 37 ^{o}C and the zone of inhibition was observed for the individual plants extracts for individual bacteria at different concentrations.

3.10 Gas Chromatography Mass Spectrometry

For GC-MS analysis 1µl of the sample is injected in split mode in the instrument. Rtx5MS-30m column with 0.25-mm ID and 0.25µm DF was used. The parameters standardized for GC-MS run are: Injection temperature: 300°C, Interface temperature: 300°C, Ion source should be adjusted to 250°C and Carrier gas used is Helium (flow rate of 1 ml/min).

Analysis was performed using the temperature program, 1 min. of isothermal heating at 100^{o}C followed by heating at 300^{o}C for 20 mins. Mass spectra were recorded at 2 scan /sec with a scanning range of 40 to 850 m/z. Each component was quantified based on peak areas and normalization based on the internal standard.

3.11 Thin Layer Chromatography Analysis

3.11.1 Preparation of Developing Chambers for Extract Separation

Scissors was used to cut a piece of filter paper slightly below chamber height, and about half the chamber perimeter in width. This paper acts as a wick to draw solvent up the chamber wall and saturate the chamber with solvent vapors, thus improving reproducibility of separations.

In a fume hood, solvents were mixed (ethyl acetate-methanol 4:1, v/v, for this study. The solvent mixture was poured into the chamber and was coveredIt was waited until the entire wick is wet with solvent, indicating chamber saturation, to put plates into chamber.

3.11.2 Loading and Development of TLC Plates

The origin was marked with pencil. If the TLC plate adsorbent is soft and easily damaged, make marks at edges. Compounds should be above the surface of the developing solvent when plates are inserted into the TLC chamber. The extracts was dissolved in enough organic solvent (in this case, methanol) to have a concentrated solution instead of a turbid suspension.Samples were loaded as narrow bands with a microliter syringe or capillary micropipettes, leaving a 1 cm border on the sides of the plate. The bands aws allowedto dry (fanning the plate or loading it in a fume hood helps). If a greater concentration of sample is needed on a plate, "overspot" by loading samples again on the dried bands.

With forceps or tongs, set plate(s) inside the saturated TLC chamber. Plates should not touch the wick because it may provide solvent to the plates at points of contact, thus altering the path of compound migration. The chamber was covered and plates was let for the develop.

3.11.3 Preparation of Plates for Bioassay

The plate was removed from TLC tank before the solvent front reaches the top of the plate; the height was marked of the solvent front with a pencil.The palte was dried in fume hood. Any remaining TLC plate’s wasdeveloped in the same TLC chamber, which is generally usable for an entire day if kept, closed. Solvent mixture was remake for TLC chamber if the amount of solvent in the chamber decreases notably.

After plates were dry, bands were visualized under visible or UV light, and delineate bands with a pencil. A viewing chamber with a portable UV lamp is convenient, especially if the lamp can detect compounds at both 254 nm (short-wave UV) and 365 nm (long-wave UV).

To aid in characterizing bands, retention factor (RF) value was calculated by measuring distances traveled by compound and solvent front, anddividing the former by the latter.

3.12 Cytotoxicity Analysis

3.12.1 Preparationof reagents

1% PBS was prepared by taking 100 ml of deionized water and one tablet of PBS and then was dissolved thoroughly by using vortex shaker.Trypsin was prepared by mixing 100 mg of trypsin with 50 ml of PBS and mixed well. And then was sterilized by using 0.22ppm filter. We used syringe filter to fitler the trypsin. Trypan blue was prepared dissolving 0.04 gm in 10ml PBSComplete RPMI media was made from incomplete RPMI media.

10 ml of the prepared trypan blue solution on a sterilized syringe was taken inside the laminar air flow cabinet. 0.22 ppm syringe filter was used to filter sterilizes the trypan solution.

By same process MTT dye was also sterilized using another syringe filter.

3.12.2 Steps of Cell Culture

100ml of incomplete RPMI media was taken and then mixed with 10% FBS (11ml) and 1% PBS (1ml) .The apparatus, chemicals, media on laminar hood was arranged in sterilized condition before starting the culture. The media and the PBS were warmed before starting the culture.

.The fresh vial of Verocell was taken out from liquid nitrogen, then put it into water bath for 1-2mins & thaws the vial properly until it detaches the frozen cells rapidly. 6-8 ml media (complete media) was kept into the falcon tube and the thawed cell was poured onto the falcon containing media by using the pipette.The media and fresh cells was centrifuged for 3 mins at 1500 rpm. The supernatant was removed and pellet was collected by pipette. About 8-10 ml media was poured on the culture flask and the pellet was discharged onto the culture flask containing the complete media and is mixed properly by using the pipette. The culture flask was shaken gently containing vero cells and the media and observe it under inverted microscope to see the cells fresh and moving. The cells were stabilized by putting in the CO2 incubator at 37°C until next passaging (about 24 to 48 hours)

3.12.3Subculture of Vero cell

Before performing the subculture, give a look at the situation of the cells whether the cells are ready for the subculture i.e. they have confluence growth. When the cells are of 80% confluent, then they are ready for the passaging. The color of media must not change. The steps are : The media, PBS and the trypsin was warmed in the 37°C water bath and arrange the required materials and apparatus in laminar hood and respective places.The cells was taken out of the CO2 incubator and observe the cells under microscope to confirm the confluency (80%) of the cells.Then in the hood, the media was discard in the discarding beaker and then 2ml of PBS solution was put and was washed twice gently. 1ml of trypsin was put in the culture flask and then shaken well from three ends of the flask gently. Then

observe the cells under microscope and incubated for 2-3 mins in the CO2 incubator. After time, cells were again observed splitted in the inverted microscope and then put the cells in the falcon tube 4-6 ml of media. Then the media containing the splitted cells was centrifuged in the falcon tube for 3 minutes at 1500 rpm. The supernatant was discraded and the pellet was collected and mixed it by pouring 1ml of complete media. 500 µl of the cells in the falcon was used for the subculture and remaining cells are cryopreserved for the stock preparation. The 500 µl of cell suspension is put into the fresh culture tube and then incubated in the CO2 incubator. When the cell becomes 80% confluent, then they can be used for tests such as MTT assay.

3.12.4 Cell Counting (Haemocytometer Count)

The chamber of haemocytometer and cover slip was cleaned with alcohol and dry using tissue paper. 20 µl of cells was added from the cell suspension and 80 µl of trypan blue was added and mixed well for successful cell counting. 10 µl of the mixed solution was taken in a micropipette and then put on the haemocytometer from the egdes of the cover slip. Then cell counting was done by observing under inverted microscope. The concentrations of drug 1 µg/ml, 10 µg/ml, and 100 ug/mland control were prepared for the MTT Assay.

3.12.5 Procedures for the MTT assay

First of all, confluent cells were observed and when they were suitable (cells reached 80% confluency), they were used for the MTT assay. Subculture processes were followed up to centrifugation and the pellets were mixed with 1ml media of which 10 µl was used for the cell counting. When the cells were determined by cell counting in a haemocytometer.100 µl cells were added in the 96-well plate and incubated in the CO2 incubator on the first day of the MTT assay. After 24 hours, known concentrations of plant extract (100 µl) were added in each well except the control (i.e. First column) and incubated for 24 hours on the second day. On third day, 10 µl MTT dye was kept in all the wells along with the control and incubated for 4 hours. Then, diluted plant extracts was added to different wells of 96 well plates according to different concentration. Each concentration of plant extracts were also placed in the plates. After time, 200 µl of the DMSO solution was kept in all the wells of interest and then incubated for half an hour. Then absorbance was taken at 540/590nm and cytotoxicity was calculated.

3.13 Statistical Analysis

All the experiments were performed in triplicates for each sample and the values were reported as mean ±SD. The obtained data were also subjected to analysis of variance and mean values were compared. All the statistical analysis was done using Excel software.

CHAPTER 4

RESULTS

4.1 Survey of medicinal plants from ethnic community

Medicinal plants list was collected from the ethnic community according to their knowledge and traditional practices of medicinal plants they had been using them. The survey from the ethnic community on the basis of their traditional knowledge 36 plants was surveyed mentioned in the table 4.1.

Table 4.1 Ethanobotanical survey of medicinal plants from tamang community

S.N	Plants local name	Parts Used	Purpose
1.	Pahelo fool	Roots and Entire Plant	Fever
2.	Luman jhaar	Whole Plant	Allergy
3.	Bhui kaafal	Whole plant	Saarki khatira
4.	Balu jhaar	Whole plant	Wound (pilo)
5.	Katus	Bark and root	Fever
6.	Dhayero	Flower	Dysentery
7	Gurche laharaa	Whole plant	Cancer/Diabetes/Pressure
8.	Laaliguras (dried)	Flowers	Diabetes/Choking
9.	Gane gujro	Tendrils	Cancer
10.	Kukur kaalo Jibro	Flower	Fever
11.	Pirre		
12.	Dhasingare	Flower	Fever
13.	Cucumber Root	Root	Diarrhea
14.	Khurpaaani	Endosperm	Cancer
15.	Tadelo	Flower(Soaked in water)	Fever (Dokh)
16.	Jaai(Single)	Flower	Mouth Sores
17.	Aaru	Seed (Dried)	Dysentery
18.	Lahare ghaas(Taangsar Kaado)	Tendrils	Mouth Sores
19.	Bilaauni jhaar	Whole plant and Latex	Mouth Sores
20.	Kanike kuro	Fruit	Wound
21.	Noon dhiki(Chiyapatti)	Whole plant	Fracture
22.	Khareto	Leaf	Antidandruff
23.	Soli fool/Nilo fool	Flower	Wound and gums bleeding
24.	Ban pidaalu	Fruit	Wound heal, reduce Inflammation
25.	Bankhhirra	Whole plant	Mouth sores
26.	Maaslahari	Whole part	Fractures
27.	Sisnu	Root	Fractures

28.	Aank	By making cigarette type	Malaise
29.	Gaaitihare	Flower	Stomach ache
30.	Aiselu	Root	Dehydration
31.	Bhaairaaz	Whole plant	Fractures
32.	Aaru	Flower	Insecticidal
33	Guava	Bark, Leaf	Diarrhea
34.	Kapoor	Whole plant	Rheumatism and Muscle pain
35.	Chilaaune	Leaf and Bark	Fever, Head lice
36.	Daaruhaldi	Bark	Blood pressure lowering

4.2 Selection of medicinal plants for study

Plants were selected which were easy to find and seasons too. The sites were chosen as Tarakeshwor municipality -4 (Chogaaun and Dhitalthok), Kathmandu district Pharping and Godawari municipality of Lalitpur district. Plants used for the study were selected on the basis of their availabilty and more usage by local people is mentioned in the table 4.2.

Table 4.2 Selected plants for the phytochemical study

S. N	Scientific name/Vernacular name)	Parts used	Purpose	Location	Flowering/ Fruiting time
1	***Cinamomum Camphora*** **(Kapoor)**	Whole part	Antispasmodic, stimulant, insecticidal	Dhitalthok	April-May
2	***Schima wallichii*** **(Chilauney)**	Leaves and bark	Antihelminthic, rubifacient	Dhitalthok	April-May
3	***Reinwardtia indica*** **(Pahelo fool)**	Entire plant	Fever	Chogaaun	March-April
4	***Woodfordia fructicosa*** **(Dhayero ko fool)**	Flower	Dysentery	Tribhuvan University(CDBT)	April-May
5	***Castanopsis indica*** **(Katus*)***	Bark	Fever	Pharping	
6	***Maesa chisia*** **(Bilaune)**	Bark	Wound healing	Chogaaun	April-May

7	***Mimulus nepalensis* (Luman jhaar)**	Whole plant	Allergy	Chogaaun	Whole year
8	***Mahonia nepalensis* (Jamanemandro/ Daaruhaldi)**	Bark and berry	Dysentery,Diarrhea	Godavari	October-April

4.3 Percentage yield of plant extracts

Eight different plants were collected from the ethanobotanical community and subjected for the different solvents extraction (i.e.n-hexane, Ethyl acetate, Methanol and Aqueous).The total amount of all the extracts isolated from 50 grams of each finely powdered whole plant material is shown below. The highest yield of extract was found on aqueous (57%) and other solvent gave alomost equal percentage of extracts.All of these extracts was found to be of sticky and greasy in nature. All the extracts were of different colour. The percentage yield is mentioned in Table 4.3 along with bar graph and extract after drying in Figure 4.1 and Figure 4.2

Table 4.3 Percentage yield of plants extracts in different solvent

S.N	Name of plants	Hexane extracts %	Ethyl acetate extracts %	Methanol extracts %	Aqeous extracts %
1	*Cinamomum camphora*	14	14	30	57
2	*Schima wallichii*	8	6	14	43
3	*Reinwardtia indica*	4	4	4	30
4	*Woodfordia fruticosa*	2	20	6	43
5	*Castanopsis indica*	3	2	6	45
6	*Maesa chisia*	10	12	14	44
7	*Mimulus nepalensis*	2	2	7	43
8	*Mahonia nepalensis*	2	2	2	43

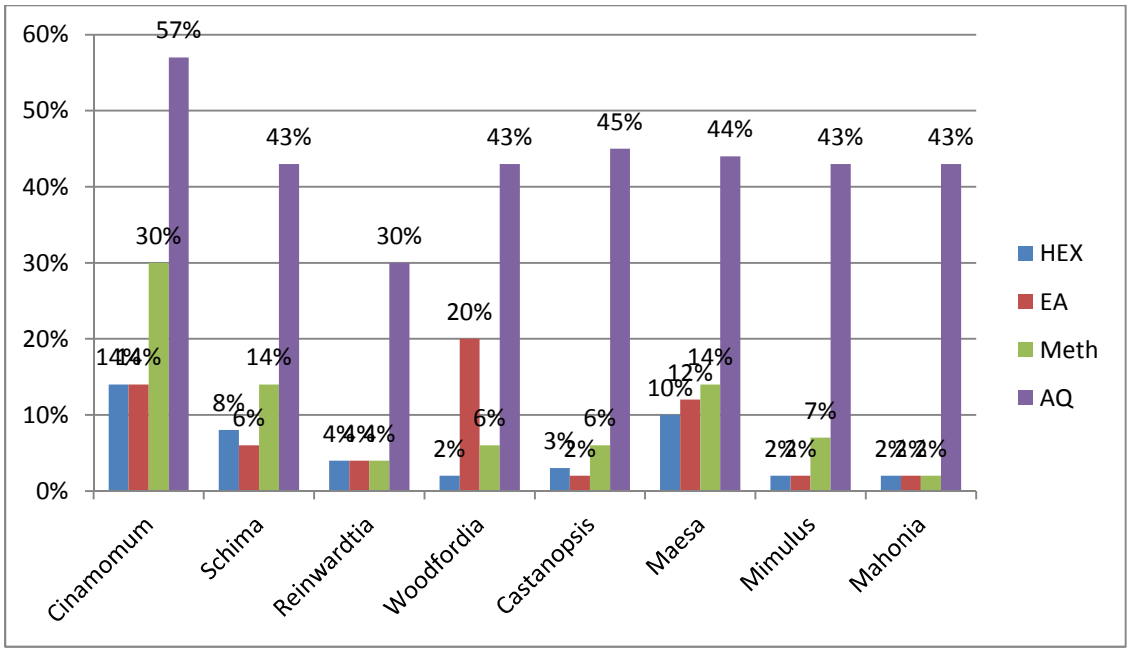

Figure 4.1 Percentage yield of plants extracts in diferent solvent

Figure 4.2 *C.camphora* extracts after drying

4.4 Preliminary Phytochemical Tests

Summary of different tests performed on n-hexane, ethyl acetate, methanolic and aqueous of different plant species is tabulated in the table 4.4 below:

Table 4.4 Preliminary Phytochemical tests of different plants extracts.

Sample	Flavonoid	Glycoside	Steroid	Terpenoid	Alkaloid	Phenols and Tannins
	Alkaline Reagent Tests(A)	Liberman Tests(B)	Steriod tests(C)	Terpenoid tests (D)	Meyers Tests (E)	Ferric chloride tests (F)
Cinamom(HEX)	-	+	+	+	-	-
Cinamom(EA)	-	+	+	+	+	-
Cinamom(MTH)	-	-	+	+	+	-
Cinamom(AQ)	-	-	+	+	+	-
Schima(HEX)	+	+	+	-	+	-
Schima(EA)	+	+	-	-	+	-
Schima(MTH)	-	+	+	+	-	-
Schima(AQ)	-	+	+	+	-	+
Reinward(HEX)	+	+	+	+	-	-
Reinward(EA)	-	+	+	-	-	-
Reinward(MTH)	-	+	+	-	-	-
Reinward(AQ)	-	+	+	-	-	-
Woodford(HEX)	+	-	-	+	-	-
Woodford(EA)	-	-	-	+	+	+

Sample	Flavonoid	Glycoside	Steroid	Terpenoid	Alkaloid	Phenols and Tannins
	Alkaline Reagent Tests(A)	Liberman Tests(B)	Steriod tests(C)	Terpenoid tests (D)	Meyers Tests (E)	Ferric chloride tests (F)
Woodford(MTH)	-	-	-	+	+	+
Woodford(AQ)	-	-	-	+	+	+
Castanop(HEX)	+	-	+	+	+	-
Castanop(EA)	+	-	-	+	+	+
Castanop(MTH)	+	+	-	+	+	-
Castanop(AQ)	+	-	-	-	+	+
Maesa(HEX)	-	+	+	+	+	-
Maesa(EA)	-	-	-	+	+	+
Maesa(MTH)	-	-	+	+	+	+
Maesa(AQ)	-	-	+	+	+	+
Mimulus(HEX)	-	+	+	+	+	-
Mimulus(EA)	-	+	+	+	+	-
Mimulus(MTH)	-	+	+	+	+	+
Mimulus(AQ)	-	+	+	+	+	+
Mahonia(HEX)	+	+	-	+	+	-
Mahonia(EA)	+	+	-	+	+	-
Mahonia(MTH)	+	-	-	-	-	+
Mahonia(AQ)	+	-	-	-	-	+

Figure 4.2 Reagent preparation and preliminary test

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4.5 Estimation of Total Phenol Content

The range of concentration for standard was 10ug/ml to 100ug/ml aftter genrating calibration curve and calculating the total phenolic content of all the extracts of plants. The results were expressed in terms of GAE/g dry weight based on standard Gallic acid.

4.5.1 Ethyl Acetate Extract Phenol Content in Plant Samples

The equation generated for the ethyl acetate standard was y = 0.0007x+0.02 and R^2= 0.9325. Highest phenol content was observed in *Cinamomum* (381±1.73) and lowest phenol content was observed in *Reinwardtia* (31±5.50). The graph obtained is plotted in the Figure 4.4 for the standard and the value of phenol content obtained in figure 4.5.

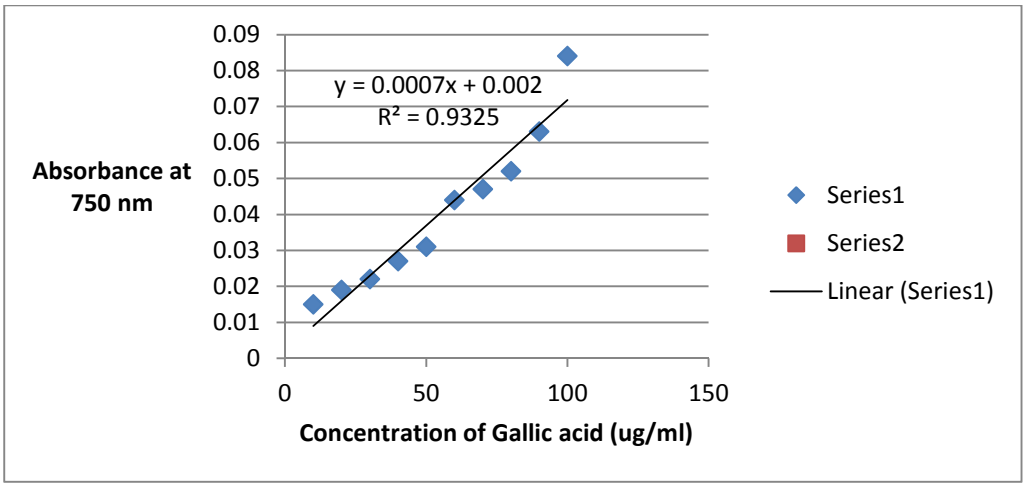


Figure 4.3 Standard gallic acid for phenol estimation

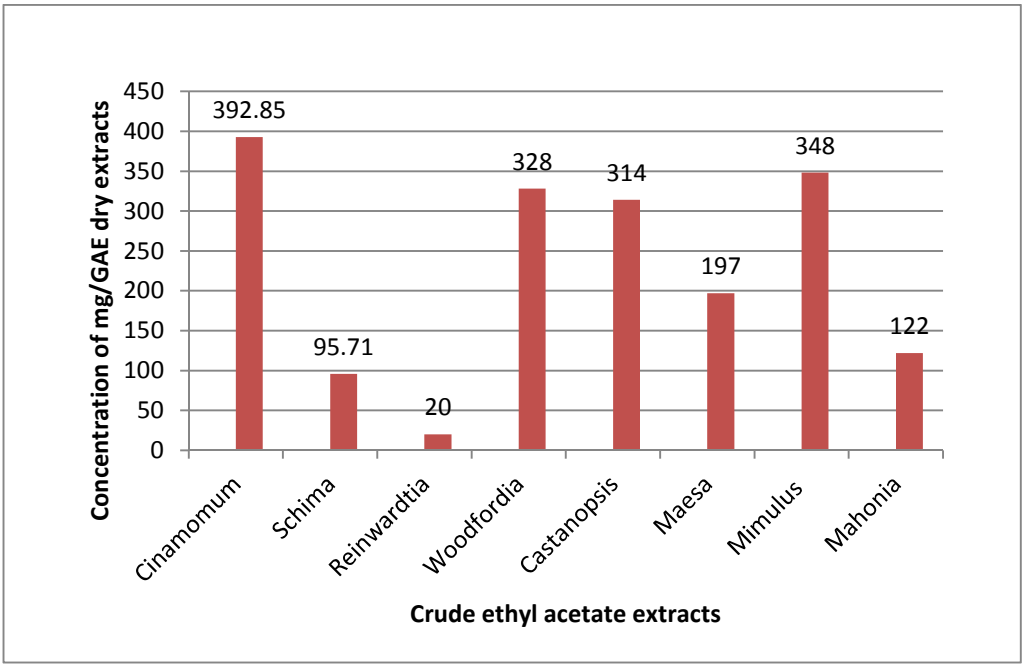


Figure 4.4 Estimation of total phenol content of Ethyl acetate extracts.

4.5.2 Methanol Standard comparison with plant samples

The equation generated for the methanol standard was y =0.123x-0.0793 and R^2= 0.9789. Highest phenol content was observed in *Cinamomum* (66.3±0.18) and lowest phenol content was observed in *Mahonia* (36.43±0.37). Standard graph is plotted in figure 4.6 while sample one is in figure 4.7.

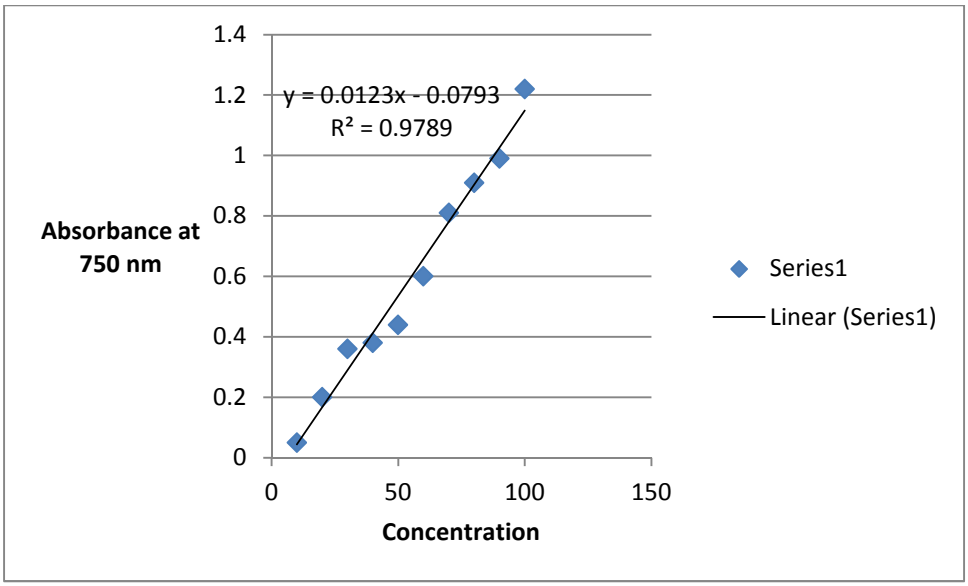


Figure 4.5 Standard graph of gallic acid for methanol extracts.

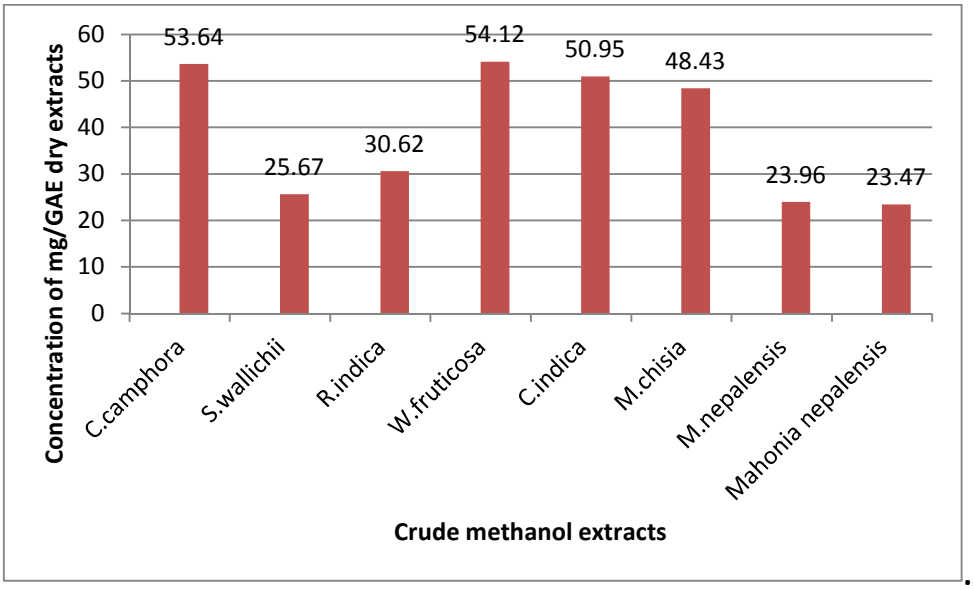


Figure 4.6 Estimation of total phenol content of crude Methanol extracts.

4.5.3 Aqueous Extracts Phenol Content in Plant Samples

The equation generated for the aqeous standard was y = 0.109x+0.0247 and R^2=0.9928. Highest phenol content was observed in *Cinamomum* (87.27±0.23) mg GAE/g dry weight and lowest in *Maesa* (53.05±0.18) mg GAE/G dry weight. Standard graph for aqeous is plotted in figure 4.8 while the sample one is mentioned in figure 4.9.

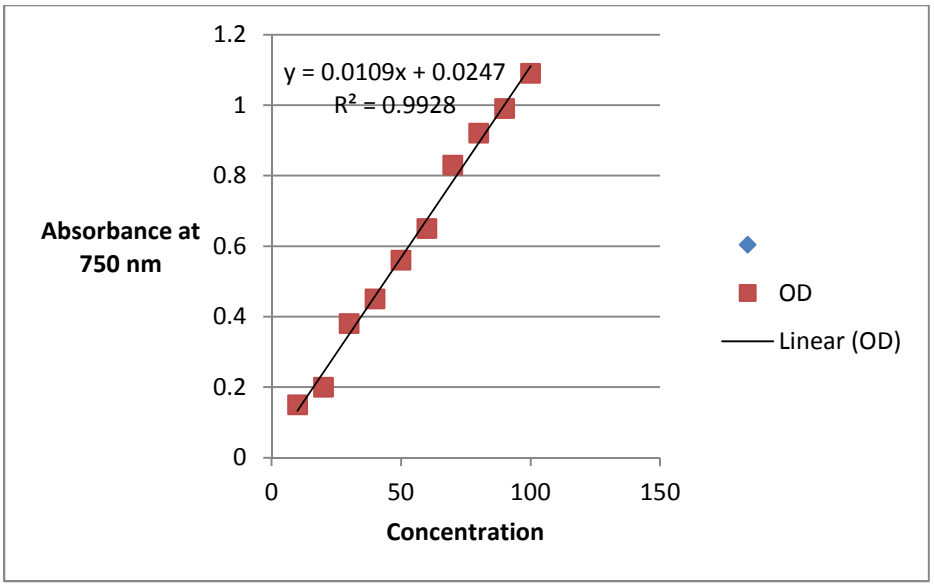


Figure 4.7 Gallic acid standard for phenol estimation of Aqueous extracts

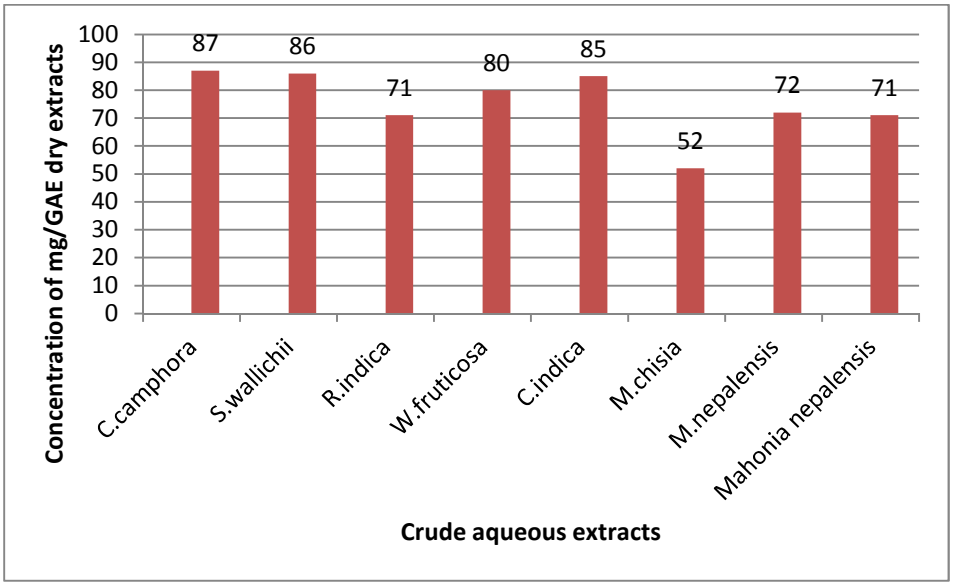


Figure 4.8 Estimation of total phenol content of crude Aqueous extracts

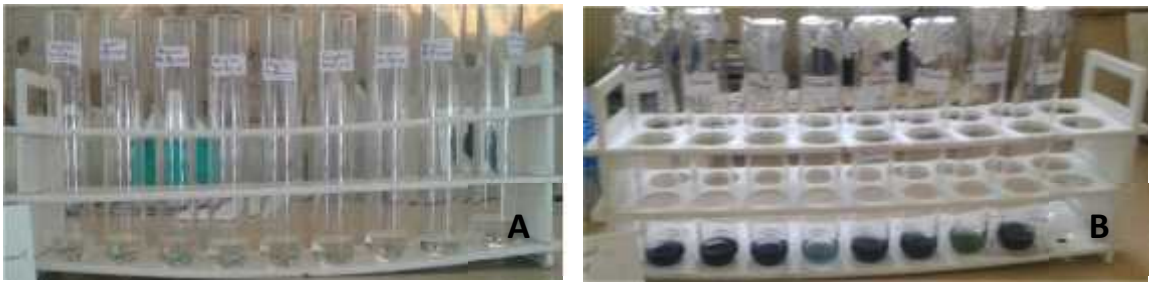


Figure 4.9 Standard preparation of gallic acid and test of phenol test

4.6 Estimation of total flavonoid content

Total flavonoid content is expressed in terms of Quercetin equivalent (mg QE/gm dry extract). Quercetin was used as standard flavonoid at concentration ranging from 10ug/ml to 100ug/ml to generate the standard curve. The generated equation of the standard curve differs from solvent to solvent.

4.6.1 Ethyl Acetate Extract comparison with plant samples

The equation generated for the ethyl acetate standard was y =0.0045x-0.0133 and R^2=0.9909. Highest flavonoid content was observed in *Castanopsis* (160±0.67) mg QE/gm dry wt and *Mimulus* (160± 0.44) mg QE/gm dry wt and lowest flavonoid was observed in *Reinwardtia* (106±0.92) mg QE/gm dry wt. The graph of standard quercetin for ethyl acetate and sample bar graph is mentioned in figure 4.12 and 4.13

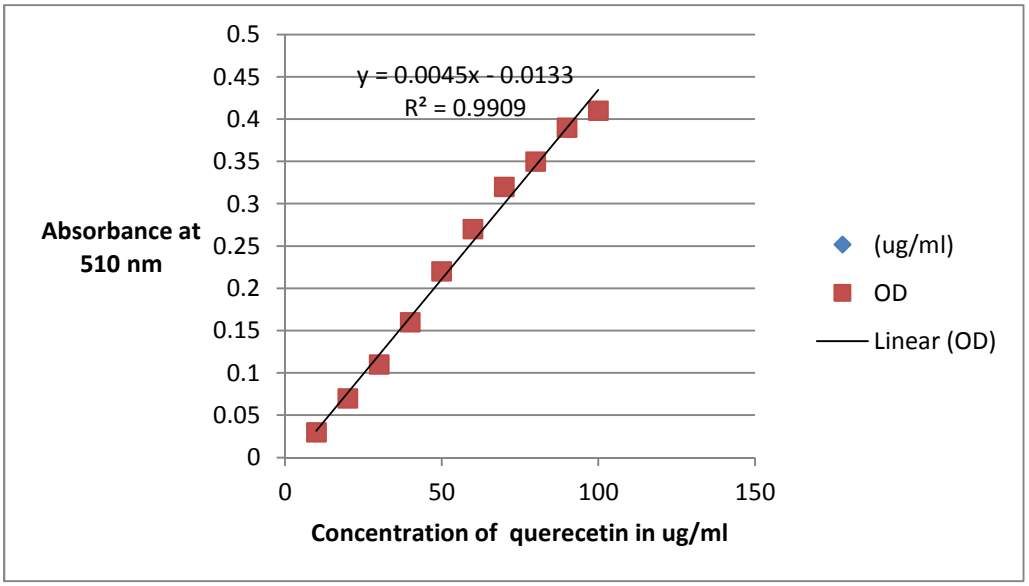


Figure 4.0.10 Standard of quercetin for ethyl acetate

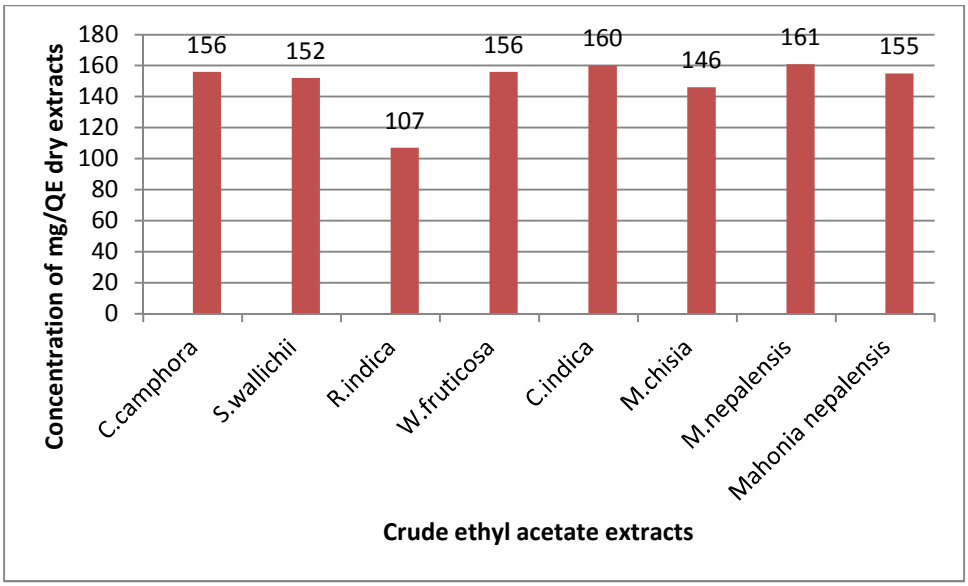


Figure 4.11 Estimation of Total Flavonoid Content of Crude ethyl acetate extracts

4.6.2 Methanol standard comparison with plant samples:

The equation generated for the methanol standard was y=0.0113x+0.1105 and R^2=0.9375. Highest flavonoid content was observed in *Maesa* (42.38±3.86) mg QE/gm dry wt and lowest in *Woodfordia* (2.5±0.38) mg QE/gm dry wt. The standard graph and test is plotted in figure 4.14 and 4.15 respectively.

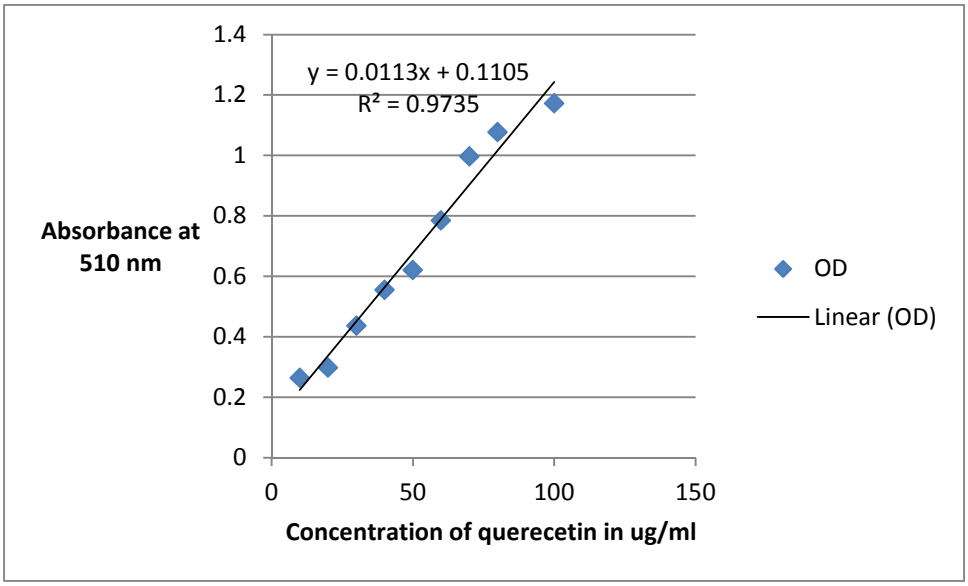


Figure 4.12 Standard of quercetin for methanol extracts

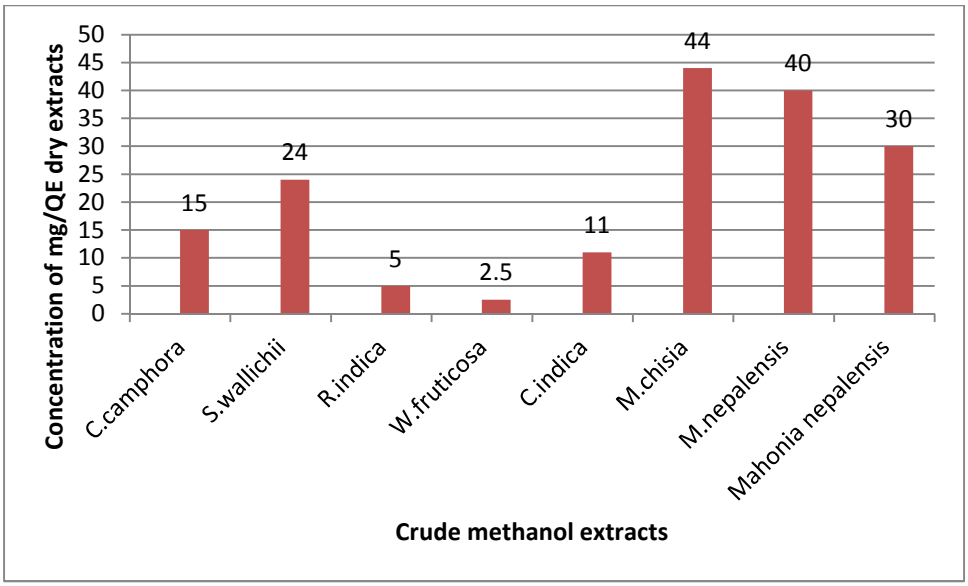


Figure 4.13 Estimation of Flavonoid Content of crude Methanol extracts

4.6.3 Aqeous standard comparison with plant samples:

The equation generated for the aqeous standard was 0.0061x-0.0573 and R^2=0.951. Highest flavonoid was observed in Schima (58.07±0.32) mg QE/gm dry wt and lowest in *Cinamomum* (11.63±0.25) mg QE/gm dry wt. The standard of quercetin and test is labelled in figure 4.16 and 4.17.

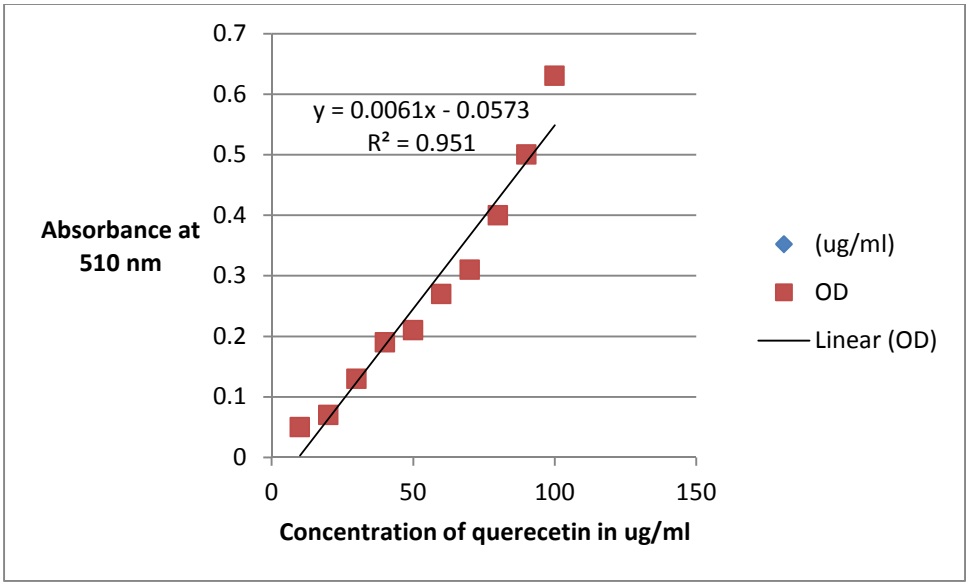


Figure 4.14 Standard of quercetin for aqeous extracts

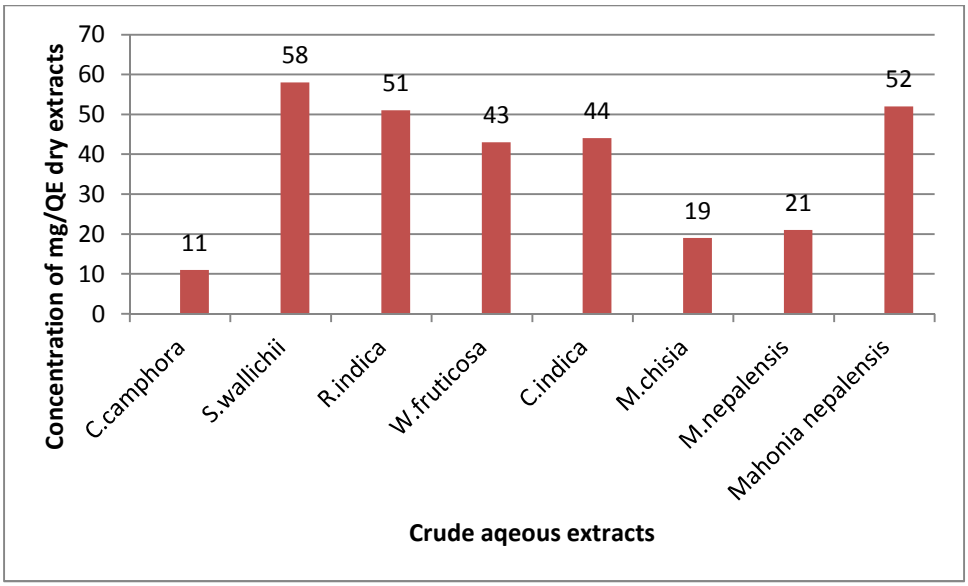


Figure 4.15 Estimation of total Flavonoid contents of crude aqueous extracts

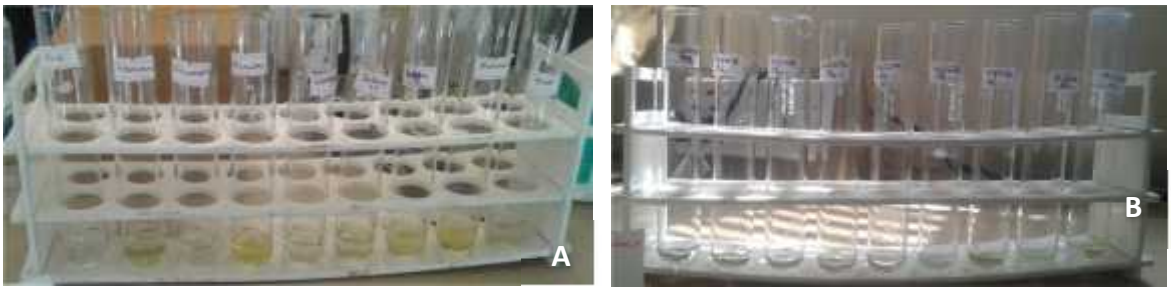


Figure 4.16 Flavonoid Standard preparation and test of flavonoid

4.7 Total DPPH Free Radical Scavenging Activity (RSA)

The graph of standard Querctin was drawn using its various concentrations ranging from 50ug/ml to 1000ug/ml against the DPPH radical scavenging percentage. The IC50value was calculated for quercetin and ethyl acetate, methanolic and aqueous were compared. For each extract IC50 value was calculated as tabulated in the table 4.5, 4.6 and 4.7.

Table 4.5 IC50 values of different plants pecies of ethyl acetate extracts

S.N	Plants extracts	IC50 value(ug/ml)
1	*C.camphora*	49.35±4.10
2	*S.wallichii*	68.44±3.23
3	*R.indica*	96.99±2.18
4	*W.frutiocosa*	69.62±5.72

5	*C.indica*	53.99±1.06
6	*M.chisia*	53.33±0.24
7	*M.nepalensis*	55.41±0.33
8	*Mahonia.nepalensis*	57.00±0.15

Table 4.6 IC50 values of different plants pecies of methanolic extracts

S.N	Plants extracts	IC50 values(ug/ml)
1	*C.camphora*	57.44±5.43
2	*S.wallichii*	66.25±7.28
3	*R.indica*	58.24±1.75
4	*W.frutiocosa*	70.71±4.70
5	*C.indica*	46.67±7.32
6	*M.chisia*	66.36±5.35
7	*M.nepalensis*	54.10±2.70
8	*Mahonia nepalensis*	57.40±2.51

Table 4.7 IC50 values of different plant species of aqeous extracts

S.N	Plant extracts	IC50 values
1	*C.camphora*	56.18±0.33
2	*S.wallichii*	74.43±0.64
3	*R.indica*	75.38±1.29
4	*W.frutiocosa*	69.28±1.60
5	*C.indica*	103.01±5.67
6	*M.chisia*	107.65±3.92
7	*M.nepalensis*	135.98±2.92
8	*Mahonia nepalensis*	138.99±7.59

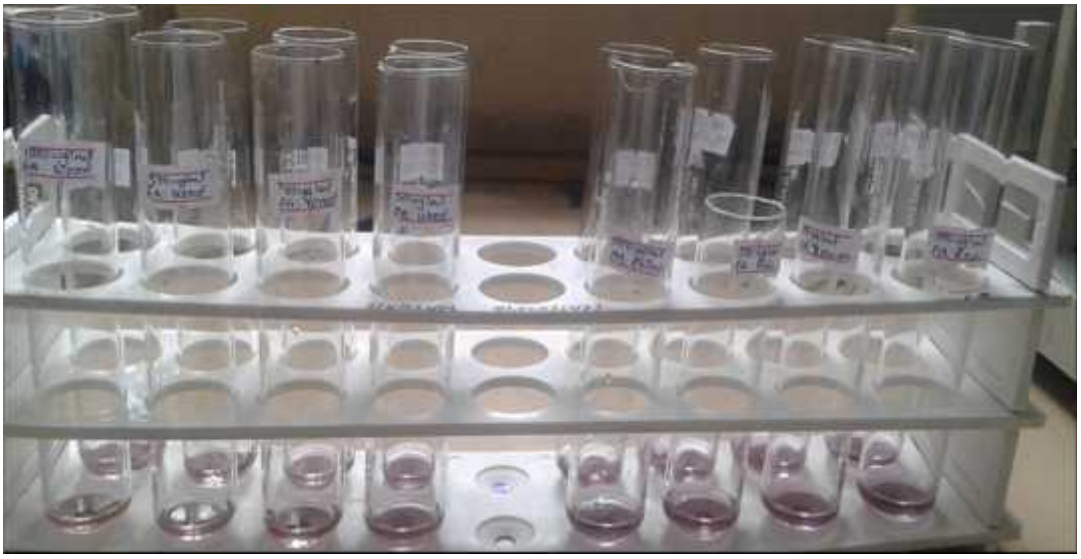


Figure 4.17 Antioxidant test of samples

4. 8 Nanoparticles Synthesis:

The nanoparticles were synthesized by using silver nitrate solution and were incubated under room temperature.

Table 4.8 Colour change and peak report after spectrophotometer reading

SN	Sample	Initial Colour	Final colour	Peak at
1	*C.camphora*	Dark Brown	Ash coloured	421 nm
2	*R.indica*	Dark Orange	Pale yellow	421 nm
3	*W.fruticosa*	Pale green	Ash coloured	423 nm
4	*S.wallichii*	Pale yellow	Ash coloured	423 nm
5	*C.indica*	Reddish Brown	Faint Brown	447 nm
6	*M.nepalensis*	Dark Brown	Ash Coloured	421 nm
7	*Mahonia.nepalensis*	Dark brown	Ash Coloured	421 nm
8	*M.chisia*	Brown	Ash Coloured	421 nm

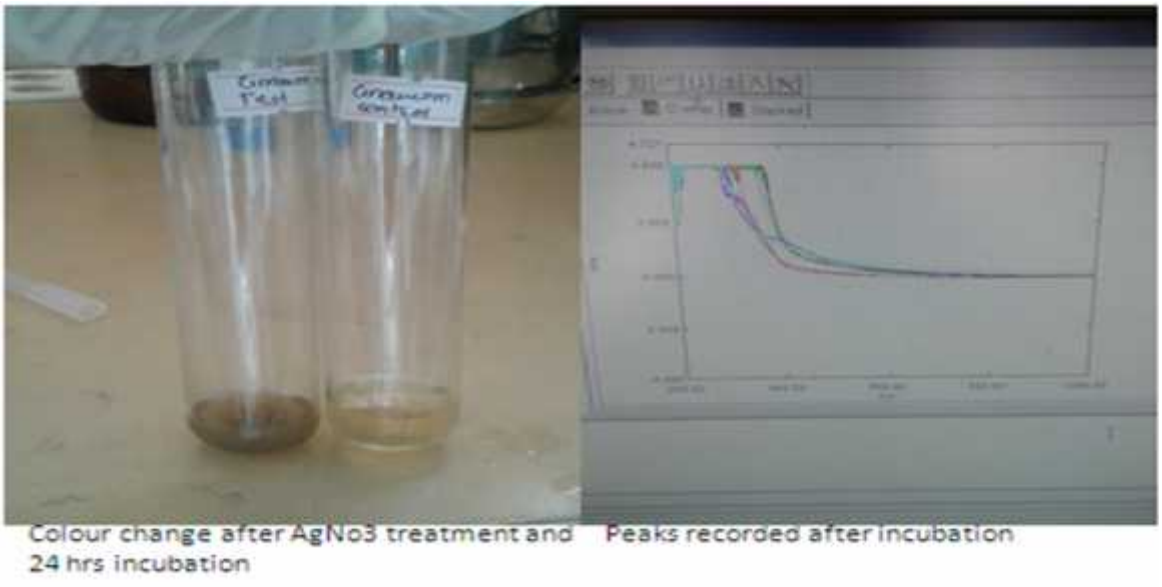


Figure 4.18 Colour changes of aqeous extract after AgNo3 treatment and peaks.

4.9 Fluorescence Study

The fluorescence study of all the plant crude powder form is subjected to the treatment with different solvent and the observation were observed in the visible light and UV light.

4.9.1 Glacial Acetic Acid

When glacial acetic aid was subjected to plant powdered form slight change in colour was observed in some sample under visaible and UV light mentioned in table 4.9.

Table 4.9 Fluorescent study using Glacial Acetic Acid

Sample	Visible light	UV light
C.camphora	Dark green (Black)	Black
R.indica	Pale green	Yellow
W.fruticosa	Light yellow	Reddish Yellow
S.wallichii	Dark Green	Black
C.indica	Yellow Shiny	Pale yellow
M.nepalensis	Pale Yellow	Pale yellow
M.nepalensis	Brown	Dark Brown
M.chisia	Light Brown	Dark Brown

Figure 4.0.19 Glacial acetic acid treatment in UV light and visible light

4.9.2. Benzene

When benzene was subjected to plant powdered form slight change in colour was observed in some sample under visible and UV light mentioned in table 4.10.

Table 4.10 Fluorescent study using Benzene

Sample	Visible light	UV light
C.camphora	Dark green (Black)	Black
R.indica	Pale green	Yellow
W.fruticosa	Light yellow	Reddish Yellow
S.wallichii	Dark Green	Black
C.indica	Yellow Shiny	Pale yellow
M.nepalensis	Pale Yellow	Pale yellow
M.nepalensis	Brown	Dark Brown
M.chisia	Light Brown	Dark Brown

Figure 4.0.20 Benzene treatment in UV light and visible light

4.9.3 Chloroform

When chloroform was subjected to plant powdered form slight change in colour was observed in some sample under visible and UV light mentioned in table 4.11

Table 4.11 Fluorescent Study Using Chloroform

Sample	Visible Light	U.V light
C.camphora	Green	Green
R.indica	Yellow	Golden
W.fruticosa	Red	Orange
S.wallichii	Dark Green	Dark Green
C.indica	Dark brown	Reddish Brown
M.nepalensis	Blackish Brown	Brownish Black
M.nepalensis	Dark Brown	Black
M.chisia	Blackish Brown	Dark black

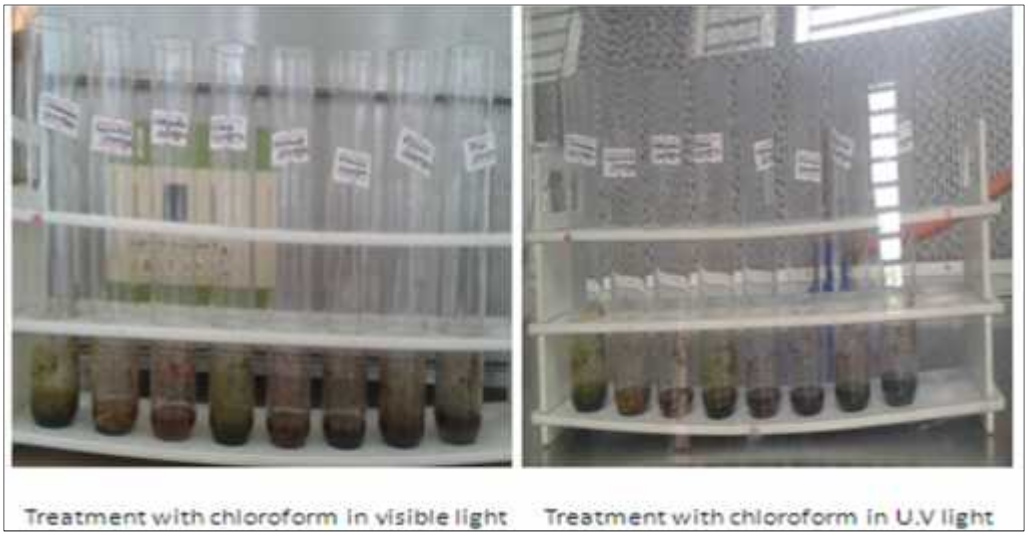


Figure 4.21 Chloroform treatments in visible ligt and U.V light

4.10 Antibacterial tests

The antibacterial activity of extracts was tested against the ATCC culture of *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa*. Antibiotic drugs Amikacin was taken as positive control and DMSO was used as negative control against four bacterial strains of each extracts and compared with antibiotic amikacin. The results were expressed with the zone of inhibition on mm and showed on the following table 4.12.

4.10.1 Antibacterial tests of n-hexane extracts in different Gram positive and negative organisms

Table 4.12 Zone of inhibition of n-hexane extracts (100 mg/ml) against *Escherichia coli* using amikacin

Plant Extracts	Zone of inhibition (mm)	Amikacin
C.camphora	-	20 mm
S.wallichii	-	20 mm
R.indica	-	20 mm
W.fruticosa	-	20 mm
C.indica	-	20 mm
M.chisia	-	20 mm
M.nepalensis	-	20 mm
M.nepalensis	-	20 mm

Table 4.13 Zone of inhibition of n-hexane extracts 100mg/ml (in mm) against bacterial strain in *Staphylococcus aureus*

Plant Extracts	Zone of inhibition (mm)	Amikacin
C.camphora	-	20 mm
S.wallichii	-	20 mm
R.indica	-	20 mm
W.fruticosa	-	20 mm
C.indica	-	20 mm
M.chisia	-	20 mm
M.nepalensis	-	20 mm
M.nepalensis	-	20 mm

Table 4.14 Zone of inhibition of n- hexane extracts 100mg/ml (in mm) against bacterial strain in *Pseudomonas aeruginosa*

Plant Extracts	Zone of inhibition (mm)	Amikacin
C.camphora	-	20 mm
S.wallichii	-	20 mm
R.indica	-	20 mm
W.fruticosa	-	20 mm
C.indica	-	20 mm
M.chisia	-	20 mm
M.nepalensis	-	20 mm
M.nepalensis	-	20 mm

Table 4.15 Zone of inhibition of n-hexane extract 100mg/ml (in mm) of bacterial strain *Klebsiella pneumoniae*

Plant extracts	Zone of inhibition (mm)	Amikacin
C.camphora	-	18 mm
S.wallichii	-	18 mm
R.indica	-	18 mm
W.fruticosa	-	18 mm
C.indica	-	18 mm
M.chisia	-	18 mm
M.nepalensis	-	18 mm
M.nepalensis	-	18 mm

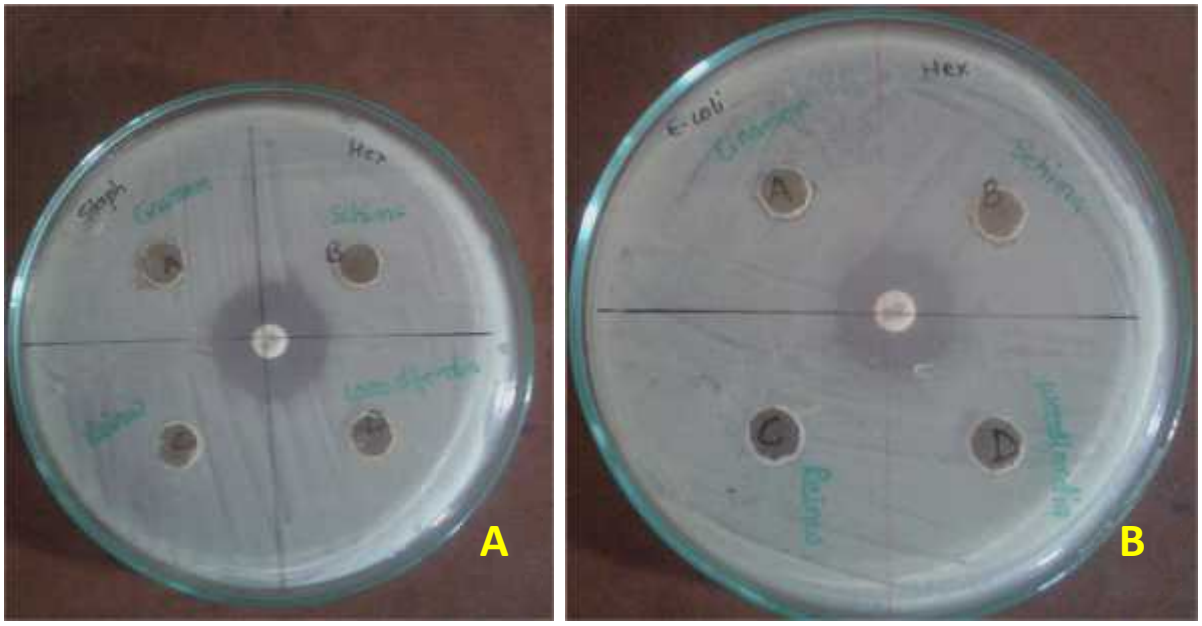


Figure 4.22 Hexane extracts of Plants in antibacterial tests

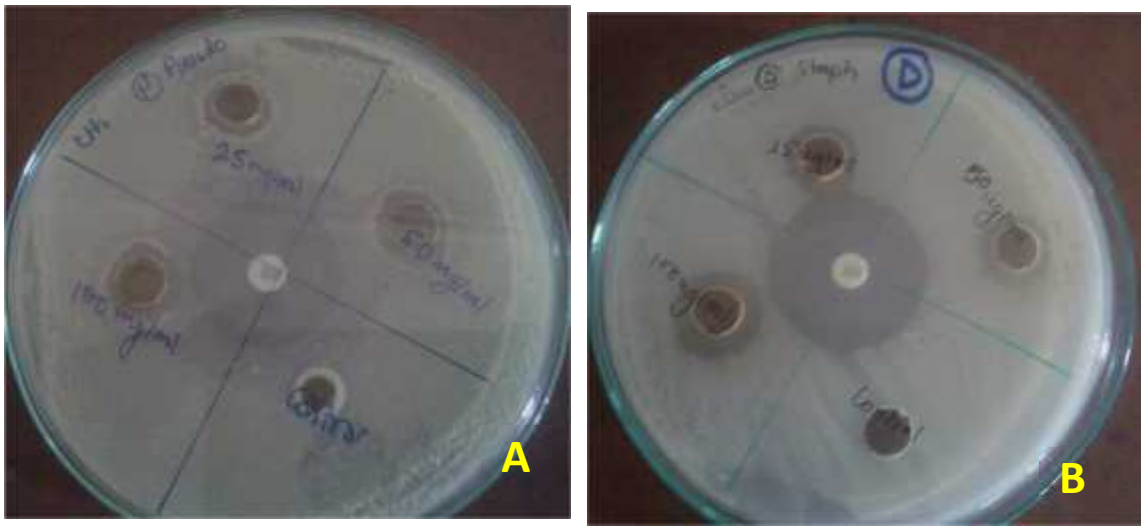
4.10.2 Antibacterial tests of Ethyl acetate extract in gram positive and Negative Organisms

Table 4.16 Zone of inhibition of ethyl acetate extract (in mm) against *Staphylococcus aureus*

Plants extracts	Zone of inhibition (mm)			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
C.camphora	-	-	-	20 mm	No ZOI
S.wallichii	9 mm	12mm	18 mm	20 mm	No ZOI
R.indica	6 mm	mm	9mm	20 mm	No ZOI
W.fruticosa	5 mm	8 mm	13 mm	20 mm	No ZOI
C.indica	-	-	-	20 mm	No ZOI
M.chisia	-	7 mm	12 mm	20 mm	No ZOI
M.nepalensis	5 mm	9 mm	12 mm	20 mm	No ZOI
M.nepalensis	2 mm	5 mm	10 mm	20 mm	No ZOI

Table 4.17 Zone of inhibition of ethyl acetate extract (in mm) against *Pseudomonas aeruginosa*

Plants extracts	Zone of inhibition (mm)			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
C.camphora	3	9	13	30	No ZOI
S.wallichii	-	-	-	30	-
R.indica	5	9	12	30	No ZOI
W.fruticosa	6	8	10	30	No ZOI
C.indica	5	9	11	30	No ZOI
M.chisia	4	7	12	30	No ZOI
M.nepalensis	5	9	11	30	No ZOI
Mahonia .nepalensis	4	10	13	30	No ZOI



Ethyl acetate extract in Pseudomonas aeruginosa and Staphylococcus aureus

Figure 4.23 Ethyl acetate extracts in antibacterial test

4.10.2 Antibacterial tests of methanolic extracts in Gram Positive and Negative Organisms

Table 4.18 Inhibition Zone of Methanolic extract (in mm) against *Escherichia coli*

Plants extracts	Zone of inhibition (mm)			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
C.camphora	10	12	13	23 mm	No ZOI
S.wallichii	No	No	12	23 mm	No ZOI
R.indica	No	No	No	23 mm	No ZOI
W.fruticosa	No	No	No	23mm	No ZOI
C.indica	10	15	20	23 mm	No ZOI
M.chisia	No	No	No	23 mm	No ZOI

M.nepalensis	No	No	No	23 mm	No ZOI
M.nepalensis	13	15	17	23 mm	No ZOI

Table 4.19 Inhibition Zone of Methanolic extract (in mm) against *Staphylococcus aureus*

Plants extracts	Zone of inhibition (mm)			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
C.camphora	No	No	No	20 mm	No ZOI
S.wallichii	No	No	No	20 mm	No ZOI
R.indica	No	No	No	20 mm	No ZOI
W.fruticosa	No	No	No	20 mm	No ZOI
C.indica	No	No	No	20 mm	No ZOI
M.chisia	No	No	No	20 mm	No ZOI
M.nepalensis	No	No	No	20 mm	No ZOI
M.nepalensis	No	No	No	20 mm	No ZOI

Table 4.20 Inhibition Zone of Methanolic extract (in mm) against *Pseudomonas aeruginosa*

Plants extracts	Zone of inhibition (mm)			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
C.camphora	6 mm	10 mm	14 mm	30 mm	No ZOI
S.wallichii	5 mm	8 mm	12 mm	30 mm	No ZOI
R.indica	6 mm	10mm	13 mm	30 mm	No ZOI
W.fruticosa	5 mm	8 mm	13 mm	30 mm	No ZOI
C.indica	3 mm	5mm	7 mm	30 mm	No ZOI
M.chisia	-	-	-	30 mm	No ZOI
M.nepalensis	-	-	-	30 mm	No ZOI
M.nepalensis	5 mm	7 mm	12 mm	30 mm	No ZOI

Figure 4.24 Methanolic extracts antibacterial tests

4.10.3 Antibacterial tests of Aqeous Extracts in gram positive and Negative Organisms

Table 4. 21 Inhibition Zone of aqueous extract (in mm) of bacterial strain E.coli using Amikacin

Plants extracts	Zone of inhibition			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
Cinamomum	-	-	-	23 mm	No ZOI
Schima	-	-	-	23 mm	No ZOI
Reinwardtia	-	-	-	23 mm	No ZOI
Woodfordia	-	-	-	23 mm	No ZOI
Castanopsis	-	-	-	23 mm	No ZOI
Maesa	-	-	-	23 mm	No ZOI
Mimulus	--	-	-	23 mm	No ZOI
Mahonia		-	-	3 mm	No ZOI

Table 4.22 Inhibition Zone of aqueous extract (in mm) against bacterial strain *Staphylococcus aureus*

Plants extracts	Zone of inhibition (mm)			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
C.camphora	-	-	-	20 mm	No ZOI
S.wallichii	-	-	-	20 mm	No ZOI
R.indica	-	-	-	20 mm	No ZOI
W.fruticosa	-	-	-	20 mm	No ZOI
C.indica	-	-	-	20 mm	No ZOI
M.chisia	-	-	-	20 mm	No ZOI
M.nepalensis	-	-	-	20 mm	No ZOI
M.nepalensis	-	-	-	20 mm	No ZOI

Table 4.23 Inhibition Zone of aqeous extract (in mm) of bacterial strain Pseudomonas using Amikacin

Plants extracts	Zone of inhibition			Positive Control(Amikacin)	Negative control (DMSO)
	25 mg/ml	50mg/ml	100mg/ml		
Cinamomum	-	-	-	30 mm	No Z0I
Schima	-	-	-	30 mm	No Z0I

Reinwardtia	-	-	-	30 mm	No Z0I
Woodfordia	-	-	-	30 mm	No Z0I
Castanopsis	-	-	-	30 mm	No Z0I
Maesa	-	-	-	30 mm	No Z0I
Mimulus	-	-	-	30 mm	No Z0I
Mahonia	-	-	-	30 mm	No Z0I

Table 4.24 Inhibition Zone of aqeous extract (in mm) of bacterial strain Klebsiella pneumonia using Amikacin

Plants extracts	**Zone of inhibition**			**Positive Control(Amikacin)**	**Negative control (DMSO)**
	25 mg/ml	50mg/ml	100mg/ml		
Cinamomum	-	-	-	29 mm	No Z0I
Schima	-	-	-	29 mm	No Z0I
Reinwardtia	-	-	-	29 mm	No Z0I
Woodfordia	-	-	-	29 mm	No Z0I
Castanopsis	-	-	-	29 mm	No Z0I
Maesa	-	-	-	29 mm	No Z0I
Mimulus	-	-	-	29 mm	No Z0I
Mahonia	-	-	-	29 mm	No Z0I

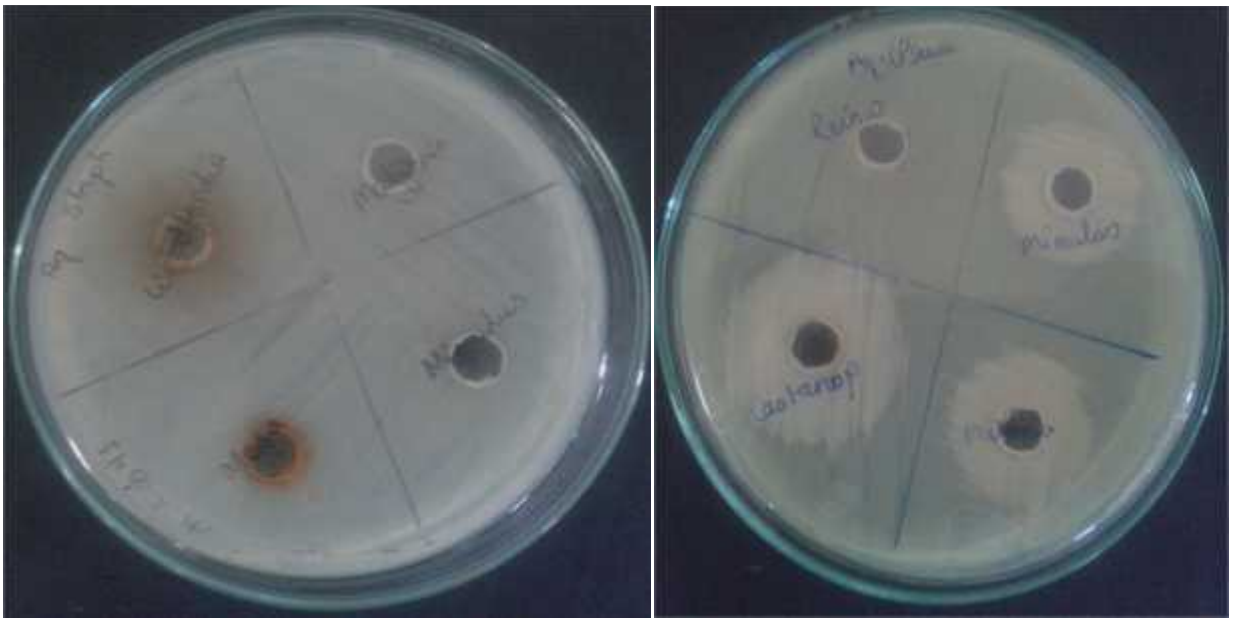


Figure 4.25 Aqeous extracts of plants in antibacterial tests

4.11 Gas Chromatography-Mass Spectroscopy

4.11.1 *Maesa chisia* extract (Methanolic)

After the GC-MS was carried out of methanolic extract of *Maesa chisia* 17 peaks were obtained of different compounds. Retention time, Area percentage, compound name along with m/z ratio is given in the tabulated form.

Table 4.25 Table from GC-MS of *Maesa chisia* methanolic extract

Peak#	R.Time	Area%	Name	Base m/z
1	4.559	22.19	1,2-Benzenediol	110.10
2	7.247	1.74	Diethyl Phthalate	149.10
3	9.217	1.41	2(1H)-Naphthalenone, 3,4,4a,5,6,7,8,8a.alph	208.15
4	9.419	12.40	2-Hydroxy-5-methylisophthalaldehyde	136.15
5	9.511	9.02	Hexadecanoic acid, methyl ester	74.05
6	10.723	14.81	8-Octadecenoic acid, methyl ester	55.05
7	10.883	8.71	Phenol, 2,6-bis(1,1-dimethylpropyl)-4-methy	219.20
8	12.078	1.46	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-e	57.05
9	12.225	2.74	Octanamide, N-(2-hydroxyethyl)-	98.10
10	13.223	1.85	9-Octadecenoic acid, 1,2,3-propanetriyl ester	55.05
11	13.371	1.82	13-Docosenoic acid, methyl ester, (Z)-	57.10
12	13.516	1.90	Nonadecanoic acid, methyl ester	74.05
13	13.579	2.67	Hexadecanoic acid, 2-hydroxy-1-(hydroxym	98.10
14	14.743	3.50	Oleoyl chloride	55.05
15	15.019	11.62	Methyl 18-methylicosanoate	74.05
16	16.759	0.85	Cholesta-3,5-diene	81.10
17	21.770	1.31	7,22-Ergostadienol	81.10
		100.00		

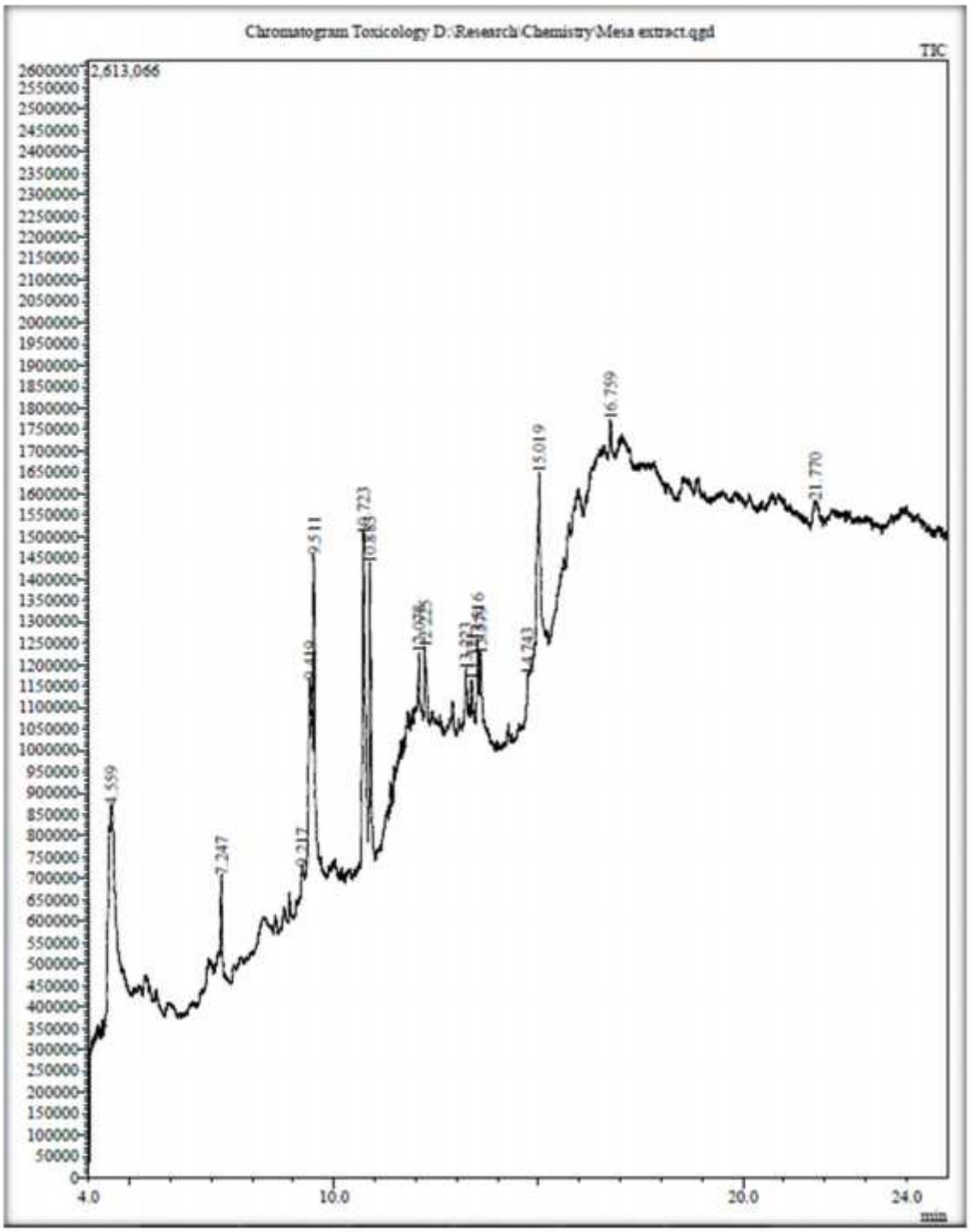


Figure 4.26 GC-MS chromatogram of *Maesa chisia* methanolic extracts

4.11.2 Mimulus nepalensis extract (Methanolic)

Table 4.25 Peaks from GC-MS of *Mimulus nepalensis* methanolic extracts

Peak	R.Time	Area%	Name	Base m/z
1	4.237	0.42	3-Undecene, 10-methyl-	56.05
2	4.998	0.30	Dodecane, 1,1-dimethoxy-	75.05
3	5.138	1.81	Heptanoic acid, 4-octyl ester	113.10
4	5.739	0.17	(2,6,6-Trimethylcyclohex-1-enylmethanesulf	137.20
5	5.925	0.40	Caryophyllene	93.10
6	6.296	4.50	Di-epi-.alpha.-cedrene	119.10
7	6.498	8.60	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methy	136.20
8	7.257	0.16	Diethyl Phthalate	149.10
9	7.717	3.73	2-Naphthalenemethanol, decahydro-.alpha.	59.05
10	7.996	4.17	alpha.-Bisabolol	43.00
11	8.926	1.91	3-Eicosyne	68.05
12	9.232	0.71	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	82.10
13	9.510	1.79	Hexadecanoic acid, methyl ester	74.05
14	10.750	2.72	11,14,17-Eicosatrienoic acid, methyl ester	79.10
15	11.414	2.91	1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-te	69.10
16	12.332	4.39	1-(5-Hexyl-2,4-dihydroxyphenyl)ethanone	165.10
17	13.202	1.46	9H-Xanthen-9-one, 1,3-dihydroxy-2-methyl-	242.10
18	13.593	1.85	Hexadecanoic acid, 2-hydroxy-1-(hydroxym	57.10
19	14.827	22.11	1,2-Bis(3,5,5-trimethyl-2-cyclohexenylidene	257.15
20	15.054	5.31	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(	287.20
21	15.207	2.89	Cholest-2-eno[2,3-a]naphthalene	179.10
22	15.503	5.33	3-Cyclohexene-1-propanoic acid, .beta.,4-di	165.10
23	16.335	2.57	1H-Naphtho[2,1-b]pyran, 3-ethenyldodecahy	249.15
24	16.584	3.76	Cannabinol	295.15
25	18.919	2.03	.alpha.-Tocopherol-.beta.-D-mannoside	165.15
26	20.946	5.42	Stigmasterol	55.05
27	21.893	7.40	.gamma.-Sitosterol	43.05
28	23.188	0.56	4,22-Cholestadien-3-one	81.10
29	24.424	0.60	Stigmast-4-en-3-one	124.15
		100.00		

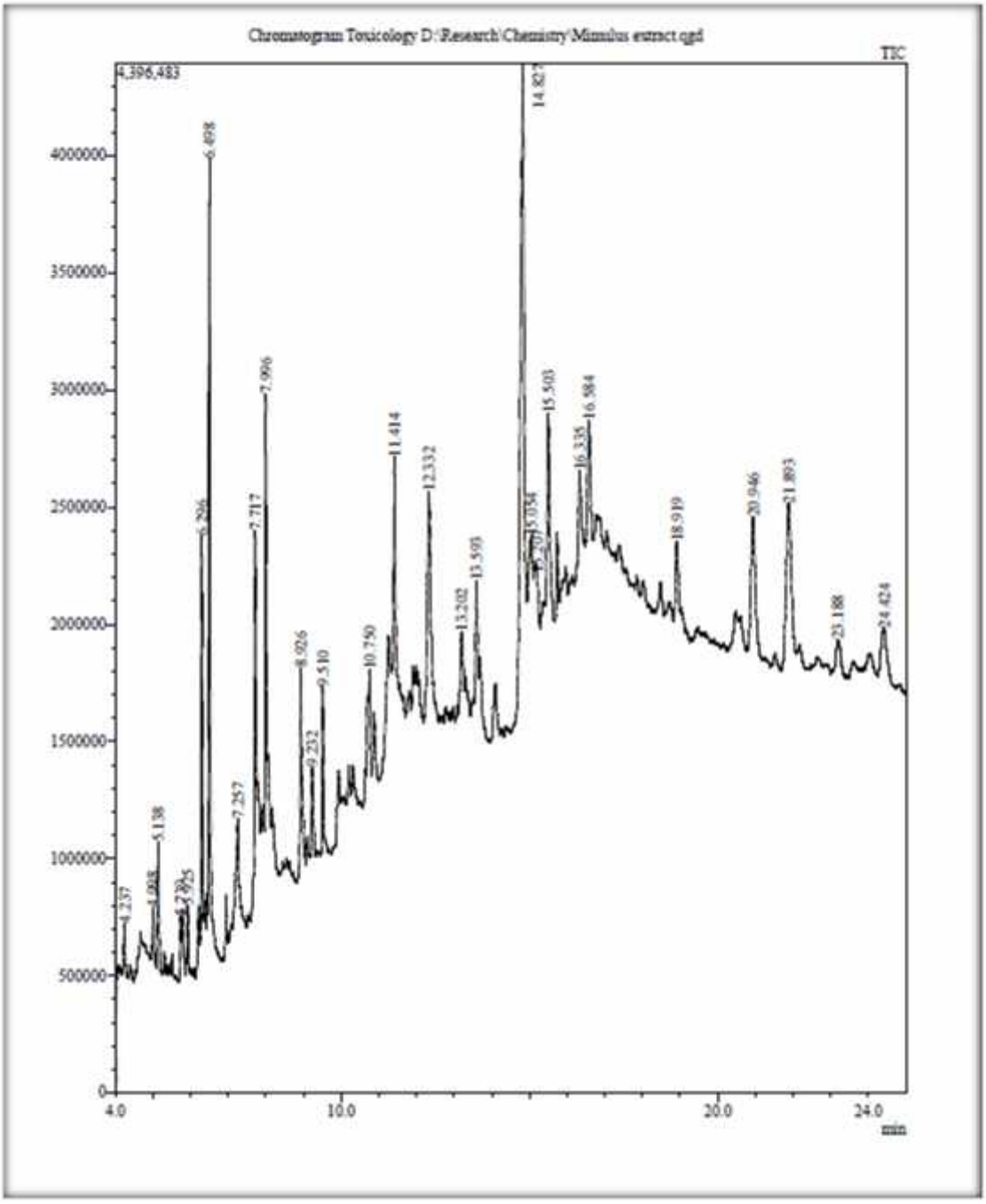


Figure 4.27 GC-MS chromatogram of methanolic extracts of *Mimulus nepalensis*

4.12 Thin Layer Chromatography

TLC was carried out for the calculation of retention factor. Two extracts namely methanolic and ethyl acetate was used for the TLC study and the solvent used were ethyl acetate and methanol 4:1, v/v

Table 4.28 Retention factor of ethyl acetate extracts

Sample	Distance by analyte	Distance by solvent	Retention Factor
C.camphora	6.2	8.3	0.74
S.wallichii	6.3	8.2	0.76
R.indica	3.3	8.0	0.41
W.fruticosa	3.5	8.2	0.42
C.indica	4.0	8.2	0.48
M.chisia	4.5	8.3	0.54
Mi.nepalensis	5.5	8.3	0.66
M.nepalensis	3.4	8.1	0.41

Table 4.29 Retention factor of methanolic extracts

Sample	Distance by analyte	Distance by solvent	Retention Factor
C.camphora	6.0	8.2	0.73
S.wallichii	5.9	8.1	0.72
R.indica	6.2	8.1	0.76
W.fruticosa	5.4	8.2	0.65
C.indica	5.3	8.3	0.63

M.chisia	5.3	8.2	0.64
Mi.nepalensis	5.5	8.2	0.67
M.nepalensis	5.7	8.2	0.69

Figure 4.28 TLC of plants extracts

4.13 Cytotoxicity

The result of cytotoxicity of methanolic extracts is presented in the figure 4.30 and of ethyl acetate is in the figure4.31

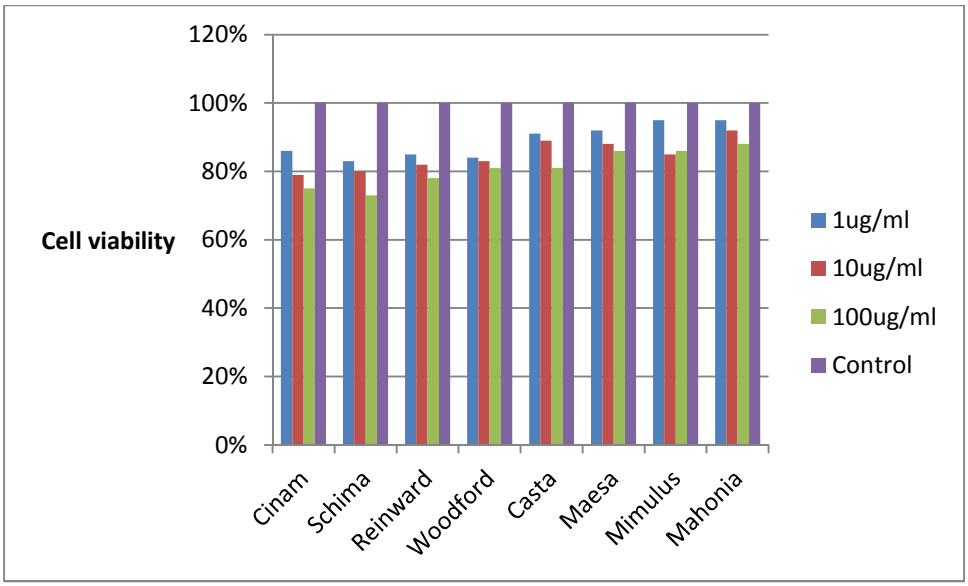


Figure 4.29 Cytotoxicity of methanolic plants extracts on Vero cell line

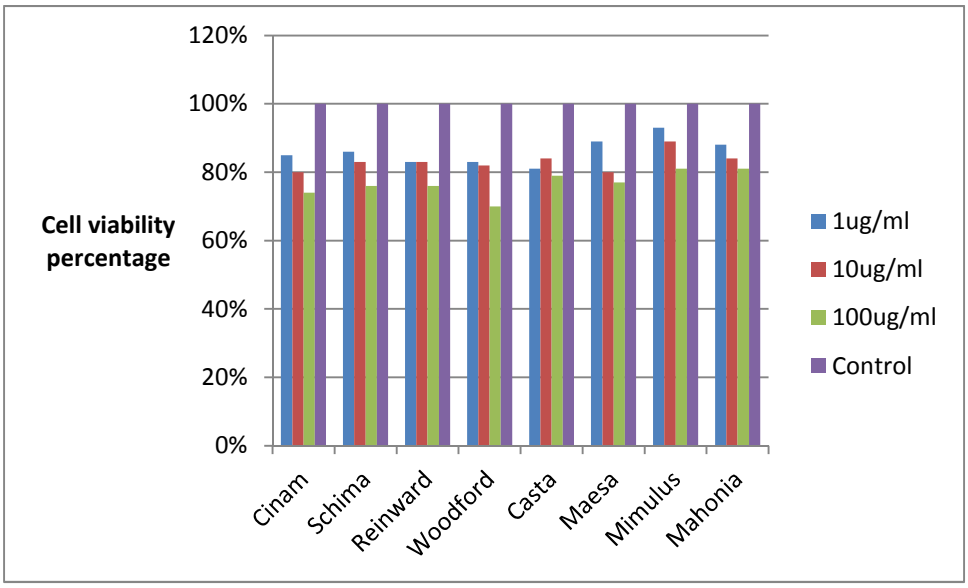


Figure 4.30 Cytotoxicity of ethyl acetate plants extracts on Vero cell line

Figure 4.31 MTT assay plate and cell proliferation

CHAPTER 5

DISCUSSION

In any kind of phytochemical investigation and analysis the first step is the extract preparation from plants.The percentage yield of any kind of extracts depend upon polarity of the solvent used and its system and all the extraction procedure.An extraction technique is that which is able to obtain extracts with high yield and with minimal changes tothe functional properties of the extract required (Quispe Candoriet al., 2008). Several studies have reported variations inthe biological activities of extracts prepared using different extraction techniques. Therefore, it is necessary to select the suitable extraction method as well as solvent based on sample matrix properties, chemical properties of the analytes, matrixanalyte interaction, efficiency and desired properties (Hayouni et al., 2007; Ishida et al., 2001).The present result also suggest and accord with the variation in percentage yield of plants extracts.This variation may be contributed to genotypic differences and their collection stages.Aqeous extracts has shown the higher extractive yield than other extracts because of high polarity of water than other solvents that results for the solubility of both polar and less polar compounds.

The preliminary phytochemical analysis and identification provides a rough outline for the presence of major secondary metabolites in the plant species. The qualitative screening of different plant specieswhich were ethanobotanically important revealed the presence of major phytochemicals.

5.1 Total Phenol Content

Folin-Ciocalteu Reagent has redox reagent. It forms blue colored complex on reaction with phenolic plants extracts (Kamboj et.al. 2015; Schofield, 2001). Fc reagent degrades quickly in alkaline solution. Excess reagent is required for the complete reaction.

Total phenolic estimation is performed by Folin-Ciocalteu method, few extracts showed good amount of phenolic contents. In this experiment higher reading of absorbance is shown by Polyphenol inhibits growth of microbes (Cowan, 1999) acts as active antioxidant, chelate metals and donate hydrogen atom (Tsao and Deng,2004).

5.2 Total flavonoid Content

For the flavonoid content determination spectrophotometric method is used and was determined by the complex formed of flavonoid-Aluminium Chloride (Fernandes et.al, 2012; Mabry at.al.,1970). This technique was originally developed for herbs analysis containing O-glycosides (Petry and Ortega, 2011). In this research, total flavonoid content was found highest in *Mimulus* and lowest in *Reinwardtia* in ethyl acetate extracts where as in methanolic extracts highest in *Maesa* and lowest in *Woodfordia*. Similarly in aqueous extracts highest in *Schima* and lowest in *Cinamomum.*

5.3 Antioxidant activity

DPPH is a free radical and it is stable at room temperature.It gets reduced in presence of any antioxidant moleculeand turns ethanol solution colorless.DPPH is a rapid method to quantify antioxidant using spectrophotometer (Huang, 2005). In this experiment highest IC50 value was shown by and lowest IC 50 value was found to be differing with the solvent used for the extraction.

5.4 Antimicrobial activity

The antimicrobial activity shown by the plants extracts may be due to the presence of bioactive compounds like polyphenol, alkaloid, terpenoid, flavonoid, tannins present in them (Cowan, 1999: Gonzalez-Lamothe, et.al., 2009). Various compounds present in the plants have the capacity to how the various multiple effects to inhibit bacterial growth like denatuaration of extracellular and intracellular proteins, deactivates the toxins, and transport the proteins disruption, (Cowan, 1999). Gram positive and gram negative both the organisms were used for the antibacterial tests (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli).*

5.5 Nanoparticles Synthesis

Bioreduction of silver ions to Ag nanoparticles was evaluated by UV–Vis spectroscopy which is the most simple and indirect method. For this experiment, 9 ml of 1 mM AgNO3 solution was taken as the initial amount to which 1 mL of aqueous leaf extract was added and incubated at room temperature (dark condition).

After overnight incubation, a visible color change was observed Different plant samples gave different colour after incubation.The intensity of the color was increased with increasing in incubation time. In the present study, we observed the appearance of absorption peak mostly at 421 nm and remaining other with 434 and 372 nm.Previous study reported that AgNPs give absorption peak at 420–450 nm as a result of its Surface plasmon resonance (SPR) character (Mohanta et al., 2016; Nayak et al., 2016). In our study, the observed absorption peak at 421 nm further confirms the biosynthesis of Ag nanoparticles.

5.6 Fluorescence Study

Fluorescent study of powdered sample using different chemical reagents showed differentcoloration under visible light and UV light. One of the important features of fluorescence is that UV light induces a fluorescent nature in many natural products (e.g.Alkaloids like berberine) where fluorescence is not seen in natural day light. Among the three solvents tested in the tests (Glacial Acetic Acid, Benzene, chloroform) they showed different kind of fluorescence in different solvents differing in the visible light and U.V light.One of the important feature of fluorescence is that UV light induces a fluorescent nature in many natural products (e.g. Alkaloids like berberine) where fluorescence is not seen in natural day light.

Some of substances may be often converted into fluorescent derivatives by using different chemical reagents though they are not fluorescent, hence we can often assess qualitatively some crude drugs using fluorescence as it is the most important parameter of pharmacognostical evaluation (Ansari et.al 2006).

5.7 Gas Chromatography-Mass analysis

In Maesa methanolic extract, there are 17 major peaks detected in samples as major compounds.All these compounds are not found to be useful from the medicinal point of view but some are found.From peak table of TIC, for each one peak, there are 5 possibly compounds related to target mass of the library which is printed as Hit#1 to Hit 5 in descending order of similarity. For example for peak #1,possibly of 5 compounds are-1,2 Benzenediol, Resorcinol,Hydroquinone, Pyridazinone and Aprocarp in descending order of similarity according to the target mass 110,64,81,92,53.So Hit# 1 compound is more probability than Hit#2 compound and so on.

5.8 Thin Layer Chromatography

This protocol simply describes the simple method for separating the extracts into subset of compounds. In the methanolic extracts the RF value is found greatest in the *Schima* (0.76) and only little less in *Cinamomum* (0.74) wheras the lowest value is found in the Reinwardtia and *Mahonia* (0.41) which is nearly equal to *Woodfordia* (0.42). Likewise in the ethyl acetate extracts,highest is found in the Reinwardtia(0.76) and lowest in the *Castanopsis*(0.63) In comparing the both ethyl acetate extracts found to be having the retention value greater in all the samples.

A small RF value indicates that the moving molecules are not very soluble in the hydrophobic (non-polar) solvent; they are larger and/or have a greater affinity for the hydrophillic paper (they have more polar groups) than molecules with a larger Rf. The temperature of the solvent and plate may also vary the value. Since the retention factor is based on the relative affinity of the chemical for the adsorbent compared to the solvent, changing the adsorbent can also greatly affect the result. The technique applied by the technicinas may also contribute for different results. If the sample is overloaded then the diffuse band is seen which makes the calculation quite difficult. Also thickness and uniformity of adsorbent layer vary from plate to plate especially if they are hand made

5.9 Vero cell cytotoxicity

First and foremost, the medicinal plant holds an important value in the field of pharmacology and traditionally it is being used in the cure of various diseases, possessing a high Ayurvedic value like antihelminthic, antibacterial, and nowadays it is mostly being researched in the field of oncology.

As the part of plant extract contains bioactive compounds such as quercitroside, isoquercitroside, rutoside, myrietol glucoside and kaempferol glycoside, it has become a essential part of medical pharmacology and many research are undergoing in it. The cell line

we used for the cell viability and cytotoxicity study was Vero. It grew well under our laboratory conditions, was lymphoblastas like in appearance and after 80% confluency, it was used for the MTT assay to see the viability due to the effect of the drug extracts.

MTT assay was used as the indicator of cell viability and cell cytotoxicity by the methanolic and ethyl acetate extract of medicinal plants extracts. Different concentrations of extract was prepared as 1 µg/ml, 10µg/ml and 100µg/ml and then used as the drug for the effect of extract on the Vero cell line. DMSO was used as the solubility reagent for the extract. The vero cell line was 8 passaged and cell was counted on haemocytometer to estimate the number of cells to put on in the MTT assay and MTT reagent as the test of viability. The MTT Cell Toxicity Assay measures the toxicity of the plant metabolites to kill the cells and the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell viability.

The MTT Reagent is ready to use and stable at 4°C in the dark for up to eighteen months, provided there is no contamination. Care should be taken not to contaminate the MTT Reagent with cell culture medium during pipetting. We recommend that the appropriate volume required for each experiment be removed and aseptically placed into a separate clean tube and the stock bottle returned to 4°C in the dark. If the MTT Reagent is blue-green, we should not use. (Wilson AP et al, 2000).

The above graph represents the viability percentage of cells with respect to different concentration which analyses the effectiveness of the extract in cell viability and cell cytotoxicity. The result indicates that the gradual decrease in viability of cell with respect to the different concentration of the cell line Vero. This suggest that the extract has a cell viability of % in concentrations of (1,10,100) µg/ml referring to cell control as 100% cell viability indicating that the extract has a impact on cell cytotoxicity and leading the cells to undergo apoptosis. This is due to the negative effect of cells in cell metabolism, thus reducing the number of cells in respective higher concentration of the extract. The nature of the graph indicates that the extract has effect in killing the cells.

Thus, it can be inferred that the plants extract is a possibility to be used as a drug for the Cell line and it needs to be further researched in other cell lines for its application in the medicine. Through purification the exact bioactive compounds can be screened and each chemical could be used in different cell lines and seen their effects in them. Hence, medicinal plants become a potent choice to study in the field of medicinal pharmacology to be used in other cell line.

CHAPTER 6

SUMMARY

Many new findings been done with this research work. In case of *Maesa chisia* only few works been done.

List of medicinal plants was collected from the ethanobotanical knowledge of ethnic community (Tamang Community). Preliminary tests were done for the plants which are first step for the general idea of the plants regarding the constituents present in the plants.

Different compounds have been identified by GC-MS techniques. Compounds with different structure are found which are important from the medicinal aspects too. Many compounds from *Maesa chisia* and four compounds from *Mimulus nepalensis* have been identified for which other research can be done for other purposes.

Similarly for phenol content the result was found with many variations such as in case of ethyl acetate highest phenol content was found in *C.camphora* and then *Mimulus nepalensis*. Likewise *W fruticosa* and *C.indica* have almost similar value.The lowest was found in the *Reinwardtia indica*. Phenol content in the methanol extract was highest in *C camphora* and *W*. fruticosa with almost similar value followed by *C. indica* and lowest was of *Mahonia nepalensis* and *Mimulus nepalensis*.For the aqueous extract highest content was found in *C.camphora* and lowest in *M.chisia*.

For the flavonoid content in the ethyl acetate extract highest was found in the *C.indica*and *Mimulus nepalensis* with similar value. Flavonoid content in this was almost high in all case except *R indica*. In the methanolic extract highest flavonoid was observed in *M chisia*and lowest *R.indica* followed by *W.fruticosa*. In aqueous, highest was observed in *S wallichii* and lowest *C.camphora.*

For the antioxidant activity, highest IC50 was found in *R.indica* and Lowest in *C.camphora* for the ethyl acetate extract. In methanolic extract Highest IC50 was observed in *M.chisia* and lowest in *C.indica* In the aqeous extract lowest IC50 was found in *C.camphora* and highest in *Mahonia nepalensis*.

For the nanoparticles synthesis, the silver nanoparticles was confirmed by the Colour change and max peak was observed in different nanometer. Almost every extract showed the peak at 421 nm except for Castanopsis which showed the peak at 447 nm.

Fluorescence test showed slightly different result in the visible light and UV light where benzene, chloroform and glacial acetic acid was used as solvent.

For the antibacterial tests different result were obtained in different extract in both gram positive and gram negative organism.

In case of TLC different Rf value was found in all the extract in which ethyl acetate and methanol was used as the solvent.

For the cytotoxicity analysis, in the methanolic extract showed highest cytotoxicity activity against the vero cell line was shown by *S.wallichii* followed by *C.camphora* and lowest by *Mahonia nepalensis* and *Maesa chisia*. In case of ethyl acetate highest cytotoxicity was shown by *W.fruticosa* and followed by *C.camphora* and lowest by *Mahonia nepalensis* and *Mimulus nepalensis.*

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusion

This present investigation deals the preliminary phytochemical analysis on different medicinal plants collected from the ethanobotanical knowledge of Tamang community. The results obtained from the different plant extracts are observed in percentage yield, phenol, flavonoid, antioxidant, nanoparticles, TLC, antibacterial, and other.

7.2 Recommendations

In regard to the present preliminary study, following recommendations can be made:

- This study is a novice that has covered only a small number of plant species from the ethanobotanical knowledge and concept. So, the remaining plants can also be studied and explored for all the medicinal properties and active bioconstituents and also for their bioactive compouds.
- With the availability of primary information on the studied plants extracts, further studies should be carried out like phytopharmacology, their standardization, identification and isolation of of active and pharmacological compounds.This may be followed by development of lead molecules for many diseases that can be validated/ tested in animal models.
- Screening of indigenous plants species and isolation of other novel/compounds should be the primary goal in the future research.
- The species of medicinal plants can be helpful in monitoring and establishing medicinal plants trade too.This will contribute to intensive pharmaceutical products development.

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