ANALYSIS OF PHYTOCHEMICAL AND BIOACTIVITY OF UNDERUTILIZED NEPALESE PLANTS

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CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Submitted by

Sandipty Kayastha

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List of Abbreviations

^0C	Degree Celsius
µl	Microlitre
µg	Microgram
λ	Wavelength
amu	atomic mass unit
ATCC	American Type Culture Collection
CDBT	Central Department of Biotechnology
DPPH	1, 1- diphenyl-2 picryhydrazyl
DW	Dry Weight
ESON	Ethno botanical Society of Nepal
g	Gram
GAE	Gallic Acid Equivalent
GC-MS	Gas chromatography-Mass spectroscopy
IC_{50}	Inhibitory Concentration 50
IUCN	International Union for Conservation of Nature
LC_{50}	Lethality Concentration 50
KRIBB	Korea Research Institute of Bioscience and Biotechnology
mg	Milligram
ml	Milliliter
MDR	Multi-drug resistance
MHA	Muller Hinton Agar
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass Spectrometry
NA	Nutrient Agar

OD	Optical Density
QE	Quercitin Equivalent
RSA	Radical Scavenging Activity
RT	Room Temperature
SD	Standard Deviation
Sp	Species
TB	Tuberculosis
TPC	Total Phenol Content
TFC	Total Flavonoid Content
UV	Ultra Violet
Wt	Weight
WHO	World Health Organization

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Abstract

Nepal, despite being rich in biodiversity, has not been able to utilize its huge resources of medicinal plants. Phytochemicals can be investigated for therapeutic purposes. The aim of the present work was to explore the medicinal values of under-utilized plants. Ten species (*Albizia chinensis* (Osbeck) Merrill, *Catharanthus roseus* (L.) G. Don, *Clerodendrum japonicum* (Thunberg) Sweet, *Euphorbia pulcherrima* Willd. ex Klotzsch, *Ficus religiosa L., Lydogium japonicum* (Thunb.) Swartz, *Punica granatum* L., *Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis, *Sapindus murokosii* Gaertner and *Camellia kissi* Wall) of plants were selected for the study. Methanolic extracts were used for the phytochemical analysis. The total phenolic and flavonoid content were estimated spectrophotometrically using Folin Ciocalteu and aluminium chloride colorimetric methods respectively. The highest and lowest amount of flavonoid was shown by *Ficus religiosa L.* (57.49±0.60 mg of QE/g) and *Albizia chinensis* (Osbeck) Merrill (22.45±0.18 mg of QE/g) respectively. The highest and lowest phenolic content was shown by *Punica granatum* L. (228.73±10.56 mg of GA/g) and *Albizia chinensis* (Osbeck) Merrill 24.67±1.99 mg of GA/g) respectively. The antimicrobial activity was tested against Gram positive (*S. aureus*) and Gram negative (*E. coli*) bacteria and fungi (*S. cerevisae* and *Pichia sp.*). *Punica granatum* L. showed antibacterial activity against Gram positive bacteria while the extracts were ineffective against Gram negative bacteria. Antifungal activity was observed only from *Camellia kissi* Wall. DPPH (2, 2-diphenyl-1-picryl hydrazyl) assay was carried out to evaluate the antioxidant activity. *Ficus religiosa L.* showed the highest antioxidant activity with IC50 value of (13.87±0.53 µg/ml) in comparison to ascorbic acid. The preliminary test for compounds with toxicity activity was performed using brine shrimp lethality assay at various concentration. Among the crude plant extracts tested *Lydogium japonicum* (Thunb.) Swartz, *Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis, *Punica granatum* L., *Catharanthus roseus* (L.) G. Don, *Camellia kissi* Wall, *Euphorbia pulcherrima* Willd. ex Klotzsch and *Ficus religiosa L.* were found to have high toxicity with LC_{50} value less between 1-100 ppm. GC-MS of *Punica granatum* L. and *Camellia kissi* Wall was performed which showed the presence of different compounds like pentadecanoic acid 16.73%, Cis-Vaccenic acid 15.85%, 03027205002 flavone 4'-oh,5-oh,7-di-o-glucoside 14.90% and 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid 12.75% and 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid 27.65%, betulin 15.64% and vaccenic acid 9.25% respectively. Thus, selected under-utilized plants showed presence of bioactive compounds which can be further identified and isolated for therapeutic activity.

Keywords: Phytochemicals, Antimicrobial, Antioxidant, Anti-cancerous, GC-MS.

CHAPTER 1. Introduction

1.1 Background

Nepal has been known for its biological diversity. Herbal plants in Nepal have been used as a medicine from the very ancient time in crude form even when the main constituents in the extract are unknown. Now days also these herbal plants are being used widely in many parts of the country especially in the areas where people do not have contact to modern medicines. We are heavily being relied on herbal medicine because of the high cost of the modern drugs available in the market. So the research in plants may lead to the development and validation of traditionally used medicinally important plants and enable full usage of the properties of these plants. According to World Health Organization (WHO), medicinal plants would be the best source to obtain variety of drugs. Drugs from the plants are easily available, less expensive, safe, and efficient and rarely have side effects.

In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Such a fact is cause for concern, because the problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. The global emergence of multi-drug resistant (MDR) bacteria is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure. Bacterial resistance to chemically unrelated antimicrobial agents is public health concern and may be caused by over-expression of MDR efflux pumps

There is a pressing need to develop new and innovative antimicrobial agents. According to World Health Organization medicinal plants would be the best source to obtain a variety of drugs. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. 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. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments.

1.1.1 Brief overview of Medicinal Plants in Nepal

Nepal is rich in biodiversity, because of extreme variation in altitude, complex topography and varied climatic conditions (Agricolture, 1996). Different types of the plants available are rich in medicinal value and have been used to cure variety of diseases from thousand years back (Prasad and Subedi, 2011). Since our country is a developing country 90% of the population solely rely upon the medicinal plants because the government is not able to equally facilitate all the regions with same medicinal facility (Prasad and Subedi, 2011). Nepal claims over 2.2% of the biological wealth of the world's natural flowering plants. Nepal ranks 25th in biodiversity richness globally and 9th richest flowering plant diversity in Asia (Dhakal *et al*., 2011). It is estimated that more than 7,000 vascular plant species are present of which 370 species are endemic and 700 species are medicinal plants. Altogether, 5,500 species have been collected, identified and preserved in the National Herbarium at Godavari, Lalitpur. The total number of edible species is estimated to be more than 500 belonging to about 70 families. There are about 200 cultivated species, which belong 150 genera of 50 families (Agricolture, 1996).

1.1.2 Phytochemicals in Medicinal Plants

Phytochemicals (phenol, flavonoid, tannin, alkaloid etc.) are the naturally occurring bioactive chemical compounds found in different parts of the plants like roots, stem, leaves etc. (Wadood *et al*., 2013). These chemicals contribute to the plants color, aroma, flavor and protect the plants against diseases, environmental stress etc.

Even though phytochemicals are not essential nutrients required by the human body for sustaining life but these chemicals play a vital role in human health to prevent or to fight some common diseases. Moreover, the biological properties present are the antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property provided by the phytochemicals to protect the humans against disorders (M. Saxena *et al*., 2001).

There are more than thousands of phytochemicals studied and many more to be known. Previously the crude drugs/extracts were made from the phytochemical rich plants on the basis of literatures available but now due to advancement in the field of pharmacognosy, various techniques have been followed for the standardization of crude drugs. Phytochemical screening is one of the techniques to identify new sources of therapeutically

and industrially important compounds like alkaloids, flavonoids, phenols, steroids, tannins, saponins etc. present in the plant extracts (Geetha *et al*., 2014).

There are many phytochemicals and each works differently. These are some possible actions:

Antioxidant - Most phytochemicals have antioxidant activity and protect our cells against oxidative damage and reduce the risk of developing certain types of cancer. Phytochemicals with antioxidant activity: allyl sulfides (onions, leeks, garlic), carotenoids (fruits, carrots), flavonoids (fruits, vegetables), polyphenols (tea, grapes).

Hormonal action - Isoflavones, found in soy, imitate human estrogens and help to reduce menopausal symptoms and osteoporosis.

Stimulation of enzymes - Indoles, which are found in cabbages, stimulates enzymes that make the estrogen less effective and could reduce the risk for breast cancer. Other phytochemicals, which interfere with enzymes, are protease inhibitors (soy and beans), terpenes (citrus fruits and cherries).

Interference with DNA replication - Saponins found in beans interfere with the replication of cell DNA, thereby preventing the multiplication of cancer cells. Capsaicin, found in hot peppers, protects DNA from carcinogens.

Anti-bacterial effect - The phytochemical allicin from garlic has anti-bacterial properties.

Physical action - Some phytochemicals bind physically to cell walls thereby preventing the adhesion of pathogens to human cell walls. Proanthocyanidins are responsible for the anti-adhesion properties of cranberry. Consumption of cranberries will reduce the risk of urinary tract infections and will improve dental health.

1.1.3 Classification of Phytochemicals

The exact classification of phytochemicals could have not been performed so far, because of the wide variety of them (M. Saxena *et al*., 2001). Phytochemicals are classified as primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary metabolites and secondary metabolites have terpenoid, alkaloids and phenolic compounds(Wadood *et al*., 2013).

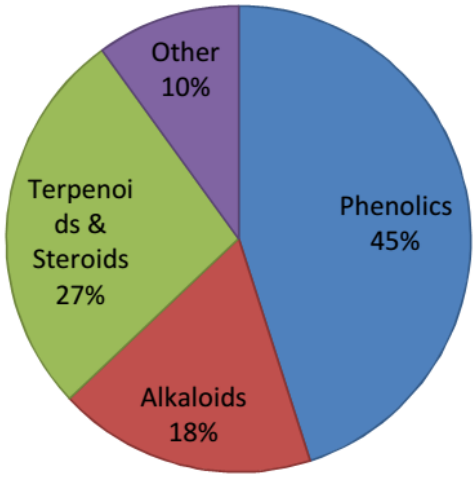

Figure 1.1 The major groups of plant Phytochemicals (Wadood *et al*., 2013)

1.1.3.1 Phenolic

Phenolic, phenols or polyphenols (or polyphenol extracts) are most abundant secondary metabolites in plants mainly responsible for the color of fruits (Doughari, 2009 ; Dai & Mumper, 2010). The three most important groups of phenolic are flavonoids, phenolic acids, and polyphenols (M. Saxena *et al*., 2001). Among the eight thousand naturally occurring plant phenolic about half this number are flavonoids (Vaidya Sala, 2012). Phenolic in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL) (Doughari, 2009). Plant parts rich in phenolics are leaves, flowering tissues and woody parts, such as stems and barks (Soni and Sosa, 2012). Phenolics are potentially toxic to the growth and development of pathogen(Khan *et al*., 2011). Other biochemical activities present in phenolics are antioxidant, anti-mutagenic, anti-carcinogenic as well as ability to modify the gene expression. The antioxidant activity of phenolics is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors (Soni and Sosa, 2012). Phenolics are the largest group of phytochemicals that account for most of the antioxidant activity in plants or plant products

(Vaidya Sala, 2012). Other examples of phenolics include flavones, rutin, naringin , hesperidin and chlorogenic (Doughari, 2009).

1.1.3.2 Flavonoids

Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts both in free state and as glycosides (Vaidya Sala, 2012). Flavonoids are ubiquitous among vascular plants and occur as aglycones, glucosides and methylated derivatives (Altok, 2011). The multiple biological property present in flavonoids are antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (M. Saxena *et al*., 2001). Other group of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, chalcones and catechin and leucoanthocyanidins (Doughari, 2009).

1.1.4 Antioxidants Activity in Medicinal Plants

Reactive oxygen species or free radicals (singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite) are metastable chemical species, which tend to trap electrons from the molecules in the immediate surroundings causing oxidative stress and leading to cellular damage (Mattson *et al*., 2006; S.N. Uddinet *et al*., 2013). Free radicals produced inside the body are neutralized by the antioxidant produced inside the body such as glutathione or catalases but the antioxidants are very less produced so the deficiency must be compensated by the use of natural exogenous antioxidants such as vitamin C, vitamin E, flavones-carotene and natural products in plants (C. K. Sen, 1995; Lindberg Madsen, 1995; Rice-Evans *et al*., 1997; Diplock *et al*.,1998). Antioxidants exert their activity by scavenging the ‘free-oxygen radicals’ thereby giving rise to a fairly ‘stable radical (S.N.Uddin *et al*., 2008). Plants contain a wide variety of free radicals scavenging molecules including phenols, flavonoids, vitamins, terpenoids that are rich in antioxidant activity (Lindberg Madsen, 1995; Cai *et al*.,2003).Many dietary polyphenol constituents derived from plants are more effective antioxidants in vitro than vitamins E or C, and thus might contribute significantly to protective effects in vivo (Rice-Evans & Miller, 1997; Jayasri *et al*., 2009).

1.1.5 Antimicrobial activity in Medicinal Plants

Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Perumal *et al*., 2008). The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. Phytoconstituents employed by plants to protect them against pathogenic insects, bacteria, fungi or protozoa have found applications in human medicine (Nascimento *et al*., 2000). In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, flavonoids, resins fatty acids gums which are capable of producing definite physiological action on body (Joshi *et al*., 2010). Plants with anti-bacterial effect are rich in polyphenol substances such as tannins, catechins, alkaloids, steroids and polyphenol acids. The anti-bacterial activity also could be due to various chemical components and the presence of essential oils in adequate concentrations, which damage microorganism. The insolubility of essential oils and nonpolar extracts make it very difficult for them to be used in an aqueous medium during the study of anti-microbial activity (Samy *et al*., 2008). A great number of factors can influence the results such as the extraction method, volume of media, culture composition and incubation temperature (Samy *et al*., 2008).

1.1.6 Brine Shrimp Lethality Assay

Different species of *Artemia* commonly known as Brine shrimp is used to elevate lethality of bioactive compounds present in the crude plant extracts. It serves as a guideline for the detection of antitumor and pesticidal compounds (Pradesh, 2015). Moreover brine shrimp is also used for detection of fungal toxins, heavy metals, cytotoxicity testing of dental materials etc. (Krishnaraju and Rao, 2005). Brine shrimps are used in all these tests because it is sensitive towards micro amount of the toxic substances (A. S. Michael *et al*., 1956).

The lethality of herbal extracts expressed as LC_{50} values is commonly valorized either by comparison to Meyer's or to Clarkson's toxicity index. According to Meyer's toxicity index, extracts with LC_{50}< 1000 µg/ml are considered as toxic, while extracts with LC_{50}> 1000 µg/ml are considered as non-toxic (Meyer *et al*., 1982). Clarkson's toxicity criterion for the toxicity assessment of plant extracts classifies extracts in the following order: extracts with LC_{50} above 1000 µg/ml are non-toxic, LC_{50} of 500 - 1000 µg/ml are low toxic, extracts with LC_{50} of 100 - 500 µg/ml are medium toxic, while extracts with LC_{50} of 0 - 100 µg/ml are highly toxic (Clarkson *et al*., 2004).

While carrying out the experiment larva hatched for 24 hours is used. The dried eggs can be stored for years and hatched when necessary. In this way there is no problem to maintain the culture. It is also easy, inexpensive, simple method. But along with the advantage comes disadvantage as well. Standardized experimental conditions in the means of temperature, salinity, aeration, light and pH, same geographical region of the cysts, etc. also must be maintained (Hamidi *et al*., 2014).

1.1.7 Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Gas chromatography (GC/MS) is normally used for direct analysis of components existing in plants (Sermakkani and Thangapandian, 2012). Gas chromatography coupled with mass spectroscopy (GC-MS) is normally used for direct qualitative and quantitative analysis of compounds present at molecular levels with very high precision. GC is used to separate the individual chemical components and the MS ionizes and identifies them by their structure and molecular weight. Further, the main advantage of GC-MS is that it can quantitative determining materials present even at very low concentrations. This feature of GC-MS has been widely utilized in forensic science, trace element analysis, pollution studies, quality control, etc. Recently GC-MS studies have been increasingly used for the analysis of non-polar components, volatile substances, alkaloids, phenols long chain and branched chain hydrocarbons, alcohols, acids, esters, and other bioactive components(Sivakumar *et al*., 2015).

Principle

Separation is due to differential distribution coefficients. In this chromatography, moving phase (or mobile phase) is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The instrument used to perform gas chromatography is called a gas chromatography (or 'aerograph', 'gas separator'). The gaseous compounds being analyzed interact with the walls of the column, which is coated with different stationary phases. This causes each compound to elute at a different time, known as the retention time of the compound. Secondly, the column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography has no such temperature control. Thirdly, the concentration of a compound in the gas phase is solely a function of the vapor pressure of the gas (Sermakkani & Thangapandian,2012).

1.2 Statement of problem

The development of antibiotics was one of the most important advances of medicine. Many bacterial infections (e.g. tuberculosis and infected wounds) that previously had no effective treatment and often killed people became treatable with antibiotics, saving millions of lives. Now, because of the overuse and misuse of antibiotics, bacterial infections that were once easily cured with antibiotics are becoming harder to treat. This is due to antibiotic resistance.

The World Health Organization has called this one of the biggest threats to human health today. Resistance is on the rise. An increasing number of pathogens are resistant to one or more drugs used to treat the diseases they cause. Indeed, many diseases common in developing countries —including malaria, pneumonia, cholera, and dysentery—are increasingly caused by strains that are resistant to multiple drugs. This is true for diseases such as tuberculosis (TB) and infections such as *Staphylococcus aureus* (*S. aureus*) that afflict rich countries as well as poor ones. The problem is global: Drug-resistant TB is spreading rapidly to countries where it has not been seen before. All currently available antimalarial, including artemisinin-based combination therapies (ACTs), have shown declining efficacy that could precipitate a global health crisis.

Many of the conditions accelerating drug resistance can be fixed, and the spread of resistance can be greatly slowed. For this the plants could be a good source for new, safe and biodegradable antimicrobial drugs, and could offer potential lead towards development of novel compounds that are active against pathogenic microbes.

1.3 Hypothesis

Plants have been used for the treatment of various diseases from ancient times. Thus, under-utilized plants may possess bioactive compound that can counter act various diseases. These active compounds can be evaluated and quantified by performing different phytochemical and bioassay.

1.4 Objectives

1.4.1 General Objectives

The overall goal of this research is to check the presence of bioactive compounds in the chosen plants and its toxic effects.

1.4.2 Specific Objectives

1) To estimate total phenolic and flavonoids content.
2) To evaluate antibacterial (*E.coli* ATCC- 25922, *S. aureus* ATCC -25923 and MRSA) properties.
3) To evaluate antifungal (*S. cerevisae, Pichia sp*.) properties.
4) To evaluate the lethality of the extracts.
5) To identify the bioactive compounds.

1.5 Rationale

Plants being a pool of chemical are believed to contain potent bioactive compounds. Being better compatibility with human body plants are taken as an alternative to modern medicine because of their complication emerging in a greater rate. Polyphenol and flavonoids are found to inhibit the prognosis of cancer by various mechanisms like scavenging of free radicals or by indirect inhibition of cancer promoting cellular metabolic product or biological chemicals. Plants under study are reported to have bioactive compounds with antioxidant and toxicity properties.

1.6 Scope

This study creates an interest among the researcher for exploration of medicinal importance of underutilized plants and if found to be effective, opens a new arena for further research and finally if approved can go for medical prescription. This research will give a scientific validation on the bioactive compounds present in the selected plants. Apart from this study would be a preliminary step towards identification of active compounds. Also the most important thing is that it will create awareness among the people to use these plants in better way, exploiting it for their own benefit or say benefit sharing.

Botanical Name	**Taxonomy**		
	Order	**Family**	**Common Names**
Albizia chinensis (Osbeck) Merrill	Fabales	Fabaceae	Kalosiris
Catharanthus roseus (L.) G. Don	Gentianales	Apocynaceae	Bahramasephool
Clerodendrum japonicum (Thunberg) Sweet	Lamiales	Lamiaceae	Dhapre
Euphorbia pulcherrima Willd. ex Klotzsch	Malpighiales	Euphorbiaceae	Lalupate
Ficus religiosa L.	Rosales	Moraceae	Peepal tree
Lydogium japonicum (Thunb.) Swartz	Schizaeales	Lygodiaceae	JanaiLaharo
Punica granatum L.	Myrtales	lythraceae	Anar
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	Gentianales	Rubiaceae	Bajrajanti
Sapindus murokosii Gaertner	Sapindales	Sapindaceae	Reetha
Camellia kissi Wall	Ericales	Theaceae	Goldhari

Table 1.1 Taxonomical classification and ethnobotanical information of plant species selected for study.

CHAPTER 2. Literature review

Antibiotics developed long time back is now showing resistance. It is very necessary to develop antimicrobials from the natural medicinal plant sources to replace the synthetic ones. Antimicrobial activity of different extracts (ethanol, methanol, petroleum ether and aqueous) of *M. azedarach* was done against some bacteria and fungus which was regarded as pathogenic to humans. All the extracts of *M. azedarach* showed antimicrobial activity. Significant zone of inhibition was shown by the methanol and ethanol extract compared to petroleum ether and aqueous. From this it was concluded that the bioactive compounds present in medicinal plants showing antibacterial property was organic in nature and was easily soluble in the organic solvents (Sen and Batra, 2012).

Flavonoids present in propolis were considered as an important element for evaluating propolis quality. Two complementary colorimetric methods: aluminum chloride method and 2, 4-dinitrophenylhydrazine method was used for quantitative determination of the flavonoids. However, the convenient colorimetric method utilizing aluminum chloride reaction to determine flavonoid contents was proved to be specific only for flavones and flavonols, while another colorimetric method utilizing 2, 4-dinitrophenylhydrazine reaction was specific for flavanones. Therefore, both analyses must be conducted so that the sum of the results may better represent the real content of total flavonoids (Chang *et al*., 2002).

The antiradical activities of various antioxidants were determined using the free radical, 2.2-Diphenyl-l-picrylhydrazyl (DPPH). In its radical form, DPPH has an absorption band at 515 nm which disappears upon reduction by an antiradical compound. The interaction of a potential antioxidant with DPPH depends on its structural conformation. Certain compounds react very rapidly with the DPPH reducing a number of DPPH molecules corresponding to the number of available hydroxyl groups. However, for the majority of the compounds tested the mechanism was more complex (Brand-Willliams *et al*., 1995).

Among the various test organisms, *Artemia salina*, a crustacean, commonly known as the brine shrimp was proved to be superior for the detection of insecticidal residues. It not only proved to be sensitive against a broad range of compounds but it also solved the problem of maintaining culture. The dry eggs of the shrimp can be stored easily for years retaining its viability. More over whenever there was a necessity of the eggs to carry out the experiment it can be hatched in less than 24 hrs at 30^0C using salt solution. When *Artemia salina* was used to test the toxicity of insecticide in acetone solution suspended in various

concentration of water or salt solution it showed sensitivity in micro amount. All age groups of *Artemia* from 24-hrs-old nauplii to adults were tested. Usually shrimp move freely in the salt water and take position in the air-liquid interface. But when the toxic material was present the movement of shrimp was affected and it becomes unable to stay at the top. This way we can find the toxicity of insecticide in 45 mins to 2 hrs (A. S. Michael *et al*., 1956).

Brine shrimp lethality assay was reported to be a very simple, inexpensive method to determine the toxicity of bioactive compounds present in the plants. Moreover it was also used to analyze the toxicity of heavy metals, pesticides, medicines especially natural plant extracts and etc. It was based on the killing ability of test compounds on a simple zoological organism-brine shrimp (*Artemia salina*).While carrying out this assay it was very important to know about the solvent used because some of the solvent itself may be lethal and give false positive result. The organic solvent that was best used was DMSO, methanol and TWEEN20. TWEEN20 is a detergent; having long alkyl chains and water-soluble functional groups. Hence was water-soluble and able to dissolve hydrophobic compounds with their alkyl chains being more toxic (Dr. Chao Wu, 2012).

Cytotoxicity evaluation of ethanolic extracts of *Lantana camara, Chromolaena odorata,* and *Euphorbia hirta* was done using the brine shrimp. The lethality concentration (LC_{50}) of *Lantana camara, Chromolaena odorata*, and *Euphorbia hirta* extracts were 55 ppm (µg/ml), 10 ppm, and 100 ppm respectively. Maximum mortalities (100%) were observed at a concentration of 1000 ppm in both *Lantana camara* and *Euphorbia hirta* extracts while that of *Chromolaena odorata* was at 100 and 1000 ppm. All the three extracts contained bioactive compounds because their LC_{50} were less than 1000ppm. Thus the pharmacological properties in these plants was due to the bioactive components present (Olowa and Nuñeza, 2013).

Brine shrimp was used as a test organism for the detection of toxicity of the active plant compounds by calculating the LC_{50}. It showed sensitivity towards micro amount of the toxic residues. Screening results with seed extracts of 41 species of *Euphorbiaceae* were compared with 9KB and 9PS cytotoxicity. This method is easy, inexpensive and simple so must be readily utilized by pharmacognosists and natural product chemists in the detection and isolation of higher plant constituents with a variety of pharmacologic activities (B. N. Meyer *et al*., 2015).

2.1 Lygodium japonicum (Thunb.)Sw

Lygodium japonicum (Thunb.)Sw, fern commonly named as Japanese climbing fern was reported native to eastern Asia, including Japan, Korea, southeastern Asia, and India, and eastern Australia. It produces a creeping stem from which grows very long leaves, the longest exceeding 30 meters (98 feet). The leaves have rachises, which are vine-like and may climb other vegetation.

It was commonly used as folk medicine in China as a diuretic (Puri, 1970) to treat colds, inflammation, kidney stones and renal ailments (Eisenberg *et al*., 2009),in India as an expectorant and to treat snakebites (Reutter, 1923; Puri, 1970; Srivastava and Uniyal, 2013) and to treat diabetes, wounds and ulcers (Yumkham and Singh, 2011) and In Nepal, paste used to treat scabies, juice used to treat herpes and wounds (Manandhar, 1995) and its juice was applied for boils, wounds, whitlow and scabies (Mall *et al*., 2015). Formal studies had been conducted to determine medicinal benefits of *L. japonicum* (Thunb.)Sw Duan *et al*., (2012) showed that this species had compounds with strong antioxidant properties.

According to literatures available its main constituents were steroidal, flavonoids, organic acid and for roots were ecdysteroside, triterpenes, flavonoids, phytosterols, glycosides and organic acids. The three new compounds isolated and identified were: lygodiumsteroside A and lygodiumsteroside B and 2-isopropyl-7-methyl-6-hydroxy-α- (1, 4) naphthoquinone. The other pharmacological properties present was antibacterial, antivirus and liver protection (Guo-gang & Ying-cui ,2012).

2.2 Randia tetrasperma (Roxb.)Benth. & Hook. f. ex Brandis

Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis is a large shrub with horizontal spines; flowers hairy, yellowish- white, fragrant; fruit berry, fleshy. It has many local names like Gangeri, Kikra, Kukal, Khukhuri (Hindko), Mainphal (Urdu), Mindla (Punjabi). Fruit was found to be irritating and emetic; used as fish poison. Pulp of fruit was used in dysentery, anthelmintic, abortifacient treatment. Moreover, leaf and stem was used for spine extraction from flesh (Samin Jan *et al*., 2008).The phytochemicals present in the leaves of *Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis was extracted using ethanol as the solvent. The extract was found to contain various phytoconstituents such as alkaloids, saponin, tannins, flavonoids, glycosides and phenols. Each of these phytoconstituents had its importance like saponins used for the treatment of cancer, tannins used to cure inflammation etc. Total phenol and flavonoid content were found to be 85.5 mg GA/g and 45 mg Rutin/g respectively. The antimicrobial activity was evaluated against four clinical

isolates *S. aureus*, and *P. aeruginosa*, *C. albicans* and *A. niger* maximum activity was observed against *S. aureus* (MIC0.187 mg/ml) followed by *P. aeruginosa* and the fugal isolates (MIC 0.375 mg/ml), and antioxidant potential was assessed by its ability to scavenge various free radicals like DPPH, Super oxide and the Hydroxyl radicals. IC_{50} values of extract and the standard Ascorbic acid for stable free radical DPPH was found to be 95 and 30 µgs, for hydroxyl radical it was 90 and 40 µgs and that of super oxide radical was found to be 60 and 30 µg/ml (Gulnaz *et al*., 2014).

2.3 Sapindus mukorossi Gaertner

Sapindus mukorossi Gaertner is a fairly large, deciduous tree, usually up to 12 m in height, sometimes attaining a height of 20 m and a girth of 1.8 m, with a globose crown and rather fine leathery foliage. It was found to be native to China and Japan. The fruit and seeds were regarded as a cure for epilepsy in northern India. A decoction of the fruit was used as an expectorant. Seeds were used in China to stop dental caries. The fruit was considered to be haemolytic (Al, 2009).

Sapindus mukorossi (Gaertn), has several commonly names such as soapnut, soapberry, washnut, reetha, aritha, dodan and doadni. Pericarps of *it* had been traditionally used as an expectorant and natural surfactant. Due to the presence of saponins, soapnut was well known for its detergent and insecticidal properties and was traditionally used for removing lice from the scalp. The leaves were used in baths to relieve joint pain and the roots were used in the treatment of gout and rheumatism. Other pharmacological effects of *Sapindus mukorossi* (Gaertn) were like anti-bacteria, insecticidal, spermicidal, anti-trichomonas, anti-tumor, hepatoprotective, anxiolytic, molluscicidal, fungicidal, anti-inflammatory and piscicidal activities. Moreover it was used for the treatment of different ailments in the indigenous system of medicine (Upadhyay and Singh, 2012).

Antioxidant activity, polyphenol compounds contents, reducing potential and lipid peroxidation activities from extracts of *S. mukorossi (Gaertn*) were evaluated by different assays. Ethanolic, methanolic and aqueous extract of leaf and fruit's pericarp were used for above assay through standard method. Maximum antioxidant activity was observed in aqueous extract of fruit. Total phenolic content was maximum in the methanolic extract of fruit (469.00±0.57 mg/g of gallic acid equivalent) and total flavonoid content was maximum in methanolic extract of leaf (540.11±0.89 mg/g of rutin equivalent).High reducing potential was observed in case of aqueous extract of both leaf and fruit. A positive correlation was observed between antioxidant activity and polyphenol compounds (total phenolic content

and total flavonoid content). Similarly, a significant correlation was observed between antioxidant activities and reducing potential, indicating that reducers present in extract are major contributors to the antioxidant potential (Singh and Kumari, 2015).

2.4 Punica granutam L.

The pomegranate, botanical name *Punica granatum* L., is a fruit-bearing deciduous shrub or small tree growing between 5 and 8 m (16 and 26 ft.) tall. The pomegranate was originated in the region of modern day Iran, and had been cultivated since ancient times throughout the Mediterranean region and northern India. The pomegranate has multiple spiny branches, and is extremely long-lived, with some specimens in France surviving for 200 years (Ahirrao *et al*., 2013).

The bioactive compounds present in ethanolic, aqueous and chloroform extracts of *P. granatum* L. peel, whole fruit and seeds were examined. The three different extracts from peel were found to contain triterpenoids, steroids, glycosides, flavonoids, tannins, carbohydrate &vitamin C. The three different extracts from whole fruit were found to contain triterpenoids, steroids, glycosides, saponins, alkaloids, flavonoids, tannins, carbohydrate & vitamin C. The three different extracts from seeds were found to contain triterpenoids, steroids, glycosides, saponins, alkaloids, tannins, carbohydrate & vitamin C. Since the whole fruit extract contains the components of both peel and seeds, it was found to contain more constituents. It would thus mean that in this study , the whole fruit extract had the highest number of bioactive compounds and among the three extracts ethanolic extract was more potent (Bhandary *et al*., 2012).

The potential therapeutic property of pomegranate was reported to be of wide-range including treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, and protection from ultraviolet (UV) radiation. Other potential applications include infant brain ischemia, Alzheimer's disease, male infertility, arthritis, and obesity (Bhandary *et al*., 2012). Different part of pomegranate like bark, leaves, immature fruits, and fruit rind had some medicinal importance (M. Hajoori, *et al*., 2014). This may be due to the presence of bioactive compounds and secondary metabolites in different parts of the plant. Moreover the antimicrobial potential of the plant may be attributed to the various bioactive compounds present in the crude extracts (Ahirrao S.D., 2013).

Among the various solvents used in this study, the methanolic extract of P. *granatum* L. leaves inhibited *S. aureus, B. cereus, S. typhi and P. mirabilis*. Moreover, only methanolic extract demonstrated antifungal activity *A. niger, A. flavus, T.rubrum, C. albicans* and

Cryptococcus sp. The extract also contained carbohybrates, reducing sugars, phenolics, flavonoids, tannins etc. (Nishita *et al*., 2012).

The ethanolic rind extract of *P. granatum* L. fruit had been subjected to identify the phytochemicals by GC-MS analysis. *In vitro* antioxidant activity of the plant extract was observed by DPPH radical scavenging assay and the FRAP assay. The GC-MS study identified the bioactive compounds like like 3, 5-Dihydroxy-6-methyl-2, 3-dihydro-4H-pyran-4-one, Nitro isobutyl glycerol, Ethyl. Alpha.-d-glucopyranoside, 3H-indole-3-carbaldehyde (4-amino-5-methyl-4H-1, 2, 4-triazol-3-yl) hydrazone, maltol etc. The extract showed significant scavenging effect on DPPH, it was increased with the increasing concentration from 50-250 µg/ml but the scavenging activity of all extracts was lower than that of standard (Zab *et al*., 2012)**.**

2.5 Catharanthus Roseus(L.) G. Don

*Catharanthus roseus (*L.) G. Don is an evergreen subshrub or herbaceous plant growing 1 m tall. In the wild, it is endangered plants due to habitat destruction by slash and burn agriculture. The species has long been cultivated for herbal medicine and as an ornamental plant.

Due to its high survivability in a variety of habitats it was found to be widely distributed around the World (Don, 2013). Alkaloids, saponins, flavanoids, carbohydrates and anthraquinone glycosides were present in *Catharanthus roseus (*L.) G. Don (Yedukondalu *et al*., 2013) resulting to its wide range of pharmacological activity like anti-inflammatory, antimalarial, antimitotic, antihypertensive, antifertility, antihypercholesterolemic, antimutagenic, antidiuretic, antifungal, antispasmodic, antiviral, cardio tonic, CNS depressant, antitumor, cytotoxic, antispermatogenic, anticancer activities (Ibrahim *et al*., 2011)

Different extracts of leaves and flowers of *Catharanthus roseus (*L.) G. Don was used to carry out the cytotoxic, antioxidant and antibacterial tests. Methanol extract of leaves and flower showed potent brine shrimp cytotoxic assay with LC_{50} values 3.18 and 12.57 µg/ml respectively. In the antibacterial assay the extract showed better result in gram positive bacteria compared to the gram negative bacteria. On the other hand, the free radical scavenging activity of the extracts of leaves and flowers were IC_{50} 24.14 µg/ml and 108.20 µg/ml respectively (Shahin Aziza *et al*., 2014).

Catharanthus roseus (L.) G. Don is a medicinal plant rich in phytochemical constituents. It consists of different types of anticancer alkaloids like Vinblastine and Vincristine. These

alkaloids have growth inhibition effect to some human tumors. Vinblastine is used experimentally for treatment of neoplasms and is recommended for Hodgkin's disease, choriocarcinoma. Vincristine another alkaloids is used for leukemia in children. Vinblastine is sold as velbanor vincristine as oncovin. The other pharmacological properties are anti-diabetic, anti helmintic, anti-diarrheal, anti-microbial etc. (Sain and Sharma, 2013).

All parts of different plants (leaf, stem and root) having medicinal value was taken for the phenol and flavonoid estimation. The methanol leaves extract of *Catharanthus roseus* (L.) G. Don showed 163.02 mg GA/100 g of phenol and 12.80 mg CE/100 g of flavonoid present in it (Srivastava *et al*., 2013)**.**

2.6 Camellia kissi Wall

Extracts of leaves from the tea plant contained polyphenols with activity against wide spectrum of microbes. Studies conducted over the last 20 years have shown that the green tea polyphenol catechins, in particular (−)-epigallocatechingallate (EGCg) and (−)-epicatechingallate (ECg), can inhibit the growth of a wide range of Gram-positive and Gram negative fungal species with moderate potency. The study was carried out on various species of fungal *Candida albicans* (MTCC No.183), *Candida tropical* (MTCC No.184), and *Saccharomyces cerevisiae* (MTCC No.170) using cup plate method (Inamdar *et al*., 2014).

Methanolic extracts of *Camellia sinensis* was taken to examine its ant proliferative property against Hella cell line. As a result *C. sinensis* decreased the viability of the cancer cell line. The viability was increased with the concentration. Thus, *C. sinensis* can be further studied to identify and isolate the anti-cancerous compound (Geetha and Santhy, 2013).

2.7 Albizia chinensis (Osbeck) Merrill

Bark of Albizia chinensis (Osbeck) Merrill was reported to contains tannin along with the other phytochemical constituents like alkaloids, steroids, flavonoids, reducing sugars and gums (Chinensis *et al*., 2015; YU, Shishan *et al*., 2014). Prominent free radical-scavenging property of the stem bark may be correlated to its high phenolic content or due to synergistic activity from various chemical entities present in the extract (Merr, 2014).

Different species of *Albizia chinensis* (Osbeck) were investigated and found the presence of secondary metabolites such as saponins, terpenes, alkaloids and flavonoids. Due to the presence of these bioactive compounds in plants, each part of the plant has its own pharmacological properties (Khatoon *et al*., 2013). The antioxidant activity of *Albizia*

procera leaves through DPPH, exhibited an IC_{50} value of about 90% among that of DPPH radicals. Novel macrocyclic alkaloids (budmunchiamines A, B and C) isolated from A. Amara found to have antiplatelet aggregation and bactericidal activity. The other pharmacological properties present are anticancer, ant diabetic, anti-inflammatory, hepato protective etc. Thus, *Albizia* species shall be considered as a promising plant with various therapeutic properties and can be further explored pharmacologically against various ailments and for free- radical related diseases (Kokila *et al*., 2013)**.**

Methanolic extract of *A. chinensis (Osbeck)* stem bark, as well as its organic and aqueous soluble fraction were studied to know its total phenolic content, antioxidant properties present in the medicinal plant. Among the different fractions used carbon tetrachloride-soluble fraction (CTCSF) showed high phenolic content (60.76 ± 0.45 mg of GA/g of sample) along with maximum free radical-scavenging activity (IC_{50} = 10.21 ±0.84 µg/ml) when compared to ascorbic acid (IC_{50} = 5.8 ± 0.21 µg/ml). High antioxidant property of the plant may be due to the high phenolic content or due to the synergistic activity of different bioactive constituents present in it. The general toxicity was determined using a brine shrimp lethality bioassay, where the carbon tetrachloride (LC_{50} 0.608 ± 0.19 µg/ml) and hexane (LC_{50} 0.785 ± 0.26 µg/ml) soluble partitionates had considerable bioactive properties. The crude extract and its chloroform- and aqueous-soluble fractions displayed significant antimicrobial activity against eighteen microorganisms with inhibition zones ranging from 7.0 mm to 26.0 mm (Merr, 2014).

2.8 Euphorbia pulcherrima Willd. ex Klotzsch

Euphorbia pulcherrima Willd. ex Klotzsch (poinsettia) is a shrub or a small tree. Its latex caused allergic reaction in sensitive individuals, irritation to the skin or stomach and diarrhea and vomiting if eaten (Rahman *et al*.,2013). It had a common herbs used as concoction against ailments such as resembling typhoid and gastroenteritis in Kano Nigeria. Potential secondary metabolite found in it was used for the treatment of ant arthritis, anticancer, anticonvulsant, ant diabetic, anti-eczema, anti-eczema, anti-inflammatory, antimicrobial and antioxidant (Rauf and Muhammad, 2013).

Methanol extract and different solvent fractions of the aerial parts of *Euphorbia pulcherrima* Willd. ex Klotzsch was used to carry out the phytochemical and pharmacological tests. It consisted of various phytochemicals like alkaloids, steroids, terpenoids, saponins, glycosides, reducing sugar and amino acid with various pharmacological properties. Methanol, ethyl acetate extracts showed significant free radical

scavenging activity of 90.22% and 91.31% respectively. In case of the antibacterial test all the solvent showed resistance against *E.coli* and *S. aureus* whereas ethyl acetate fraction and methanol extract showed moderate antibacterial property especially against *K. pneumonia, S. epidermidis B. stearothermophihu s*and *S. Typhimuriun*. Searching new antimicrobial agent is a big challenge for the scientist of the present modern era and plants the biggest source of these agents (Rauf and Muhammad, 2013).

2.9 Clerodendrum japonicum (Thunberg) Sweet

Clerodendrum japonicum (Thunberg) Sweet is an important medicinal plant in Southwest China. Its roots and beautiful red flower was used to treat arthritis, liver problems, eye dysfunction, hemorrhoids, hernia and insomnia. A plant with a multitude of beneficial functions and yet a marvelous ornamental flower for the warm humid garden.

Antimicrobial and antioxidant activities of methanol extract as well as n-hexane, chloroform, ethyl acetate and aqueous fractions of *Clerodendrum viscosum Vent*. Leaves were evaluated. The maximum antibacterial activity was shown by ethyl acetate fraction. Moreover ethyl acetate fraction of methanol extract also showed highest total phenolic content (TPC) and total flavonoid. The ethyl acetate fraction of methanol extract of *C. viscosum* has significant amount of total phenolic and flavonoids and has shown higher in vitro antioxidant and antimicrobial activity compared to the crude extract and other fractions. The highest scavenging activity may be due to the phenolic, flavonoid and tannins present. *C. viscosum* can be used as natural antioxidant in slowing down ageing and to cure other damages caused by reactive oxygen species (Ghosh *et al*., 2014).

Brine shrimp lethality bioassay was done using ethyl alcohol and chloroform extracts of root, leaf and stem of *C. infortunatum*. The result was analyzed calculating the LC_{50} value in mg/l. Along with the LC_{50} value 95% confidence limits; regression equation andχ2values were also calculated. The LC50values of the crude extracts of root, leaf and stem were found to be 20.845, 24.017 and 31.379 mg/l for ethyl alcohol, 30.702, 32.907 and 42.559 mg/l for chloroform respectively and 14.675 mg/l for the tetracycline. All the values of extract was found to be lethal thus proving the presence of bioactive compound. This is just a preliminary test so to determine the extract compound further process must be done (Waliullah *et al*., 2015).

2.10 Ficus religiosa L.

Ficus religiosa L. is a medicinally important tree. Nearly every part of the tree can be used as medicine because stem bark of *Ficus religiosa* L. Contains phenols, tannins, steroids, alkaloids and flavonoids etc., leaves contains tannic acid, arginine, serine, aspartic acid, glycine, threonine, alanine, etc., fruit contains asgaragine, tyrosine, undecane, tridecane, tetradecane, etc. Alanine, threonine, tyrosine have been reported in seeds whereas the crude latex shows the presence of a serine protease, named religiosin (Makhija *et al*., 2010). Leaf juice has been used for the treatment of asthma, cough, sexual disorders, diarrhoea, haematuria, earache and toothache, migrane, eye troubles, gastric problems and scabies (Salem *et al*., 2013).

Ficus religiosa L. had been used to cure different diseases like dysentery, diarrhea, nervous disorders, tonic, astringent and many more. Each part of the plant had its own significance and was used separately to cure distinct disorders. *F. religiosa* L. had so many pharmacological properties due to the presence of the phytochemicals like tannins, saponins, flavonoids, steroids, terpenoids and cardiac glycosides. According to Ayurvedic system of medicine, *F.religiosa* L. (Peepal tree) was well known to be useful in diabetes (Makhija *et al*., 2010).

Antibacterial activity of diethyl ether and methanol extract of bark and leaves of *Ficus religiosa* L. plant against three bacteria (*E. coli, S. aureus* and *P. aurignosa*) and one fungus (*A. niger*) was studied. The result showed that the methanol extracts of both leaves and bark showed antimicrobial activity on the three bacteria and no effect on fungi. In methanol extracts *S.aureus* showed maximum sensitivity (inhibition zone 28mm) followed by *E.coli* (inhibition zone 16mm) and *P. aurignosa* (inhibition zone 12mm).Diethyl ether extracts of leaves also showed maximum inhibition on *S.aureus* followed by *E.coli* and *P. aurignosa*. Both methanol and diethyl ether extracts of bark showed antimicrobial activity on three types of tested bacteria and very less inhibition activity on *A. niger*. But comparatively bark extracts of both the solvents showed less antimicrobial activity than leaves extracts (Ramakrishnaiah *et al*., 2012).

CHAPTER 3. Materials and methods

3.1 Materials

3.1.1 Chemicals and Reagents

All the chemicals and reagents (Appendix A) that were used during the research work were of analytical grade (Merck Co., Mumbai). The total research work was conducted at the Central Department of Biotechnology, TU.

3.1.2 Instruments

The instruments that were used during this thesis are Centrifuge, Autoclave, Microwave oven, Incubator, UV spectrophotometer, GC-MS and all of them were in good working condition.

3.1.3 Plant Samples

The plant materials have been supplied by "Bioprospecting on bioresources" (Nepal project) conducted by KRIBB (Korea), CDBT and ESON. Collected plants were identified and verified by Central Department of Botany, Tribhuvan University, Kirtipur, Nepal.

3.1.4 Bacterial Strains

ATCC cultures of *Escherichia coli* (25922), *Staphylococcus aureus* (25923), MRSA

3.1.5 Fungal Strains

Sacharomyces cerevisae, Pischia sp.

3.2 Methods

3.2.1 Selection of the plant samples

Less studied and underutilized plants in Nepal were selected.

3.2.1.1 List of Plants Selected

Scientific Name	Plant parts Used	Life Form	Collection Date	Collection Site
Lydogium japonicum (Thunb.) Swartz	Whole plant	Climber	01-07-14	Phulkharka-3 Dhading
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	Leaf, Stem	Shurb	29-06-14	Phulkharka-3 Dhading
Sapindus murokosii Gaertner	Leaf	Tree	13-07-14	Vetenary, Dhangadi
Punica granatum L.	Stem,Leaf	Tree	14-07-14	Taranagar-5, Dhangadi
Catharanthus roseus (L.) G. Don	Stem, Leaf	Herb	13-07-14	Taranagar-5, Dhangadi
Camellia kissi Wall	Stem	Tree	25-06-14	Pulkharka-3, Dhading
Albizia chinensis (Osbeck) Merrill	Bark	Tree	04-16-14	Budhathum-8, Dhading
Euphorbia pulcherrima Willd. ex Klotzsch	Leaf	Tree	10-08-14	Golang, Againchok, Dhading
Clerodendrum japonicum (Thunberg) Sweet	Leaf	Shrub	10-08-14	Golang, Againchok, Dhading
Ficus religiosa L.	Bark	Tree	02-10-14	Narayansthan-7, Atherikhola, Baglung

Table 3.1 Description of plants species under study including plant parts used for the study along with their site of location and life forms of the plants

Scientific Name	GPS Coordinates	Elevation (meters)	Difficulty of Collection
Lydogium japonicum (Thunb.) Swartz	N 28°03.840', E 84° 55.225'	1375	Medium
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	N 28° 04.358', E 84° 55.172'	1670	Medium
Sapindus murokosii Gaertner	N 28° 42' 10.5'', E 80° 35'00.1''	154	Medium
Punica granatum L.	N 28° 42' 52.8'', E 80° 35' 11.3''	160	Hard
Catharanthus roseus (L.) G. Don	N 28° 42' 52.8'', E 80° 35’ 11.3’’	160	Easy
Camellia kissi Wall	N 28° 04.319', E 84° 55.265'	1660	Easy
Albizia chinensis (Osbeck) Merrill	N 28° 04' 48.1'', E 84° 50' 14.6''	755	Easy
Euphorbia pulcherrima Willd. ex Klotzsch	N 28° 01.057', E 84° 50.674'	850	Medium
Clerodendrum japonicum (Thunberg) Sweet	N 28 01.097', E 84 50.329'	770	Medium
Ficus religiosa L.	N 28° 12.560', E 83° 38.752'	975	Medium

Table 3.2 Description of plants under study

3.3 Preparation of the plant material

The collected plant material were air/shade dried at 32-35^{o}C for 6 days to remove all their moisture. The dried plant material was powdered with the help of grinder, and the fine powder was collected on sterile and dry polyethylene bag for extraction**.**

3.4 Preparation of plant extracts and Extracts dilution

About 100 g of fine powder of each plant sample was taken separately and dissolved in 750 ml of 100 % methanol and left to percolate for 24 hrs. After percolation, these samples were subjected to ultra-sonication for 3 days (2 hrs each day). The solvent was removes after filtration. Remaining methanol was removes by evaporation on the Rotatory Evaporator and finally incubated at 37^0C till the solid mass was obtained. Obtained solid mass was weighed carefully to express the gram of extract extracted per 100 g of the plant powder. For each sample extract were prepared individually. The extracts were kept at 4^0C for further analyses.

Each 100 mg of methanol extract was weighed accurately and dissolved on 1 ml methanol. This 100 mg/ml stock of each plant extract was used for antimicrobial tests, antioxidant activity, quantification of the total phenol and total flavonoids.

$$\text{Percentage Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant material}} \times 100$$

3.5 Qualitative phytochemical analysis (Richardson and Harborne 1985)

The methanol extracts were used to screen for the presence of various secondary metabolites.

3.5.1 Test for flavonoids

3.5.1.1 Alkaline reagent test

Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow color was formed which turned colorless on addition of few drops of diluted acid which indicated the presence of flavonoids.

3.5.2 Test for glycosides

3.5.2.1 Liebermann's test

Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice. Carefully concentrated $H_2SO_4$was added. A color change from violet to blue to green indicated the presence of steroidal nucleus, i.e., glycone portion of glycoside.

3.5.3 Test for steroids

Crude extract was mixed with 2ml of chloroform and concentrated H_2SO_4 was added sidewise. A red color produced in the lower chloroform layer indicated the presence of steroids. Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of each of concentrated H_2SO_4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids.

3.5.4 Test for terpenoids

Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for about 2 mins. A grayish color indicated the presence of terpenoids.

3.5.5 Test for alkaloids

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

3.5.6 Test for phenols and tannins

Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

3.6 Quantitative phytochemical analysis

3.6.1 Total polyphenol content (McDonald *et al*., 2001)

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

3.6.2 Total flavonoid content (Chang *et al*.,2002)

The total flavonoid content in the plant extract was estimated using the Aluminium chloride ($AlCl_3$) colorimetric method. 0.25 ml of extract (10 mg/ml) was separately mixed with the 0.75 ml of ethanol, 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium acetate (CH_3COOK) and 1.4 ml of the distilled water. The reaction mixture was incubated for 30 mins at room temperature. Then absorbance of the mixture was measured at 415 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained with the help of the quercetin (Sigma) as standard dissolved in methanol with the concentration from 10-100 µg/ml. The total flavonoid content was expressed in terms of the mg of quercetin equivalent per g of the dry mass (mg QE/g). The test was triplicated for the reproducibility of results.

3.6.3 Determination of Total Antioxidant activity via DPPH free radical scavenging assay (Brand-Willliams *et al*., 1995)

The antioxidant activity of extracts and standard (Ascorbic acid) was assessed on the basis of the radical scavenging effect of the stable 1, 1- diphenyl-2 picryhydrazyl (DPPH) - free radical activity. Different concentration of plant extract (10-150 µg/ml) and ascorbic acid (1-200 µg/ml) were prepared in methanol on clean test tubes. 0.5 ml of samples of plant extract as well as ascorbic acid of each concentration was taken separately in clean test tubes. To this sample 0.5 ml of the 0.2 mM DPPH solution was added. The tubes were shaken uniformly for proper mixing and incubated in dark for half an hr. The control was prepared as above but without the plant extract or ascorbic acid and methanol was taken as blank. The absorbance was taken on spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5) at 517 nm.

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

$$\%\, Radicalscavengingactivity \;=\; [(Controlabs - sampleabs)/Controlabs)]\; x100\%$$

Then a standard graph was plotted taking the concentration of Ascorbic acid on the X-axis and percentage scavenging activity on the Y-axis. Based on this standard graph, IC_{50} value of each sample was calculated based on the formula $IC50 = EXP\,(LN\,(conc.>50\%) - ((pi>50\%-50)/\,(pi>50\%-pi<50\%) * LN\,(conc.>50\%/conc.<50\%)))$using Maes *et al.,* (2010). The IC_{50} value of the different species was compared. The species having the lowest IC_{50} was considered to have the best antioxidant property.

3.7 Determination of Antibacterial activity

3.7.1 Preparation of Nutrient agar (NA)

NA plates and NA broths were prepared for the antibacterial tests. About 28 g of the powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and poured in distilled water. The contents were dissolved on the water completely and the final volume was maintained to 1000 ml followed by boiling for uniform mixing. This media was sterilized on an autoclave at 15lbs pressure at 121^0C for 15 mins. The autoclave tape was used as an indicator for the completeness of sterilization. After cooled to about 45-50^0C, the media was poured on sterilized and properly labeled petriplates. About 25 ml of the media was poured on each petriplate of 9 cm diameter in sterile conditions under a laminar flow hood. All of the plates were left for the solidification of media. For the preparation of the NB broth, screw tight bottles were filled with NB media and auto-claved. It was then cooled and used.

3.7.2 Preparation of Mueller Hinton Agar (MHA)

The Mueller Hinton Agar (MHA) Miller media was required for antimicrobial tests. About 38 g (Hi Media Laboratories Pvt. Ltd, Mumbai, India) from the powder supplied was carefully weighed and transferred on a conical flask. The content was dissolved in distilled water and final volume was maintained to 1000 ml. This media was transferred to the screw bottles and sterilized on autoclave at 15 lbs pressure and 121^0C for 15 mins. Autoclave tape was used for the indication of the completeness of the sterilization. Finally this media was cooled in laminar airflow and dispensed in sterile and dry petriplates.

3.7.3 Preparation of the standard bacterial culture Inoculums

The individual pure ATCC cultures of *Escherichia coli, Staphylococcus aureus*, was obtained from Tribhuvan University Teaching hospital and were inoculates in broth with the help of the sterilized inoculating loop. The inoculated culture bottles were kept on the incubator at 37^0C for overnight. The turbidity of the sub-cultured bacterial suspension was adjusted at the 0.5 McFarland standards (freshly prepared on the other day for the antibacterial tests. These bacterial inoculums were used for the swabbing on the MHA plates to test the antimicrobial effects of the plant extracts.

3.7.4 Antibacterial test

First, all the sterile and dry plates were properly labeled with name of bacteria, name of the plant species and the concentration of the plant extract to be added. The MHA plates were

inoculated with the appropriate bacterial culture by a sterile cotton swab aseptically. The culture plates were allowed to dry for about 15 to 20 mins.

The antimicrobial tests were performed by modified agar well diffusion method. On the above prepared MHA plates five wells were prepared on the solid MHA media with the help of the sterile cork borer (4 mm diameter). Five different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml) of the plant sample were prepared on methanol. With the help of the sterile pipette the 20 µl of the each individual plant extract were poured in the above prepared wells. The methanol was taken as negative control while the ampicillin 10mg/ml or piperacilin 10mg/ml was taken as the positive control. The plates were incubated on the microbial incubator overnight at 37^0C and the zone of inhibition was observed for individual plant extract for individual bacteria at different concentrations.

3.8 Determination of Antifungal activity

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

About 25 g of PDA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed, dissolved in distilled water and final volume was maintained to 1000 ml. This media was autoclaved at 15 lbs pressure and 121^0C for 15 mins, was cooled in laminar airflow and dispensed in sterile petriplates. Likewise, PD broth was prepared in culture tubes excluding agar.

3.8.2 Preparation of the standard fungal cultures

The individual pure and characterized cultured *of Sachharomyces cerevesiae* and *Pischia sp.* was obtained from CDBT, TU and were sub-cultured in PD broth with the help of the sterilized inoculating loop and kept on the shaking incubator at 37^0C and 120 rpm for overnight. The turbidity of the sub-cultured fungal suspension was adjusted to 0.5 McFarland standards. These bacterial inoculums were used for the swabbing on the MHA plates to test the antimicrobial effects of the plant extracts.

3.8.3 Antifungal test

Properly labeled PDA pertiplates were taken and test cultures were inoculated by modified agar well diffusion method. The petriplates were allowed to dry for about 15 to 20 mins.

On the above prepared PDA plates, six wells were prepared with the help of the sterile cork borer (4 mm diameter). Five different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml,

12.5 mg/ml and 6.25 mg/ml) of the plant extracts were prepared on methanol. With the help of the sterile pipette, 20 µl of the each individual plant extract was poured in the above prepared wells. The methanol was taken as negative control. Fluconazole at the concentration of 10 mg/ml was taken as the positive control. The plates were incubated on the microbial incubator overnight at 28^0C and the zone of inhibition was observed for individual plant extract for individual bacteria at different concentrations.

3.9 Brine Shrimp Toxicity Assay

Brine Shrimp toxicity assay was employed to find out potential toxicity of the plant extracts. The protocol given in a book entitled "Bioassay Techniques For The Drug Development" by (Atta-ur-Rahman *et al*., 2005) was modified and carried out in the experiment. The toxicity was carried out in a microtitre plate.

1 g of brine shrimp (*Artemis salina*) eggs were allowed to hatch in one liter of artificial sea water prepared in laboratory. The temperature condition was maintained at 22-28^0C with continuous aeration for 48 hrs. Soon after hatching, the larvae were harvested using glass pipette into small vial. Each of the larvae was picked from the vial and placed into the wells of the microtitre plate. A total of five larvae were placed in each well. Then after the crude plant extracts were prepared at three different concentrations of 1000 ppm, 100 ppm and 10 ppm. For this, initially the extracts were dissolved in minimum amount of methanol (10 mg/100 µl methanol). Then the resulting solution was made to desired concentrations by serial dilution with the same sea water. To each of the wells containing 5 brine shrimp larvae, 100 µl of each of the extracts of each concentration were added. The inoculated microtitre plate was then incubated at 22-28^0C for 24 hrs. Potassium dichromate ($K_2Cr_2O_7$ at 1 mg/ml) was used as positive control while methanol was used to check the solvent toxicity and plain sea water was taken as negative control. After 24 hrs of incubation the viability of the larvae was observed with naked eyes on lamp light and the number of live larvae was counted. The percentage of mortality was calculated as:

$$\text{Percentage of mortality (PM)} = \frac{\text{Live count in control} - \text{Live count in test}}{\text{Live count in control}} * 100$$

Then the LC_{50} values for each of the extracts were also calculated according to the given formula below:

$$\text{LC50} = \text{EXP}\frac{\text{LN (conc> 50\%) - (signal>50\%} - 50)}{(\text{Signal>50\%} - \text{signal <50\%})} * \frac{\text{LN (conc>50\%)}}{(\text{conc<50\%})}$$

EXP: exponential function; LN: Natural log function, both used in graph pad prism software

Signal >50%: PM value just above 50%; signal <50%: PM value just below 50%

Conc >50%: Concentration of signal >50%; conc <50%: Concentration of signal <50%.

3.10 Gas Chromatography-Mass Spectrometry analysis (GC-MS)

50 mg of extracts of *Punica granatum* L. and *Camellia kissi* Wall were dissolved in 1ml of methanol and the samples were analyzed using GC-MS. GC-MS analysis was performed using Shimadzu QP 2010. The inert gas helium (99.9995%) was used as carrier gas, at flow rate of 1.21ml/min, split ratio 10:1; sample size, 1µL injected using the split less injection technique; fused capillary silica column HP-5 (30m × 0.25mm × 0.25µm). The sample was injected into the column with the injector temperature at 260^0C. GC oven temperature started at 100^0C and holding for 3mins and it was raised to 250^0C at the rate of 10^0C /min and holding for 5mins. Then the temperature was raised to 280^0C at the rate of 15^0C /min and was hold for 20mins. The injector and detector temperatures were set at 260^0C and 300^0C respectively. Ion source temperature was maintained at 230^0C. The mass spectrum of compounds in samples was obtained by electron ionization at 70eV and the detector was operated in scan mode from 45-450 amu (atomic mass units). A scan interval of 0.5sec and fragments from 45 to 450 Da was maintained. The total running time was 60mins. Identification of compounds was conducted using the database of NIST11 and WILEY8 libraries. Mass spectrum of individual unknown compound was compared with the known compounds stored in the software database libraries. The name, molecular weight and structure of the components of the test samples were ascertained.

3.11 Satistical Analysis

All the experiments were performed in triplicates for each sample and the values were reported as mean ± SEM. The obtained data were also subjected to the analysis of variance and mean values were compared. All the statistical analysis was done using Graph pad Prism5 and Excel software.

CHAPTER 4. Results

4.1 Percentage yield of plant extracts

All the plant samples collected were subjected to methanol extraction. The total amount of methanol plant extract isolated from 100 g of the finely powdered whole plant material is shown in the following figure 4.1**.** The highest and lowest yield of extract was found in *Punica granatum* L. and *Albizia chinensis* (Osbeck) Merrill respectively.

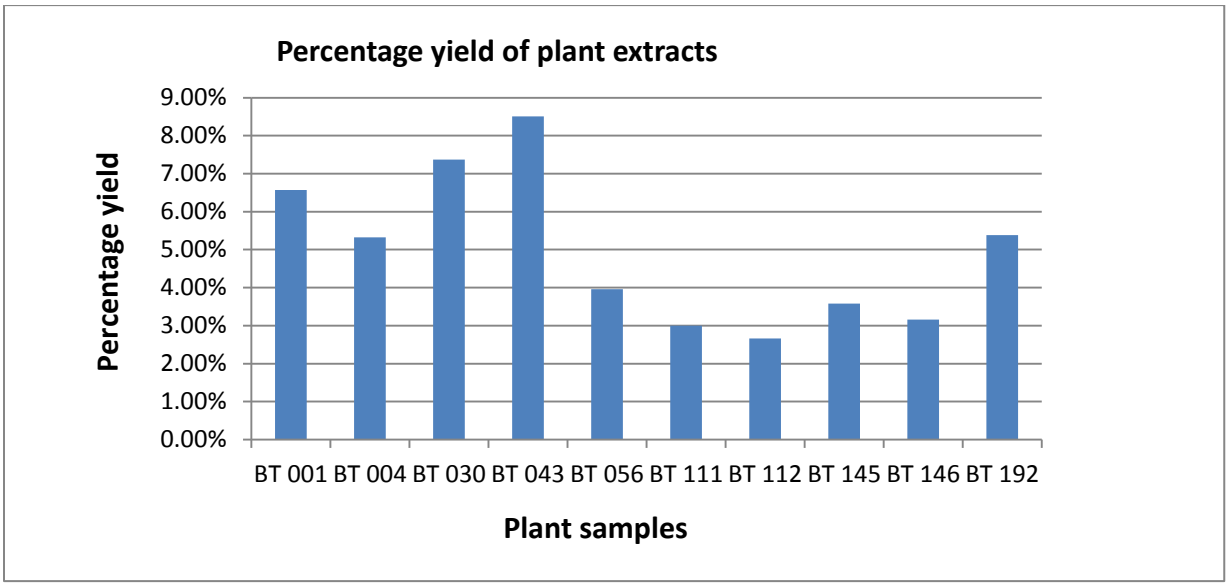

Figure 4.1: Total percentage yields of methanol extracts of plant samples

Indexing

S.N.		**Scientific Name**
1	BT 001	*Lydogium japonicum* (Thunb.) Swartz
2	BT 004	*Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis
3	BT 030	*Sapindus murokosii* Gaertner
4	BT 043	*Punica granatum* L.
5	BT 056	*Catharanthus roseus* (L.) G. Don
6	BT 111	*Camellia kissi* Wall
7	BT 112	*Albizia chinensis* (Osbeck) Merrill
8	BT 145	*Euphorbia pulcherrima* Willd. ex Klotzsch
9	BT 146	*Clerodendrum japonicum* (Thunberg) Sweet
10	BT 192	*Ficus religiosa L.*

4.2 Qualitative Phytochemical Analysis

Methanol extract was subjected to various reagents to observe the presence of secondary metabolites present in the samples.

Plant Extracts	**Alkaloid**	**Phenol**	**Flavonoid**	**Glycoside**	**Terpenoid**	**Tannin**	**Sterol**
	Mayer's Test	**Ferric Chloride Test**	**Alkaline Reagent Test**	**Modified Brontrager's Test**	**Copper Acetate Test**	**Gelatin Test**	**Salkowski's Test**
Lydogium japonicum (Thunb.) Swartz	+	+	+	+	+	-	+
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	+	+	+	-	+	+	+
Sapindus murokosii Gaertner	+	+	+	+	-	+	+
Punica granatum L.	+	+	+	-	+	+	+
Catharanthus roseus (L.) G. Don	+	+	+	-	+	+	+
Camellia kissi Wall	+	+	+	-	-	+	-
Albizia chinensis (Osbeck) Merrill	+	+	+	-	-	+	+
Euphorbia pulcherrima Willd. ex Klotzsch	+	+	+	+	-	-	+
Clerodendrum japonicum (Thunberg) Sweet	+	+	+	-	-	+	-
Ficus religiosa L.	+	+	+	+	-	+	+

(+) = present; (-) = absent

Table 4.1 Preliminary phytochemical analysis of screened plant species.

Methanol extract depicted the presence of alkaloids, phenols, flavonoids, glycosides, terpenoids, and sterols as shown in table 4.1. All the plant extracts showed the presence of alkaloids, phenols and flavonoids.

4.3 Total phenol and flavonoid content

S.N.	Samples	Total Phenolic content mg GA equivalent/g of extract	Total Flavonoid content mg QE equivalent/g dry weight
1	*Lydogium japonicum* (Thunb.) Swartz	35.95±4.65	24.38±0.50
2	*Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis	40.11±2.44	50.01±0.20
3	*Sapindus murokosii* Gaertner	41.22±2.68	34.30±4.45
4	*Punica granatum* L.	228.73±10.56	33.53±0.26
5	*Catharanthus roseus* (L.) G. Don	279.55±2.34	37.79±1.85
6	*Camellia kissi* Wall	152.08±24.77	37.04±4.59
7	*Albizia chinensis* (Osbeck) Merrill	24.67±1.99	22.45±0.18
8	*Euphorbia pulcherrima* Willd. ex Klotzsch	117.37±2.01	35.75±3.69
9	*Clerodendrum japonicum* (Thunberg) Sweet	46.86±9.92	50.23±1.55
10	*Ficus religiosa L.*	136.84±1.24	57.49±0.60

Table 4.2 Total phenolic and flavonoid content.

Each Value is the average of three values ± SEM.

Using the equation y=0.005412*x+0.2025 from the standard graph (appendix) and formula for mg Gallic acid equivalent/ gram of extract= c.v/w. Where c is the concentration of Gallic acid from the equation, v is volume of extract used during assay and w is dry weight of extract used in the assay, the total phenol contents in the examined plant was determined spectrophotometrically at 765 nm. The values obtained for the total concentration of phenols are expressed as mg of GA/ g of extract. (Table 4.2).The highest amount of phenol 279.55±2.34 mg of GA/g was shown by *Catharanthus roseus* (L.) G. Don while the lowest amount of phenol 24.67±1.99 GA of /g was shown by *Albizia chinensis* (Osbeck) Merrill. The phenol content of other plant extracts remained in between these two extremes.

Using the equation y=0.009747*x-0.09816 obtained from the standard graph of quercetin (appendix) and formula for mg quercetin equivalent/ gram of extract= c.v/w. Where c is the concentration of quercetin from the equation, v is volume of extract used during assay and w is dry weight of extract used in the assay, the total flavonoid contents in the examined plant was determined spectrophotometrically at 415 nm. The values obtained for the total concentration of flavonoids are expressed as mg of QE/ g of extract. (Table 4.2).The highest amount of flavonoid 57.49 ± 0.60 mg of QE/g was shown by *Ficus religiosa L.* while the lowest amount of flavonoid 22.45 ± 0.18 mg of QE/g was shown by *Albizia chinensis* (Osbeck) Merrill. The flavonoid content of other plant extracts remained in between these two extremes.

4.4 Total DPPH Antioxidant activity

Total Antioxidant activity of different extracts was determined using DPPH Free Radical Scavenging Assay. For this, Ascorbic acid (appendix) was taken as the pure antioxidant reference compound. IC_{50} value was calculated for each sample taking the concentration vs. % radical scavenging activity following (Ames *et al*., 1993). First RSA was calculated for the standard Ascorbic acid and other samples were compared to this standard graph.

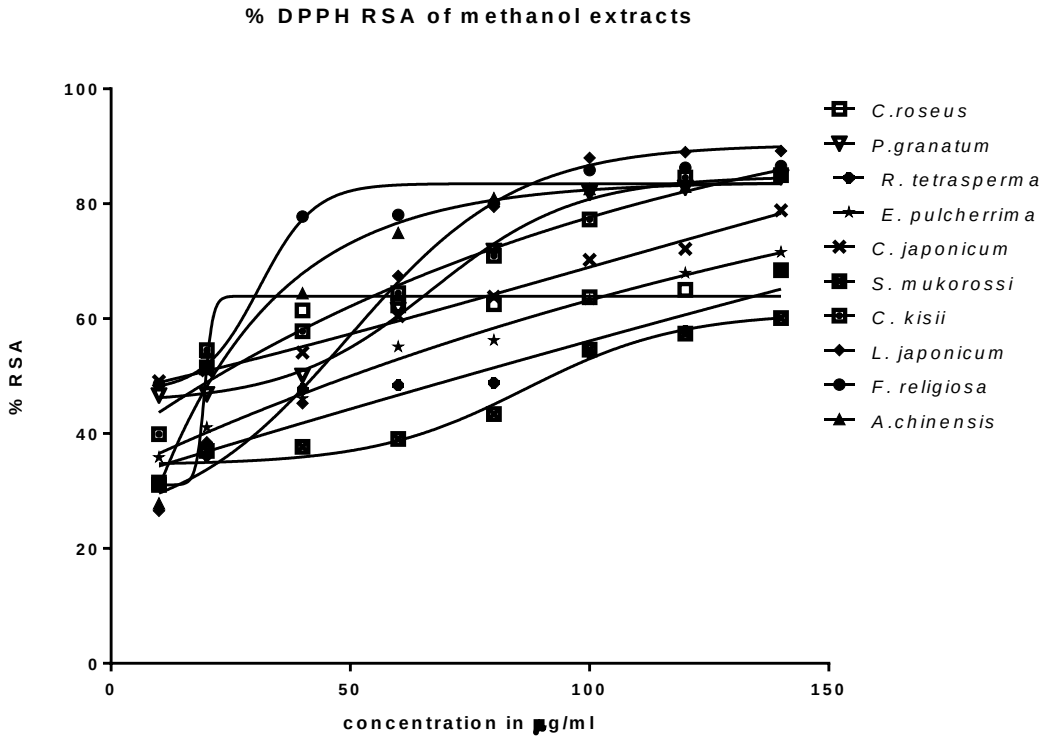

Figure 4.2 Percentage DPPH RSA of methanol extracts of different plant species

Highest scavenging activity of 89.17% and lowest scavenging activity of 26.55% was seen in *Lydogium japonicum* (Thunb.) Swartz. In case of the other species the % RSA was found between these values. Higher concentrations of plant extracts showed an increasing percentage of radical scavenging activity in DPPH.

4.5 Determination of IC_{50} Values

S.N.	Plant Samples	IC_{50} values (µg/ml)
1	*Lydogium japonicum* (Thunb.) Swartz	43.81±1.01
2	*Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis	84.77±1.74
3	*Sapindus murokosii* Gaertner	91.83±.089
4	*Punica granatum* L.	25.53±0.41
5	*Catharanthus roseus* (L.) G. Don	19.10±0.91
6	*Camellia kissi* Wall	16.28±1.5
7	*Albizia chinensis* (Osbeck) Merrill	17.99±0.71
8	*Euphorbia pulcherrima* Willd. ex Klotzsch	47.94±0.48
9	*Clerodendrum japonicum* (Thunberg) Sweet	14.15±8.12
10	*Ficus religiosa L.*	13.87±0.53

Table 4.3 IC_{50} values of methanol extracts of different plant species
The error bars represent the standard error of the mean from the mean values

The IC_{50} value for ascorbic acid was found to be 6.75±2.13 µg/ml. In case of extracts the highest and lowest IC_{50} values were observed in *Sapindus murokosii* Gaertner (91.83±.089 µg/ml) and *Ficus religiosa L.* (13.87±0.53 µg/ml) as shown in table 4.3. The species with lower IC_{50} are considered as better antioxidants. So*, Ficus religiosa L.* has the best antioxidant activity among the other plant species. The IC_{50} value of the other extracts was observed in between these two extremes.

4.6 Antibacterial Assay

The antibacterial activity of methanol extracts of different plant species was tested against ATCC cultures of *Staphylococcus aureus, Escherichia coli*. Antibiotic drug ampicillin or piperacillin was taken as a positive control and methanol (the solvent of the plant extract) was taken as a negative control. Different concentrations of plant extract (100 mg/ml, 50mg/ml, 25mg/ml, 12.5 mg/ml and 6.25 mg/ml) were tested against bacterial strains. The assay was done in the MHA plates swabbed with respective bacterial culture and the zone of the inhibition was measured after 24 hrs. The results were expressed with Zone of

inhibition on mm and shown on following table 4.4.

Plant Extracts	**Zone of Inhibition (mm)**						
	100 mg/ml	**50 mg/ml**	**25 mg/ml**	**12.5 mg/ml**	**6.25 mg/ml**	**+ve control**	**-ve control**
Lydogium japonicum (Thunb.) Swartz	-	-	-	-	-	25	6
Randia tetrasperma (Roxb.) Benth. & Hook. f.ex Brandis	-	-	-	-	-	25	6
Sapindus murokosii Gaertner	9	6	-	-	-	25	6
Punica granatum L.	6	4	-	-	-	25	6
Catharanthus roseus (L.) G. Don	4	4	-	-	-	25	6
Camellia kissi Wall	-	-	-	-	-	25	6
Albizia chinensis (Osbeck) Merrill	-	-	-	-	-	25	6
Euphorbia pulcherrima Willd. ex Klotzsch	7	-	-	-	-	25	6
Clerodendrum japonicum (Thunberg) Sweet	-	-	-	-	-	25	6
Ficus religiosa L.	-	-	-	-	-	25	6

Table 4.4 Zone of Inhibition (in mm) of different methanol extracts in gram negative (*E. coli*)organism

Plant extracts did not show any significant results in *E. coli* (table 4.4). Few plants *Sapindus murokosii* Gaertner , *Punica granatum* L., *Euphorbia pulcherrima* Willd. ex Klotzsch and *Catharanthus roseus* (L.) G. Don showed result in 100 mg/ml and 50 mg/ml concentration only.

Plant Extracts	Zone of Inhibition (mm)						
	100 mg/ml	**50 mg/ml**	**25 mg/ml**	**12.5 mg/ml**	**6.25 mg/ml**	**+ve control**	**-ve control**
Lydogium japonicum (Thunb.) Swartz	8	5	3	-	-	29	6
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	3	3	-	-	-	29	6
Sapindus murokosii Gaertner	7	7	-	-	-	29	6
Punica granatum L.	18	14	13	10	7	29	6
Catharanthus roseus (L.) G. Don	4	3	3	-	-	29	6
Camellia kissi Wall	7	5	5	3	3	29	6
Albizia chinensis (Osbeck) Merrill	7	4	3	-	-	29	6
Euphorbia pulcherrima Willd. ex Klotzsch	6	5	3	3	3	29	6
Clerodendrum japonicum (Thunberg) Sweet	-	-	-	-	-	29	6
Ficus religiosa L.	5	4	3	3	3	29	6

Table 4.5 Zone of Inhibition (in mm) of different methanol extracts in gram positive(*S. aureus*) organism

Punica granatum L. showed significant result in gram positive bacteria whereas other extracts showed a moderate result. *Clerodendrum japonicum* (Thunberg) Sweet did not show any result in gram positive bacteria as shown in table 4.5.

Plant Extracts	Zone of Inhibition (mm)						
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	Methicillin 10mg/ml	-ve control
Lydogium japonicum (Thunb.) Swartz	-	-	-	-	-	-	6
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	7	-	-	-	-	-	6
Sapindus murokosii Gaertner	-	-	-	-	-	-	6
Punica granatum L.	9	6	4	-	-	-	6
Catharanthus roseus (L.) G. Don	-	-	-	-	-	-	6
Camellia kissi Wall	-	-	-	-	-	-	6
Albizia chinensis (Osbeck) Merrill	-	-	-	-	-	-	6
Euphorbia pulcherrima Willd. ex Klotzsch	17	15	9	9	7	-	6
Clerodendrum japonicum (Thunberg) Sweet	-	-	-	-	-	-	6
Ficus religiosa L.	-	-	-	-	-	-	6

Table 4.6 Zone of Inhibition (in mm) of different methanol extracts in MRSA

Euphorbia pulcherrima Willd. ex Klotzsch showed significant result in MRSA. *Punica granatum* L. showed results in 100 mg/ml, 50 mg/ml and 25 mg/ml concentrations. *Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis showed result only in 100 mg/ml concentration. Remaining extracts did not show any results (table 4.6).

4.6.1 Antifungal Assay

Different methanol extracts were tested for their antifungal activity. Two characterized fungal strains *S. cerevisae* and *Pichia sp.* were tested using fluconazole as standard drug against different concentrations of plant extracts (100 mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml and 6.25 mg/ml). The assay was done in the PDA plates swabbed with respective fungal culture and the zone of the inhibition was measured after 24 hrs. The results were expressed with Zone of inhibition in mm and shown on following table 4.7 and table 4.8.

Plant Extracts	**Zone of Inhibition (mm)**						
	100 mg/ml	**50 mg/ml**	**25 mg/ml**	**12.5 mg/ml**	**6.25 mg/ml**	**+ve control**	**-ve control**
Lydogium japonicum (Thunb.) Swartz	4	-	-	-	-	30	6
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	-	-	-	-	-	30	6
Sapindus murokosii Gaertner	4	4	-	-	-	30	6
Punica granatum L.	9	-	-	-	-	30	6
Catharanthus roseus (L.) G. Don	-	-	-	-	-	30	6
Camellia kissi Wall	10	9	7	4	4	30	6
Albizia chinensis (Osbeck) Merrill	-	-	-	-	-	30	6
Euphorbia pulcherrima Willd. ex Klotzsch	-	-	-	-	-	30	6
Clerodendrum japonicum (Thunberg) Sweet	-	-	-	-	-	30	6
Ficus religiosa L.	-	-	-	-	-	30	6

Table 4.7 Zone of Inhibition (in mm) of different methanol extracts in fungal culture (*S. cerevisae*)

Camellia kissi Wall showed a significant result. *Sapindus murokosii* Gaertner showed result in 100 mg/ml and 50 mg/ml concentration and *Lydogium japonicum* (Thunb.) Swartz and *Punica granatum* L. showed result in 100 mg/ml concentration. Remaining extracts did not show antifungal activity.

Plant Extracts	Zone of Inhibition (mm)						
	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	+ve control	-ve control
Lydogium japonicum (Thunb.) Swartz	-	-	-	-	-	30	6
Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis	-	-	-	-	-	30	6
Sapindus murokosii Gaertner	-	-	-	-	-	30	6
Punica granatum L.	-	-	-	-	-	30	6
Catharanthus roseus (L.) G. Don	-	-	-	-	-	30	6
Camellia kissi Wall	14	13	9	5	4	30	6
Albizia chinensis (Osbeck) Merrill	4	-	-	-	-	30	6
Euphorbia pulcherrima Willd. ex Klotzsch	5	4	-	-	-	30	6
Clerodendrum japonicum (Thunberg) Sweet	-	-	-	-	-	30	6
Ficus religiosa L.	-	-	-	-	-	30	6

Table 4.8 Zone of Inhibition (in mm) of different methanol extracts in fungal culture (*Pichia sp.*)

Camellia kissi Wall showed a significant result. *Euphorbia pulcherrima* Willd. ex Klotzsch showed result in 100 mg/ml and 50 mg/ml concentration and *Albizia chinensis* (Osbeck) Merrill showed result in 100 mg/ml concentration. Remaining extracts did not show antifungal activity.

4.7 Brine shrimp cytotoxicity assay

S.N.	Plant extracts	LC_{50}ppm±SEM
1	*Lydogium japonicum* (Thunb.) Swartz	69.40±0.00
2	*Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis	17.81±0.00
3	*Sapindus murokosii* Gaertner	561.15±0.00
4	*Punica granatum* L.	4.26±0.00
5	*Catharanthus roseus* (L.) G. Don	4.26±0.00
6	*Camellia kissi* Wall	4.26±0.00
7	*Albizia chinensis* (Osbeck) Merrill	>1000
8	*Euphorbia pulcherrima* Willd. ex Klotzsch	4.26±0.00
9	*Clerodendrum japonicum* (Thunberg) Sweet	>1000
10	*Ficus religiosa L.*	4.26±0.00

Table 4.9 LC_{50} values in brine shrimp cytotoxicity assay
All the results are expressed as mean ± SEM and all the tests have been performed in triplicates.

The mortality of the freshly hatched brine shrimp larvae was used to assess cytotoxicity of the crude plant extracts. In our experiment five larvae were used in each microtitre plate well. Among the crude plant extracts tested *Lydogium japonicum* (Thunb.) Swartz*, Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis*, P. granatum* L. *, Catharanthus roseus* (L.) G. Don*, Camellia kissi* Wall*, Euphorbia pulcherrima* Willd. ex Klotzsch and *Ficus religiosa L.* were found to have high toxicity while in other extracts the toxic effects were found to be low. Thus obtained LC_{50} values (table 4.9) were then tallied with the interpretation chart to define their toxicity. The brine shrimp toxicity assay interpretation chart has been given in appendix.

4.8 GC-MS Analysis

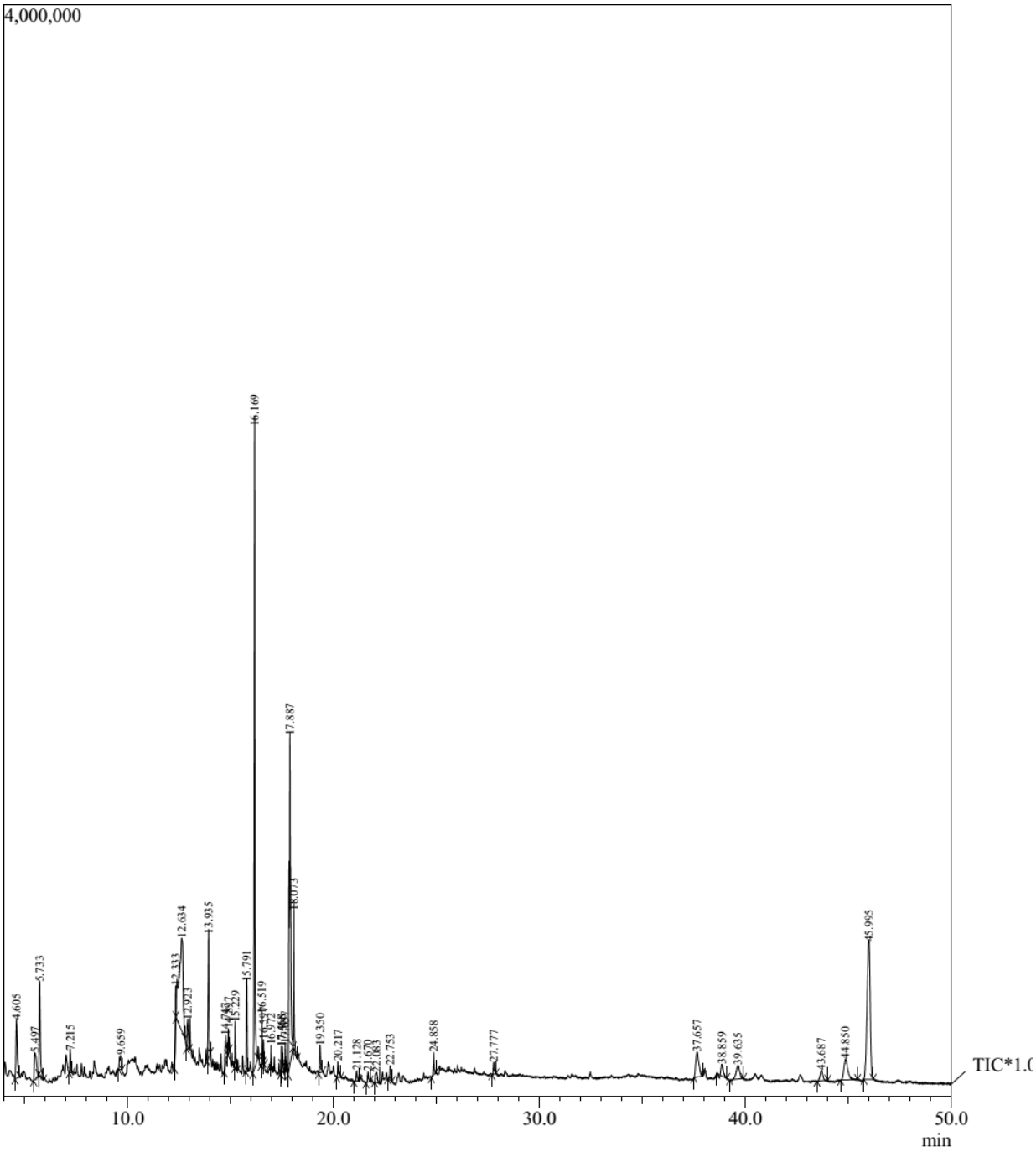

Figure 4.3 Chromatogram of *Punica granatum L.*

Peak	Retention Time	Name of compounds	Molecular formula	Molecular weight	Peak area%
1	4.605	1,3,5-triazine-2,4,6-triamine	$C_3H_6N_6$	126	2.92
2	5.497	1,2,3-propanetriol	$C_3H_8O_3$	92	1.82
3	5.733	2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one	$C_6H_8O_4$	144	3.53
4	7.215	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	134	0.65
5	9.659	4-Hydroxy-2-methoxybenaldehyde	$C_8H_8O_3$	152	0.76
6	12.333	Phenol, 3,4,5-trimethoxy	$C_9H_{12}O_4$	184	0.56
7	12.634	1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{12}O_6$	192	12.75
8	12.923	Methyl-(2-hydoxy-3-ethoxy-benzyl)ether	$C_{10}H_{14}O_3$	182	1.24
9	13.935	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180	4.00
10	14.747	3-Hydroxy-4,5-dimethoxybenzoic acid	$C_9H_{10}O_5$	198	1.40
11	14.897	Neophytadiene	$C_{20}H_{38}$	278	0.54
12	15.229	2-cyclohexen-1-one, 5-methyl-2-(1-methylethyl)	$C_{10}H_{16}O$	152	1.10
13	15.791	Hexadecanoic acid	$C_{17}H_{34}O_2$	270	1.91
14	16.169	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	16.73
15	16.519	3-(3',5'-dimethoxy-4'-hydroxyphenyl)-e-2-propenal	$C_{11}H_{12}O_4$	208	1.00
16	16.597	2,4-dimethoxybenzyl acetate	$C_{11}H_{14}O_4$	210	0.47
17	16.972	Hexadecanoic acid	$C_{19}H_{40}O_2Si$	328	0.38
18	17.468	Linoleic acid,methyl ester	$C_{19}H_{34}O_2$	294	0.27
19	17.516	Cis-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.44
20	17.657	Phytol	$C_{20}H_{40}O$	296	0.41
21	17.887	Cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	15.75
22	18.073	Octadecanoic acid	$C_{18}H_{36}O_2$	284	3.01
23	19.350	1-heneicosanol	$C_{21}H_{44}O$	312	0.99
24	20.217	Stearic acid	$C_{18}H_{34}O_2$	282	0.66
25	21.128	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	$C_{19}H_{34}O_2$	294	0.45
26	21.670	Abieta-8,11,13-trien-18-oic acid	$C_{20}H_{28}O_2$	300	0.35
27	22.083	Acetic acid, chloro-, octadecyl	$C_{20}H_{39}ClO_2$	346	0.73

		ester			
28	22.753	Heptadecanal	$C_{17}H_{34}O$	254	0.48
29	24.858	7-(3,4-Methylenedioxy)-tetrahydrobenzofuranone	$C_{15}H_{12}O_5$	272	0.69
30	27.777	Hexadecane	$C_{16}H_{34}$	226	0.47
31	37.657	Stigmasta-7,22-dien-3-ol, (3.beta.,5.alpha.,22e,24r)	$C_{29}H_{48}O$	412	2.67
32	38.859	Beta.-amyrin	$C_{30}H_{50}O$	426	0.78
33	39.635	Stigmast-7-en-3-ol, (3.beta.,5.alpha.,24s)	$C_{29}H_{50}O$	414	1.54
34	43.687	03027205002 flavone 4'-oh,5-oh,7-di-o-glucoside	$C_{27}H_{30}O_{15}$	594	0.88
35	44.850	17.alfa.,21.beta.-28,30-bisnorhopane	$C_{28}H_{48}$	384	2.76
36	45.995	03027205002 flavone 4'-oh,5-oh,7-di-o-glucoside	$C_{27}H_{30}O_{15}$	594	14.90
					100.00

Table 4.10 Phytochemicals identified in the methanol extracts of *Punica granatum* L.

Chromatographic analysis of *Punica granatum* L. enabled the identification of 36 compounds which are listed in table 4.10 in order based on their retention times and peak area percentage. The main constituents in extract were pentadecanoic acid 16.73%, Cis-Vaccenic acid 15.85%, 03027205002 flavone 4'-oh,5-oh,7-di-o-glucoside 14.90% and 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid 12.75%.

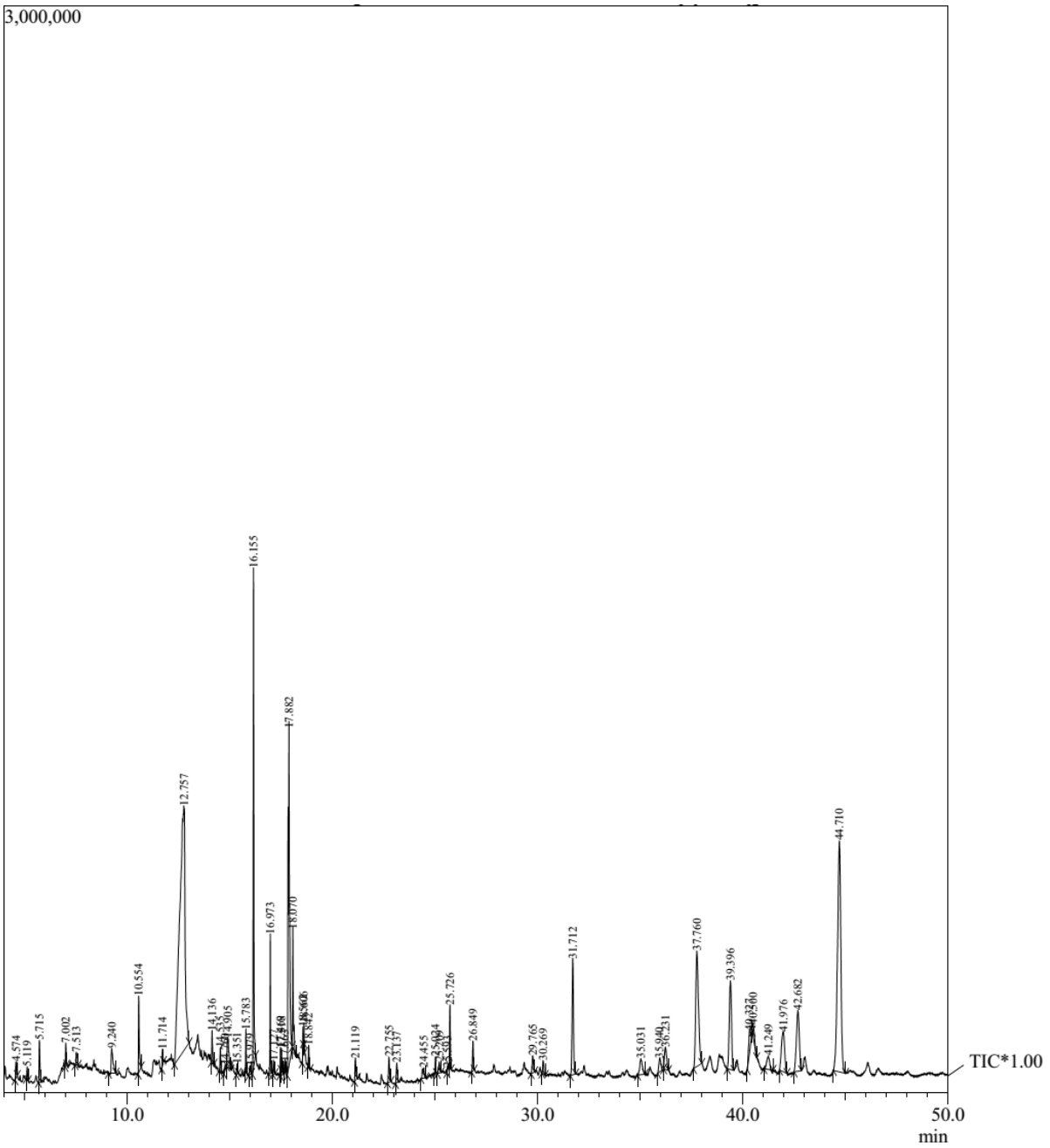

Figure 4.4 Chromatogram of *Camellia kissi* Wall

Peak	Retention Time	Name of compounds	Molecular formula	Molecular weight	Peak area%
1	4.574	Cyclopentane, 1-acetyl-1,2-epoxy	$C_7H_{10}O_2$	126	0.29
2	5.119	Ethanethioic acid	$C_4H_6N_2$	82	0.17
3	5.715	2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one	$C_6H_8O_4$	144	0.74
4	7.002	2-Heptanone, 1-ethoxy	$C_9H_{18}O_2$	158	0.28
5	7.513	6-undecanone	$C_{11}H_{22}O$	170	0.26
6	9.240	Phenol, 2-propyl	$C_9H_{12}O$	136	0.95
7	10.554	Phenol, 4-[2-(dimethylamino)ethyl]	$C_{10}H_{15}NO$	165	1.24
8	11.714	1h-2-benzopyran-1-one	$C_{10}H_{10}O_3$	178	0.21
9	12.757	1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid	$C_7H_{12}O_6$	192	27.65
10	14.136	1-Phenyl-1-(trimethylsilyloxy)ethylene	$C_{11}H_{16}OSi$	192	0.49
11	14.535	2,3-Bis(1-methylallyl)pyrrolidine	$C_{12}H_{21}N$	179	0.35
12	14.760	(1,5-dimethyl-1h-pyrazol-4-ylmethyl)-methyl-(7-nitro-benzo[1,2,5]oxadiazol-4-yl)-amine	$C_{13}H_{14}N_6O_3$	302	0.15
13	14.905	3-methyl-4-nonenoic acid	$C_{10}H_{18}O_2$	170	1.05
14	15.351	Neophytadiene	$C_{20}H_{38}$	278	0.14
15	15.783	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	0.43
16	15.979	(8z)-14-methyl-8-hexadecen-1-ol	$C_{17}H_{34}O$	254	0.20
17	16.155	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	6.47
18	16.973	Palmitic acid, rimethylsilyl ester	$C_{19}H_{40}O_2Si$	328	1.46
19	17.127	9-octadecenoic acid	$C_{18}H_{34}O_2$	282	0.19
20	17.469	Linoleyl alcohol	$C_{18}H_{34}O$	266	0.23
21	17.518	9-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	0.26
22	17.663	Phytol	$C_{20}H_{40}O$	296	0.13

23	17.882	Vaccenic acid	$C_{18}H_{34}O_2$	282	9.25
24	18.070	Steric acid	$C_{18}H_{36}O_2$	284	1.83
25	18.562	Linolsaeure, trimethylsilylester	$C_{21}H_{40}O_2Si$	352	0.22
26	18.606	Trans-9-Octadecenoic acid, trimethylsilyl ester	$C_{21}H_{42}O_2Si$	354	0.15
27	18.842	Octadecanoic acid, trimethylsilyl ester	$C_{21}H_{44}O_2Si$	356	0.28
28	21.119	Palmitoyl aldehyde	$C_{16}H_{32}O$	240	0.47
29	22.755	Heptadecanal	$C_{17}H_{34}O$	254	0.53
30	23.137	Oxalic acid, cyclohexyl tetradecyl ester	$C_{22}H_{40}O_4$	368	0.43
31	24.455	2,2,3,7-tetramethyltricyclo(5.2. O.o.(1,6))undec-3-ene	$C_{15}H_{24}$	204	0.36
32	25.024	Delta.-guaiene	$C_{15}H_{24}$	204	0.24
33	25.209	Trans-limonenoxid	$C_{10}H_{16}O$	152	0.30
34	25.593	Pentadecanal	$C_{15}H_{30}O$	226	0.08
35	25.726	Isolongifolol, acetate	$C_{17}H_{28}O_2$	264	0.91
36	26.849	Squalene	$C_{30}H_{50}$	410	0.50
37	29.765	3-Hydroxypropanoic acid, 3-(2,2,6-trimethylbicyclo[4.1.0]hept-1-yl)-, ethyl ester	$C_{15}H_{26}O_3$	254	0.47
38	30.269	Acetate, (2,4a,5,8a-tetramethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenyl) ester	$C_{16}H_{26}O_2$	250	0.31
39	31.712	5,12-Dihydroxyergost-25(27)-ene-3,6-dione	$C_{28}H_{44}O_4$	444	3.34
40	35.031	5-(7a-isopropenyl-4,5-dimethyl-octahydro-inden-4-yl)-3-methyl-penta-2,4-dien-1-ol	$C_{20}H_{32}O$	288	0.84
41	35.940	26,27-dinorcholesta-5,22-dien-3-ol, (3.beta.,22e	$C_{25}H_{40}O$	356	0.53
42	36.231	Lanosterol	$C_{30}H_{50}O$	426	0.99
43	37.760	Beta-sitosterol	$C_{29}H_{50}O$	414	6.60

44	39.396	Lanosterol acetate	$C_{32}H_{52}O_2$	468	4.16
45	40.327	Silane	$C_{33}H_{58}OSi$	498	0.88
46	40.500	Methyl commate b	$C_{31}H_{50}O_3$	470	0.50
47	41.249	Lanosta-8,24-dien-3-yl acetate	$C_{32}H_{52}O_2$	468	0.72
48	41.976	Dimethyl{bis[(4,8,8-trimethyldecahydro-1,4-methanoazulen-9-yl)methoxy]}silane	$C_{32}H_{56}O_2Si$	500	2.59
49	42.682	9,19-cyclolanost-24-en-3-ol, (3.beta.)	$C_{30}H_{50}O$	426	3.50
50	44.710	Betulin	$C_{30}H_{50}O_2$	442	15.64
					100.0

Table 4.11 Phytochemicals identified in the methanolic extracts of *Camellia kissi* Wall

Chromatographic analysis of *Camellia kissi* Wall enabled the identification of 50 compounds which are listed in table 4.11 in order based on their retention times and peak area percentage. The main constituents in extract were 1, 3, 4, 5 -tetrahydroxy-cyclohexanecarboxylic acid 27.65%, betulin 15.64% and vaccenic acid 9.25%.

CHAPTER 5. Discussion

Different parts of the plant such as leaf, stem, root and bark contain various solute molecules with more than one functional group. Therefore, the different solutes have the ability to solubilize in different solvents. While solubilizing the plant material in a particular solvent to obtain its bioactive compounds an alternative way is to use the concept of polarity. Polarity is the sum of all possible interactions. Mostly used solvents are polar organic solvent containing hydroxyl group, namely methanol, ethanol etc. (Kumoro *et al*., 2009).Therefore in the study, methanol has been chosen as the solvent to extract the active compounds in the studied plant. Methanol extracts most of the active constituents from plant materials. So it is more often used as the starting solvent even in experiments involving a wide array of solvent usage. Another advantage of using methanol is that it evaporates easily.

Potent bioactive compounds contained in plants can elicit both poisonous and medicinal value. Whether it causes a beneficial or an adverse result may depend on the amount eaten and the context of intake (Paulsen, 2010). Alkaloids, glycosides, flavonoids, saponins, tannins, steroids are important bioactive constituents present in plants. Alkaloids are toxic against micro-organisms (anti-bacterial and anti-fungal), herbivores and insects so it insures protection to humans and animal health (M. Saxena *et al*., 2001). The other pharmacological applications are: CNS stimulants, anesthetics etc. (Doughari, 2009). Steroidal saponins are cardiac glycosides (useful in heart medication) that inhibit N^+, K^+-ATPase but are strong toxin. Medicinal properties of tannins are: antifungal, bind to proteins, used to cure diarrhea, skin bleeding etc. (Paulsen, 2010). Flavonoids and phenolics act as antioxidants, anti-inflammatory, anti-cancerous so plays a vital role to cure different diseases (Thamaraiselvi, Lalitha *et al*., 2012). Therefore presence of all these active compounds in the tested plant extracts proves that the use of these plants can be an alternative to use as medicine.

The total flavonoid content estimation method followed was the Aluminium chloride chelation method involves chelation of aluminium atom of $AlCl_3$ by the flavonals and flavones thereby forming deep yellow colored complexes (Chang *et al*., 2002). Compared to the TPC values the flavonoid content data were not above 100mg QA per g extract. The lower flavonoid content of these plants might be because the plant extracts in phenols. Other reasons for the poor flavonoid content might be due to some inherent weakness in the estimation method followed. In the plant materials the flavonoids are not usually found

in free and isolated form such that they could chelate $AlCl_3$ without any restrictions. In fact the flavonals and flavones in plant exist as glycosides such that their hydroxyl groups often remain sequestered and not readily available for chelation. Eventually this might mislead the tested plant extract lacked any flavonoids though they are rich in them in reality (Mammen and Daniel, 2012). The content of flavonoid in *Randia tetrasperma* (Roxb.) Benth. & Hook. f. ex Brandis was found to be similar in finding of other researcher (Gulnaz *et al*., 2014). In case of *Sapindus mukorossi* Gaertner moderate amount of flavonoid was obtained in our study. Similar flavonoid quantification done by (Singh and Kumari, 2015) in *Sapindus mukorossi* Gaertner reported a large variation in the flavonoid content, this variation may be due to the different standard used. *Punica granatum* L. was found to contain 33.53±0.26 mg/g while *Punica granatum* L. contained 135.33±8.08 mg/g of flavonoid in (Rajan *et al*., 2011). This deviation in data may be due to the use of fruit rind as sample. Flavonoids present in *Catharanthus roseus* (L.) G. Don in our experiment was 37.79±1.85 mg/g whereas the flavonoid content in the experiment done by (Srivastava *et al*., 2013) was found to very less (32.60 mg CE/100 g). The high value of flavonoid in our experiment may be due the use of leaf and stem both as sample whereas in the experiment done by (Srivastava *et al*., 2013) only leaf has been used. Very less flavonoid (19.7 mg/ml) was present in *Camellia sinensis* (Rajeswari, 2015). Due to the difference in species used *Camellia kissi* Wall in our experiment than (Rajeswari, 2015) the flavonoid present is a bit high (37.04±4.59 mg/g). The highest flavonoid content was shown by *Ficus religiosa* L. followed by *Clerodendrum japonicum* (Thunberg) Sweet. These plants might be responsible for alternative therapeutic purposes because flavonoids are found to be capable of free radical scavenging inhibition of various hydrolytic and oxidative enzymes like lipoxygenase, phospholipase A_2. The also reported to have anti-inflammatory roles (Ghosh *et al*., 2014).

The estimation of polyphenol content was done by using Folin-Ciocalteu method. The Folin-Ciocalteu method is based on the transfer of electrons from the phenols compounds to the phosphomolybdic/phosphotungstic acid present in F-C reagent in alkaline condition (Ainsworth and Gillespie, 2007). During the reaction yellow color of the F-C reagent changes to blue colored complex between the F-C reagent and the phenolate ion. The intensity of the blue colored complex corresponds to the amount of the phenolate ions generated during the reaction (Ainsworth and Gillespie, 2007). So in the experiment the higher absorbance at 765nm showed higher content of phenols in the tested plant extracts. Our data of polyphenol content in *Randia tetrasperma* (Roxb.) Benth. & Hook.f.ex Brandis varies from the study of (Gulnaz *et al*., 2014) were they found polyphenol content of 85.5 mg

GAE/g. This variation may be due to the difference in the species and the use of ethanol as solvent in their experiment. Similar work was performed by (Singh and Kumari, 2015), in *Sapindus mukorossi* Gaertner showed high content of polyphenol 205.16±0.44 mg/g than the polyphenol content in our experiment . This difference may be due to the difference in extraction method, time of sample collection, altitudinal variation. In the research done by (Srivastava *et al*., 2013) in *Catharanthus roseus* (L.) G. Don the polyphenol content is 163.02 mg GAE/100 g, which is less than our experiment. The reason behind this may be because they have only used leaf as their sample and in our research we have used leaf and stem. The polyphenol (122.33±6.42 mg/g) present in *Punica granatum* L. (Rajan *et al*., 2011) is less than our experiment because the part (fruit rind) of the plant and solvent (ethanol) used are different. *Camellia kissi* Wall showed less result of polyphenol content (62.3±0.6 mg/ml) in an experiment done by (Rajeswari, 2015) than our experiment due to the difference in the part (leaf) of the plant used. Similar work was done by (Ghosh *et al*., 2014) in *Clerodendrum viscosum* but the result was different then our due to the difference in the plant species used. The polyphenols estimation in the remaining plants may have been done for the first time. Since the polyphenols are known to have both antimicrobial and antioxidant properties these plants can be exploited in treatment of various disorders (Cowan, 1999). In fact the polyphenols are considered as active antioxidants because of their ability to donate hydrogen atom, quench singlet oxygen and metal chelating potential (Tsao and Deng, 2004).

The antioxidant power of the polyphenols and flavonoids also give an insight to pharmacological efficacy of the plant extracts they are derived from. Basically there are two reaction pathways by which the plants are found to exert antioxidant activity, namely hydrogen atom transfer (HAT) reactions and electron transfer (ET) reactions. A number of methods have been developed based on these two pathways. The DPPH free radical scavenging activity assay has been followed in this research. DPPH is one of the stable nitrogen centered free radicals, violet in color, having maximum absorption of the UV-light at 515-517nm. During the assay the successive scavenging of the free radical is indicated by progressive fading of the color from violet to yellow. The actual mechanism of the antioxidant activity in DPPH assay is still a controversy; some claim that it is the hydrogen atom transfer reaction while there are studies saying that it is based on electron transfer (Huang *et al*., 2005). Antioxidant property was inferred on the basis of percentage RSA and IC_{50} value. Antioxidant activity DPPH inhibition of the plant extracts was expressed as percentage of inhibition of stable radical or inhibition concentration fifty (IC_{50}) in reference

to a standard compound. The plant with higher %RSA has the lower IC_{50}. The plant with lowest IC_{50} value of the studied plant species was found. Among the samples *Ficus religiosa L.* showed significant antioxidant activity. In a work done by (Makhija *et al.,* 2010) also have said that *Ficus religiosa L.* showed significant antiradical activity by DPPH method and suggest that this result may be due to the rich phytochemicals present in it. Followed by *Ficus religiosa L., Clerodendrum japonicum* (Thunberg) Sweet showed good antioxidant activity. Similar work was done by (Khalil and Zagloul n.d.) Using *Clerodendrum splendens* by isolating compounds in different extracts. It has been said that it showed high antioxidant activity. Antioxidant activity in *Camellia kissi* Wall is first time determined. Most work have been done in *Camellia sinensis* (green tea) (Khalaf *et al*., 2008) which shows high antioxidant activity. The activity is less in our studied species than *Camellia sinensis*, it may be due to the difference in the species used (Merr, 2014) found IC_{50} 10.21±0.84 µg/ml of *A. chinensis* using carbon tetrachloride soluble fraction which is even less than our result. It must be due to the difference in the solvent used. *Catharanthus roseus* (L.) G. Don was found to be rich in alkaloid (Sain and Sharma, 2013) so have antioxidant activity and can be used in cancer therapy as well but the specific compound must be isolated. The remaining plants showed very high IC_{50} value i.e. less antioxidant value. It may be due to the variation in the bioactive compounds present in it.

The present study was conducted to obtain preliminary information on the antimicrobial activity of methanol extracts of different under-utilized plants found in Nepal. The agar well diffusion method was applied for the study. In this research the antimicrobial activity was tested by measuring the diameter of the clear zone around the wells in the MHA plates. The chemistry behind these amazing antimicrobial activities might be due to the presence of bioactive compounds like tannins, terpenoids, flavonoids, alkaloids and polyphenols in plants (Cowan, 1999). The plant extracts and their active constituents are found to act both directly and indirectly against the pathogenic microorganisms. Directly they are able to denature extracellular and intracellular proteins, inactivation of toxins and disruption of transport proteins. Besides, activation of macrophages, rendering the substrates unavailable to the microorganisms, chelation of metal ions and eventual inhibition of oxidative phosphorylation also exert inhibitory effect against the bacterial growth (Cowan, 1999). Each of the plants showed different antimicrobial activity due to the difference in the chemical composition of the extracts. Among all the plants *Punica granatum* L. showed significant antibacterial activity against Gram's positive bacteria. The methanol extracts of *Punica granatum* L. was found to be rich in flavonoids (M.Hajoori *et al*., 2014) which must

be the reason to show its antibacterial activity. All the remaining plants showed moderate results except *Clerodendrum japonicum* (Thunberg) Sweet against Gram's positive bacteria. All the plants were found to contain different phytochemical constituents which may be the result to show antibacterial activity. All the extracts did not show antibacterial activity against the Gram's negative bacteria. Gram-negative bacteria have been found to be less susceptible to plant extracts and Gram positive bacteria have been found to be more susceptible to plant extracts in earlier studies done by other researchers (Rahman *et al*., 2009). This high sensitivity to Gram-positive bacteria could be attributed to their outer peptidoglycan layer which is not an effective permeability barrier as the outer phospholipid membranes of Gram-negative bacteria (Ngoci *et al*., 2015). We are now days facing a lot of problem due to MRSA. So in this research antibacterial assay against MRSA was performed using the plants under study for the first time. Only *Euphorbia pulcherrima* Willd. ex Klotzsch showed result. The leaves of this plant contain alkaloids (Yakubu and Mukhtar, 2011) which may be the reason to show antibacterial result. In case of antifungal activity also only one plant *Camellia kissi* Wall showed result. This is result is similar to the work done by (Rajeswari, 2015).The use of some antibiotics is no longer recommended because of the potency of the widespread resistance to them. Thus, these plants, like many other plants, can be used instead of antibiotics (Ertürk, 2006). Further work must be done for the compound isolation from these plants.

Pharmacologically active compounds are always toxic in higher doses; therefore it is necessary to determine the toxic limit before they can be applied for effective treatment. So toxicity is yet another important parameter to qualify any drug or drug formulation regarding safety. Any such formulation if has toxic effects to the host, then the drug is not preferred, rather discarded. Preliminary monitoring of the toxicity of natural products has been carried out via brine shrimp toxicity assay. The freshly hatched nauplii between 24-48 hrs of hatching are considered highly sensitive to toxins and therefore this stage of brine shrimp larvae are used for toxicity assay. In this, most of the plant extracts tested *Lygodium japonicum* (Thunb.) Swartz, *Randia tetrasperma* (Roxb.) Benth. & Hook.f.ex Brandis *, P. granatum* L. *, Catharanthus roseus* (L.) G. Don *Camellia kissi* Wall*, Euphorbia pulcherrima* Willd. ex Klotzsch and *Ficus religiosa L.* were found to have high toxicity with LC_{50} value below 1000 ppm. *Sapindus mukorossi* Gaertner was found to be weakly toxic and *Clerodendrum japonicum* (Thunberg) Sweet was found to be partially nontoxic. Similar study in *Catharanthus roseus* (L.) G. Don was also done by (Shahin Aziza *et al*., 2014) using only the leaves of *Catharanthus roseus* (L.) G. Don. In his experiment the LC_{50} value obtained

was 3.18 µg/ml. These observed toxicities might be exploited for their use as anticancer drugs. Therefore these plants might be subjected to further rigorous bioassays for confirmation of specific toxicity.

Among the 36 and 50 different types of the compounds characterized in *Punica granatum* L. and *Camellia kissi* Wall, the major compounds present are 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid, betulin, vaccenic acid, pentadecanoic acid and 03027205002 flavone 4'-oh,5-oh,7-di-o-glucoside. 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid have also been found in Camellia sinensis species studied by (Sr *et al*., 2015). 1,3,4,5-tetrahydroxy-cyclohexanecarboxylic acid shows antimicrobial, anti-inflammatory activity which induces the antioxidant activity . Moreover it stimulates the antioxidant mechanism of other compounds and increases the efficiency of antioxidant activity. Betulin is a triterpenoid compound having diverse therapeutic uses like anti-human immunodeficiency virus (anti-HIV), anticarcinogenic, anti-flu, anti-inflammatory, immune-omodulator, hepatoprotector, antihypoxic, anti-allergen, anti-tuberculosis, antitumor, anti-viral, aphidifuge, cytotoxic, hypolipemic, detoxicant (detoxicating agent), adaptogenic and anti-oxidant activities. It also prevents hyperlipidosis and acts as prostaglandin-synthesis and topoisomerase II-inhibitor (Tijjani *et al*., 2012). Vaccenic acid is a trans-fatty acid found in milk fat. Mammals convert it into rumenic acid, a conjugated linoleic acid, where it shows anticarcinogenic properties. Pentadeconic acid is also a saturated fatty acid found in trace amount in dairy products. It is due to the presence of these bioactive compounds *Punica granatum* L. and *Camellia kissi* Wall showed good antioxidant and cytotoxicity tests. However, isolation of individual phytochemical constituents and study of their biological activity will definitely give fruitful results and open a new avenue for discovery of a novel drug.

CHAPTER 6. Conclusion

The use of herbs for treatment of various health complications has started to fascinate even the people in the developed world and therefore the demand of these plants has increased rapidly. Moreover due to the antibiotic resistance increasing now days it has been must to search an alternative for the antibiotics. The study of the herbs can be the alternative for antibiotics. Therefore some of the underutilized herbs were studied for isolation and identification of the bioactive compounds present in it. These bioactive compounds are the basic compounds for drug development.

The herbs have chosen in this research aims to give a vision on these plants with regard to photochemistry and pharmacology. The plants used showed the presence of flavonoid and phenols in various amount. Talking about the antibacterial test, the extracts showed result against gram positive bacteria only and not against gram negative bacteria. *Euphorbia pulcherrima* Willd. ex Klotzsch only showed significant result in MRSA and *Camellia kissi* Wall only showed the antifungal result. Similarly the antioxidant and cytotoxicity property of these plants were also found to be good.

On the basis of the results obtained only two plants *Camellia kissi* Wall and *Punica granatum* L. was selected for GC-MS. This research is only the preliminary study but it has given powerful insight to the pharmacological values of these plants with scientific evidence.

Further research towards isolation, identification and characterization of specific bioactive compounds, their rigorous testing in animal models, assessment of safety via sophisticated tests regarding these plants could ultimately lead to discovery of novel drug for an array of health complications.

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APPENDIX - A (List of Reagents and Culture media)

1. Preparation of 1 M Na2CO3 -100 ml

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

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

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

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

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

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

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

5. Preparation of 0.2 mM DPPH solution - 100 ml

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

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

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

7. Composition of Nutrient agar media

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

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

8. Composition of Mueller Hinton Agar (MHA)

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

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

9. Composition of Potato Dextrose Agar (PDA)

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

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

10. Composition of Artificial Sea Water

S. N.	Salt	Molecular wt. (g/mol)	Amount in gram Per liter
1.	NaCl	58.44	23.50
2.	Na_2SO_4	142.04	4.00
3.	KCl	74.55	0.68
4.	H_3BO_3	61.83	0.026
5.	$MgCl_2.6H_2O$	203.33	10.78
6.	$CaCl_2$	110.98	1.47
7.	$NaHCO_3$	84.00	0.196
8.	Na_2EDTA	372.24	0.0003

PH =8.0±0.2.

11. General classification of brine shrimp cytotoxicity

LC_{50} values (ppm)	**Classification**
<1	Extremely toxic
1-100	Highly toxic
100-500	Moderately toxic
500-1000	Weakly toxic
>1000	Practically non- toxic

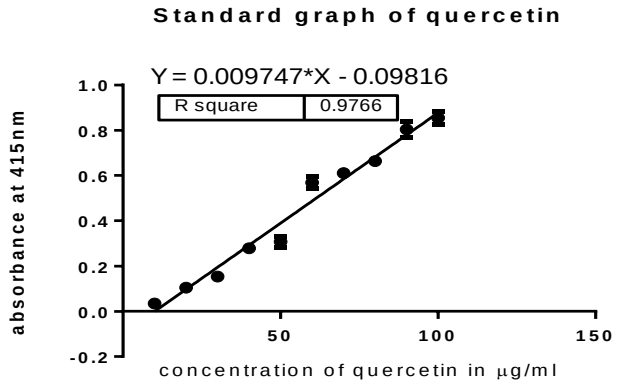


Figure: Standard graph of quercetin for total flavonoid content

The error bars represent the standard error of the mean from the mean values.

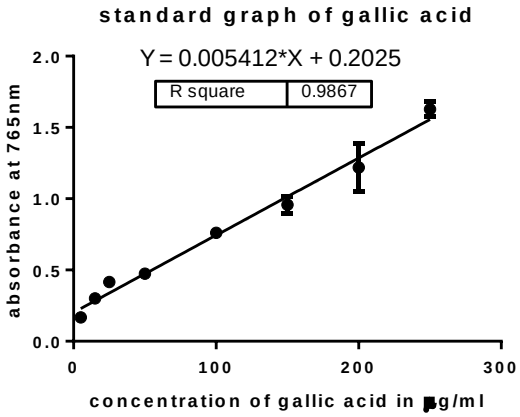


Figure Standard graph of Gallic acid for total polyphenol content

The error bars represent the standard error of the mean from the mean values.

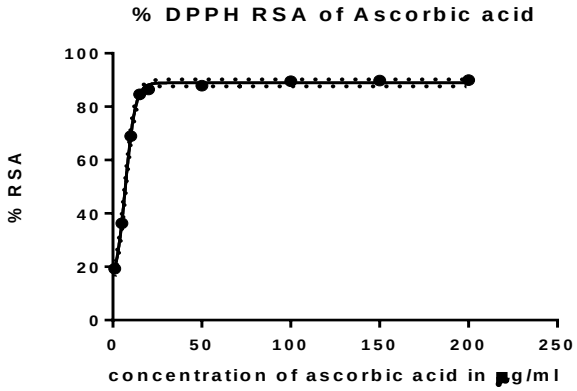


Figure Percentage Radical Scavenging Activity of Ascorbic Acid

Plants selected for research

Albizia chinensis (Osbeck) Merrill

Catharanthus roseus (L.) G. Don

Camellia kissi Wall

Sapindus murokossi Gaertner

Randia tetrasperma (Roxb.) Benth. & Hook. f. ex Brandis

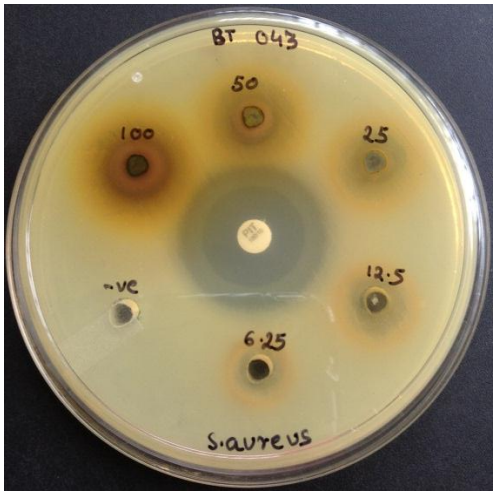
Punica granatum L.

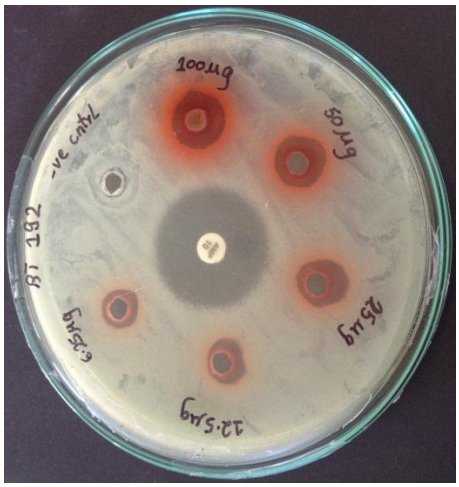
Euphorbia pulcherrima Wild. Ex Klotzsch

Ficus religiosa L.

Clerodendrum japonicum (Thunberg) Sweet

Lygodium japonicum
(Thunberg) Swartz





Antimicrobial screening

GC MS instrument Shimadzu QP 2010

Brine shrimp