ENZYMES INHIBITION AND ANTIOXIDANT ASSAY OF ETHNO MEDICINAL PLANTS OF NEPAL

M.Sc. Thesis

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Acronyms

ATCC	American Type Culture Collection
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
DPPH	1, 1-Diphenyl-2-Picryl-Hydrazyl
ELISA	Enzyme-linked Immunosorbent Assay
F-C	Folin Ciocalteu
HSI	Human Small Intestine
IC_{50}	Dose of a drug that gives half-maximum response
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistance
MRSA	Methicillin Resistance *Staphylococcus aureus*
mgQE/g	Milligram Quercetin Equivalent per gram
mgGAE/g	Milligram Gallic acid Equivalent per gram
MHA	Muller Hilton Agar
NA	Nutrient Agar

NB	Nutrient Broth
NCCLS	National Committee for Clinical Laboratory Standards
NADPH	Nicotinamide Adenine dinucleotide phosphate
NAST	Nepal Academy of Science and Technology
OD	Optical Density
PBS	Phosphate buffer system
PNPG	4-Nitrophenyl beta-D-glucoside
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
TPC	Total Polyphenol Content
TFC	Total Flavonoid Content
WHO	World Health Organization
ZOI	Zone of Inhibition

Table of Content

ABSTRACT

In this research, both chemical and biological assays performed. *Terminalia chebula, Terminalia bellerica,* and *Eucalyptus alba* were found to be effective against *Staphylococcus aureus* with the zone of inhibition 18, 11 and 7 ug/ml respectively, rest of the selected plants were not found to be active against *Pseudomonas aeruginosa. Staphylococcus aureus* has been found to cause different disease like bacteremia, endocarditis, meningitis, skin infection and toxic shock syndrome. *Eucalyptus alba* showed highest polyphenol content (138.86±9.62 mg GAE/g dry wt.) while flavonoid content were even lower than polyphenol content while flavonoid content were even lower than polyphenol content. Phytochemical screening showed presence of tannins, polyphenol, flavonoids, and cardiac glycosides. IC_{50} value of antioxidant activity for most of the selected plants were below 50 µg/ml. Least IC_{50} value was found to be of *Terminalia chebula* that is 19.63±0.89 µg/ml. Such results indicated the efficacy against disease due to free radical like rheumatic arthritis. In enzymatic assays, low IC50 values by *Terminailia chebula, Ampelocissus divaricata, Terminalia bellerica, Eucalyptus alba*, and *Woodfordia fruticosa* for inhibition of α-glucosidase and 15-lipoxygense indicated their efficacy against disease due to free radicals like diabetes. Result of this research awares us about the use of these medicinal plants.

Keywords: Medicinal plants, phytochemical screening, antioxidant, antibacterial activity, enzymatic assays.

CHAPTER I: INTRODUCTION

1.1 Background

Natural product derived from plants have versatile applications. Number of medicinal plant derived drugs are practiced in traditional system of medicine, folk medicines, nutraceuticals, food supplement, modern medicine, synthetic drugs and pharmaceutical intermediates (Ncube *et al.,* 2008; Tiwari *et al.,* 2011).

Natural product obtained from medicinal plant are combination of secondary metabolites in solvent or in powder form and are used orally or externally. Natural products or plant extracts include different types of product known as decoction, infusion, fluid extract, pilular (semi-solid) extract or dry powder extracts. Such types of preparations are called galenicals, which is named after Galen, the second century Greek physician (Lippincot 21st edition; Tiwari *et al.,* 2011). Purpose to standardize extraction procedures of crude drugs is to get therapeutically desired part and to remove remaining material by treatment with a selective solvent known as menstrum. Such obtained extract after standardization are used as crude drugs or medicinal agent as such or in form of tinctures and fluid extract further processes to be incorporated in any dosage form like capsules and tablets. These natural products contain complex mixtures of many medicinal plants secondary metabolites such as alkaloid, glycosides, terpenoids, and flavonoid (Handa *et al.,* 2008; Tiwari *et al.,* 2011).

Humans have been using medicinal plants since ancient times. Numerous drugs in modern medicine system have come from use of plants by indigenous cultures and folk medicines (Anonymous, 1994). Near about seventy thousands species of plants are used for different (Comer, 1996). All of the Asian countries have abundant medicinal plants. Many people from rural areas of Asian countries have knowledge about medicinal properties of various plants, especially of those nearer to their houses, forest or in garden. Traditional herbal doctors have knowledge of medicinal properties of plants, time to harvest, method formulate,ways to serve and many others details as well (Burkill, 2002).

1.2 Medicinal Plants of Nepal

Only 15 to 20% Nepalese people live in and around urban areas. They have access to modern medicinal facilities and the rest are dependent on traditional medicine (Sharma *et al.,* 2004). Nepal has a huge natural resource of medicinal plants (Shrestha *et al.,* 2001). Nepal exports thousands of tons of raw material to Asia, Europe and America each year (Edwards *et al.,* 1996). Government of Nepal encourage use of medicinal plants and conserve these plants for livelihood improvement (Cox *et al.,* 1996). In Nepal, many ethno-pharmacological studies have been conducted (Subrat, 2002). In Himalayan part of Nepal, traditional medicine plays very important role. Now interest in traditional herbal medicine has increased in recent years (Burlakoti & Kuwar, 2008). Variety of plant species have very important role in traditional system of medicine (Khan and Balick, 2001) and they are in use from the beginning of civilization (Kunwar *et al.,* 2006). Herbal medicines are valuable and have therapeutic (Bailey and Day, 1989) and toxic side-effect property (Keen *et al.,* 1994).

1.3 Antioxidant properties in Medicinal Plants

It's a well-known fact that herbs and spices possess antioxidant activity. Caffeic acid derivates, flavonoids and terpenoids are also responsible for this effect (Madsen & Bertelsen, 1995). In recent years consumers are more aware about the addition of synthetic additives to food and the two most commonly used antioxidants are butylated hydroxyanisole and butylated hydroxytoluene. They have shown DNA damage induction (Sasaki *et al.,*2002). It can be clearly observed that an increasing interest in natural food additives, such as spices or spice extracts, which can function as natural antioxidants besides seasoning the food. Selecting suitable extraction procedure can increase the antioxidant concentration. Difference in antioxidant activity between the plant extracts indicate the polarity of the compounds mediating antioxidant effect. There are different analytical methods to determine the antioxidant capacity of natural products in vitro. They can be classified into two groups: (i) assays for radical-scavenging ability, (ii) assays for lipid oxidation inhibitory effect.

1.4 Antimicrobial Activities of Medicinal Plants

The antimicrobial activity of natural product or extracts and pure compounds can be detected by observing the growth activity of various micro-organisms to samples that are placed with

them. Different methods to detect activity are available, all the methods are not equally sensitive or not based upon the same principle, and results will be profoundly influenced by the method (Vanden Berghe and Vlietinck, 1991; Cole, 1994; Rios *et al.,* 1988; Hadacek and Greger, 2000), so focus must be on the correcting implementation and interpretation of the diverse laboratory models. The antibacterial and antifungal test methods can be classified into three different groups, they are: diffusion, dilution and bioautographic methods. A new test method is the conductimetric assay, detecting growth of microbes as a change in the electrical conductivity or impedance of the growth medium (Sawai *et al.,* 2002). It should be emphasized that many research groups have modified these methods for specific samples, such as essential oils and non-polar extracts and these small modifications make it almost impossible to directly compare results. It is therefore a 'must' to include at least one, but preferentially several reference compounds in each assay (Cole, 1994). By agar diffusion technique, a well containing the plant extract or test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir is measured after the incubation period. To improve the detection limit, the inoculated petri-plate is kept at lower temperature for several hours before incubation to favor compound diffusion over growth of microbes, which increases the inhibition diameter. Different types of sources can be used, such as filter-paper discs, stainless-steel cylinders placed on the surface and holes punched in the media. The hole-punch method is suitable with diffusion technique for aqueous extracts, because it interferes by particulate matter which is much less than with other types of reservoirs or sources. Fixed agar is left on the bottom of the hole so as to ensure that the sample does not leak under the agar layer (Cole, 1994).

1.5 Enzymes inhibition by plant extracts

A therapeutic approach to treat diabetes is to control postprandial glucose level based on α-glucosidase inhibitor. **α-glucosidase** can convert oligosaccharide and disaccharide become monosaccharide in the digestive tract. α-Glucoside inhibitor can inhibits α-glucosidase and it helps in reducing the digestion and absorption of glucose. It means post-prandial glucose level

can be decreased. **15-Lipoxygenase (15-LO)** is an enzyme present in different systems that reacts with poly-unsaturated fatty acids which produces active lipid metabolites that are involved in many diseases such as cancer, atherosclerosis and diabetes (Zou *et al.,* 2014; Schneider and Bucar, 2005; Dobrian *et al.,* 2011). **Xanthine oxidase (XO)** is a pro-oxidative enzyme that generates reactive oxygen species (ROS) in vascular cells (Pacher *et al.,* 2006).

1.6 Gas Chromatography - Mass Spectrometry (GC/MS) analysis.

To identify the metabolites which show antioxidant property, the samples can be subjected to GC-MS analysis. Identification of metabolites can be carried out using a QP2010 gas chromatography with Thermal Desorption System TD 20 coupled with mass spectroscopy. At ionization voltage of 70 eV gas chromatography can be conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial temperature of column should be 80^{o}C for 1 min, then it is increased linearly at 7^{o}C min−1 to 220 ^{o}C, it has to be hold for 3 min following by linear increase in temperature by 10^{o}C min−1 upto 290^{o}C hold for 10 min. The temperature for injection port is 290^{o}C and the GC/MS interface has to be maintained at 290^{o}C. The samples should be introduce via an all-glass injector working in the split mode, with helium carrier gas flow rate was 1.2 ml min−1. Component identification is accomplish by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library or any reference library stored in the computer software (version 1.10 beta, Shimadzu) of the GC-MS. The relative percentage of each extract constituent is express as percentage with peak area normalization (Grover and Patni, 2013).

1.7 Research Plan

1.7.1 Hypothesis

- The selected medicinal plants for the research are frequently used by indigenous people against diseases diabetes, cancer and rheumatic arthritis. So they must have bioactive compounds.

- The claimed benefits to health by these plant may be due to antioxidant and antibacterial properties, and inhibition of α-glucosidase, 15-lipoxygenase and xanthin oxidase enzym

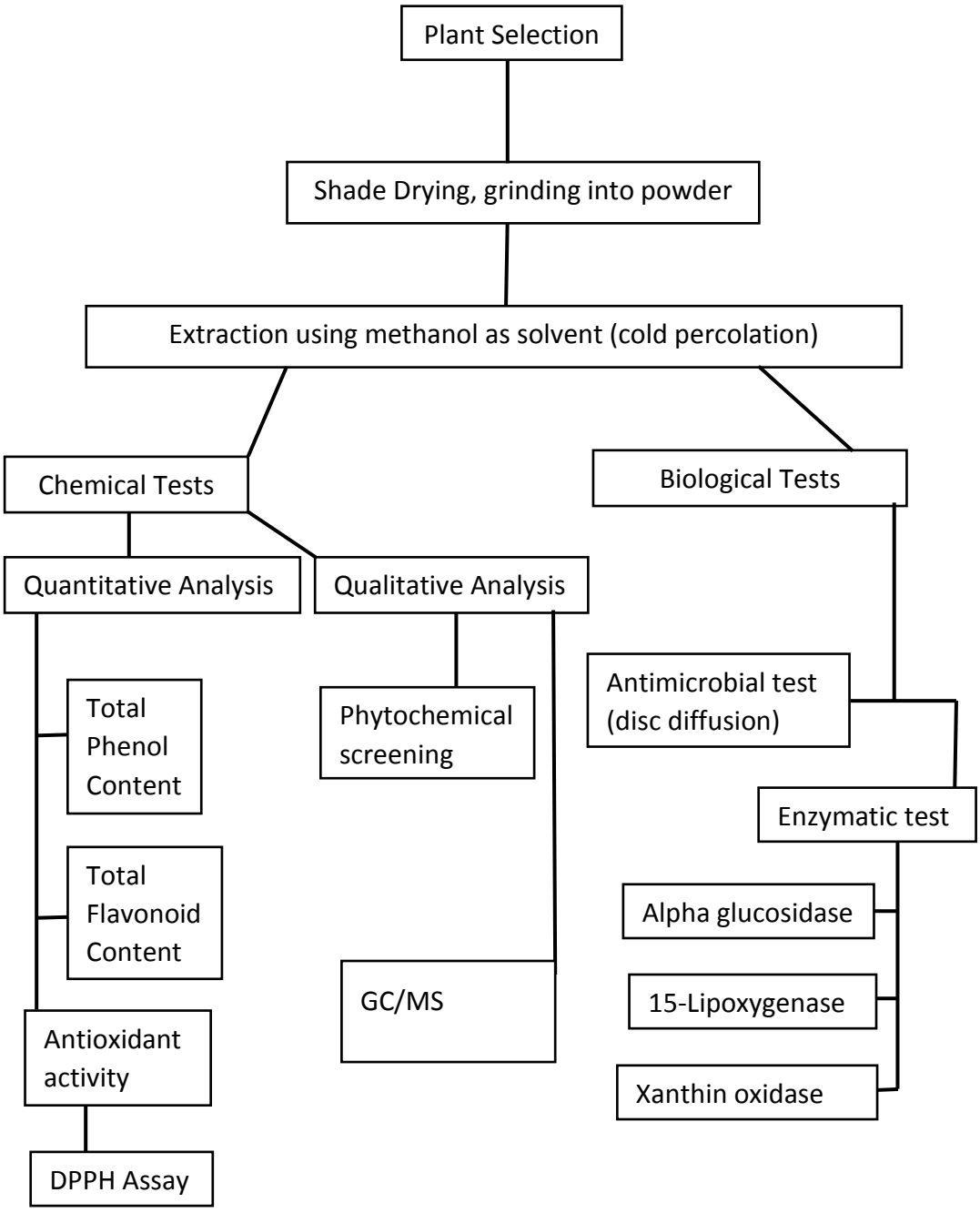

Fig 1.7.1: Research Flow-chart

1.7.2 Objectives

1.7.2.a General Objective

- Evaluation of medicinal plants having ethno medicinal values.

1.7.2.b Specific Objectives

- Methanolic extraction of medicinal plants.
- Phytochemical screening for bioactive compound in extracts of medicinal plants.
- Estimation of the total phenol and flavonoid content in the extracts.
- Test antimicrobial property of plants against pathogenic bacteria.
- Determination of enzymes inhibition.
- GC/MS analysis of the plant extract and predict the structure of compound present in it.
- Comparative analysis of the finding using statistical tools and techniques.

1.7.3 Rationale

α-glucosidase, 15-lipoxygenase and xanthin oxidase inhibitor are the drugs of target that helps in the prevention of diabetics, cancer and gout respectively. There exist many synthetic drugs for these disease but they are not fully successful eliminating disease and also have side effects. Therefore, inhibitors for enzymes (α-glucosidase, lipoxygenase and xanthin oxidase) will contribute safer treatment of disease like diabetes, cancer and gout. In this research, focus is mainly on screening of medicinal plant which possess antidiabetic, anti-cancer and anti-gout properties. Medicinal plant are rich in natural compound such as polyphenol, flavonoid, and antioxidant. Bioactive compounds in plant may show enzyme inhibition activity. So, it can be hypothesized that plant consisting bioactive compound will show higher antioxidant and enzyme inhibition properties. This research may help people all over the world as people are more prone to cancer and diabetes complication.

1.7.4 Scope

Identification and characterization of different compounds present in medicinal plants.

Structure prediction of compounds present in medicinal plant.

This study can display the importance of under-utilized medicinal plants.

This study validates under-utilized medicinal plants of Nepal.

Development of new drugs from medicinal plants of Nepal.

CHAPTER II: LITERATURE REVIEW

Medicinal plants have gained attention of many people including drug discovery researcher, pharmacologist and pharmaceutical companies. Herbal doctors or traditional healers can derive different treatments from these medicinal plants. And it is claimed that herbal remedies do not have any toxicity or side effects. Therefore medicinal have gained attention. Researchers on natural product have found antidiabetic, antimicrobial, anti-inflammatory, antimalarial and anticancer properties.

Medicinal plants have been described with their taxonomical position, antimicrobial and antioxidant properties, and presence of different compound them are given below:

2.1 *Terminalia chebula* Retz.

2.1.1 Taxonomic position

Kingdom: Plantae

Division: Magnoliopphyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: *Terminalia*

Species: *chebula*

Vernacular name: Harro

2.1.2 Ethno-medicinal practices

Ethno-medicinal practices of herbal medicines or natural products are in high demands, almost in all countries. Natural products or herbal-medicine have wide biological and medicinal applications. They are very safe to use and available at lower costs. *Terminalia chebula* is a medicinal plant. It is widely distributed throughout South-Asia. Ripen and dried fruit of *Terminalia chebula* is known as Black Myrobolan. It is used for treatment and control of various diseases like common-cold, asthma, sore-throat, vomiting, hiccough, beeding, piles, diarrhea, gout, heart and bladder disease (Kirtkar and Basu, 1935; Manoj *et al.,* 2009).

2.1.3 Phytochemistry

Black myrobolan have been found to possess antioxidant and free radical scavenging activity (Cheng *et al.,* 2003; Manoj *et al.,* 2009). It has been found to be active against cancer cell 3 and *Helicobacter pylori* (Malekzadeh *et al.,* 2001; Manoj *et al.,* 2009), beneficial as an anti-carries agent (Sugina *et al.,* 2002; Manoj *et al.,* 2009). It is used in skin or dermal wound healing (Jagpat & Karkera, 1999; Manoj *et al.,* 2009) and also improves gastrointestinal mobility (Tamhane,1997; Manoj *et al.,* 2009). There are evidences of use *Terminalia chebula* in anaphylactic shock 8 and diabetes mellitus (Sabu & Kuttan, 2002; Manoj *et al.,* 2009). Extracts of *Terminalia chebula* from two different solvents show significant effect against growth of uro-pathogenic *E.coli* (Chatopadhya, 2007; Kannan *et al.,* 2009). Ethyl ester of Gallic acid and both can be isolated from ethanolic extract of *Terminalia chebula*. The extract shows effect against growth of methicillin-resistant *Staphylococcus aureus* (MRSA). It also shows effect against growth of *Salmonella typhi* and other intestinal bacteria. But there are no such published articles or reports related to antimicrobial growth effect against *Bacillus subtilis, Staphylococcus aureus, and Escherichia coli,* by aqueous extract of *Terminalia chebula* fruit (Farag, 1989; Manoj *et al.,* 2009).

The research data indicates *Pseudomonas aeruginosa*, a Gram's negative bacteria is sensitive against *Terminalia chebula* among tested organism. The extract showed strongest zone of inhibition against *Pseudomonas aeruginosa*. The extract with 100% concentration showed highest activity against growth of *Escherichia coli*. It showed moderate level of activity against *Staphylococcus epidermidis*, Bacillus subtilis, *Staphylococcus aureus, and Shigella flexneri.* Extract of *Terminalia chebula* with the concentration of 75% showed strongest zone of inhibition against various strains of microbes. Data of the research shows growth effect against bacteria by inhibition zone of 14 mm to *Pseudomonas aeruginosa*, *Escherchia coli*, and *Staphylococcus epidermidis*. By comparing the effect against microbial growth due to extract with standard antibiotic (amoxicillin, tobramycin, erythromycin, cephalexin), it can be concluded that the extract has demonstrated better activity than cephalexin and tobramycin. But the plant extract is not better than cephalexin and tobramycin. But the plant extract is not better than erythromycin and amoxicillin. After analyzing complete research, *Terminalia*

chebula has been found to possess growth inhibiting property against different microorganism. The whole extract of *Terminalia chebula* shows protection or growth against inhibitory effect against Gram's positive and Gram's negative bacteria. Further studies and researches on extracts are required to pinpoint or specify the finding (Manoj *et al.,* 2009). *Terminalia chebula* is used by herbal medicine system in Tamil Nadu to cure disease and ailments such as diarrhea, skin disease, cough, fever, urinary-tract infection, candidiasis, wound infections (Dash, 1991; Kannan *et al.,* 2009). The IC_{50} for *Staphylococcus aureus* and *Escherichia coli* was found to be 1mg/ml. Growth of *Bacillus subtilis* and *Staphylococcus aureus* was found to be inhibited by the extract of *Terminalia arjuna* (Perumal and Ignacimuthu, 2001; Kannan *et al.,* 2009). Enzyme inhibition of alpha-glucosidase was observed to be strong in extract of *Terminalia macroptera.* Extracts from hot water also showed activity against some assays (Zou *et al.,* 2014; Pham *et al.,* 2011a).

2.2 *Ampelocissus divaricata* (Wall. ex M.A.Lawson)

2.2.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Vitales

Family: Vitaceae

Genus: *Ampelocissus*

Species: *divaricata*

Vernacular name: Pureni jhar

2.2.2 Ethno-medicinal practices

Natural products are the sources of antioxidant. They can prevent different pathological conditions like cancer, neurodegenerative and cardiovascular problems. People in developing countries of western Africa cannot afford drugs. Almost 80% people are dependent on medicinal plant for treatment of health problem (Zongo *et al.,* 2010; Karou *et al.,* 2006; Hostettnann & Marston, 2002; Kirby, 1996). It has been reported that Shigellosis and malaria

can be treated by Ampelocissus grantii or in mixed form with others (Zongo et al, 2010; Adjanhoun *et al.,* 1980). Schistosomiasis can be treated by this plant (Zongo *et al.,* 2010; Bah *et al.,* 2006). Old wound (2-3 years) can be cured by the use of this plant (Zongo *et al.,* 2010; Inngjerdingen *et al.,* 2004).

2.3 *Terminalia bellirica* (Gaertn.) Roxb.

2.3.1 Taxonomic position

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: *Terminalia*

Species: *bellirica*

Vernacular name: Barro

2.3.2 Ethno-medicinal practices

Nature is infinite resource of herbal remedy for treatment of human's ailments. Higher plants have potential for discovery of a new drug. But they are largely unexplored till the date (Devi *et al.,* 2014; Oke & Hamburger). From the times of Rig veda, medicinal plant have been used by traditional medicinal system to cure many diseases. Number of microbial diseases are treated and cured by medicinal plants. These plants don't show any side effects and are less expensive (Devi *et al.,* 2014; Nithya *et al.,* 2004).

2.3.3 Phytochemistry

Development of multiple drug resistance towards many antimicrobial compounds has led to use natural drugs (Devi *et al.,* 2014; Kavitha & Padma, 2011). A clear symptoms appears in case of infectious diseases. Traditional healers can recognize such infectious diseases. They have developed number of therapies effective against infectious diseases. Exponential growth for consumption of herbal products can be observed, globally. Various phytochemical are present

in plant which are responsible to show antimicrobial activities. Most of the people in the world are dependent upon traditional system of medicine for primary health care (Devi *et al.,* 2014; Prabuseenivasan *et al.,* 2006). *Terminalia bellirica* belongs to Combreteceae is a large type of deciduous tree. They are found throughout in India. It has various medicinal properties. It can be applied on painful swollen part, skin diseases, and premature gray hair. For the treatment of astringent, conjunctivitis, impart black color to hair, boost hair growth, arrest bleeding, asthma, premature voice, cold and cough, fruit of bellerica can be used. Blood pressure falls at the concentration of 70 mg/kg weight of the body. The plant also help in curing loss of appetite, blood pressure, lowering cholesterol, prevent ageing and boost immunity. It prepares against diseases by enhancing body resistance. Bellerica is being used by traditional medicinal system to get all disease cured mentioned above by the people of Coimbatore, India (Devi *et al.,* 2014; Kritikar, 1999). One of the major cause of public health issue is infectious disease. Emerging strains with developed resistance against drugs show low susceptibility to antibiotic because mutation in genes. This is the challenge for any researcher to discover new drug (Devi *et al.,* 2014; Rani *et al.,* 2013). Evaluating antimicrobial property from different medicinal plant sources is of great importance at present scenario. The observed activity against bacteria on which test has been performed, provide evidence for usage of the plant in treatment of different disease. The plant extracts were found to be active against bacteria and fungi. Such tests indicate the extracts has a broad spectrum antimicrobial property. This observation is of higher significance. This shows the possibility to develop substance of therapeutic value which are active against those organism with developed multi drug resistant. Many studies on fruit extract of this plant describes its traditional application and indicates the extract composed of antimicrobial substance which can be used as antibacterial or antifungal in novel drugs to treat microbial diseases (Devi *et al.,* 2014; Rana *et al.,* 1997). Fruit aqueous extract of *Terminalia bellirica* showed antimicrobial activity on bacteria and fungi. This indicates phytochemical present in fruit extract can deactivate numbers of cellular enzymes that has role in metabolic pathways of the microbes. It has been reported, proteins of the cells may be denatured by phytochemicals, which results abnormal cellular processes. Various non nutrient phytochemical compounds are present in the plant extract which shows biological property that are of

valuable and desirable therapeutic index. Varieties of phytochemicals have been found to consist a broad range of activities, this may protect from chronic disease (Devi *et al.,* 2014; Gurib & Fakim, 2006). Flavonoid, tannins, phenol and alkaloid have been found to be present during phytochemical screening. The possible reason for inhibition of microbial growth by the presence of phenol are enzymes involved for production of energy may have impaired, interference in integration of cell membrane and synthesis of structural component. Fungal growth might have inhibited by phenol which induced swelling, distortion, plasma seeping and leakage, wrinkling of hyphae and abnormal branching or fusion. Tannins are present in fruit extract of *Terminalia bellirica*. Tannins might have interfered the development of microorganism by precipitation of microbial protein. This causes unavailability of nutritional proteins to microbes (Devi *et al.,* 2014; Hung & Chung, 2006). In reports, it has been found that tannins from such complexes with proteins rich in proline, which cause inhibition in synthesis of cell protein (Devi *et al.,* 2014; Hagerman and Butler, 1981).

2.4 *Eucalyptus alba* (Reinw.)

2.4.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Myrtales

Family: Myrtaceae

Genus: *Eucalyptus*

Species: *alba*

Vernacular name: Lyptis

2.4.2 Ethno-medicinal practices

Many identified medicinal plants have been used throughout the history of human. For the discovery of future medicine, ethnobotany could be an effective way. The ability of plant to synthesize various chemical compound can be utilized for defense action against the attack of predators like mammals, herbivores, fungi and insects, and to perform some biological

functions. About 12,000 compounds have been identified till the date. This is estimated to be less than 10 percent of total (Shayoub *et al.,* 2015; Tapsell *et al.,* 2006; Lai & Roy, 2004). 122 compounds have been identified by researcher (in 2001) are derived from ethno-medical plant sources and they are being used in modern medicine. 80% of these compounds have an ethno-medicinal use. The active elements from these plants are being used currently (Shayoub *et al.,* 2015; Fabricant & Fransworth, 2001).

2.4.3 Phyto-chemistry

Many drugs which are being used currently such as aspirin, digitalis, quinine, and opium have history for its uses in herbal remedy. Conventional drugs or chemical compounds from plants show their effect on human body are identical. So, herbal medicine are similar to conventional drug in term of the way they work. This implies herbal remedy or medicine to be as effective as conventional drugs, and same with the potential of its side effects (Shayoub *et al.,* 2015; Tapsell, 2006; Lai & Roy, 2004). Creation of tablets by the Sumerians dates back to 5000 years in the written history, with hundreds of medicinal plant lists (Shayoub *et al.,* 2015; Sumner & Judith, 2000). Ancient Egyptians in 1500 BC wrote Ebers Papyrus, which has information on more than 850 medicinal plants. This includes: Mandrake, aloe, bean, castor, cannabis, juniper, garlic (Shayoub *et al.,* 2015; Sumner & Judith, 2000). Sudan has extreme geographical conditions in terms of meteorology, climate, and topography. This results in growth of flora with variations. These different types of medicinal plants are flora of many countries. In Sudan, such medicinal plants are grown which have therapeutic value and drug isolated from these plants are used by pharmacopeias of many countries. The main purpose of these study is to focus on anti-hyperglycemic effect of an important tropical plant with medicinal value. Eucalyptus genus has around 800 genus. Eucalyptus is Australia's native and spread widely (Shayoub *et al.,* 2015; Slee *et al.,* 2006).

2.5 *Woodfordia fruticosa* (L.) Kurz

2.5.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Myrtles

Family: Lythraceae

Genus: *Woodfordia*

Species: *fruticosa*

Vernacular name: Ras dhairo

2.5.2 Ethno-medicinal practices

It is commonly known found throughout Northern part of India. Its height is 1 to 3 metre sometimes upto seven metres. It is cultivated widely for its ornamental purpose. In gardens, it is cultivated for flowers. It is also known as fire flame bush because color is like flame. It is used for manufacture of red dye which colors fabric. In India, it is commonly known as Dhavri, Dhatki etc. This plant has medicinal properties. And belongs to Lyrthraceae family. For fermentation purpose in Ayurvedic medicine, these plant's dried flowers are considered to be very effective (Kroes *et al.,* 1990; Grover & Patni, 2013). It is used for internal and external purposes. Its dried flower have been reported in use for treatment of diseases like herpes, headache, fever, skin diseases, burning sensation, ulcers, wounds, menorrhagia, mucous membrane leucorrhoea disorder, piles, liner disease, dysentery diarrhea, hemorrhoids (Chadha, 1976; Grover & Patni, 2013). Often, they are added to Ayurvedic Arishtas to cause alcoholic fermentation (Atal *et al.,* 1982; Grover & Patni, 2013).

2.5.3 Phyto-chemistry

Powder made up of dried form of flower is sprinkled externally on wounds alleviates sensation of burning, arrest bleeding, and promotes healing. Fresh-flower juice of the plant is applied on forehead which reduce headache due to pitta. Powder prepared from dried flower are used in massage of gums which facilitates dental eruption in case of children. Leaves of *Woodfordia fruticosa* have antimicrobial activity in "in vitro" condition. It shows such effect against *Micrococcus pyogenes* var. aureus and also has sedative properties. This medicinal plant have curative value may be due to different secondary metabolites present in it such as sterols, saponin, phenol, glycosides, flavonoids, alkaloid etc. For screening, preliminary tests are

performed which can detect bioactive compound. This helps in discovery of drug and their development. Screening of phytochemical is the most important and significant in identification of new source of therapeutics which could be valuable compound industrially and have medical significance. Such process is best and justified way to use natural wealth which are available. This research was performed for the determination of possible phyto-constituent from *Woodfordia fruticosa* by analysis of GC/MS. Characterizing organic compound of plants are area of interest in recent years. So this research was organized for screening and isolation of bioactive compound. It was observed that more bioactive compounds were obtained from methanolic extract of the plant leaves (Audu *et al.,* 2004; Grover & Patni, 2013).

2.6 *Cheilanthes dalhousidae* Sw.

2.6.1 Taxonomic position

Kingdom: Plantae

Division: Pteridophyta

Class: Polypodiopsida

Order: Polypodiales

Family: Pterideceae

Genus: *Chelanthes*

Species: *dalhousidae*

Vernacular name: Rani sniko

2.6.2 Ethno-medicinal practices

Information from traditional medicine system are very important for the development of any drug and health care (Ghorpade *et al.,* 2015; Pei, 2001). According to WHO 80% of people in world mostly from developing countries are dependent on plant derived medicine or drugs for the treatment of disease (Ghorpade *et al.,* 2015; Gurib, 2006). Pteridophytes are also known as “reptile group of plants”. Primarily, pteridophytes are vascular plant groups. There is less information available on the literature of pteridophytes related to medicinal value except some studies. Pteridophytes are not easily available as flowering plants. They play important role in biodiversity of earth (Ghorpade *et al.,* 2015; Caius, 1953; Manandhar, 1996; Kumar & Kaushik,

1999; Sharma, 2002; Benjamin and Manikam, 2007). Cheilanthes dalhousidae is a herb known as Rani Sinka in Nepal and its raw leaves have been reported for use in snake bite (Rai and Singh, 2015).

2.6.3 Phyto-Chemistry

Plant contents number of phytochemical molecules. They are: betalains, amines, alkaloids, coumarins, quinones, tannins, flavonoids, stilbenes, lignin, phenolic acids, terpenoid, vitamin and some additional metabolites, and these are rich source of antioxidant activity (Ghorpade *et al.,* 2015; Zheng & Wang, 2001; Cai *et al.,* 2003). According to many researches, so many antioxidant compound possess antiviral, antiathero-sclerotic, antibacterial, anticarcinogenic, antimutagenic, antitumour, anti-inflamatory properties (Ghorpade *et al.,* 2015; Sala *et al.,* 2002; Rice –Evans *et al.,* 1995). Eating natural antioxidant is related to reduce of hazard for cancer, cardiovascular diseases, diabetes and related to ageing (Ghorpade *et al.,* 2015; Ashokkumar *et al.,* 2008; Veerapur *et al.,* 2009). Natural phytochemical found in different fruits like berry crops, herbs, oil seed, fruit and vegetable beans, teas etc. are being used universally in modern years (Ghorpade *et al.,* 2015; Kitts *et al.,* 2000; Muselik *et al.,* 2007; Wang & Jiao, 2000). Pharmacological effects of plant secondary metabolites are being studied widely for therapeutic values in modern centuries (Ghorpade *et al.,* 2015; Krishnaraju *et al.,* 2005). It can be concluded that plant extract or phytochemical showing higher antibacterial activity can be used against bacterial infections (Ghorpade *et al.,* 2015; Balandrin *et al.,* 1985). Microbial pathogen do not infect pteridophytes, this could be significant reason of evolutionary progress for pteridophytes. There is an information available that pteridophytes stay alive for 350 million year and even more than that. India has rich diversity of medicinal plant including pteridophytes. It can be concluded that the screening for plant extract with antibacterial activity may help curing plant and human disease (Ghorpade *et al.,* 2015; Sharma & Vyas, 1985). The main for this study was for evaluation of phytochemical from extracted from methanol of four Cheilanthes species (Ghorpade *et al.,* 2015; Brinda *et al.,* 1981).

2.7 *Centella asiatica* (L.) Urb.

2.7.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Apiales

Family: Apiaceae

Genus: *Centella*

Species: *asiatica*

Vernacular name: Ghodtapre

2.7.2 Ethno-medicinal practices

It is native to many countries of Asia (Matsuda *et al.,* 2001; James & Dubery, 2009; Thangavel *et al.,* 2011). *Centella asiatica* is known as Ghodtapre in Nepal (Rai, 2003). According to the medicine system of Ayurveda, it can be used as brain-tonic, to treat many chronic and mental diseases.

2.7.3 Phytochemistry

It contains various important compound, they are: sceffoleoside, centellasaponin, asiaticoside and madecassoside (Matsuda *et al.,* 2001; James & Dubery, 2009; Thangavel *et al.,* 2011). It contains pectin (Wang *et al.,* 2009; Thangavel *et al.,* 2011). It contains castilliferol 1 and castellicetin 2 (Subban *et al.,* 2008; Thangavel *et al.,* 2011). Isolated fatty-oil from *Centella asiatica* contain glycerides of steric, palmitic, lignoceric, centoic and oleic acid. Leaves of *Centella asiatica* contain 7-glycosyl kaemferol, 3-glycosyl kaemferol, 3-glycosyl quercetin and triterpene madaciatic acid (Martin, 2004; Thangavel *et al.,* 2011). Some bitter compound like pectic acid, vellasine and resin are present in the root and leaves. Compound like asiaticoside and oxyasiaticoside are active against leprosy and tuberculosis (Chopra *et al.,* 1980; Thangavel *et al.,* 2011). The plant shows varieties of pharmacological effects. They are used for healing, antibacterial, mental disorder, anticancer and antioxidant purposes. This plant highly active in ulcer preventive (Cho, 1981; Thangavel *et al.,* 2011). It is used as anti-depressive sedative. It also has property of neutralizing venom poisoning (Zheng & Qin, 2007; Thangavel *et al.,* 2011).

Centella asiatica have been found to be responsible for increase in concentration power, behaviors and general abilities of mental retardness in children (Appa Rao *et al.,* 1973; Thangavel *et al.,* 2011). It is used for treatment of rheumatic diseases (Howes and Houghton, 2003; Thangavel *et al.,* 2011). Asiaticoside is a type of triterpene saponin is found in leaves. It can be utilized as a "wound healing" agent because of its anti-inflammatory effects (Pointel *et al.,* 1987; Shukla *et al.,* 1999; Thangavel *et al.,* 2011). Drug resistance has developed against some common antibiotics by human pathogens. This indicates for the search of new antibiotics from different sources like plants. So many plant species possess antibacterial properties. Many of these plants are not evaluated systematically. Antimicrobial properties of plant extract attract attention highly for their evaluation of antimicrobial properties against resistant plant pathogen. Protocol of callus proliferation is very important and has to be developed efficiently to start in-vitro method of cultivation. To produce cells and secondary compound in large scale, there are many developed techniques of plant tissue culture available (Lee *et al.,* 2011; Thangavel *et al.,* 2011). By this approach, the desired active component can be isolated from callus without harming natural resources of plants. For screening of bioactive compound, antimicrobial activity of leaf extract and development of in-vitro callus from leaf of *Centella asiatica*, this particular study has been conducted. Phytochemical evaluation of the plant revealed presence of reducing sugar, alkaloid, tannin, glycosides, flavonoids, terpenoids and steroids. These compounds can be used against pathogen of humans and those organisms which are responsible cause of enteric infections. It has been reported to possess curative properties against many pathogens so, it can be used to treat many diseases (Hassan *et al.,* 2004; Thangavel *et al.,* 2011). The antioxidant activity of this plant is due to total amount of phenolic compound present in leaf, root and petiole (Zainole *et al.,* 2003; Thangavel *et al.,* 2011).

2.8 *Citrus maxima* (Burm. f.) Merr.

2.8.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Rutaceae

Genus: *Citrus*

Species: *maxima*

Vernacular name: Bhogate

2.8.2 Ethno-medicinal practices & Phyto-chemistry

The two common Gram negative bacteria are Escherichia coli and Salmonella typhimurium and these bacteria frequently cause infections in Gastro-intestinal tract in humans and animals. Most of time in human cases, infections due to Escherichia coli and Salmonella are food borne by nature. Escherichia coli and Salmonella cause food-borne infections. They are reason for higher consequences on the status of public health because they increase the cause of mortality and morbidity. These are well known bacteria which are factors to determine the cause, control and distribution of Gram negative enteric and have changed with time. According to the report, emerging pathogen mere responsible for increment in prevalence and they were also responsible for association with newer food vehicles and cause of systemic disease which cause long term complication and develop antibiotic resistance. Eschericha coli and Salmonella typhi are resistant to different class of antibacterial agent which been reported world-wide (Friedman *et al.,* 2004; Geornaras *et al.,* 2007; Azu *et al.,* 2008; Duan & Zhao, 2009; Barrion *et al.,* 2014). There is growth in interest on public health. Food safety issue should not be emphasis highly. Newer economic plant have gained attentions from which antimicrobial extract can be derived. They have possibility to be used as to secure food security. Many recent reports are there related to safeness and health, natural food are preferred over food items. New pathogens may have potential develop resistance against antibiotics. There are issues related to traditional antibiotics and its misuse. Many Citrus species are the subject for researches related to antimicrobial activity. There are little or very less work have been done on the evaluation of antimicrobial property or potential of un-edible part of the fruit. One of the common Citrus species in the country is Pumelo. It is said to be the grape fruits local version. Analyzing previous researches, there are higher antioxidant and antimicrobial activities for different phytochemicals present in fruits like Citrus. It can be concluded that different Citrus

fruits have same complexity in structure of all cultivars. So, synthetic preservatives can be replaced by pummelo which has healthy side effects to consumers. It can be used for medicine, food technology and therapeutic purpose (Ladiya, 2008; Cowan, 1999; Barrion *et al.,* 2014). By examining probable mechanism of resistance developed by Gram negative bacteria like Escherichia coli and Salmonella typhimurium against natural antibiotic potential of Citrus maxima extract would give some hints on the mechanism pattern of developed antibiotic-resistance mechanism. Such information or finding are helpful to control growth of the microbes effectively and eliminate or minimize high load of infectious disease which might affect the population. Identification of phytochemical composition and the determination of antibacterial activity for the inedible part of pumelo fruits were the objectives of this study. Antibacterial activity was performed against Escherichia coli and Salmonella typhimurium (Sarian, 2009; Barrion *et al.,* 2014).

2.9 *Hedyotis diffusa (Willd.) Roxb.*

2.9.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Gentianales

Family: Rubiaceae

Genus: *Hedyotis*

Species: *diffusa*

Vernacular name: Majithe jhar

2.9.2 Phyto-chemistry

Compounds like polysaccharide, anthraquinone (2-hydroxy-3-methoxy-7-methyl anthraquinone, 2-hydroxy-1-methoxyanthraquinone), flavonoid (quercetin, rutin, quercetin-3-O-β-D-glucopyranoside, quercetin-3-O sambubioside, kaempferol, kaempferol-3-O-β-D pyranside), phenolic acid (p-coumaric acid and ferulic acid), asperuloside acid, diacetyl asperulosidic acid methyl ester, geniposidic acid, triterpenes (ursolic acid and oleanolic acid),

iridoids ((E) -6-O-p-coumaroyls candoside-methyl ester) and a miscellaneous compound are identified as marking compound for quality control of *Hedyotis diffusa* which are confirmed by series of analytic methods like UV, HPLC, TLC, and LC/MS. Wide variation were present in these compound. Samples were collected from different places and time. There is an urgent requirement of a method to ensure the quality of *Hedyotis diffusa*. There is very less information or known about pharmacokinetics investigation of this plant. *Hedyotis diffusa*, when administered orally in case of renal inflammation induced by lipopolysaccharide in mice showed most compound like iridoids glycoside, anthraquinone, flavonoid present in plasma and twelve compounds (which include eight flavonoids and four iridoid-glycoside) present in kidney according to the analysed result of UPLC-Q-TOF-MS/MS. (Chen et. al., 2016; Ye et. al., 2015). Biological availability of *Hedyotis diffusa* was investigated by producing post absorption sample with the use of Laco-2 cell model and it confirmed good permeability of decoction (Chen et. al., 2016; Ganbold *et al.,* 2010).

2.10 *Albizia lebbeck* (L.) Benth.

2.10.1 Taxonomic position

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Albizia*

Species: *lebbeck*

Vernacular name: Shirish

2.10.2 Ethno-medicinal practices

It is known as Shirish in India. In Indian subcontinent, it is very familiar and well known for its uses in medicine. It is well explained in Ayurveda, Indian folk and traditional medicine system.

Inflammatory diseases like arthritis, asthma and burns can be cured by the use of *Albizia lebbeck* (Faisal *et al.,* 2012; Malla *et al.,* 2014).

2.10.3 Phyto-Chemistry

Antihistaminic property also have been reported for its alcoholic extracts. Histamine is directly neutralized by *Albizia lebbeck* or by action of corticotrophin resulting rise in level of plasma corticsol (Babu *et al.,* 2009; Malla *et al.,* 2014). Diseases like Alzheimer and Parkinson can be treated by the use of saponin obtained from *Albizia lebbeck* (Sanjay *et al.,* 2003; Malla *et al.,* 2014). When free radicals are released, these are responsible for the creation of oxidative stress. These oxidative stress and release of free radicals are among the major reasons for disease and disorder (Shrotri *et al.,* 2012; Malla *et al.,* 2014). Free radicals are fundamental in biochemical pathways. They play important part in metabolism and aerobic life (Fang *et al.,* 2002; Malla *et al.,* 2014). Super oxide anion, hydrogen peroxide, hydroxyl and peroxy radical are the most common reactive oxygen species (ROS). Peroxy nitrite anion and nitric oxide are the free radicals derived from nitrogen (Nagendrappa, 2005; Malla *et al.,* 2014). For more than hundreds of diseases ROS are responsible. Disease like infection, arthritis, connective tissue disorder, cardiovascular malfunction, physical injury are due to ROS. (Ray & Hussain, 2002; Malla *et al.,* 2014). In order to treat these diseases, antioxidant therapy play an important role. To evaluate the antioxidant, antimicrobial and preliminary phytochemical tests of *Albizia lebbeck* this research was organized (Leggett & Westerman, 1973; Malla *et al.,* 2014).

CHAPTER III: MATERIAL & METHODOLOGY

3.1 Chemicals and Equipments

Enzymes and chemicals were purchased from Sigma-Aldrich, Fischer Chemical. The rotary evaporator used for concentration of the extract was from IKA werke, Germany. And sonicator used was from Indosati. All the media used were from HiMedia Laboratories Pvt. Ltd. ELISA was from Thermo Electron.

3.2 Plant Material

Table 3.2: Name and Parts of Plants selected.

Scientific Name	Vernacular name	Parts used
Terminalaia chebula	Harro	Fruit
Ampelocissus divaricate	Pureni jhar	Whole Plant
Terminalia bellirica	Barro	Fruit
Eucalyptus alba	Lyptis	Bark
Woodfordia fruticosa	Ras dhairo	Stem
Cheilanthes dalhousidae	Rani sinko	Whole Plant
Centella asiatica	Ghodtapre	Whole Plant
Citrus maxima	Bhogate	Young shoot & leaves
Hedyotis diffusa	Majithe Jhar	Stem
Albizia lebbeck	Shirish	Bark

3.3 Extraction of plant materials (by cold percolation method)

Plant materials were dried in shade. Fine powders of the materials were prepared using electric crusher. Cold percolation technique with sonication was followed. Weighed dried plant materials were taken in air-tight jar. Methanol was added to the material such that the ratio of the volume of solvent in ml to the gram weight of the plant material would be 1:6. Such prepared solvent system was then allowed to stand for three days at room temperature. And

the solution was subjected to sonication. Sonication was done for 75 minutes at 50^{o}C, each day for three days. On day 4, the supernatant was slowly poured into round bottom flask via whatman no 1 paper filtration.

Marc or the remaining solid residue was pressed gently to squeeze out all the absorbed solvent. The obtained solvent then contained the phytochemicals from respective plant materials. The solution collected in round bottom flask was then subjected to evaporation at reduced pressure in a rotatory vaccum evaporator. To the remaining solid material of plant, the process of extraction was repeated to obtain ethyl acetate and methanol extract respectively. After evaporation, the reduced amount (about 25-30 ml) of solution was transferred to petri-plates. The plates were then incubated at 37^{o}C for two days such that solvent in the extract completely get evaporated. Thus obtained extract is called crude extract of the respective plant material.

Percentage yield (%) = Dry wt. of extract * 100 / Dry wt. of plant material

The crude extract were then stored in cryovial at 4^{o}C until use.

3.4 Phytochemical Screening

Crude extract of the plants were subjected to preliminary phytochemical screening to detect the major phyto-constituents present in them. The procedure for this has been followed from different research papers. A detail protocol followed for detection of major group of compound has been described below:

3.4.1 Detection of Phenolic compounds

3.4.1.a Ferric Chloride Test:

To the aqueous fraction of the extract as prepared earlier, few drop of alcoholic Ferric chloride were added. Change in the color of the solution to dark green occurs as a result of presence of phenolic.

3.4.2 Detection of flavonoid

3.4.2.a. Alkaline Reagent Test:

Development of yellow fluorescence upon addition of 10% Ammonium Hydroxide solution to aqueous fraction of the extract confirms presence of flavonoids.

3.4.2.b. Magnesium and Hydrochloride Acid Reduction

50 mg of the extract was dissolved in 5 ml of ethanol and to it few fragment of magnesium ribbon were added. Upon drop wise addition of conc. HCl, development of pink to crimson color occurs due to presence of flavonol glycoside.

3.4.2.c. Schinoda Test

A piece of magnesium ribbon was added to few ml of methanolic extract and then 1 ml of concentrated sulfuric acid was added to it. Development of pink or red coloration indicates the presence of flavonoids.

3.4.3. Detection of carbohydrates (Raaman, 2006)

3.4.3.a Molich's Test

Test solution i.e., aqueous fraction of plant material was taken in a test tube. To it 2 drops of alcoholic solution of of α-napthol was added and shaken well, then 1 ml of conc sulfuric acid was added along the wall of the test tube and kept undisturbed. Formation of violet ring is positive result.

3.4.4 Detection of Protein and amino acids

100 mg extract was dissolved in 10 ml distilled water and filtered to obtain aqueous fraction of extract.

3.4.4.a Biuret Test

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

3.4.4.b Ninhydrin test

Two drops of Ninhydrin reagent to 2ml of the filtrate. Appearance of characteristic purple color indicates presence of amino acid.

3.4.5 Detection of Tannins

3.4.5.a Braemer's Test (Kumar et al., 2007)

Methanolic extract and 10% Ferric Chloride solution was added to test tube in equal volume. Formation of dark blue or greenish blue of the solution confirmed presence of tannins.

3.4.6 Detection of Cardiac glycoside

3.4.6.a Kellar-Killani test (SV,2007)

0.5 ml of methanolic extract was added to 2 ml glacial acetic acid. One drop of 5% ferric chloride solution was added to it. 1 ml of conc sulfuric acid was added to overlay the solution. Brown ring formation at surface indicates presence of cardiac glycoside. Greenish ring or a violet ring below bwron ring is also considered as a positive result.

3.5 Antibacterial Screening

The antimicrobial activity of the crude extracts was performed by Agar well diffusion Technique (Perez C, 1990). Equal size of wells were bored on the agar plates, along with positive and negative controls. Halo zone was marked as zone of inhibition. The diameter of the halo zone shows antibacterial activity of the crude extract.

3.5.1 Micro-organism

Clinical isolates pathogen one Gram positive *Staphlococcus aureus* ATCC 2593 and one Gram negative *Pseudomonas aeruginosa* ATCC 27853 pathogen were used for antibacterial assay.

3.5.2 Culturing of Bacteria

Biochemical tests was performed for all the bacterial samples to identify the organism. All the organisms were sub-cultured every fifteen days. Before testing the bacterial inoculums were sub-cultured in Nutrient Broth for 12-18 hours at 37^{o}C. The cell suspension in the culture was maintained at 1.0-1.5×10^{8} CFU/ml.

3.5.3 Preparation of extract for Antibacterial Screening

50mg/ml plant extract in DMSO was prepared. Then, the extract was stored in refrigerator at 4^oC.

3.5.4 Anti-bacterial screening via Agar well diffusion Technique

Autoclaved molten MHA was poured in the sterile petri-plates and allowed to set. The thickness of the media maintained in each of the plates was 4mm. The prepared bacterial inoculum was compared with 0.5 McFarland standard to obtain inoculums of 1.5×10^8 CFU/ml. Sterile cotton swab was dipped into the standard microbial inoculum, then the swab was gently rubbed on surface of media (MHA) which ensures a uniform lawn of microbial growth. All inoculated media plates were allowed to diffuse for 20 minutes in laminar air flow. Using sterile borer, seven wells of 6 mm each were bored in the inoculated media. In each plate, DMSO as solvent control, antibiotic disc (Chloramphenicol, 50 mcg) as positive control and plant extracts were added to seven wells. Those inoculated plates were incubated at 37^oC in incubator at upright position for 18-24 hrs.

The plates were observed for the formation of halo zone around the seven wells, after the incubation for 18-24 hrs. Halo zone represents the antibacterial activity of the corresponding plant extract and clear zone's diameter represents the strength of the antibacterial activity.

3.6 Determination of Total Polyphenol Content

Folin-Ciocalteu method of was followed for the determination of total phenolic content in the plant materials (Ainsworth and Gillespie, 2007). Crude methanolic extract at concentration of 1 mg/ml in absolute methanol were prepared. In a clean test-tube 100µl of these samples were taken and 1570µl of sterile distilled water was added to each test-tube such that final concentration of methanol becomes 6%. Then, 200 µl of Folin-Calteu reagent of 10% v/v was added to each of them. 800 µl of Sodium Carbonate (7.4%) was also added to it. The solution was shaken well and incubated at 37^oC for one hour. Then the absorbance of the resulting blue/violet coloured solution were measured by UV-Spectrophotometer at wavelength of 765 nm. For the blank, 100 µl of absolute methanol was used replacing the test solution. Gallic acid

was used as standard. The total phenolic content in the respective plant extracts were quantified by using the standard calibration curve generated. The results were expressed as Gallic Acid Equivalent, milligrams per grams of dry weight. Each of the assays were carried out in triplicate (n=3).

3.7 Determination of Total Flavonoid content

The total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method Chang et al., 2002). In brief, 50 L of crude extract (1 mg/mL methanol) were made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg quercetin equivalent per g dry weight.

3.8 Determination of Antioxidant Activity

3.8.1 DPPH Assay

The antioxidant activity of the extract was determined by the 2, 2-diphenyl-2-picryl-hydrazyl (DPPH) assay. The stable 2, 2-diphenyl-2-picryl-hydrazyl (DPPH) radical was used to assay the antioxidant potential of the crude methanolic extract. A range of varying concentration from 0-10mg/ml of the samples was taken for the assay. A 50 µl of the crude extract was added to 450 µl of tris-HCl buffer (0.05M, pH 7.4) and 1 ml of 0.1 mM DPPH was added to the resulting mixture. The solution in the test tubes were then shaken well and incubated in dark for 30 minutes at ambient temperature. Then after the absorbance of the solution in each tube were measured in UV-spectrophtometer at 517nm. Gallic acid was used as positive control. Sample blank and a control containing only buffer and DPPH were also taken for the experiment.

The free radical scavenging activity (RSA) of the sample was calculated in percentage by using the formula:

DPPH scavenging effect = (Control OD − Sample OD) × 100 /Control OD

The IC_{50} values of each extract were calculated by using the formula given below:

IC50 = EXP [LN(conc>50%) –(Signal>50%-50)/(signal>50% - signal<50%) × LN (conc>50%/(conc<50%)]

EXP: exponential function; LN: Natural log function, both used in Microsoft Excel 2007 software

Signal>50%: RSA value just above 50%; Signal<50%: RSA value just below 50%

Conc>50%: Concentration of Signal> 50%; Conc<50%: Concentration of Signal <50%

3.9 α-Glucosidase Inhibition

α-Glucosidase inhibition assay was performed according to the procedure given by Matsui et al., 1996 with slight modification. This protocol was modified to perform test in 96 well ELISA plate. 100µl buffer (PBS, pH 6.8) was added to each well followed by 60 µl of 0.5 mg (in 30 % DMSO) and 20 µl of 2U/ml enzyme. ELSIA plate was incubate at 37^{o}C for 10 minutes. Then 20µl of 0.7 mM PNPG was added. Again it was incubated at 37^{o}C for 15 minutes. Finally, reaction was stopped by addition of 30 µl 0.5M TRIS. Acarbose was positive control. Absorbance was taken at 400nm.

Calculation of % inhibition: $\frac{A_0 - A_t}{A_0} \times 100\%$

Where, A_0 is absorbance of enzyme substrate reaction with DMSO and A_t is absorbance is of enzyme.

3.9 15-Lipoxygenase

Enzyme iinhibition was performed according to Lykander and Malterud, 1992 with silght modification. Substrate solution was prepared by adding 50 µl linoleic acid to 150 µl ethanol

followed by addition of 50 ml borate buffer. Of this cloudy solution, 10 ml was diluted with 150 ml borate buffer, which made substrate solution. Now in 96 well ELISA plate, 63 µl buffer (0.2M borate buffer, pH 9.00) was mixed with 134 µl of substrate solution and 3 µl of plant extract (in DMSO). To this 3 µl if enzyme (10,000 U/ml in borate buffer) was added. Quercetin was used as positive control. Absorbance (234nm) was taken at 30, 60, 90 seconds. Percentage inhibition was calculated using formula,

µl Where A_0 is absorbance of enzyme substrate reaction with 30% DMSO and A_t is absorbance of enzyme substrate with plant extract.

3.10 Xanthine Oxidase

Xanthin Oxidase was performed according to Pham et al., 2011. Enzyme solution of 1.8 U/ml was prepared in 0.05M pH 7.5 phosphate buffer. Hypoxanthine, the substrate was dissolved in distilled water to make up final concentration of 0.2mg/ml. 123 µl of buffer was mixed with 3 µl of plant extract (in DMSO). To it 3 µl of enzyme was added. Finally 67 µl of was substrate added. Quercetin was used as positive control. Absorbance was taken at 290 nm. Percentage inhibition was calculated using formula,

Calculation of % inhibition: $\frac{A_t - A_0}{A_t} \times 100\%$

Where, A_0 is absorbance of enzyme substrate reaction with 30% DMSO and A_t is absorbance of enzyme substrate with plant extract.

3.11 Gas Chromatography – Mass Spectroscopy

Composition of methanolic extracts were analysed by GCMS instrument (AccuTOF GCV). The system of GCMS was equipped with FID detector and capillary column of HP-1 (30m*0.2mm; thickness of film was 0.25µm). Helium acted as carrier gas at a flow rate of 1ml/min. The temperature of oven was set at 80-260^{o}C at the rate of 10^{o}C /min and held at this temperature for 3 minutes. Then, it was increased to 280^{o}C at 5^{o}C per minute and held at this temperature for 9 min. The injector and detector temperature were set as 25^{o}C and 280^{o}C respectively.

Methanolic extract sample (0.1µl) was injected into GCMS instrument for its analysis. Ion source temperature were maintained at 200^{o}C and the mass spectra were taken at 70 eV. GCMS was done at National Forensic Science Laboratory.

CHAPTER IV: RESULTS

4.1 Extraction of plant materials (by cold percolation method)

For extraction, the parts of plants were selected according to their ethno medicinal practices. Different parts of plants leaves, stem, fruits, and whole plant were used for extraction. Plant extract percentage yield varied from 5.76 % to 29.78 %. *Terminalia chebula* showed highest percentage yield while *Cheilanthes dalhousidae* had the lowest. Variation was observed with the texture and consistency of obtained extracts. Most of the extracts were solid and semi-solid, some were sticky. Characteristics and yield percentage for the obtained extract are mentioned below. None of the plant extract had aroma.

Table 4.1: Percentage Yield and Physical characteristics of the crude methanolic extract

Plant	Dry Weight (gm)	Wt. of extract (gm)	Percentage yield (%)	Characteristics of extract	
				Color	Consistency
Terminalia chebula	50	14.89	29.78	Dark Brown	Semi-solid
Ampelocissus divaricata	50	3.015	06.03	Reddish Brown	Solid
Terminalia bellerica	50	12.69	25.39	Dark Brown	Semi-solid
Eucalyptus alba	50	5.217	10.43	Brown	Solid
Woodfordia fruticosa	50	6.033	12.06	Light Brown	Solid
Cheilanthes dalhousidae	50	2.88	05.76	Dark Green	Sticky
Centella asiatica	50	9.005	18.01	Dark Green	Semi-solid
Citrus maxima	50	3.578	07.15	Dark Green	Semi-solid
Hedyotis diffusa	50	8.405	16.81	Light Green	Sticky
Albizia lebbeck	50	3.389	06.77	Brown	Solid

4.2 Phytochemical Screening

Preliminary phytochemical screening of the crude methanolic plant extract indicated the presence of different pharmacologically active compounds like phenol, flavonoid, flavonol glycoside, carbohydrates, protein, tannin and cardiac glycoside. Presence of these bio-active secondary metabolites is the reason for having medicinal values. Proteins have been detected in six plants while amino acids were not detected in any of the extract. Cardiac glycosides were present only in *Ampelocissus divaricata, Eucalyptus alba*, and *Woodfordia fruticosa*. Flavonoids were present in all plant extracts except in *Hedyotis diffusa*. Nihydrin test showed negative result in all the plant extracts. Abundance of carbohydrates were present in fruits of *Terminalia bellerica.* Phenols were not present in *Citrus maxima, Hedyotis diffusa, Centella asiatica.* Woodfordia fruticosa and *Ampelocissus divaricata* showed positive result for all of the preliminary tests except in Nihydrin.

Table 4.2: Phytochemical screening of the methanolic extracts of the plants

Tests/ Chemical conpound	Crude Methanolic Extract of the plant materials									
	T.chebula	***A.divaricata***	***T.bellerica***	***E.alba***	***W.fruticosa***	***C.dalhousidae***	***C.asiatica***	***C.maxima***	***H.diffusa***	***A.lebbeck***
Carbohydrate Test										
Molisch's test	-	+	+++	-	+	-	-	-	++	+
Protein & Amino acid										
Ninhydrin	-	-	-	-	-	-	-	-	-	-
Biuret	+	+	+	++	++	-	-	-	-	+
Phenolic Test										
Ferric Chloride	++	+	+	++	+	++	-	-	-	+++
Flavonoid Test										
Alkaline reagent test	+++	++	+++	+++	++	++	+	+	-	+
Schinoda	-	+++	-	++	+++	-	+	-	++	+++
Flavonol Glycoside Test										
Mg&HCl Reduction	-	++	-	++	++	-	-	++	-	+
Tannin Test										
Braemer's	+++	+	+	++	++	+	-	-	-	-
Cardiac Glycoside Test										
Keller-Killani	-	+	-	+	+	-	-	-	-	-

+++: extremely positice ++: moderately positive +: psositive - : negative/absence

4.3 Antimicrobial screening

Agar well diffusion method was performed for antimicrobial screening. Methanol extracts of plants was able to exert inhibitory effect against Gram's positive *Staphylococcus aureus* with

concentration of 50 µg/ml and 25 µg/ml. Similarly, no zone of inhibition with any of the concentrations indicated the plants had no effect against growth of Gram's negative *Pseudomonas aeruginosa.* These plants extracts were not effective against growth of Gram's negative *Pseudomonas aeruginosa* at the concentration less than equal to 50 µg/ml. 18 mm zone of inhibition exerted by the extract of *Terminalia chebula* against *Staphylcoccus aureus* indicated that the plant could be potent source of antimicrobial compounds.

4.3.1 Table: Anti-bacterial activity of the crude methanolic extracts

	Zone of inhibition (mm) for 50 µg/ml extracts	
Plant	***S. aureus* ATCC 25923**	***P. aeruginosa* ATCC 27853**
T. chebula	18	5
A. divaricata	5	5
T. bellerica	11	5
E. alba	7	5
W. fruticosa	5	5
C. dalhousidae	5	5
C. asiatica	5	5
C. maxima	5	5
H. diffusa	5	5
A. lebbeck	5	5
Ofloxacin (30 µg/ml)	27	20
DMSO	5	5

4.3.2 Table: Anti-bacterial activity of the crude methanolic extracts

Plant	Zone of inhibition (mm) for 25 µg/ml extracts	
	***S. aureus* ATCC 25923**	***P. aeruginosa* ATCC 27853**
T. chebula	15	5
A. divaricata	5	5
T. bellerica	10	5
E. alba	9	5
W. fruticose	5	5
C. dalhousidae	5	5
C. asiatica	5	5
C. maxima	5	5
H. diffusa	5	5
A. lebbeck	5	5
Ofloxacin(30 µg/ml)	30	16
DMSO	5	5

4.4 Estimation of Total Phenol Content

In order to calculate total phenolic content of methanolic extracts of plants, calibration curve was generated. Gallic acid was used as standard. The range of concentration for standard was from 10 to 50 µg/ml. The results were expressed in terms of mg GAE/g dry weight. The equation generated for standard was y = 0.0057x + 0.0105 (R^2 = 0.9957). It has been given in Appendix. Highest phenol content 138.86±9.62 mg GAE/g dry weight was found in *Eucalyptus alba*, while *Hedyotis diffusa* showed lowest phenol content that is 15.47±1.57 mg GAE/g dry weight followed by *Citrus maxima* with 19.69±1.51 mg GAE/g dry weight. Sixty percent plant showed phenol content lower than 50 mg GAE/g dry weight. Total phenolic content estimation represented in graph has been given below:

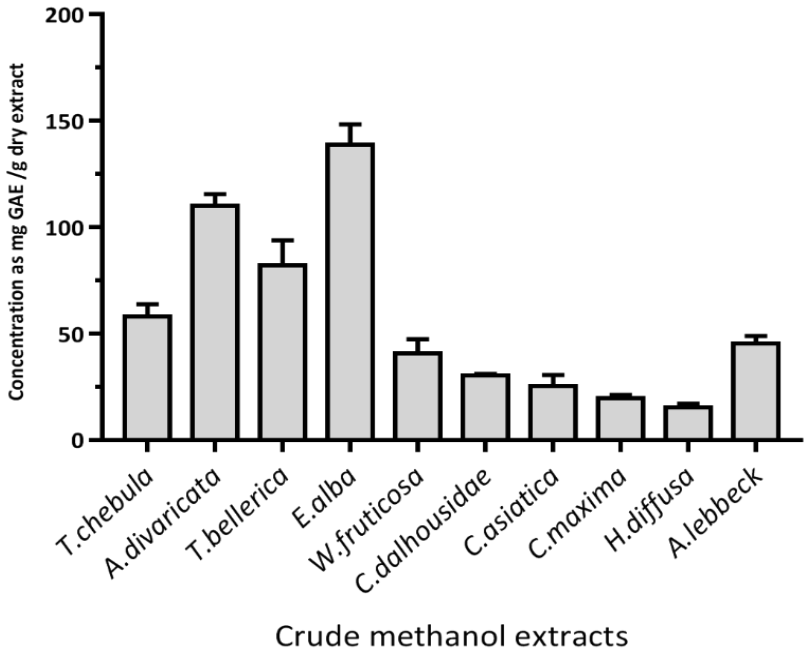

Fig 4.4: Estimation of Total Phenol content of crude methanolic extracts

The error bars represent the standard deviation from the mean value.

4.5 Estimation of Total Flavonoid Content

Total flavonoid content is expressed in terms of Quercetin equivalent (mg QE/gm dry extract). Quercetin was used as standard flavonoid at concentration ranging from 10 to 60 µg/ml to generate standard curve. The generated equation of standard curve is y = 0.0034x - 0.0018 (R^2 = 0.9897). The standard curve has been given in appendix. Flavonoid content of all plants extracts were found to be within the range of 17 to 30 mg QE/gm dry weight except *Cheilanthes dalhousidae* which weighed 50.52±0.86 mg QE/gm dry weight. Flavonoid content was found to be least in *Citrus maxima* 17.78±0.77 mg QE/gm dry weight.

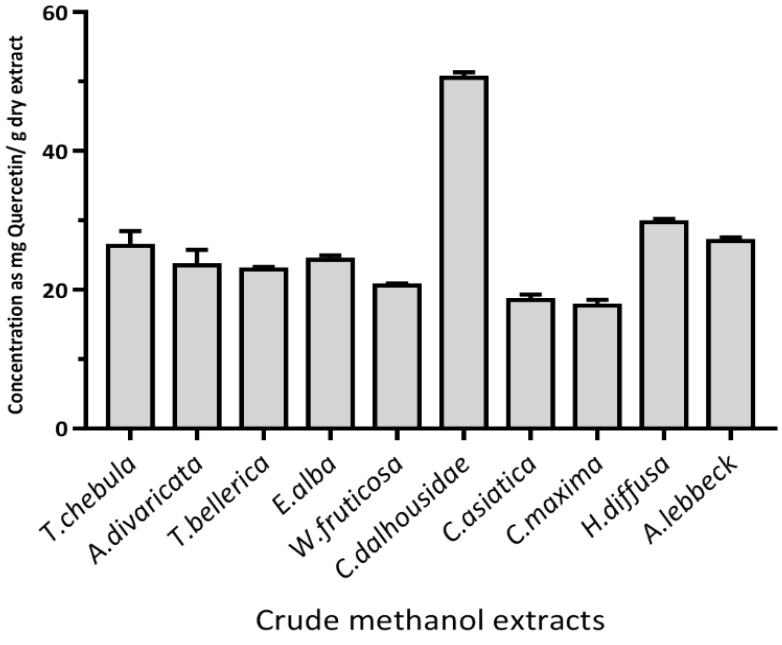

Fig 4.5: Estimation of Total Flavonoid Content of Crude methanolic extracts

The error bars represent the standard deviation from the mean value.

4.6 Estimation of Antioxidant Capacity

4.6.1 DPPH Free Radical Scavenging

To determine antioxidant activity, the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used. Ascorbic acid was used as standard. Test compound was mixed in DPPH solution, absorbance was measured at 517 nm and the percentage of radical scavenging (RSA) was calculated. IC_{50} values for all samples were calculated. IC_{50} value of standard (Ascorbic Acid) was found to be 0.136 mg/ml. Among the samples, *Centella asiatica* showed highest IC_{50} value while the value was lowest for *Ampelocissus divaricata* i.e., 0.146mg/ml. The table showing IC_{50} value of each of the plant extract has been given below along with a graphical representation to compare radical scavenging activity.

Table 4.6.1: IC_{50} values of crude methanolic extracts in DPPH Free Radical Scavenging Assay

S.No.	Plant extract	IC_{50} values (µg/ml)
1	***T. chebula***	19.63±0.89
2	***A. divaricate***	14.66±0.76
3	***T. bellerica***	48.86±1.47
4	***E. alba***	32.83±0.98
5	***W. fruticosa***	28.01±4.29
6	***C. dalhousidae***	53.42±0.56
7	***C. asiatica***	85.78±0.81
8	***C. maxima***	75.80±1.01
9	***H. scandens***	29.87±2.42
10	***A. lebbeck***	40.77±1.54
11	**Ascorbic acid**	13.64±0.66

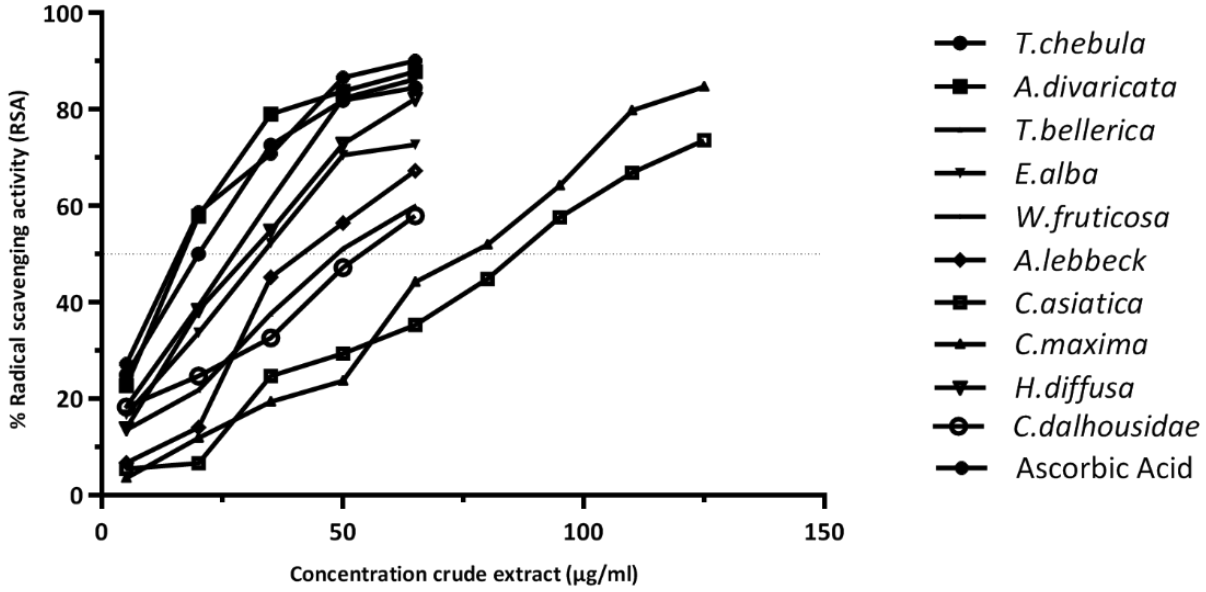

Fig 4.6.1: DPPH Free Radical Scavenging Activity

4.7 Alpha-glucosidase inhibitory activity

Ampelocissus divaricata showed highest inhibitory activity i.e., 89.02±61 % while *Cheilanthes dalhousidae* showed 53.59±2.17 % activity, which lowest among all. Positive control acarbose inhibited upto 80.63±4.57 %. IC_{50} values for *A.divaricata, C.dalhousidae* and Acarbose are 28.38±0.1 µg/ml, 139.13±5.64 µg/ml, and 42.8±4.8 µg/ml. *Terminalia chebula* showed lowest IC_{50} value i.e., 22.8±0.31 µg/ml.

Table 4.7.1: IC_{50} values of crude methanolic extracts for Alpha-glucosidase inhibition

S.No.	Plant extract	IC_{50} values (µg/ml)
1	*T. chebula*	22.8±0.31
2	*A. divaricata*	28.38±0.10
3	*T. bellerica*	39.74±1.57
4	*E. alba*	24.14±1.13
5	*W. fruticosa*	39.30±0.84
6	*C. dalhousidae*	139.13±5.64
7	*C. asiatica*	124.6±1.27
8	*C. maxima*	108.87±0.77
9	*H. scandens*	94.46±3.08
10	*A. lebbeck*	27.82±3.55
11	Acarbose	42.80±4.85

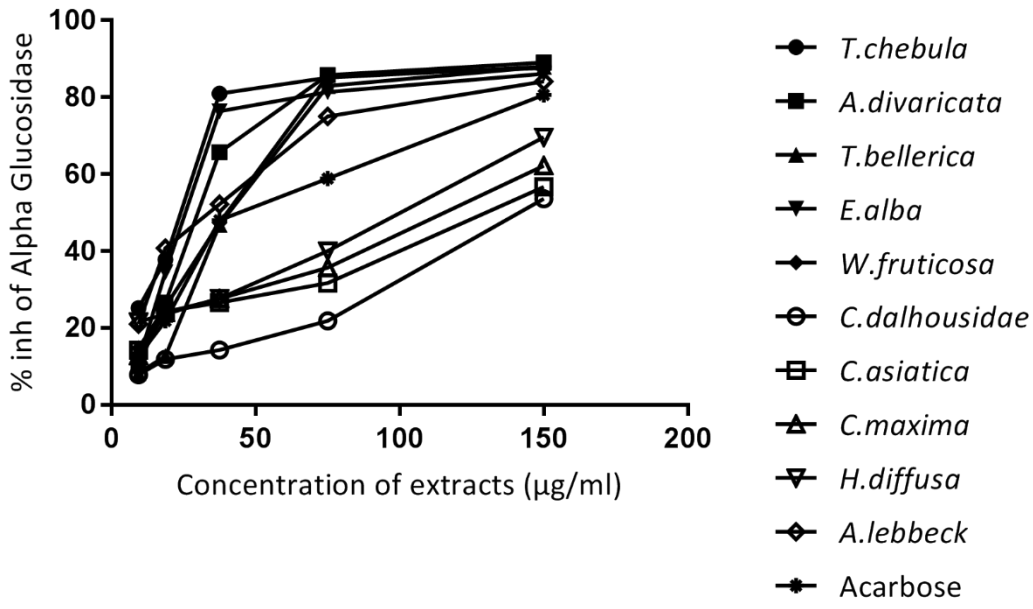

Fig 4.7.1: Alpha-glucosidase inhibitory activity

4.8 15-Lipoxygenase Inhibitory Activity

Ampelocissus divaricata showed highest inhibition i.e., 96.52±1.23 µg/ml while *Citrus maxima* inhibited 55.32±2.44 µg/ml which is least among these plants. *Terminalia bellerica* and *Eucalyptus alba* inhibited 93.51±2.05 µg/ml and 92.19±4.10 µg/ml respectively, which are closer to highest inhibition percentage. *E.alba* showed least IC_{50} value i.e., 27.60±6.59 µg/ml while *Citrus maxima* showed highest i.e., 113.52±2.82 µg/ml. Positive control quercetin had IC_{50} value 37.99±6.09 µg/ml which is close to that of *T.bellerica.*

Table 4.8.1: IC_{50} values of crude methanolic extracts for 15-lipoxygenase inhibition

S.No.	Plant extract	IC_{50} values (µg/ml)
1	***T. chebula***	40.00±5.08
2	***A. divaricata***	83.66±1.79
3	***T. bellerica***	39.96±1.64
4	***E. alba***	27.60±6.59
5	***W. fruticose***	45.37±1.47

6	***C. dalhousidae***	96.86±5.99
7	***C. asiatica***	88.29±6.26
8	***C. maxima***	113.52±2.82
9	***H. scandens***	88.15±8.76
10	***A. lebbeck***	68.09±7.22
11	**Quercetin**	37.99±6.09

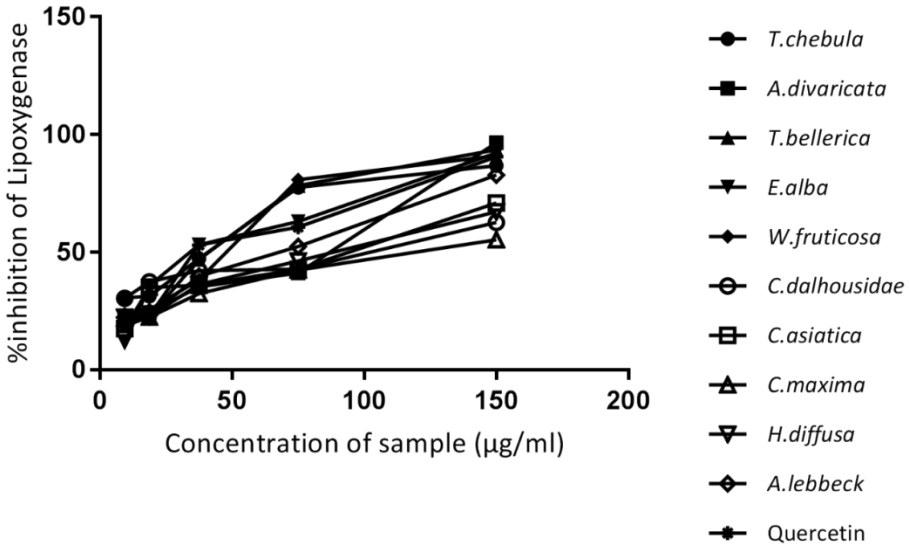

Fig 4.8.1: 15-Lipoxydase inhibitory activity

4.9 Xanthin Oxidase Inhibitory Activity

Xanthin Oxidse tests were performed upto the concentration of 150 µg/ml for all samples which could not give IC_{50} value. For higher concentrations of samples, absorbance of sample reached beyond upper detection limit of spectrophotometer. Even though the test could not be carried out at higher concentration than 150 µg/ml, *Ampelocissus divaricata* showed highest inhibition percentage i.e., 27.15±16.17 µg/ml. *Terminalia chebula* showed least inhibition percentage i.e., 8.10±3 µg/ml. Inhibition percentage of positive control quercetin is 21.62±7.74 µg/ml.

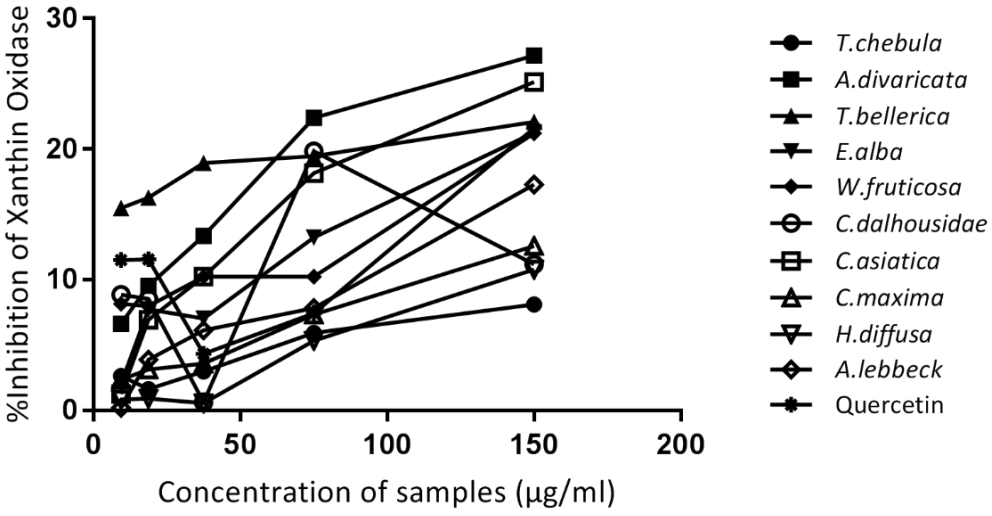

Fig 4.9.1: Xanthin Oxidase inhibitory activity

4.10 Gas Chromatography - Mass Spectroscopy (GC-MS)

The phytochemical compounds were identified and confirmed from data base of National Institute of Science and Technology (NIST). Retention time (RT), molecular formula, molecular weight and peak are percentage are represented in **Table 4.10.1**. Total numbers of compounds identified for *Terminalia chebula, Ampelocissus divaricata* and *Terminalia bellerica* rescpetively 10, 14, 10 compounds respectively.

Table 4.10.1: GC-MS Analysis of Methanolic Extracts of Plants

Peak No.	R.Time (RT)	Name of Compound	Formula	Mol. Wt.	Peak area %
Terminalia chebula					
1.	3.053	Furfural	$C_5H_4O_2$	96	0.84
2.	4.097	Phenol	C_6H_6O	94	0.84
3.	5.122	2,3-Dimethylfumaric acid	$C_6H_8O_4$	144	0.61

4.	5.453	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	0.67
5.	5.817	1,2-Benzenediol	$C_6H_6O_2$	110	0.69
6.	6.162	2-Furancarboxaldehyde,5 (hydroxymethyl)-	$C_6H_6O_3$	126	2.73
7.	7.348	1,2,3-Benzenetriol	$C_6H_6O_3$	126	90.97
8.	7.644	Phosphoric acid, bis(trimethylsilyl)monomethyl ester	$C_7H_{21}O_4PSi_2$	256	0.64
9.	8.042	D-Allose	$C_6H_{12}O_6$	180	0.95
10.	9.666	Butanoic acid, octyl ester	$C_{12}H_{24}O_2$	200	1.07
					Total= 100%
Ampelocissus divaricate					
1.	3.145	2-Propanone, 1,3-dihydroxy-	$C_3H_6O_3$	90	1.97
2.	4.117	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	144	1.52
3.	4.871	Cyclopentane, 1-acetyl-1 ,2-epoxy-	$C_7H_{10}O_2$	126	2.09
4.	5.452	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	8.30
5.	6.060	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	126	3.46
6.	6.167	1,2,3-Propanetriol, 1-acetate	$C_5H_{10}O_4$	134	1.70
7.	6.503	Heptanoic acid, 6-oxo-	$C_7H_{12}O_3$	144	0.85
8.	7.310	1,2,3-Benzenetriol	$C_6H_6O_3$	126	64.72
9.	7.912	Sucrose	$C_{12}H_{22}O_{11}$	342	9.46
10.	8.381	D-Allose	$C_6H_{12}O_6$	180	2.67
11.	9.392	2,2,4-Trimethyl-3-hydroxy-n-valeronitrile	$C_8H_{15}NO$	141	0.35
12.	13.451	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	2.14
13.	19.5	Pentadecanal-	$C_{15}H_{30}O$	226	0.28
14.	21.906	6-Methyl-11-propenyl-5-(toluene-4-sulfonyloxy)-12,13-	$C_{24}H_{32}O_7S$	464	0.49

		dioxatricyclo[7.3.1.0(1,6)]tridecane-8-carboxylic ac id, methyl ester			
					Total= 100
Terminalia bellerica					
1.	4.1	Phenol	C_6H_6O	94	1.99
2.	4.162	2-Ethylacrolein	C_5H_8O	84	1.55
3.	4.505	7-Oxabicyclo(2.2.1)hept-5-ene-2-one	$C_6H_6O_2$	110	1.11
4.	6.087	2-Furancarboxaldehyde	$C_6H_6O_3$	126	1.60
5.	7.305	1,2,3-Benzenetriol	$C_6H_6O_3$	126	75.66
6.	7.640	Phosphoric acid, bis(trimethlysilyl)monomethyl ester	$C_7H_{21}O_4PSi_2$	256	1.80
7.	8.359	D-Allose	$C_6H_{12}O_6$	180	3.95
8.	13.787	Rhodium, acetylacetonato-bis((E)-cycloocetene)	$C_{12}H_{35}O_2Rh$	422	1.20
9.	20.050	Stigmasterol	$C_{29}H_{48}O$	412	2.68
10.	21.619	.Beta.-Sitosterol	$C_{29}H_{50}O$	412	8.45
					Total= 100%

4.11 Correlation of Total Phenolic Content and DPPH Radical Scavenging Activity

Correlation between Total Phenolic Content (TPC) and DPPH Radical Scavenging Activity (%RSA) was calculated using MS-Excel. The calculated value of correlation coefficient was 0.376. This was not satisfactory and considered to be weak correlation.

4.12 Statistical Analysis

One way Analysis of Variance (ANOVA) test among the mean total phenolic content values did not showed significant difference in mean values ($P>0.05$). Similar was the result for total flavonoids content ($P>0.05$).

CHAPTER V: DISCUSSION

Plants are used to cure ailment in many of indigenous medicine system. Plants are being more popular in modern societies as alternative source of modern medicine. Herbal medicines are also known as phyto-medicine or botanical medicine. Such medicines are accessible, available and culturally more acceptable because people believe these medicines have less side effects than synthetic drugs (Dey & De, 2015; Carlson, 2002). Most productive source of drug development is natural products (Harvey, 2008).

Nepal is located between Tibet and India. Himalayan region is at northern part of the country. In rural parts of Nepal, modern and prepacked Ayurvedic medicines of India are not available. So, people use traditional medicines. (Taylor et. al., 1995; Shrestha and Joshi, 1993; Bhatterai, 1993; Manandhar, 1985, 1986, 1987, 1989a,b,c, 1990a,b).

Drugs derived from natural products have gained attention. Such drugs have low production cost and diverse structure. Active compounds from natural product are effective against number of diseases. Traditionally, generations have used medicinal plants. These plants can cure symptoms of different diseases which directs attention towards plants having medicinal values (Alvin et. al., 2014). Novel compound can to treat increased number of diseases. Microbes develop drug-resistance rapidly. Number of new cases of infections, life threats and constant recurring number of diseases have challenged the area of drug discovery to improve. (Alvin et. al. 2014; Strobel et al., 2004; Demain, 2000).

Fractionation technique has improved. It has simplified analytical (Harvey, 2007). Two weeks are enough to isolate bioactive compound from fermentation broth (Singh and Barret, 2006). 1 milligram of sample is needed to solve complex structure by NMR technique (Quinn et. al., 2008).

Folin-Ciocalteu Reagent have redox regent. It forms blue colored complex on reaction with phenolic of plant extracts (Kamboj et. al., 2015; Schofield, 2001). F-C reagent degrades quickly in alkaline solutions. Excess reagent is required for a complete reaction (Kamboj et. al., 2015; Folin and Ciocıteu, 1927).

Total phenolic estimation was performed by Folin-Ciocalteu method, few extracts showed good amount of phenolic content. In this experiment, higher reading of absorbance at 765nm showed higher phenolic content in the tested extract of plant. In this research two plant out of ten showed total phenolic content more than 100 mg GAE program extracts which is fair and shows that amount of the plants have low phenolic content.

Polyphenol inhibits growth of microbes (Cowan, 1999) acts as active antioxidant, chelate metals and donate hydrogen atom (Tsao and Deng, 2004).

Technique applied for estimation of total flavonoid content is based on spectrophotometric determination of complex of flavonoid-Aluminium chloride (Farnandes et. al., 2012; Mabry et. al., 1970). The technique was developed for analysis of herb containing O-glycoside (Petry and Ortega, 2001). Flavonols and flavonoids chelate aluminium atom of aluminium chloride resulting formation of deep-yellow colored complexes (Chang et al., 2002).

In this research, total flavonoid content was found to be less than total phenolic content. *Cheilanthes dalhousidae* showed highest flavonoid content that is 50.52 gm Quercetin/dry extract while *Citrus maxima* showed lowest phenolic content i.e, 17.78 gm Quercetin/gm extract. 70% of plants showed flavonoid content within range of 20-30 gm Quercetin/ gm extract.

DPPH is a free radical and stable at room temperature. It gets reduced in presence of any antioxidant molecule and turns ethanol solution colorless. DPPH is a rapid method to quantify antioxidant using spectrophotometer (Huang, 2005). In this experiment, lowest IC_{50} value was shown by *Ampelocissus divaricata* that is 14.66µg/ml very close to IC_{50} value (13.64µg/ml) of standard ascorbic acid. IC_{50} value of three plants were found to be more than 50 µg/ml.

The probable reason for antimicrobial activity shown by plant extracts may be due to presence of bioactive compounds like polyphenol, alkaloid, terpenoid, flavonoid and tannins present in them (Cowan, 1999; Gonzalez-Lamothe, et al., 2009). Bioactive compounds present in plant extract sample act directly or indirectly against pathogenic bacteria. Compound in plant extract show multiple effects to inhibit bacterial growth like denaturation of extracellular and intracellular proteins, deactivates toxins, and transport protein disruptions (Cowan, 1999).

Menthol, a terpenoid was found to be able of eliminating resistant plasmids (Schez et al., 2006). For this research, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected because these two bacteria exhibit most forms of resistance. *Terminalia chebula* and *Terminalia bellerica* were found to be active against *Staphylococcus aureus* while none of the plant extract were found to be active against *Pseudomonas aeruginosa.* Therefore, *T. chebula* and *T.bellerica* might lead to discovery of drug against *Staphylococcus aureus.* However, none of the plant extract could inhibit growth of *P.aeruginosa*. Multiple drugs resistant strain of *P.aeruginosa* was used for the research.

Alpha glucosidase is an extra cellular enzyme. It is distributed widely in plants, animal tissues, and microbes (Kumar, et. al., 2011; Kimura et. al., 2004). It breaks complex carbohydrates and release alpha glucosidase from non-reducing end. Inhibition of this enzyme slows rise of glucose level in blood after carbohydrate meal (Kumar, et. al., 2011; Lebovitz, 1997). Alpha glucosidase inhibition slows process of hydrolytic cleavage of complex carbohydrates. Then, the digestion process of carbohydrate spreads to lower part of small intestine. Such digestion process slows overall glucose absorption rate into the blood. So, alpha glucosidase inhibition is considered as best method to decrease post prandial increase in blood glucose level (Baig, 2002). In this research, least IC_{50} value was found to be of *Terminalia chebula* that is 22.80 µg/ml. It could be a possible source of antidiabetic drug. However, IC_{50} values of *Ampelocissus divaricata, Eucalyptus alba, and Albizia lebbeck* were also found to be below 30 µg/ml. These plants can also be considered for potent source of antidiabetic properties.

15-Lipoxygenase play important role in promoting cancer by amplification of PPARγ transcription activity (Sadeghian and Jabbian, 2015). Prostate cancer cell lines human prostate tumors express 15-LOX-1. They produce 15-LOX-1 metabolite 13HODE (Sadeghian and Jabbian, 2015; Kelavkar et al., 2000; Spindler et al., 1997). In human prostate cancer expression of 15-LOX-1 mRNA is significantly higher in human prostate cancer tissue as compared to normal prostate tissue (Kelavkar and Landsittel, 2006). In this research, *Eucalyptus alba* showed lowest IC50 value for 15 lipoxygenase inhibition that is 27.60 µg/ml followed by *Terminalia bellerica* 39.96 µg/ml and *Terminalia chebula* 40.00 µg/ml. These plant could potent sources of anticancer drugs.

Xanthin Oxidase enzymes catalyse hydroxylation of Xanthine and converts xanthine to uric acid. This uric acid is excreted by kidneys. Due to excess production and/or inadequate excreation of uric acid hyperuricemia is caused (Kostic et al., 2015). Xanthine Oxidase can blocks synthesis of uric acid from purine in human body (Kostic et al., 2015; Unno et. al., 2004). In this experiment, inhibition was less than 30%in all plant extract sample. This is because, absorbance at concentration higher than 150µg/ml could not be detected by spectrophotometer or reading lies above detective range.

GC-MS is a technique to determine phyto-constituent present in plant extract. Less than one milligram sample is required to identify compounds in the sample (Theng and Korpenwar, 2015; Sahu and Saxena, 2013). In this research, GC-MS was performed for *T.chebula, T.bellerica*, and *Ampelocissus divaricta*. Till the date there is no research article published on phytochemical analysis, enzyme inhibition and GCMS analysis on *Ampelocissus divaricata.*

CHAPTER VI: SUMMARY

Many new discoveries have been made regarding all of the plants. As for *Ampelocissus divaricata* , there are no published article available. None of the plant extract could inhibit multiple drug resistant strain of *Pseudomonas aeruginosa. Terminalia chebula, T.bellerica* and *Eucalyptus alba* showed good inhibition against *Staphylococcus aureus*. Among these three plants, *T.chebula* showed good inhibition against *Staphylococcus aureus*. This research indicated that the fruit extract of both species of *Terminalia* had better antibacterial activity against Staphylococcus aureus as compared to that bark extract of *Euclaypts alba.*

The high phenol content of bark extract of *Eucalyptus alba* and low phenol content of rest of the plant were in agreement with earlier studies. But in this research, radical scavenging activity of all the plants were found to be good. This varying results of phenolic content indicates polyphenols not only compound responsible for antioxidant property. Poor correlation between phenolic content and antioxidant activity indicates plant having higher antioxidant property be due to phytochemicals have antioxidant property beyond phenols. Method of total phenol determination was not specific for polyphenols. It can't be confirmed that total polyphenol content implies polyphenols only. Flavonoid content was lower than previous researches.

For enzymatic assays, plants like *T.chebula, A.divaricata, E.alba, T.bellerica, W.fruticosa, A.lebbeck* showed lower IC_{50} value which indicates antidiabetic properties. Similarly, for 15-lipoxygenase assay, *T.chebula, E.alba, T.bellerica,* and *W.fruticosa* showed less than 50 µg/ml which is considered to be good. For xanthine oxidase assay, IC_{50} value could not be found in the range upto 150 µg/ml. Absorbance above the concentration 150 µg/ml could not be detected by spectrophotometer. Such a result was found to be similar to previous research.

Different compounds have been identified by GCMS techniques. Compounds having benzene rings have also been identified from extracts of *T.chebula* and *T.bellerica*. Compounds have been identified for *A.divaricata* but there is no any research articles till the date. So, results of *A.divaricata* remained uncompared.

CHAPTER VII: CONCLUSION

Medicinal plants have been in use since ancient times. Vaidhyas and Amchis use the knowledge of medicinal plants for the treatment of various ailments. But the knowledge of medicinal plants is not found to be documented anywhere. Vaidhyas and Amchis exchange their knowledge of medicinal plants to the people of their community. There no such document regarding collection and use of medicinal plant. They acquire knowledge orally and pass it to next generation similarly. So it is required to maintain and preserve written form of document regarding identification, collection and uses of medicinal plants.

Many of the synthetic drugs in modern medicine system are derived from natural products. Use of herbal remedy has also increased in all over the world. Demand of these medicinal plants has increased even in developed world. These days, medicinal plants are synonymous to fast cash. Nepal people are not aware of medicinal uses of plants. They have been selling such plants to local collector for livelihood. Haphazard way of medicinal plant collection may lead to their extinction in near future. It has been found that most of the plant has good antioxidant activities. Few plants showed anti-diabetic and anti-cancerous properties. It can be concluded that these plant can be used in several chronic disorders like atherosclerosis and diabetes. This research is preliminary study and shows pharmacological values of these plants with scientific evidence.

Further recommendation is to test on animal model, evaluation of safety via sensitive tests. These plants could be lead novel drug discovery for number of diseases.

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Appendices

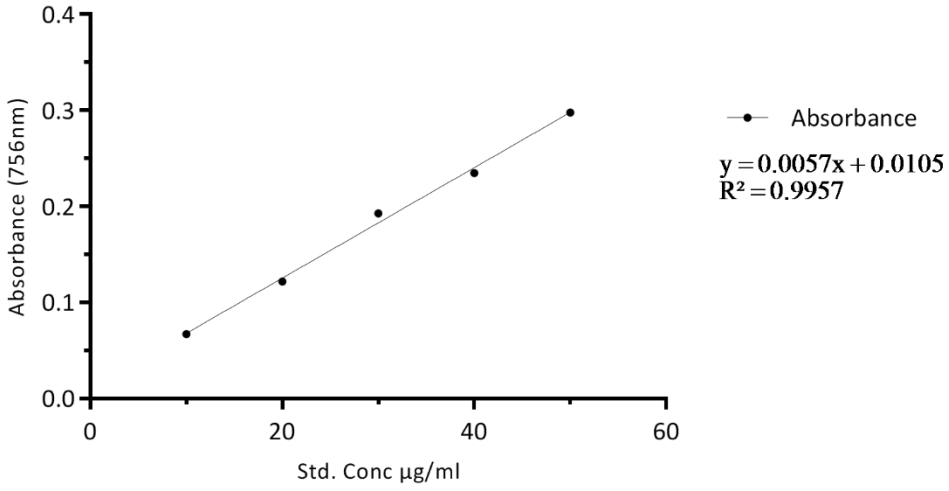


Fig: Standard Curve of Standard Gallic Acid for phenol estimation

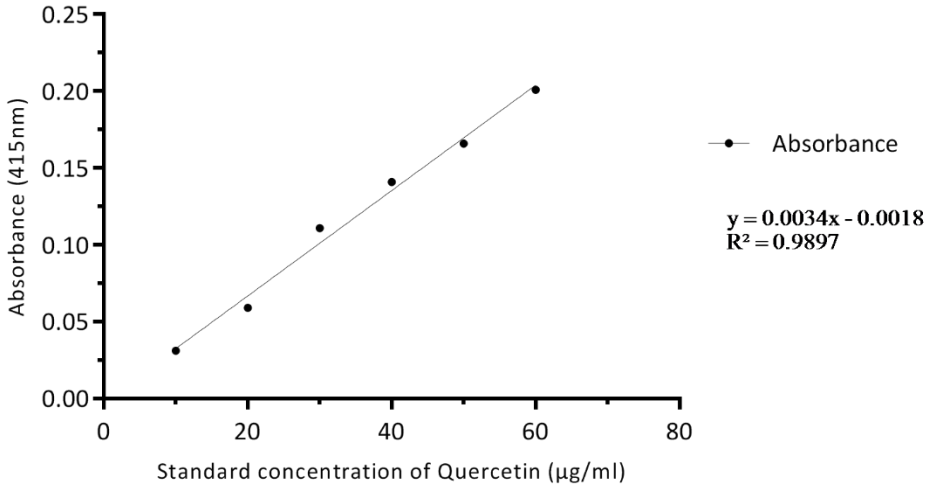
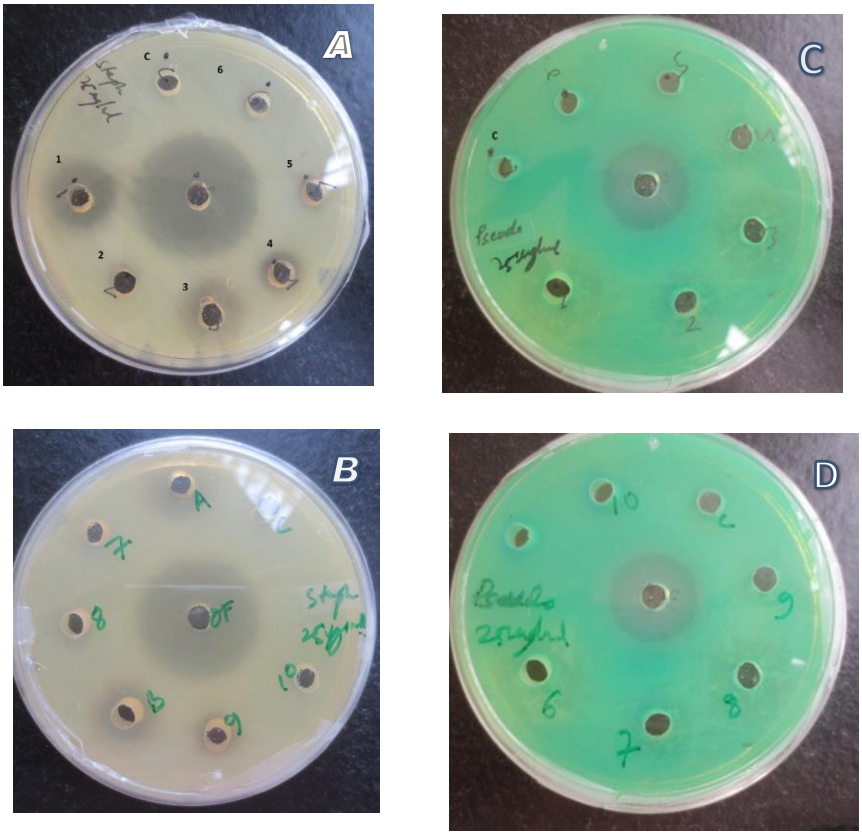


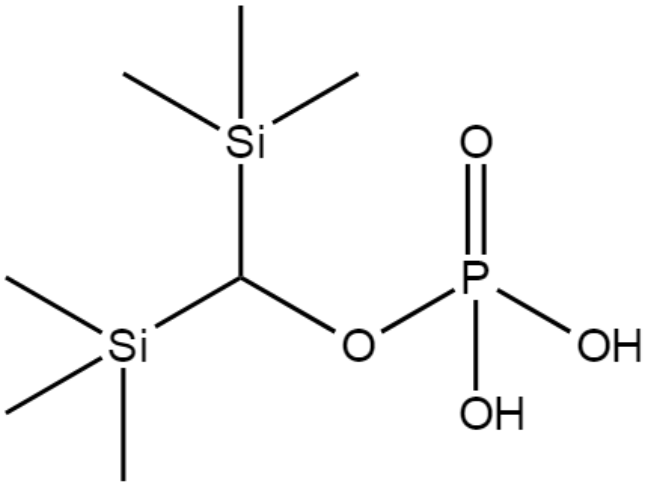
Fig: Standard Curve of Quercetin for flavonoid estimation



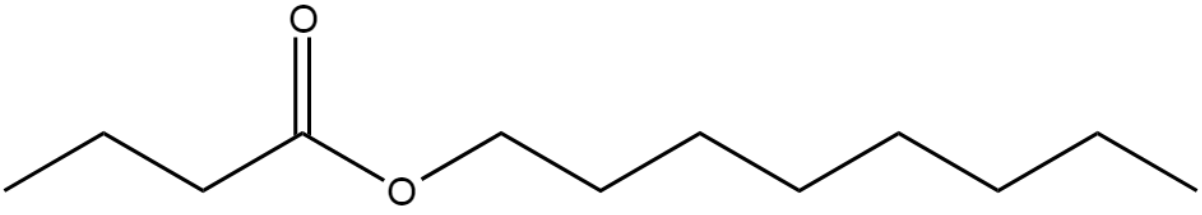
1 *T.chebula*
2 *A.divaricata*

3 *T.bellerica*
4 *E.alba*
5 *W.fruticosa*
6 *C.dalhousidae*
7 *C.asiatica*
8 *C.maxima*
9 *H.diffusa*
10 *A.lebbeck*
C control DMSO
OF Ofloxacin
Organism (pic. A&B): *Staphylococcus aureus* ATCC 25923
Organism (pic. C&D): *Pseudomonas aeruginosa* ATCC 27853

Fig 4.3.1: Anti-bacterial Tests by Disc Diffusion method



Phosphoric acid, bis(trimethylsilyl)monomethyl ester



Butanoic acid, octyl ester

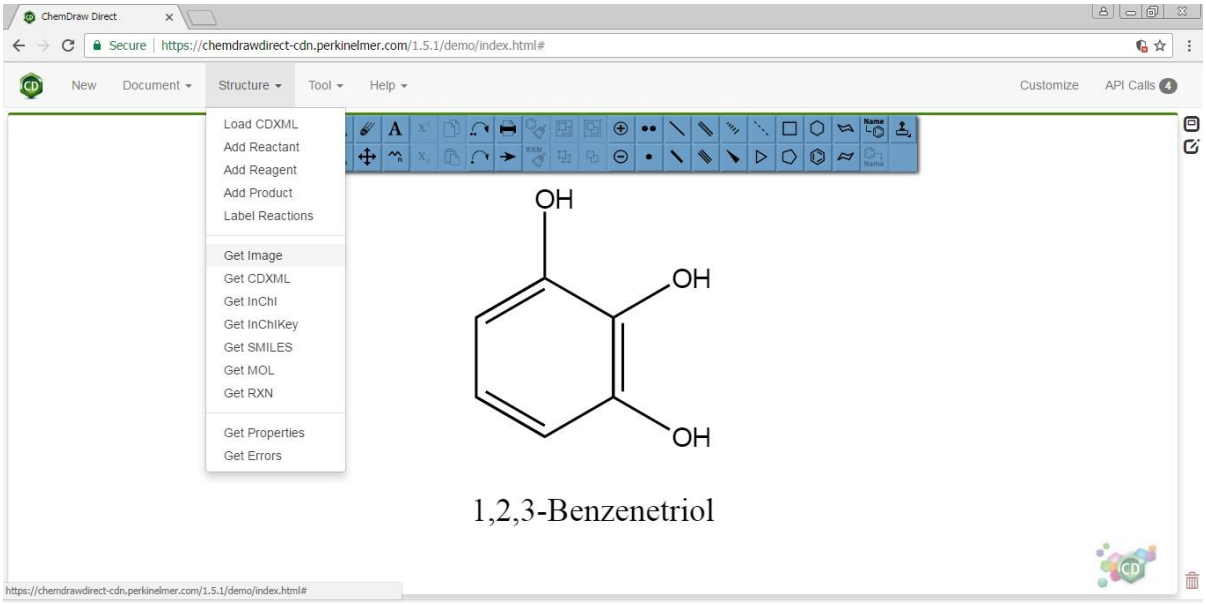


Fig: Structures of Compounds drawn on chemdrawdirect-cdn.perkinelmer.com

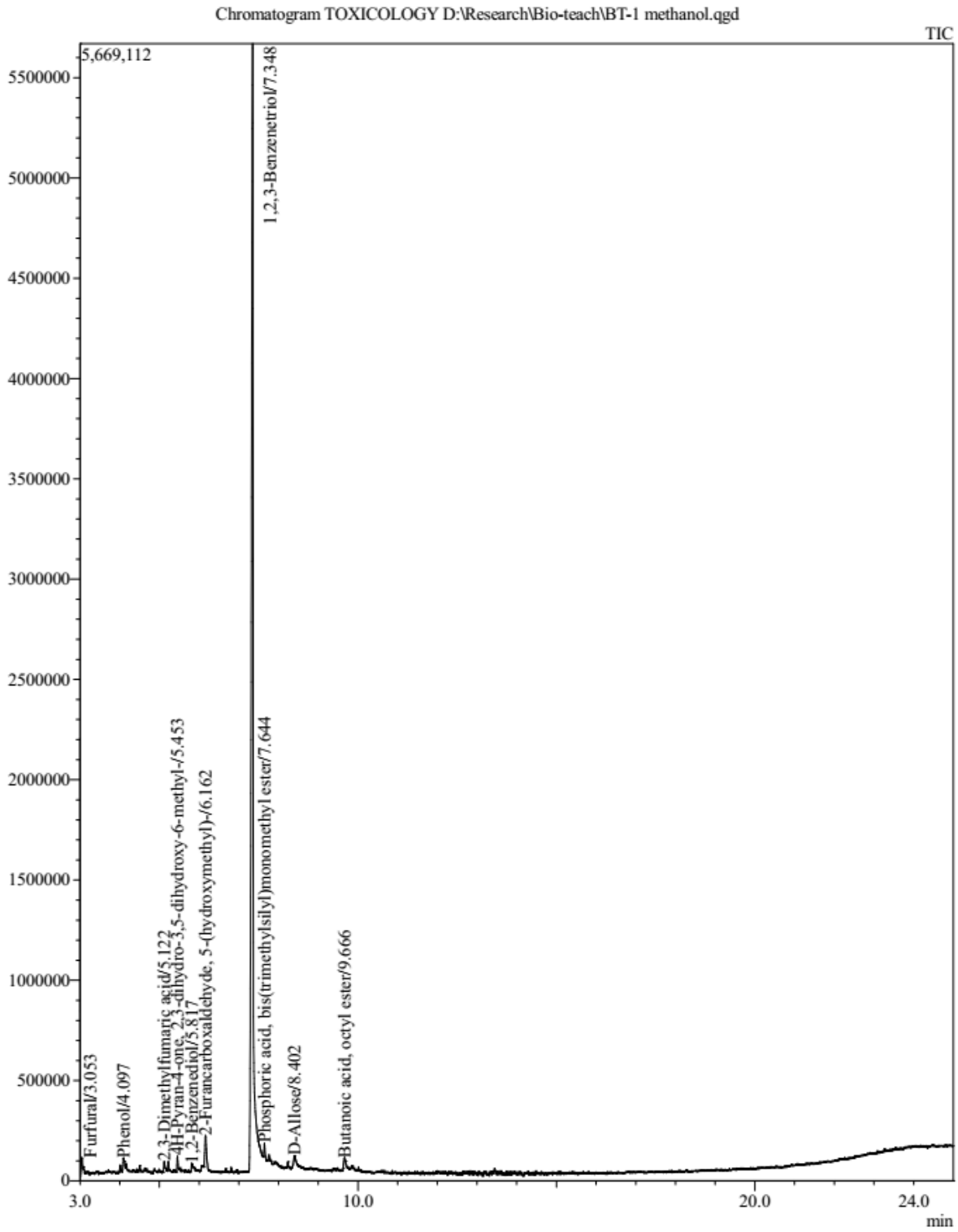


Fig: *Terminalia chebula* methanolic extract GCMS Analysis

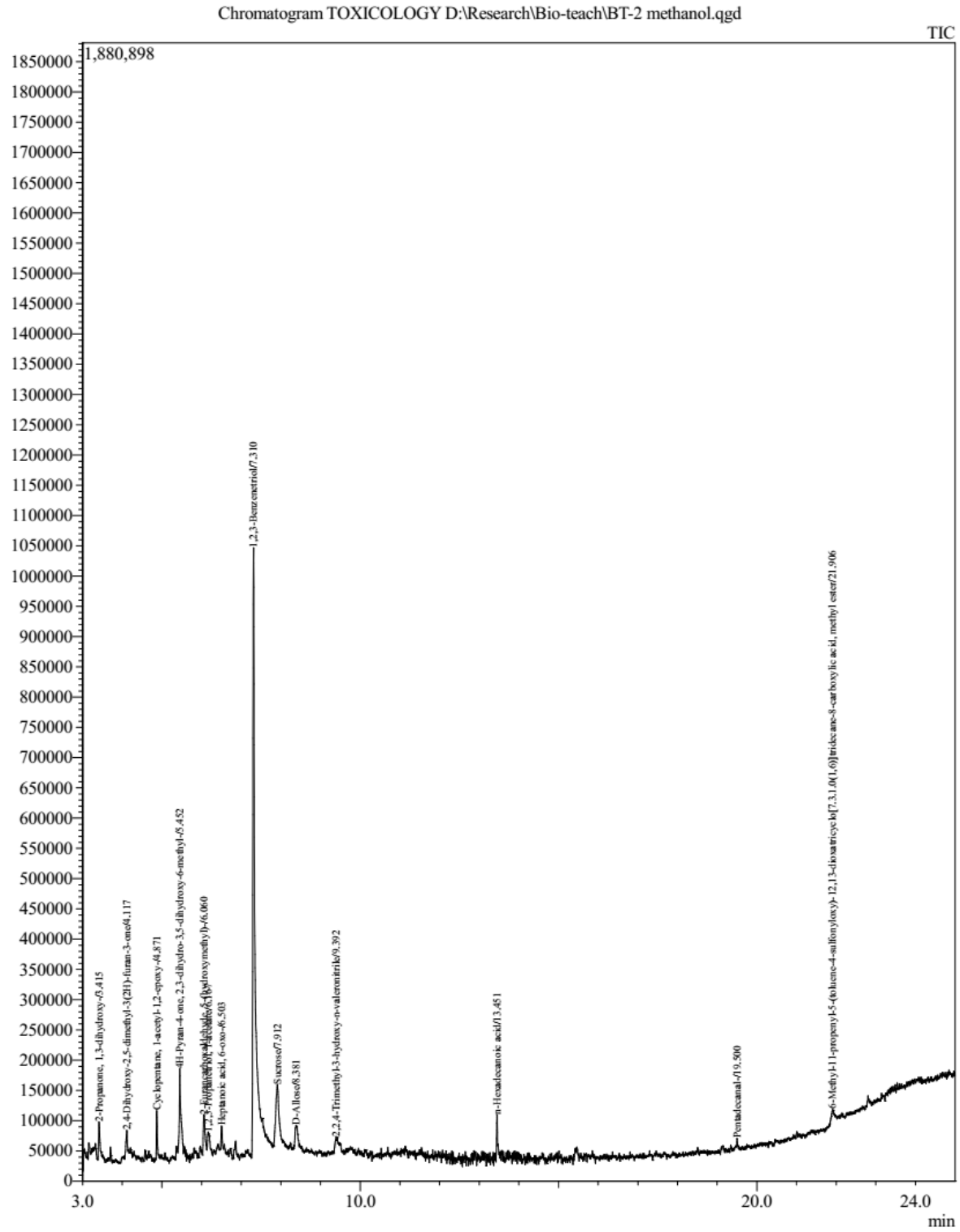


Fig: *Ampelocissus divaricata* methanolic extract GCMS analysis

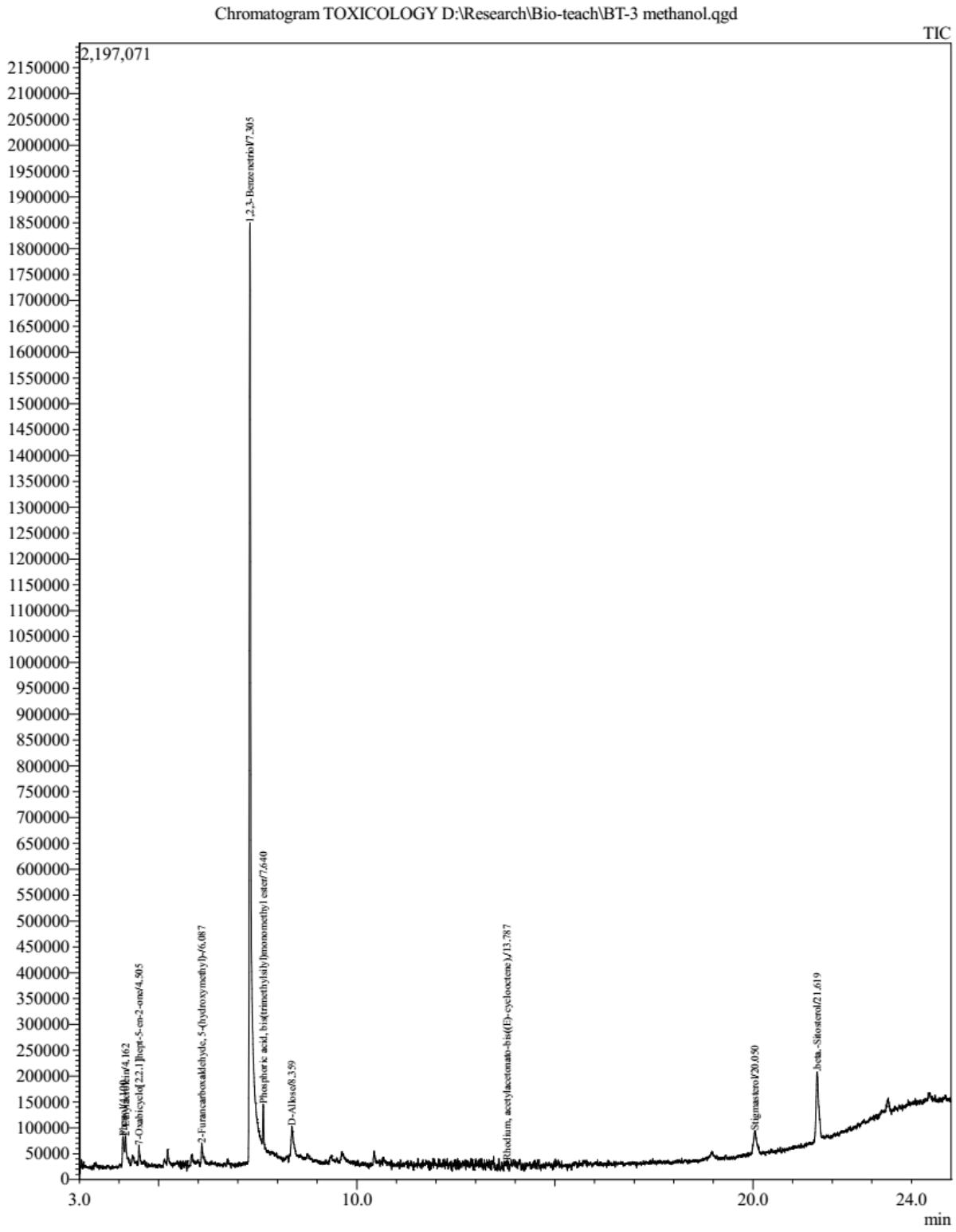


Fig: *Terminalia bellerica* methanolic extract GCMS analysis