ANTIOXIDANT, ANTIMICROBIAL, CELL CYTOXICITY ASSAY AND PHYTOCHEMICAL SCREENING OF SOME MEDCINAL PLANTS

M.Sc. Thesis

(2017)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuwan University

Kirtipur, Kathmandu, Nepal

In the partial fulfillment for the Degree of Masters of Science in Biotechnology

Dipesh Thapa

Exam Roll no: BT 204/071

T.U. Registration no: 5-3-28-107-2014

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List of abbreviations

µl	Microliter
µg	Microgram
ATCC	American Type Culture Collection
DMSO	Dimmethyl Sulfoxide
DPPH	1, 1- diphenyl-2-picrylhydrazyl
ELISA	Enzyme Linked Immuno sorbent Assay
FRAP	Ferric Reducing antioxidant power
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectroscopy
IC50	Inhibitory Concentration 50
MTT	3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
OD	Optical Density
QE	Quercetin Equivalent
RPMI	Rosell's Park Memorial Institute
RSA	Radical Scavenging Activity
SD	Standard Deviation
Spp	Species
WHO	World Health Organisation

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ABSTRACT

The plant parts are used from very prehistoric period of time. All living beings are completely dependent on the plant as food to cure different types of problem they faced. Similarly *Rosa spp*. that are always around us used as decorative, fencing, aromatic, ornamental to sign of love because of its importance on the physiological and physical effects on the human and organisms. Methanol extracts of the compound was used because large number of plant are screened under the methanol so that, methanol as the solvent of the plant extraction. *Tinospora cordifolia* was also used for observing the comparative study as it has been widely used for medicinal effects. Maximum effects was observed in the *Rosa spp* (purple) was hybrid plant that has 217.19 ± 4.49. Similarly, flavonoids contents in them *Rosa brunonii* has 161.75± 14.22.Other spp. also have similar types of readings. So that the study of radical scavenging activity was carried out where the IC50 value was calculated in the mean weight and SD so that all plant extracts has similar range value was obtained which shows the similar type of the radical scavenging activity by all the plant extracts. *Rosa spp* (white) has effects of 60.95 ± 3.42 for *Tinospora cordifolia* has 52.54 ± 0.73. Antimicrobial effects are also shown by the all plant extract in 50 to 100µl/gm which was observed in zone of inhibition by paper disc methods using both positive and negative control. The VERO cells with passage no 28 was used for observing cell cytoxicity effect that was also done by MTT assay and reading was taken spectrophotometrically so that the effect was seen by calculating % inhibition which gives the value of IC50 value that helps to find the inhibiting value for which *Tinospora cordifolia* has 128.47 ± 44.42 for Rosa spp (white) 110.76 ± 21.54.Those species required high concentration of extracts compare to *Rosa spp* and *Rosa brunoii.*GC-MS analysis was carried out to know the types of chemical compounds that are present in plant extracts. Those compounds are responsible for the various effects.

The results shows the importance of ethanomedicinal plants that are responsible for curing various disease. The bioactive compounds are responsible for the therapeutic properties. Different types of analysis and characterisation of each compounds can leads to upgrade the major findings.

Key words: Rosa species, Tinospora cordifolia, antioxidant, antimicrobial, cell cytotoxicity,GC-MS, phenol, flavonoid.

1. INTRODUCTION

Medicinal plants hold broad spectrum of the nature and primarily they have been used for the cure of various diseases from the time immemorial by our forefathers knowingly or unknowingly. It has been a boon for them as medical development at that time was too far. Medicinal plants constitute of various biochemical compounds which are used to cure the wounds from any injury or the diseases. The plant kingdom has vast depth of hidden importance which can turn the miracles on the medicinal science as our planet is gifted with billions of species among them kingdom plantae was important for the bioactive compound with the effective effect on pathogens and tumors so that it was very important for researchers for the discovery of the hidden importance for the responsibility of mankind (Kunwar et al., 2008).

Since time immemorial, people have gathered plant resources for their needs .Examples include edible nuts, mushrooms, fruits, herbs, etc. Even today, hundreds of millions of people, mostly in developing countries, derive a significant part of their subsistence income from gathered plants (Malla et al., 2008).

Nepal is a country that lies between India and China with different geographical variation and climatic conditions. It is a small country with tremendous geographic diversity that ranges from 59 meter in elevation in the tropical Terai to over 8000 meter in the Himalayan region (Hack and John, 1960) with nearly a fourth of it covered with forest (FAO, 2014). The variation of plant from different elevation zone arises due to different types of climatic zones, so Nepal is a rich country in plant diversity and as a result, various types of plant are used for medicinal purposes.

It was recorded that over 6600 species of flowering plants and about 530 ferns are found in Nepal (Poudel et al., 2011). Nepal has always been the center of herbal plants. Medicinal herbs database listing for Nepal shows 1624 species of medical and aromatic species. These herbs have been an integral part of traditional medicine practices of indigenous committee in Nepal (Bhattarai et al., 2011).

Nepal is the country blessed with diversity, so different species of plant are available in the basis of their medicinal values also. Since thousands of years, they are used as medicinal plants to cure different diseases (Dhakal et al., 2011). Though the development of modern medical science has advanced still the use of medicinal plant has been practiced which has been effective (Kumar, 1998). They contain thousands of low molecular weight bio-compounds, also termed as secondary metabolites which play important role in plant defense mechanism (Prasad et al., 2011). Medicines based on plant are dispensed earlier in the form of crude drugs such as tinctures, teas, powders and other herbal formulations which now serve as the basis of novel drug discovery (Watanabe et al., 2005).

WHO describes medicinal plant as any plant which is one or more of its organs, contains substances that can be used for therapeutic purposes or which are used for chemo para-pharmaceutical semi-synthesis (Pichersky et al., 2005). Due to low toxicity of medicinal plants and increasing inefficacy of many modern drugs resistance possessed by several bacteria to various antibiotics, medicinal plants are increasingly gaining acceptance even in urban areas (Joy et al., 1998). Traditional medicine of Nepal is handed from generation to generation by oral method rather than through documentation. Recently there are 33 Nepalese ayurvedic and Unani medicine manufacturing industry in operation (DDA, 2005). From this report, we can observe that the interest of people regarding Ayurvedic and contemporary medicine is growing and lots of Nepalese are accepting.

Rosaceae the rose family, is a medium-sized family of flowering plants, including 4,828 known species in 91 genera. The name is derived from the type genus Rosa. Among the most species-rich genera are Alchemilla, Sorbus, Crataegus, Cotoneaster, Rubus and Prunus (plums, cherries, peaches, apricots, and almonds) with about 200 species. However, all of these numbers should be seen as estimates much taxonomic work remains (Sharafi, 2011).

The family Rosaceae includes herbs, shrubs, and trees. Most species are deciduous, but some are evergreen. They have a worldwide range, but are most diverse in the Northern Hemisphere.

A large number of species in Rosaceae or rose family, have a medicinal value. Most of these are trees or shrubs with variable characteristics. This family is popular for its edible and juice fruit shrubs and trees. Some examples of this family include bramble, rose (Rosa gallica),

wood strawberry, quince, round pear, loquat, hawthorn, peach, almond and apricot Khan and Khaton, 2007).

The wild rose (Rosa gallica) and related roses are thorny shrubs with flowers that vary in color according to the species or variety. The wild rose has deep pink flowers are in bloom between April and June, and bright red fruit. It is mainly distributed in the Mediterranean, Central Europe and West Asia. (Mahmood et al., 2011).

Different species of Rosa are also reported to have various medicinal uses are Rosa Damascena," a review of the literature on the effectiveness of rose medicine which referenced nearly 100 studies. Anti-microbial, anti-infective, anti-diabetic, anti-HIV, antioxidant, and anti-inflammatory actions have all been verified using science. One study cited even claims rose to have possible anti-aging properties. In the study male and female flies whose diet was supplemented with rose extract had statistically significant decreased mortality, and longer lives. It is thought that perhaps the anti-oxidant properties of rose are also responsible for the longevity benefits (Valiollah et al., 2010).

Rose petals are used to prepare rose oil that is steam distilled. The byproduct of steam distillation is rose water, which is very good relaxing agent, pacify the nerves and adds flavor to a variety of dishes across the world. Rose essence is rich in flavanoids, tannins, antioxidants, and vitamins A, B3, C, D and E, making it beneficial in skin care (Yi-Zhong et al., 2005).

There are various uses of rose. Rose water is a good agent that reduces swelling of capillaries beneath the skin. Rose petal tea is very good in clearing gall bladder and liver, and it helps improve bile secretion. Rose petals are dried and crushed to make tea. Rose tea also reduce mild sore throats and bronchial infections. The tea cools the body and reduces fever-related rashes. Rose petals are an important ingredient in eye washes as well, as it is antiseptic in nature. Rose water beneficial for nourishing the scalp and improving hair growth. It is medicinally used as an antibacterial, antiseptic, and anti-inflammatory product. Rose essential oil is used along with carrier oils such as almond or grape fruit to treat various illnesses like hemorrhage, liver problems, nausea, fatigue, ulcers, asthma, dehydration, and bacterial infections of the stomach, colon, and urinary tract (Mohammad et al., 2011).

They have good medicinal values so that the effect of this family Rosa species is important for us to study and screen it for getting the information of different chemical compounds and their medicinal and therapeutic effects. There are some 300 chemical constituents of which only about 100 have been identified (Yassa et al., 2009).

1.1 Phytochemical analysis of medicinal plants

Phytochemicals are naturally occurring bioactive compounds which are found in different parts of the plants that are highly useful for the medicinal values so that these chemicals help to contribute the plants color, odor, aroma, ornamental value and also act as plant defense mechanism for different insects and herb (Saklani and Kutty, 2008).

Though the compound is not important for the human health primarily but can be used in case of disease for curing. The range of curing was varied from common diseases to complex cases, also they are much important for us to utilize their other medicinal benefits. They play important biological properties as antioxidant activity, antimicrobial effect, antifungal effect, anti-parasitic effect, stimulation of immune system, modulation of hormone metabolism and also help in disorders of human health. (Mwangi et al., 2009).

Phytochemical screening can lead to identify the new sources of drugs that are important from both therapeutically and industrially which are present in the different parts of the plants (Newman, 2008).

It takes long term research and plan to develop the effective compounds that can target to specific pathogens so that its analysis and screening process was important for different types of tools, chemicals and techniques are used which leads researcher to deal with bioactive compounds that play vital role for diseases and complexity. Numerous types of bioactive compounds are generally obtained from the analysis and their effect on the immune system was also different with different mechanism. So, the classification of the compound was of great importance for the analysis (Gomez, 2002).

Generally, phytochemicals are classified as primary and secondary compounds. Chlorophyll, proteins, common sugars are primary and terpenoid, alkaloids and phenolic compounds are secondary compounds of plant kingdom (Kadir et al., 2012).

Phenolic compounds are generally abundant in the plants. They are very important forms of the metabolites that are found in the different parts of plants and are responsible for color of fruits .Different types of phenolic compounds are flavonoids, phenolic acids. Among them, flavonoid compounds are naturally occurring phenolic compounds in maximum proportion. Phenolic compounds are synthesized from phenylalanine via the action of phenylalanine ammonia lyase (Elbing et al., 2005). These types of compounds generally show the effect of anti-oxidant, anti-mutagenic and cancer related deformities are modified by alteration of gene expression. The anti-oxidant activity is due to redox reaction which helps phenol to act as a reducing agent and hydrogen donor so they show most of the anti-oxidant activity in plants (Lambert, et al., 2005).

Flavonoids are naturally occurring phenolic compounds that occur in plant parts both in free-state and glycosides. They shows many biological effects such as cytotoxicity, anti-microbial, anti-tumor and also act as powerful anti-oxidant that helps to protect human cells from reactive oxygen species and free radicals. This effect depends on molecular structure of flavonoids. Position of hydroxyl groups and other features helps to enhance free radical scavenging activity (Hussain et al., 2005).

1.2 Aims and Objectives

Our main objective is to purpose the medicinal effects of different *Rosa species* and *Tinospora cordifolia.* In the context of Nepal, the species of wild *Rosa* are studied less so that to identify about different bioactive compounds that are found in those species and also concerned on their anti-oxidants, phenols, flavonoids and other properties. The main objectives of using *Tinospora* is just for comparative study with *Rosa* species because it has high medicinal properties studied so far.

To perform qualitative test of plant extracts for presence of various secondary metabolites.

To estimate total phenolic and flavonoid compounds.

To evaluate antioxidant properties and anti-microbial effects of plant extract.

To observe inhibition on VERO cell Lines.

1.3 RATIONALE

There are different solutions for any kind of problem in sense different types of disease and solution as medicinal plants as their use as medicine. Different plants and their parts contain different proportion of bioactive compounds so selection of parts is also important for effectiveness. Thus, it is important for screening to evaluate different components for different types of pharmacological activity. They have abundant source of antioxidant, antibacterial and other bioactive components that are used for healing diseases traditionally. So that it's important for pharmaceutical applications and medicinal prospective was much relevant for drug discovery. The study of Rosa species was important because they are wildly found in forest which has good aroma and good ornamental properties so people love to cultivate this type of plants around them. Thus, we can say it must have some medicinal value so that it is always loved by people of every generations and different geography.

In the context of Nepal, the domestic rose was used for economic importance because of its decorative, cosmetic and ornamental values. The research of wild species of *Rosa* has not been studied so far in Nepal so to explore the different components that can help in medicinal value is main concern of my study. *Tinospora cordifolia* is used as a reference for the proposed study because of it high medicinal values.

The present study focuses on preliminary phytochemical analysis as rose is a woody perennial plant belonging to the genus *Rosa* in the family Rosaceae. Known for its sweet smell and beauty, generally is used as a decorative in household and public gardens. Besides being used as a decorative, very few know its use as a medicinal herb. The petals, stems, leaves and roots of a rose plant contain various secondary metabolites and nutrients in the form of vitamins and minerals. Extracts from different parts of rose plant have also been reported to show substantial anti-bacterial and anti-fungal activity. The parts of the plant especially the petals have high antioxidant property that helps in curing many ailments.

2. Literature Review

Despite its relatively small size, Nepal is well-known for its biodiversity. The different physiographic zones resulting from attitudinal variation have given rise to vegetation ranging from the subtropical to alpine. About 7,000 species of plants have been reported in the country so far, out of which approximately 700 species have medicinal properties (HMG/IUCN, 1986). Some of these medicinal plants are also aromatic.

The *Rosa species* shrubs are erect and diffuse or climbing, pricky, bristly, or rarely unarmed. Stipules persistent and adnate to petiole or free except base and caducous, rarely absent. Leaves alternate, odd pinnate, usually bracteates. Rosa taxonomy is complicated because of hybridization. There are huge commercial rose industry that produces numerous new hybrids every year, combining different cultivars to produce the desired combination of traits. The origination of Rosa was from China because of its use and producing cultivars are started early from china. Hybrid *Rosa x odorata* was formed by *Rosa chinensis* and *Rosa gigantean.* Five species native to Nepal with another eight species reported to be cultivated. They are *Rosa sericea, Rosa webbiana, Rosa brunonii, Rosa macrophylla, Rosa clinophylla.* They are cultivated in the temperate regions of Nepal (Watson et al., 2011).

Rosa brunonii, commonly known as Bhainsikanda in Nepal is found in 1300-3000m of altitude of Nepal. They are distributed in Nepal, Tibetan Plateau, Assam-Burma an East Asia. Flowering starts from April to June and fruiting from July to November (Khan et al., 2017). *Rosa Brunonii* is a very effective plant for curing different types of gastrointestinal problems. Various parts such as leaf, stem, bark, flower and root are useful for pharmaceutical purposes. They can also be effective for improving the condition of skin and hair. Mainly essential oils can be prepared from flowers of *Rosa Brunonii* (Kashana et al., 2011). Essential oil includes major compounds such as Eugenol, Citronellol, Geraniol and Terpien-4-ol (Kaul et al., 1999). There are many fatty acids in this plant such Linoleic acid, Linolenic acid, Oleic acid and Palmitic acid. About 7 fatty acids were observed (Sharma et al., 2012). These Rosa species contains carboxylic acid, myrcene, vitamin C, kaempferol and quarcetin. Flowers also contain a bitter principle, tanning matter, fatty oil and organic acids (Ashrafzadeh et al., 2007).

Antiradical activity of the plant extract is attributed to their hydrogen donating ability. It is well known that free radicals cause auto-oxidation of unsaturated lipids so that extract plant

extract can be accepted as strong free radical inhibitors and anti-oxidants (Kaur and Perkins 1991). So Rosa species has numerous healing effects on health of human beings. The plant extract has very good effect on microbes and that is why it was a suitable choice for its observation.

The methanol leaf extracts of Sida cordifolia, Tinospora cordifolia showed significant antibacterial activity against Bacillus subtilis, Escherichia coli, Pseudomonas fluorescens, Staphylococcus aureus and Xanthomonas axonopodis pv. malvacearum and antifungal activity against Aspergillus flavus, Dreschlera turcica and Fusarium verticillioides when compare to root/ bark extracts (Afolayan et al., 2002). S. cordifolia leaf extract showed highest antibacterial activity against B. subtilis. Root and leaf extract of S. cordifolia recorded significant activity against all the test bacteria. Tinospora cordifolia recorded significant antifungal activity against D. turcica. The methanol extract of Sida cordifolia exhibited significant antifungal activity against F. verticillioides (Mahesh and Satish, 2008).

Tinospora Cordifolia is a very important plant with multipurpose such as anti-periodic, anti-inflammatory, anti-spasmodic, anti-arthritic, anti-allergic and anti-diabetic properties. The root of this plant is known for its anti-stress, anti-leprotic and anti-malarial activities (Soliman et al., 2002). A variety of constituents have been isolated from *Tinospora cordifolia* plant and their structures were elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compunds and polysaccharides (Maurya et al., 1995). Consequently, this plant can help us for comparative study with Rosa species.

2.1 Reactive oxygen species and antioxidants

Free radicals induce different types of diseases by lipid peroxidation and DNA damage. It is reported that numerous plant extracts have antioxidant activities to scavenge free radicals. In the present study, the antioxidant properties of crude methanol extract of *Rosa webbiana* were studied in six *in vitro* models by radical scavenging activity by DPPH reduction Assay, Scavenging of SO, H_2O_2 and NO, reducing power, FRAP assay. The extract was found to contain large amounts of phenolic Compounds and flavonoids. Methanol extract of *Rosa webbiana* possessed significant antioxidant activity as compare to aqueous extract. These

results suggest that hibiscus has potential to develop a new functional dietary agent to treat chronic metabolic diseases, such as diabetes and hyperlipidemia (Garg, 2012).

A plant extract capable of accepting electron and donating electrons is called oxidizing and reducing agent for the process of redox reaction. This process of accepting and donating electrons takes place in the form of hydrogen donation or removal of oxygen. In scientific term reducing agent is an antioxidant and oxidizing agent is pro-oxidant. This redox reaction process is necessary for survival of the cell. So, these oxidants are generally reactive oxygen species (ROS). These ROS are found inside cells to combat its pathogens or as an incomplete or insufficient process takes place in signaling (Prior and Cao, 1999). During the process of respiration and photosynthesis, the electrons flow in cyclic electron transport chain where electrons are leaked and converted to molecular form of oxygen to the superoxide form and acts as pro oxidant which can damage proteins and DNA (Turrens, 2003). These cells can undergo respiratory burst characterized by 20 fold increase in oxygen consumption, here increase in glucose utilization and production of reduced nicotinamide phosphate dinucleotide (NADPH) in pentose phosphate pathway which helps to reduce electron to NADPH oxidase. This NADPH oxidase is found in phagocytes and B-lymphocytes (Babior et al., 2002).

There are exogenous sources of reactive oxygen species that are produced in body have physiological significance and important effect depending on cellular state. The exogenous source of reactive oxygen species include radiations, ultrasound, food, drugs, toxins, pollutants, etc. In order to defense the harm done by reactive species our body has developed defense mechanism by enzymatic or non-enzymatic is called endogenous antioxidant. Likewise, the antioxidants obtained from plant and other sources are known as exogenous antioxidant. Oxidative stress can cause cell damage whereas cell membrane is made of phosphor lipid bilayer.

Different types of endogenous antioxidant are superoxide dismutase, glutathione reductase, catalase, glucose 6 phosphate dehydrogenase, etc. The exogenous antioxidant are vitamin E, ascorbic acid, polyphenols, carotenoids, etc. that are found in plant products (Bouayed and Bohn, 2010).

Antioxidant activity is a capacity that indicates oxidation reaction in a definite condition. Plants that may differ in their small molecular constituents and that make variations in

antioxidant activity of those plants. Different types of organic solvent systems are used also effect the antioxidant activity. We use the DPPH assay (2,2-diphenyl-1 picrylhydrazyl) to determine the antioxidant properties (Ng et al., 2005).

Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant by their hydrogen donating ability. The electrons become paired off and solution loses color stochiometrically depending on the number of electrons taken up (Aghdam et al., 2011).

DPPH assay was the important method that was followed to determine the antioxidant activity. This method was developed by Blois in 1958. DPPH is the stable free radical that delocalize the spare electrons over the molecule as a whole so that the molecule does not dimerise as the other most free radicals. This type of delocalization gives deep violet color with absorption in methanol solution at 520 nm (Singh, 2011). The free radical that accept electron or hydrogen radical and convert it to non-radical form and color was changed from deep violet to colorless form.

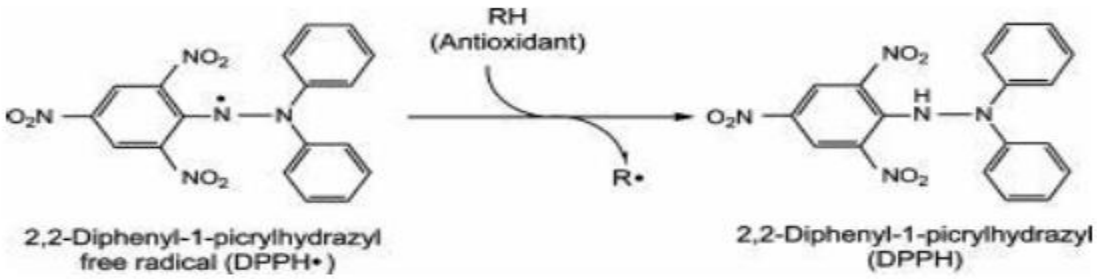


Figure 2.1: DPPH redox reaction

$$X^* + AH = XH + A^*$$

Where X* is represented as DPPH radical and AH as the donor molecules which is shown in the reaction. XH is the reduced form and A* is free radical produced in the first step. This later undergo further reaction which control the overall stoichiometry, the number of molecules of DPPH is reduced to colorless by one molecule of reductant (Molyneux, 2003). Since it is rapid and widely used method to measure antioxidant capacity. It is also convenient for the

glutathione, tocopherol, ascorbic acid and polyhydroxy aromatic compounds (Masahiro et al., 2005).

Cell membrane is made of phospholipid lipid bilayer consisting of poly unsaturated fatty acids chain. These fatty acids are sensitive to reactive oxygen and cause lipid peroxidation. This occurred in attack of methylene group by free radical which leads to weaken the bond between hydrogen and carbon which leads to formation of conjugate diene (Smirnoff, 1995). In the presence of oxygen in the vicinity, this dimer is converted to peroxide radical which is unstable. So, to maintain stability abstract hydrogen form the nearby lipid molecule, thus starting chain reaction. This reaction results in peroxidation of all unsaturated fatty acids in the membrane probably these peroxide radical are converted to cyclic peroxides.α-tocopherol terminate reactions by breaking lipophilic molecules (Baud and Ardaillou, 1986). Protein and DNA are also possible target of ROS, they can undergo direct and indirect damage following interactions with ROS including peroxidation, damage to specific amino acid residues, and change in tertiary structure, degradation and fragmentation of protein. For ROS interaction with DNA cause damages like modification of DNA bases, strand breakage, loss of purines, DNA protein cross linkage, damage to deoxyribose sugar and DNA repair system.

Folin ciocalteau method for Phenolic and antioxidant compounds are commonly used in extracts of natural products from plants and fungi. These compounds may exhibit many biological effects including antioxidant activity. The phenolic compound content of extract can be approximated by Folin Ciocalteu reagent and expressed as gallic acid equivalents in mg (or µg or mmol) /g of crude extract. The Folin Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic/antioxidant compounds to phosphomolybdic and phosphotungstic acid complexes, which can be determined by measuring the absorbance at 765 nm. Owing to the general nature of the Folin Ciocalteu chemistry, it is a measure of total phenols and other oxidation substrates. However, the F–C assay is simple and reproducible and has been widely used for studying phenolic anti-oxidants (Ainsworth and Gillespie, 2007).

2.3 Methods for preparation of plant extract

Sonication is the act of applying sound energy to agitate particles in a sample, for various purposes. Ultrasonic frequencies (>20 kHz) are usually used, leading to the process also being known as ultrasonication (Suslick, 1990).

Sonication has numerous effects, both chemical and physical. The chemical effects of ultrasound are concerned with understanding the effect of sonic waves on chemical systems, this is called Sonochemistry. The chemical effects of ultrasound do not come from a direct interaction with molecular species. Studies have shown that no direct coupling of the acoustic field with chemical species on a molecular level can account for Sonochemistry Instead, Sonochemistry arises from acoustic cavitation: the formation, growth, and implosive collapse of bubbles in a liquid (Azmira et al., 2013).

Sonication can be used for the production of nanoparticles, such as nanoemulsions nanocrystals, liposomes and wax emulsions, as well as for wastewater purification, degassing, extraction of plant oil, extraction of anthocyanins and antioxidants, production of biofuels, crude oil desulphurization, cell disruption (Peshkovsky and Peshkovsky, 2007).

2.4 Use of Rotavapor for the extract preparation

A Rotary evaporator is a gentle and efficient device which was used in chemistry laboratories for removal of organic solvent by evaporation. It was invented by Lyman C. Craig .Vacuum system is also used in it harmonically in evaporators .Vacuum evaporators is a class function because lowering the pressure above a bulk liquid lowers the boiling points of the component liquids in it. Generally, the component liquids of interest in applications of rotary evaporation are research solvents that one desires to remove from a sample after an extraction, such as following natural product isolation or a step in an organic synthesis. Use of a "rotavapor" therefore allows liquid solvents to be removed without excessive heating of what are often complex and sensitive solvent-solute combinations. (Okan and Mang , 2013)

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

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

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

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

2.5 Analysis tools for phytochemicals

The qualitative method that helps to identify the responsible components of extracts was also the queries for drugs development. Phytochemicals responsible for different types of effects and activity that can be screened to find the medicinal value component's chemicals structure by different types of analytical tools, GC-MS HPLC etc.

Gas chromatography (GC) is undoubtedly useful techniques used for screening /identification / quantification of many groups of non-polar and/or semi-polar food toxicants and plants bioactive compounds. The high attainable separation power in combination with a wide range of the detectors employing various detection principles to which it can be coupled makes GC an important, often irreplaceable tool in the analysis of (ultra)trace levels of toxic food and constituents that may occur in such complex matrices as foods and phytochemicals (Jana and Tomas, 2007).

2.6 In vitro cytotoxicity of plant extracts

Cell culture is a very systematic technique that was performed under the controlled condition or culture laboratory. In its simplest form, cell culture involves the dispersal of cells in an artificial environment composed of nutrient solutions, a suitable surface to support the

growth of cells, and ideal conditions of temperature, humidity, and gaseous atmosphere. In general practices cell culture of multicellular eukaryotes (Animals), plants, fungi's, bacteria's, microbes including viruses is done by isolating cell from them (Sassa et al., 1987).

African green monkey kidney Normal cell Vero cell line is a continuous cell line, which is aneuploid and will grow indefinitely in culture. This cell line is generally used as a vaccine cell substrate. It is also used extensively for virus replication studies and plaque assays as they are sensitive to infection withSV-40, SV-5, measles, arboviruses, reoviruses, rubella, simian adenoviruses, polioviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, vaccinia, and others. Cytotoxicity studies are a useful initial step in determining the potential toxicity of a test substance, including plant extracts or biologically active compounds isolated from plants. Minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role. The concept of basal cytotoxicity, where deleterious effects are noted on structures and functions common to all human cells, is relevant when considering the relationship between acute toxicity and cytotoxicity. The selectivity index is an important measure to identify substances with promising biological activity and negligible cytotoxicity. Various bioassays and a number of different cell lines have been used to assess cytotoxicity. VERO cells are continuous and easy for growth and maintenance so they can be used for cytotoxicity.

2.7 MTT assay

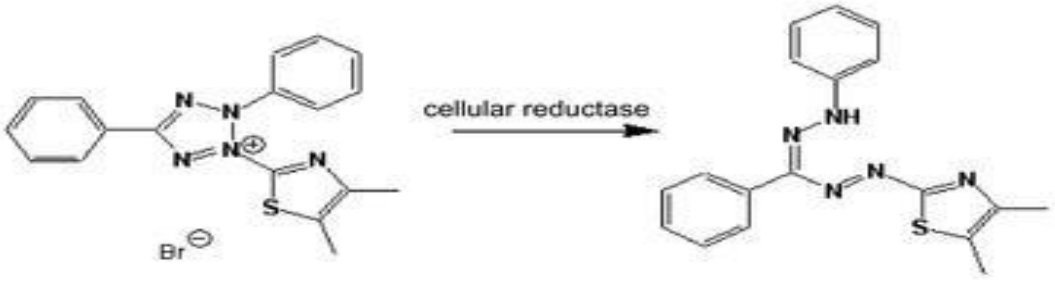


Fig 2.2: MTT reaction

MTT is (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. A solubilization solution (usually eitherdimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. (Mosmann et al., 1983).

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g. Isopropanol or DMSO; we used DMSO) and the released, solubilized formazan reagent is measured spectrophotometrically (Vanicha and Kanyawim, 2006).

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

results in an increase in the amount of MTT formazan formed and an increase in absorbance (Mosmann, 1983).

Nevertheless, the MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves. The MTT method of cell determination is most useful when cultures are prepared in multi well plates. For best results, cell numbers should be determined during log growth stage. Each test should include a blank containing complete culture (Cole, 1986).

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation (Sargent and Taylor, 1989).

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

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory setting

The research of phytochemical screening of medicinal plants was carried out in the Central Department of Biotechnology, Tribhuwan University.

3.1.2 Sample collection and identification

Plants of wild rose were collected from southern hills of Kathmandu. These plants barks were taken and they were processed for the extraction. All the samples were collected in the month of February 2017; the starting season for flowering.

3.1.3 Extract preparation

The samples were cleaned and dried in air and were grinded with help of grinder machine to make the powder forms of the bark of the sample plants.

50gm of fine powder of sample was weighed. 350ml of HPLC methanol (Fischer scientific) was mixed with the powder and sonication was started.

Sonication was done with sonicator thrice. During each step sonication was done 20Hz for 120 minutes. After sonication the mixture was filtered. The residue was subjected to sonication. Each sample the process was repeated, the extracts were mixed and concentrated by evaporation 38 degree celsius under reduced pressure using rotary evaporator. The dried extract was collected in 2 ml polypropylene reaction tubes and stored in a freezer until further use.

3.2 Qualitative phytochemical analysis

Testing of the presence of secondary metabolites of the methanol extract of the plant sample was done by using the protocol suggested by Harbone and Baxter (1973) and Trease and Evans, 1989.

Testing flavonoids: The crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow color was seen and then turned to colorless form by adding few drops of diluted acid. This change in color indicated presence of flavonoids in plant extract.

Test for Glycosides: The crude extract was mixed with each of 2ml of acetic acid and 2ml of chloroform. Then, the mixture was cooled in ice and concentrated H2SO4 was added carefully. A violet color appeared and changed to blue and again changed to green color. Hence, presence of steroid was seen.

Test for steroid: The crude extract was added with 2ml of chloroform and concentrated H_2SO_4. A red color was appeared in the lower chloroform layer indicating the presence of steroids. Another test was performed by mixing crude extract with 2ml of chloroform. Then 2ml of concentrated H_2SO_4 and 2ml of acetic acid were poured into the mixture. The greenish color appeared which showed the presence of steroid. The crude extract was dissolved in chloroform and evaporated to dryness. To this, 2ml of concentrated H_2SO_4 was added and heated for 2 minutes. Greyish color appeared showing the presence of terpenids.

Test for alkanoids: The crude extract was mixed with 2ml of 1% HCL and was heated gently. Mayer's reagent was added and then mixed. Turbidity of the resulting precipitate was taken as evidence for presence of alkaloids.

Test for phenols and tanins: The crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue green or black color was seen indicating the presence of phenols and tannins.

3.3 Quantitative phytochemical analysis

3.3.1 Determination of total phenol content

(Ainsworth et al., 2007; Lu et al., 2011)

Preparation of Reagents

1:10 v/v Folin-Ciocalteu reagent (2N)

1 M sodium carbonate

Stock solution of 0.5 mg/ml

Standard gallic acid solution (10, 20, 30, 40, 50, 60, 70 and 80 µg/ml)

(Plant extract 1mg/ml in absolute methanol. 100µl sample in testube, + 1570 µl sterile distilled water dropwise such that concentration of methanol drops to 6%. + 200µl FC reagent + 800 µl of sodium carbonate (7.4%). Incubated for one hour at 37 ^{o}C. Taking absorbance at 765nm. For blank, 100 µl of absolute methanol was used instead of the test solution).Freshly prepared solution was used for the test.0.5ml of each extract (0.5mg/ml) was mixed separately with 5ml Folin-Ciocalteau's reagent and 4ml of aqueous sodium carbonate 1M solution was added. The mixture was allowed to stand for 15 minutes at room temperature. The absorbance was measured at 765 nm using spectrophotometer, using gallic acid for constructing the standard curve (10-80 µg/ml) expressing total polyphenolic compound concentration in the extracts milligrams of gallic acid equivalent per gram of dry weight (mgGAE/g) of the extract using gallic acid standard curve. All the readings were done triplicate for each concentrations of extracts and standards.

3.3.2 Determination of total flavonoid content

(Chang et al., 2002)

Preparation of Reagents

10 % Aluminium trichloride

1 M Potassium acetate

Stock solution of 0.1 mg/ml i.e. 100 µg/ml

Standard quercetin solution (10, 20, 30, 40 and 50 µg/ml)

Plant extracts 0.5 mg/ml i.e. 500 µg/ml(Crude extract = 1mg/ml,,,, for standard = different concentration 0.5 ml sample+1.5 ml methanol+ 0.1 ml (10% aluminium chloride)+ 0.1 ml 1M potassium acetate + 2.8 ml+ shaked well and incubated at room temperature for 30 minutes. Absorbance was taken at 415 nm. Quercetin as standard. For blank, all chemical except absolute methanol instead of sample.0.25 ml of each extract (5 mg/ml) was taken and was mixed with 0.75 ml methanol.0.05ml aluminium trichloride (AlCl3, 10 %) was added along with 0.05 ml of 1 M potassium acetate. Then1.4 ml distilled water was added and the reaction

mixture was allowed to stand for 30 minutes. The absorbance was measured at 415 nm with UV-visible spectrophotometer. Quercetin was used for constructing the standard curve (10-50 µg/ml) expressing the total flavonoid compounds concentration in the concentration in the extracts in milligrams of quercetin equivalent per gram of dry weight (mg QE/g) of extract .All the readings were done triplicate for each concentrations of extracts and standards.

3.3.3 Determination of antioxidant activity

Preparation of DPPH solution

(0.1mM) 3.94 mg of DPPH methanol was dissolved to produce final volume of 100 ml solution.

Preparation of quercetin stock solution

Stock solution of 5mg/ml i.e. 5000 µg/ml was prepared by dissolving 50 mg of quercetin in 10 ml of methanol. Final concentrations by diluting the stock solution was prepared. For preparation of 1000, 500, 100 and 50 µg/ml concentration, 400µl, 200µl, 100µl, 50µl from stock solution was added and the final volume was made to 2ml.For the preparation of plant extracts 1mg/ml in 50%DMSO, 0.5ml 0.1mM DPPH was taken initially. Then 0.5 ml 1mg/ml plant extract was added to it. Similarly, as control, 0.5 ml of 0.1mM DPPH was mixed with0.5ml 50%DMSO.The mixture was kept in dark for 30 minutes.

The absorbance was measured at 517 nm. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\%\text{scavanging} = \frac{(Ao - At)}{Ao} \times 100$$

Ao = Absorbance of DPPH solution

At = Absorbance of test or reference sample

The % scavenging was then plotted against concentration and regression equation was obtained from which IC50 (micro molar concentration required to inhibit DPPH radical formation by 50%). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity (Sabudak et al., 2013; Subedi et al., 2012).

Free radical scavenging activity plant was calculated by above formula. (IC50) was calculated using the formula described by Louis and Paul (2010).

IC50=exp[ln(conc>50%)-[(inb%>50%-50)/(inb%>50%-inb%<50%)*ln(conc>50%/conc<50%)]

Exp: exponential log ln: natural log function both used in Microsoft Excel 2007 software

All the readings were done triplicate for each concentrations of extracts and standards.

3.4 Determination of Antibacterial activity

Preparation of nutrient agar broth

NA plates were prepared for antibacterial testing. 28gm of NA powder (Hi Media Laboratories Pvt. Ltd., Mumbai, India) was carefully weighted and was poured in distilled water 1000 ml and was mixed uniformly. For sterilization, the media was autoclaved at 15lbs pressure at 121 degree centigrade for 15 minutes. Then, it was allowed to cool for 45 to 50 degree centigrade and was poured into sterilized petri dishes. It was then used for culture of bacteria.

Preparation of Luria Barbertani Miller (LB) broth and Muellar Hinton Agar (MH)

LB broth was required for sub culturing microorganisms prior to antimicrobial tests. 25gm of LB powder (Hi Media Laboratories Pvt. Ltd., Mumbai, India) was weighed and was dissolved to 1000ml distilled water and the media was autoclaved at 15lbs pressure at 121 degree centigrade for 15 minutes. After that the cooled media was dispensed in sterile and dry culture tubes.

38gms of MHA powder (Hi Media Laboratories Pvt. Ltd., Mumbai, India) was added to 1000ml distill water and the media was autoclaved at 15lb pressure at 121 degree centigrade for 15 minutes. Then, it was allowed to cool for 45 to 50 degree centigrade and poured into sterilized petri dishes.

Preparation of standard bacterial culture Inoculums

ATCC culture of *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* was obtained from CDBT, TU. The culture was inoculated in LB broth and left for 37 degree centigrade incubator for overnight. The turbidity of the sub culture bacterial suspension was

adjusted at the 0.5 McFarland standards. These bacterial inoculums were used for swabbing on MHA plates for antimicrobial test of plant extracts.

Antimicrobial test

Plant extracts of different concentrations (1000, 500, 100 and 50 µg/ml) were prepared and paper disc was also prepared and was dissolved in each extract and was placed in the petri dishes where the microorganisms was swapped which was done in complete sterilized condition. Then the petri dishes was placed for the incubation for 37 degree centigrade for 24 hrs. The inhibition zone was measured as the control of antibiotics of 30mgc/disc concentration were placed.

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

50 mg of plant extracts was taken of each sample and was dissolved in 1 ml of methanol and samples are analyzed using the GC-MS.GC-MS analysis was performed using column-RTX 5 MS. The inert gas helium (99.9995%) was used as the carrier's gas, at flow rate. (Shimadzu GCMS-QP2010 Ultra Gas Chromatography Mass Spectrometer)

[GCMS-QP2010 Ultra]

[GC-2010]		
Column Oven Temp.	:100.0 °C	
Injection Temp.	:280.00 °C	
Injection Mode	:Splitless	
Sampling Time	:1.00 min	
Flow Control Mode	:Linear Velocity	
Pressure	:68.3 kPa	
Total Flow	:4.0 mL/min	
Column Flow	:0.95 mL/min	
Linear Velocity	:36.2 cm/sec	
Purge Flow	:3.0 mL/min	
Split Ratio	:0.0	
High Pressure Injection	:OFF	
Carrier Gas Saver	:OFF	
Oven Temp. Program		
Rate	Temperature(°C)	Hold Time(min)
-	100.0	0.00
15.00	250.0	1.00
30.00	280.0	2.00
15.00	300.0	10.00

Conditions of GC-MS Analysis

3.6 In vivo cytotoxicity of plant extracts on VERO cells

The cells lines was centrifuged at 1500rpm and the supernatant was discarded and pellet was suspended with RPMI media.

10µl was taken and 80µl trypan blue was added and cell count was done with haemocytometer using the formula:

Viable cell /ml = (average viable cells per count * dilution factor * 10^4)

Now media was added with calculating amount of cells. Then, 100 microliter of the media was added in each well of micro titer ELISA plate and then was incubated in 37 degree incubator for 24 hrs. After that different extracts with different concentration of extracts were added with triplicate order and again incubated for 24hrs next day. MTT dye 10 microliter was added and was incubated for 4hrs and DMSO was added and incubated for 30mins. Finally absorbance reading was taken with 551nm.

Result was interpreted using the formula:

Percentage inhibition(PI)=100-{(At-Ab)/(Ac-Ab)}*100

Percentage inhibition (PI) = 100

At=absorbance value of test compound

Ab= absorbance value of blank

Ac= absorbance value of control

The cytotoxicity/Inibitory concentration of the extracts required to inibit the 50% of the VERO cells proliferation(IC50) were calculated using the formula described by Prof. Louis Maes and Prof. Paul Cos (Louis and Paul, 2010).

IC50=exp[ln(conc>50%)-[(inb%>50%-50)/(inb%>50%-inb%<50%)*ln(conc>50%/conc<50%)]

Exp: exponential log ln: natural log function both used in Microsoft Excel 2007 software.

Graphical illustration was done to observe the inhibition%.

All the readings were done triplicate for each concentrations of extracts, control and blank.

96 well ELISA plate was used to perform cytoxicity.

Celllines: VERO cells

Passage: 28

Seeding density:1*10^5cells/well

Medium: Complete RPMI

Extracts treatment: 24hrs

MTT treatment: 4hrs

Extracts dissolved in DMSO

Absorbance at 551nm

4. RESULTS

Different species obtained from the rotavapor with given amount

a. Rosa spp. (purple) (wt 1=55gm, 62gm) b .Rosa brunonii (wt2+=88gm, 92gm)

c. Rosa spp. (white)(wt 3=88gm, 96gm) d. Tinospora cordifolia(wt4=55gm, 57gm)

4.1 Qualitative phytochemical analysis

Methanol extract was subjected to various reagents to analyze the presence of different kinds of secondary metabolites (no alkaloids).

Plant extracts	Flavonoids	Glycosides	Steroids	Terpenoids	Phenols &tannins
Rosa sp. (purple)	+	_	+	_	++
Rosa brunonii	+	-	+	_	+
Rosa sp. (white)	+	-	+	_	+
Tinospora cordifolia	++	++	-	-	+

(+)for fairly present (-)for absent (++)highly present

Table 4.1: Qualitative analysis of plant extracts

4.2 Total phenol content

Standard solution of gallic acid ranging from 10µg/ml to 100µg/ml was used to obtain standard curve and equation as shown in the figure below. Based on the equation of the concentration of the total phenol content, the methanol extracts of 4 different plants was determined. The result was expressed as mgGAE/g±SEM.

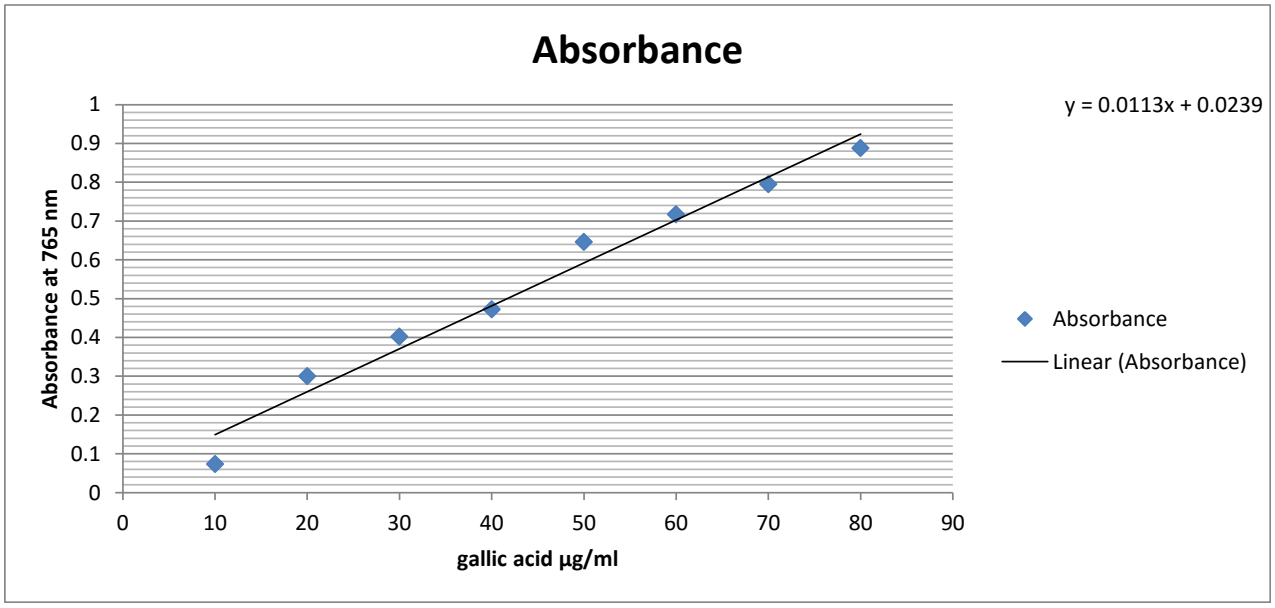
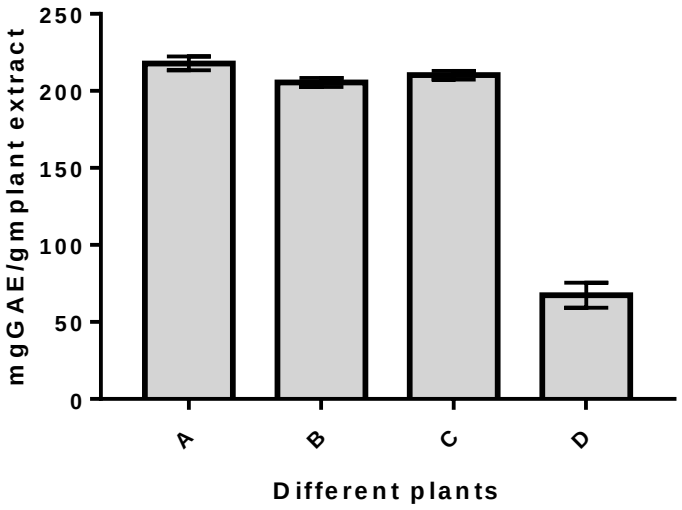


Figure 4.1: Standard curve of gallic acid

	Name of Plant	Mean Wt.	SD
a.	*Rosa* sp. (purple)	217.91	4.49
b.	Rosa brunonii	205.52	2.82
c.	*Rosa* sp. (white)	210.30	2.80
d.	Tinospora cordifolia	67.38	8.16

Table 4.2: Total Phenol content in plant extracts



A: Rosa species (purple)	C: Rosa species (white)
B: Rosa brunonii	D: Tinospora cordifolia

Figure 4.2: Bar diagram of Phenol content

The total phenol content, present in the methanol extracts of the different species of *Rosa* and a species of Tinospora for the methanol extracts, the highest amount of the phenol on the *Rosa species* (purple) was found to be 217.91±4.49 mgGAE/g. Similarly, the lower amount of phenol content was 67.38±8.16 mgGAE/g. The phenol content of the other plants extracts remained in between two extremes.

Using equation y=0.0113x+0.0239 obtained from the graph of galic acid concentration and formula for mg galic acid/gm of extract= c.v/w, where c is the concentration of galic acid, v is volume of extract and w is dry weight of extract. This was examined by spectrophotometrically at 765 nm.

4.3 Total flavonoids contents

To determine the total flavonoid content in the sample standard graph was plotted using standard solution of the quercetin ranging in concentration from 10 to 100. Based on the

standard graph equation i.e. y=x, total amount of flavonoid present in the 4 different samples of extracts was determined. The result was expressed in mgQE/g±SEM.

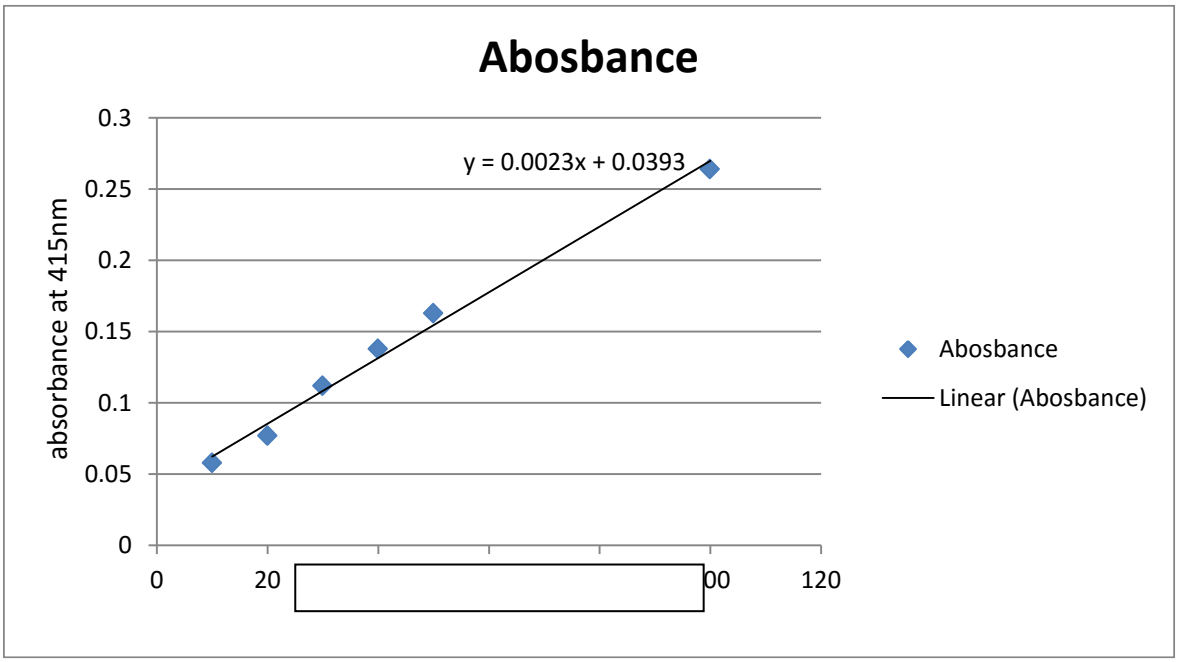
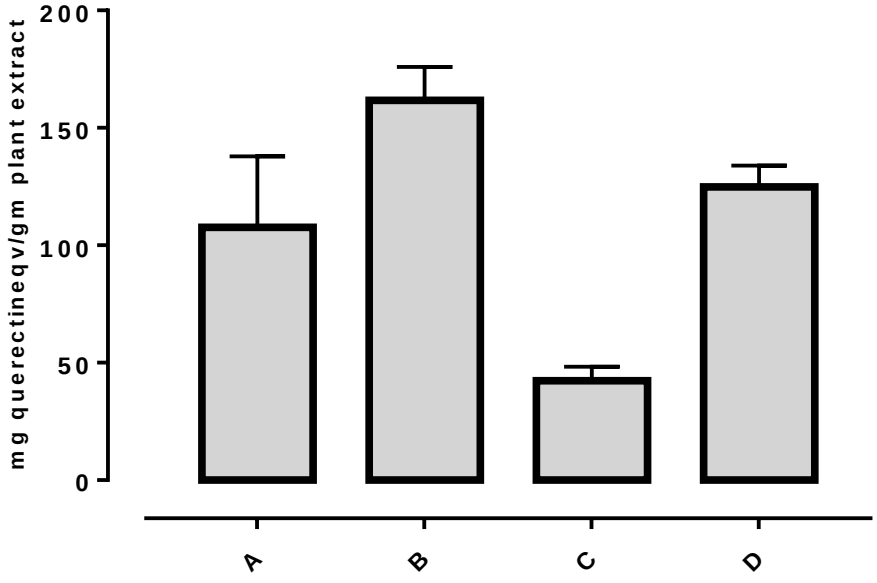


Fig 4.3: Standard curve of Querectin

S.N	Name of Plant	Mean Wt.	SD
a.	*Rosa spp*(purple)	107.69	30.19
b.	Rosa brunonii	161.75	14.22
c.	*Rosa spp*(white)	42.33	6.03
d.	Tinospora cordifolia	124.79	9.13

Table 4.3: Result of flavonoid content



A: Rosa species (purple)	C: Rosa species (white)
B: Rosa brunonii	D: Tinospora cordifolia

Figure 4.4: Bar diagram of flavonoid content

The total flavonoid content present in the methanol extracts of the different species of *Rosa* and a species of *Tinospora* for the methanol extracts, the highest amount of the flavonoid found on the *Rosa* *brunonii* was 161.75±14.12 mgQE/g. Similarly, the lower amount of flavonoid content was 42.33±6.03 mgQE/g in *Rosa* species (white). Similarly, the value for *Tinospora* species was 124.76±9.13 mgQE/g.

Using equation y=0.0023x+0.0393 obtained from the graph of quercetin concentration and formula for mg quercetin /gm of extract= c.v/w, where c is the concentration of quercetin, v is volume of extract and w is dry weight of extract. This was examined by spectrophotometrically at 415 nm.

4.4 Antioxidant activity of the plant extract

Antioxidant activity of methanol extract of *Rosa* species and *Tinospora cordifolia* collected from Kathmandu district of Nepal was determined by using the solution of DPPH (0.1mM) and taking quercetin as the pure antioxidant reference compound, IC50 value was calculated for each sample taking the concentration vs. % radical scavenging activity. There was gradual increase in % radical scavenging activity as the increase in the concentration of the extract increased.

S.N	Name of Plant	Mean wt.	SD
a.	*Rosa* sp. (purple)	56.69	5.27
b.	Rosa brunonii	55.67	5.42
c.	*Rosa* sp. (white)	60.95	3.42
d.	Tinospora cordifolia	52.54	0.73

Table 4.4: Result of IC50 value of plant extracts

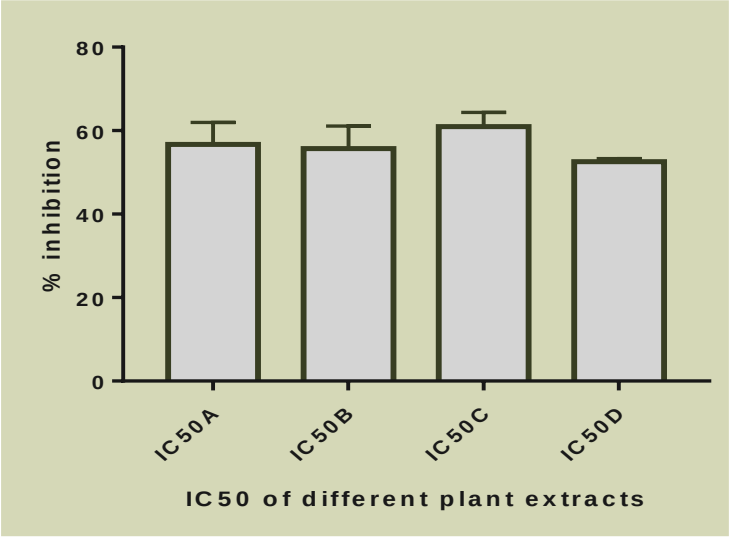


Figure 4.5: Bar diagram of IC50 value of plant extracts

A: Rosa species (purple)	C: Rosa species (white)
B: Rosa brunonii	D: Tinospora cordifolia

There was gradual increase in percentage radical scavenging activity as the concentration of the extract increased. The maximum IC50 was found in *Rosa* species (white) was 60.93± 3.42 and minimum value of *Tinospora* was 52.54±0.73.

4.5 Antimicrobial susceptibility test of different bacteria

Plant	Bacteria	Antibiotics	control	met	10	100	500	1000
A	Klebsiella spp	Cefotaxine	1cm	0.2	0.24	0.30	0.24	0.28
	E.coli	Gentamycin	0.8mm	0.22	0.25	0.22	0.22	0.24
	Staphylococcus aureus	Penicillin	-	0.25	0.35	0.48	0.51	0.51
B	Klebsiella spp	Cefotaxine	1.2cm	0.3	0.40	0.45	0.45	0.6
	E.coli	Gentamycin	1.6	0.2	0.24	0.24	0.25	0.30
	Staphylococcus aureus	Penicillin	-	-	0.23	0.38	0.44	0.51
C	Klebsiella spp	Cefotaxine	1.5cm	-	-	-	-	-
	E.coli	Gentamycin	0.8mm	0.40	0.45	0.50	0.50	0.50
	Staphylococcus aureus	Penicillin	-	0.25	0.25	0.33	0.33	0.25
D	Klebsiella spp	Cefotaxine	1.5cm	0.2	0.45	0.40	0.42	0.40
	E.coli	Gentamycin	1cm	0.3	0.4	0.45	0.42	0.44

	Staphylococcus aureus	Penicillin	-	0.25	0.25	0.20	0.34	0.30

Methanol-ve control Antibiotics +ve control

Table 4.5: Results of antimicrobial tests

A:Rosa species (purple)	C:Rosa species (white)
B: Rosa brunonii	D: Tinospora cordifolia

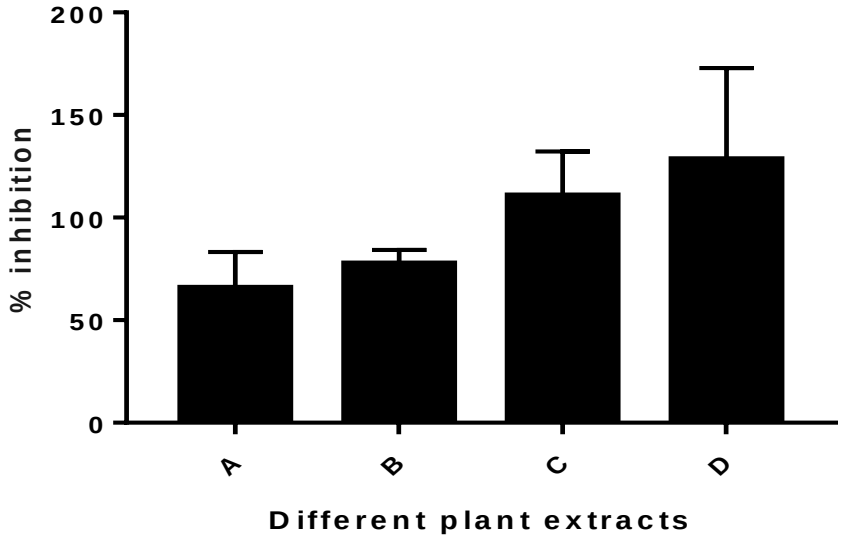
The diameter of paper disc was 0.3 mm so the maximum inhibition was shown by concentration between 50 µg/ml to 100 µg/ml. For other concentration the result was not so effective and the table shows some inhibition effect of plant extracts against gram positive and gram negative bacteria from above table.

4.6 Cytotoxicity effects of plants extracts on the VERO cells

The Cytotoxicity effects of *Rosa species (*purple*), Rosa species (white), Rosa brunoii, Tinospora cordifolia* were found to have lower inhibition value with Vero cells with different concentration

S.N	Name of Plant	Mean wt.% inhibition	SD
a.	Rosa species(purple)	65.76	17.51
b.	Rosa brunonii	77.63	6.59
c.	Rosa species(white)	110.76	21.54
d.	Tinospora cordifolia	128.47	44.42

Table 4.6: %inhibition value of VERO cell lines in different plant extracts



A: Rosa species (purple)	C: Rosa species (white)
B: Rosa brunonii	D: Tinospora cordifolia

Figure 4.6: Bar diagram showing %inhibition of VERO cells

The methanol extract of different 50µg/ml to 100µg/ml Rosa species and *Tinospora* exhibited antiproliferative activity. The antiproliferative activity of *Tinospora* with IC50 value was 128.47±44.42. The antiproliferative activity by *Rosa species* (white) was 110.76±21.54 which shows good results for antiproliferative activity on cell lines with higher concentrations and low concentrations on the *Rosa species* (purple).

4.7 Different types of compounds are identified using the GC-MS analysis methods

Different extracts

A: Rosa species (purple)	C: Rosa species (white)
B: Rosa brunonii	D: Tinospora cordifolia

GC-MS Analysis of Rosa species

The results of GC-MS analysis of the plant extracts of Rosa species (purple), the maximum retention time shown by Stigmast-4-en-3-one, gamma- Sitosterol, 2H-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-t . The result shown by 16 different compounds are shown below:

Peak Report TIC

Peak#	R.Time	Area%	Name	Base m/z
1	4.038	7.12	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	41.05
2	4.247	2.27	4-Hexen-3-one, 4,5-dimethyl-	41.05
3	5.569	4.96	1,2,3-Benzenetriol	126.00
4	6.532	14.80	.beta.-D-Glucopyranose, 1,6-anhydro-	60.00
5	7.349	7.72	Phosphoric acid, dibutyl 3-trifluoromethyl-3-	98.95
6	8.742	3.73	2-Hydroxy-5-methylisophthalaldehyde	136.00
7	9.500	7.48	Pentadecanoic acid	43.05
8	10.688	8.97	9-Hexadecenoic acid	55.05
9	10.720	4.80	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	79.05
10	10.829	5.03	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl	43.05
11	11.431	2.60	Nickel, tris(.eta.5-2,4-cyclopentadien-1-yl)[.r	39.05
12	12.882	2.19	Benzyl oxy tridecanoic acid	91.00
13	13.082	5.06	o-(p-(Dimethylamino)benzylideneamino)phe	239.90
14	17.930	3.23	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-t	165.05
15	20.425	17.60	.gamma.-Sitosterol	43.05
16	22.555	2.43	Stigmast-4-en-3-one	124.10
		100.00		

Table 4.7: Phytochemicals identified in methanolic extract of Rosa spp (purple)

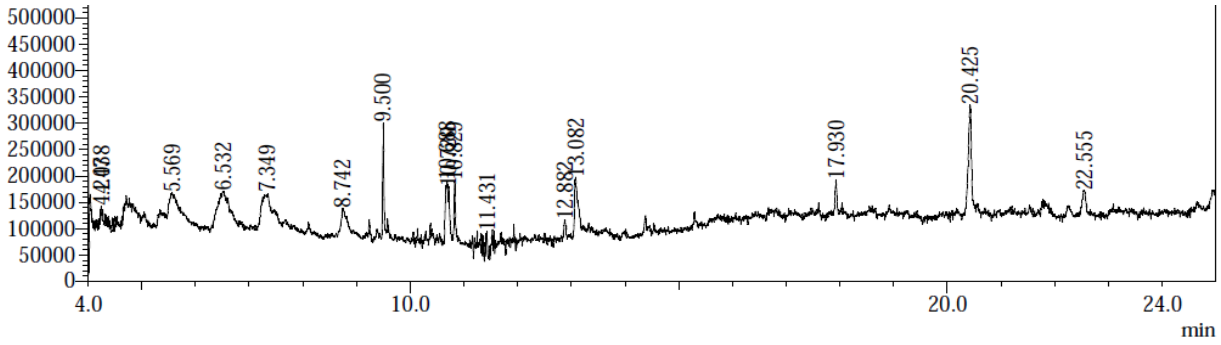


Figure 4.7: Chromatogram of *Rosa species* (purple)

GC-MS Analysis of Rosa brunonii

Different types of compounds identified of plant extracts *Rosa brunoii*, the maximum retention time shown by dl-alpha-Tocopherol, gamma-Tocopherol, 2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimer. The result shown by 16 different compounds are shown below.

Peak#	R.Time	Area%	Name	Base m/z
1	4.027	4.06	Benzoxazol, 2,3-dihydro-2-thioxo-3-diallyla	31.05
2	4.127	30.46	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	41.10
3	4.247	3.06	2H-Pyran-5-carboxylic acid, 2-oxo-, methyl	39.05
4	4.310-	2.59	3-(1-Methyl-2-pyridiniothio)-1-propanesulfo	97.00
5	5.480	11.04	1,2,3-Benzenetriol	126.00
6	6.517	8.34	.beta.-D-Glucopyranose, 1,6-anhydro-	60.00
7	7.448	10.14	.alpha.-D-Glucopyranoside, methyl	60.00
8	8.660	2.44	Pentadecanal-	68.05
9	9.501	4.51	Pentadecanoic acid	43.05
10	10.673	5.29	11,14-Eicosadienoic acid, methyl ester	67.05
11	10.725	3.90	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	79.05
12	12.877	0.98	Benzonitrile, m-phenethyl-	91.05
13	15.298	3.98	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15	69.05
14	16.277	0.94	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dim	137.05
15	17.158	2.22	.gamma.-Tocopherol	151.05
16	17.944	6.05	dl-.alpha.-Tocopherol	165.05
		100.00		

Table 4.8: Phytochemicals identified in methanolic extract of *Rosa brunoii*

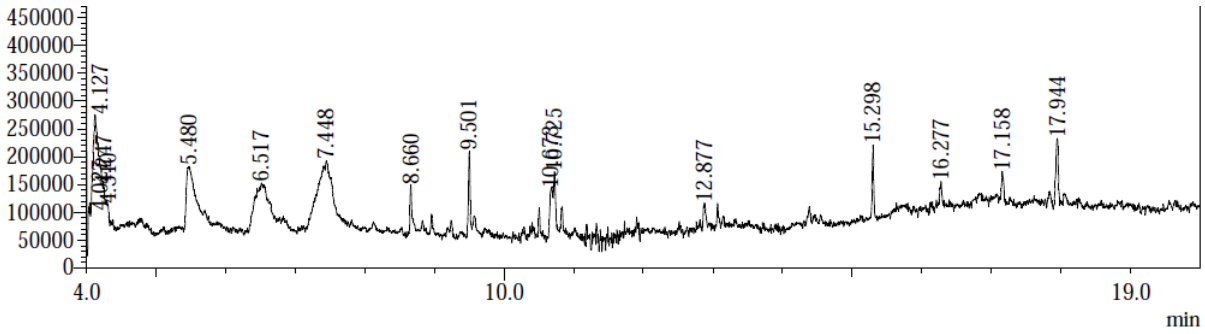


Figure 4.8: Chromatogram of *Rosa brunoii*

GC-MS Analysis of Rosa species

Different types of compounds identified of plant extracts Rosa species(white), the maximum retention time shown by Lupeol;4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5;gamma-Sitosterol. The result shown by 15 different compounds are shown below.

Peak#	R.Time	Area%	Name	Base m/z
1	5.610	9.29	1,2,3-Benzenetriol	126.05
2	6.624	3.51	L-Mannose	60.00
3	7.944	8.91	.alpha.-Methyl-D-mannopyranoside	60.00
4	9.513	17.28	3,4,5-Trihydroxybenzhydrazide	153.00
5	10.521	4.30	Phytol	71.05
6	10.711	7.43	cis-Vaccenic acid	55.10
7	11.073	3.42	17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,	43.05
8	12.893	0.45	1,2-Propanediol, 3-benzyloxy-1,2-diacetyl-	91.05
9	14.405	1.45	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy	67.05
10	16.293	0.57	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dim	137.05
11	17.978	3.29	dl-.alpha.-Tocopherol	165.05
12	20.478	4.63	.gamma.-Sitosterol	43.10

13	21.161	3.29	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,	218.10
14	21.498	2.85	Lupeol	95.05
15	21.924	29.33	Lupeol	95.10
		100.00		

Table 4.9: Phytochemicals identified in methanolic extract of *Rosa spp* (white)

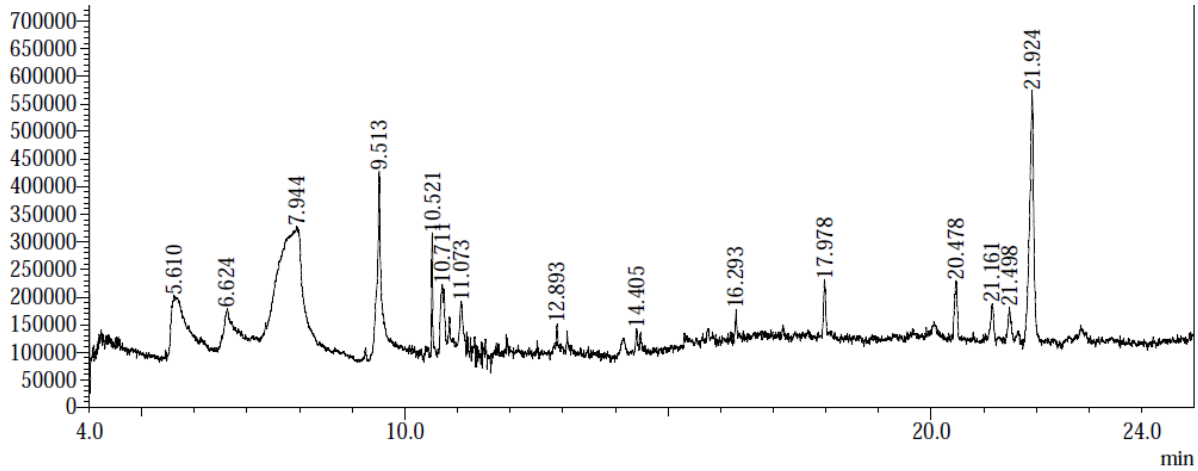


Figure 4.9: Chromatogram of *Rosa species* (white)

GC-MS Analysis of Tinospora cordiofolia

Different types of compounds identified of plant extracts *Tinospora cordiofolia*, the maximum retention time shown by 5H-3,5a-Epoxynapth[2,1-c]oxepin,dodecah; 9,19-Cyclolanost-24-en-3-ol,(3,beta); Urs-12-ene. The result shown by 30 different compounds are shown below.

Peak#	R.Time	Area%	Name	Base m/z
1	7.827	0.78	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	194.05
2	8.199	2.72	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyp	137.10
3	8.670	0.84	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	68.10
4	9.600	4.38	Pentadecanoic acid	43.10
5	9.944	1.29	Benzenemethanol, 2,5-dimethoxy-, acetate	210.00

6	10.393	0.67	9,12-Octadecadienoic acid, methyl ester	67.05
7	10.447	0.88	11,14,17-Eicosatrienoic acid, methyl ester	79.05
8	10.526	1.11	Phytol	71.05
9	10.829	7.07	Oxacycloheptadec-8-en-2-one	67.05
10	12.916	0.71	Trifluoroacetoxy hexadecane	57.10
11	13.122	2.42	Hexadecanoic acid, 2-hydroxy-1-(hydroxym	43.10
12	14.357	2.28	1-Heneicosanol	57.10
13	14.461	2.90	9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy	55.05
14	14.529	1.51	Methyl (Z)-5,11,14,17-eicosatetraenoate	79.10
15	15.846	18.32	1-Octacosanol	57.10
16	16.345	0.66	Carinol	137.10
17	17.310	0.73	3-Chloro-5-cholestene	43.05
18	17.542	22.72	1-Octacosanol	57.10
19	18.041	1.45	dl-.alpha.-Tocopherol	165.10
20	18.313	0.80	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,7,8-tri	151.10
21	18.602	0.78	1,30-Triacontanediol	55.05
22	19.397	2.56	Campesterol	43.10
23	19.731	4.89	1-Octacosanol	57.10
24	20.226	1.83	Ergosta-8,24(28)-dien-3-ol, 4,14-dimethyl-, (	55.05
25	20.610	4.92	.gamma.-Sitosterol	43.10
26	20.864	1.72	Lupeol	69.10
27	21.173	3.68	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimet	55.05
28	21.281	2.06	Urs-12-ene	218.10
29	21.746	1.69	9,19-Cyclolanost-24-en-3-ol, (3.beta.)-	69.05
30	21.977	1.64	5H-3,5a-Epoxynaphth[2,1-c]oxepin, dodecah	218.10
		100.00		

Table 4.10: Phytochemicals identified in methanolic extract of *Tinospora cordifolia*

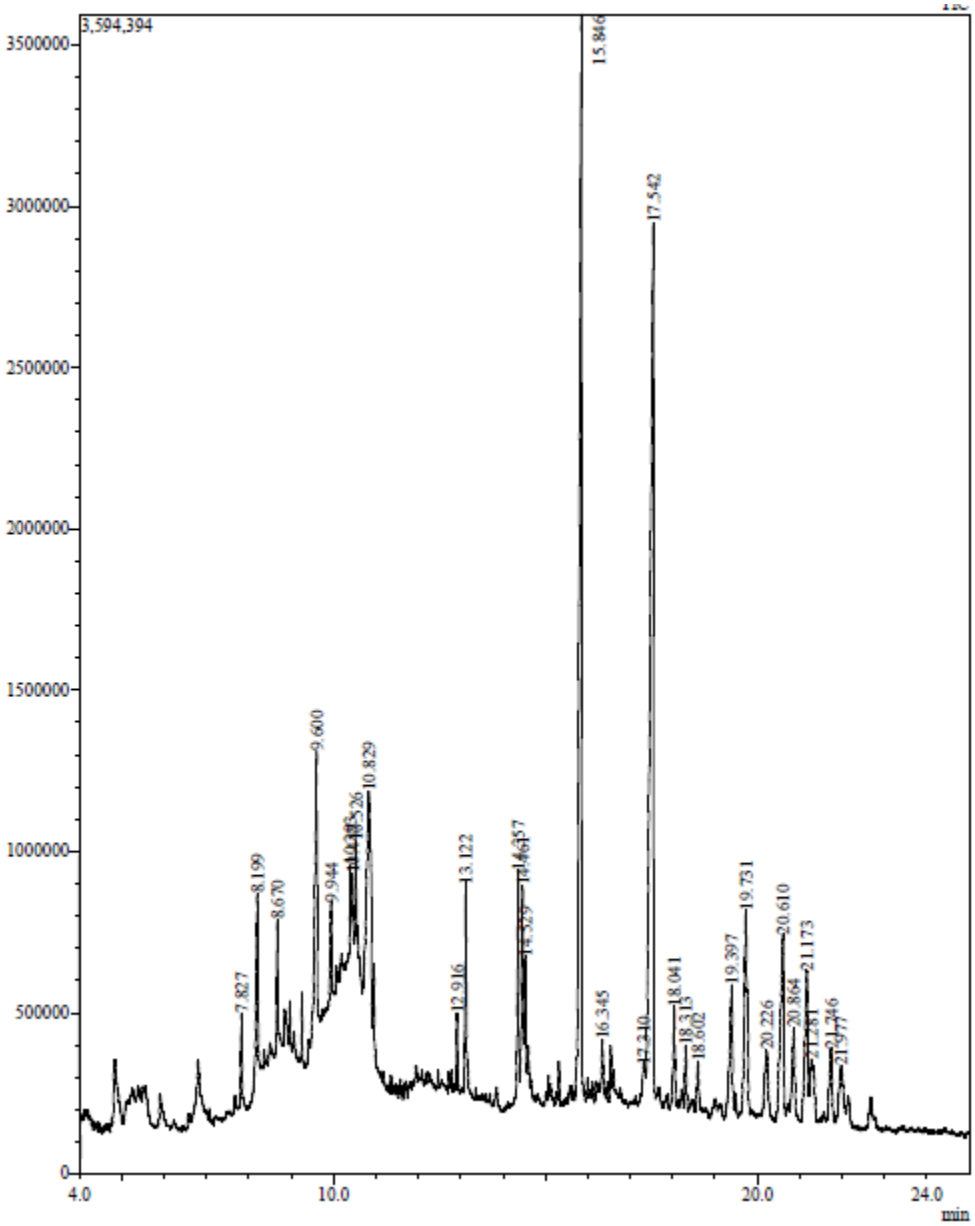


Figure 4.10: Chromatogram of *Tinospora cordifolia*

5. Discussion

Due to the diversity of geographical condition a lots of medicinal plants are found. Different plant parts are used as extracts. Those extracts are soluble to different organic solvents. The bioactivity of extract is relatively preserved and used for the investigation. Methanol is more polar and ethanol and slightly less polar then water so that it can dissolve both polar as well as nonpolar compounds. Methanol also has less boiling point so it is easy to evaporate in short period of time (Nayak et al., 2014).

Extract was subjected for different types of estimation and testing so that different types of interpretation can be done. Different types of solvent can be used but methanol was used as it is the most effective organic solvent comparatively. From the preliminary study of the different types of testing that signifies that the plant extracts contains secondary metabolites, these types of test help to move ahead for different analysis that can be carried out for the research on the respected plants. Extraction method is influenced by many factors like plant parts, geographical locations, harvest season storage conditions as well as choice of solvent extraction (Gong et al., 2012).

5.1 Phenolic and Flavonoid content

Phenolics are widely found chemicals in all parts of plants including flowers, fruits, seeds, bark, root, stem, leaves. They are considered as secondary metabolites formed against predation and protection these secondary metabolites may consist of broad range of phyto chemicals such as flavonoids, alkaloids, polysscharides, saponins, glycosides, steroids, lipids and tannins (Mothana et al., 2010). There are about 8000 phonolics are known which form stable phenoxyl redicals, hence they play important role in antioxidant activity by reducing oxidative stress and reduces risk of different types of disease (Vita, 2005).

Flavonoides are present in edible fruits as plant glycosides they are exhibiting antioxidative anticarcinogenic antic inflammatory antiaggregatory property (Erlund, 2004). Phenolic hydroxyl groups present in flavonoid is effective for free radical scavenging activity (Panat et al., 2016). We determine total flavonoid content by spectrophotometrically using aluminium chloride in presence of potassium acetate. Lower amount of flavonoid detection might be because of different factors like reaction time, the concentration of the reagent and the chemical structure of polyphenol (Fernandes et al., 2012).

Rosa species (purple) has the highest phenol content from the Spectro photometrical analysis. The total flavonoid content estimation method followed was alumunium chloride method which involves chelation of aluminium atom of AlCl3 by the flavonals and flavonas therby forming deep yellow coloration (Chang et al., 2002). Lower flavonoid content of this plant may be because of phenol in plant extracts. The estimation method can also be the reason for some kind of fluctuation in result due to handling errors. The plant flavonoid is not found in free state, so that AlCl3 is required to chelate. Flavonals and flavonas exist in glycosides such that their hydroxyl groups often remain sequestered and are not readily available for chelation which may lead to plant extract lacking any flavonoid (Mammen and Daniel, 2012).

The estimation of polyphenol content was done by using Folin-Ciocalteu method. This method was based on transfer of electron from phenols compound to phospomolybidic acid present in F-C reagent in alkaline condition (Ainsworth and Gillespie, 2007). During the reactions, yellow color of the F-C reagent changes to blue color complex between the F-C reagent and the phenolate ion. The intensity of blue color complex correspond to amount of phenolate ion generated during the reaction, so the experiment is in higher absorbance at 765nm showing higher content of phenol. In *Rosa species*, the phenol content was high and similar type of reading was observed. *Rosa species* has higher phenol content but flavonoid content was fluctuating from higher to lower reading. *Tinospora cordifolia* has good flavonoid content as per the observation.

5.2 Antioxidant activity

Among four different plants, the IC50 value was observed in mean weight with SD so all those plants has similar types of reading between 50µg/ml to 60µg/ml. In *Tinospora cordifolia*, the value was 52.54±0.73 that shows that it has good antioxidant activity in DPPH aasay. A good correlation between total phenol content and antioxidant potential of the extract was seen in the study suggesting antioxidant potential showed by plants due to their polyphenolic content (Satio et al., 2004).

DPPH scavenging potential is quiet lower than other assays but electron transfer based on assay are not much comparable. The result obtained because of different mechanisms, redox potential, pH and solvent dependencies, etc. (Thaipong et al., 2006). Antioxidant property

can be inferred on the basis of % Radical scavenging activity and IC50 value. Antioxidant activity of DPPH inhibition is expressed as % inhibition of stable radical or inhibition concentration 50 (IC50) in reference to standard compound. The plant with higher % RSA has lower IC50, the plant extract with lower IC50 value consider having better antioxidant property (Abou-Gazar et al., 2004).

5.3 Antimicrobial activity

Plants are rich source of chemical compounds which are already identified by identification test. Each constituent possess its effect against microorganisms, tannins and flavonoids are well known constituent of plants that shows effective activity against bacteria and fungi (Laxmi et al., 2011).

Antimicrobial screening of plant extracts was conducted by various authors and their activities were reported against various organisms. The activity of methanol extract crude rectified spirit extract and ethanol extract shows effective against bacteria. Different types of bacteria like *E.coli*, *Staphylococcus*, *Klebseilla* are selected for this study to observe effect of plant extract against them. *Rosa species* show good effect against micro bacteria on concentration of 100µg/ml. Similarly, *Tinospora cordifolia* also shows its effect against microorganisms so that we took positive control as antibiotic and negative control as methanol. This helped to analyze whether the effect was due to extract or methanol so we can compare them against antibiotic by observing inhibition zone (Shanti et al., 2013).

Tinospora cordifolia shows effect against Urinary tract infection causing bacteria so this type of plant helps to inhibit growth of microorganisms. They are more susceptible to *E.coli* and less susceptible to *S. aureus* (Priyanka et al., 2015).

Herbal medicines are valuable and readily available resource for primary health care though the exact activity was to be known, the possible activities are shown by these plants to get some results. Extracts from almost all parts of the plants can show inhibition zone, it should be further studied to determine the active compounds that are responsible (Umesh et al., 2012).

5.4 Cytotoxicity effect

Cancer was responsible for 8.8 million deaths (WHO 2015) and about 70% of deaths from low and middle income countries. Due to drugs resistance and toxic effects of chemotherapeutic drugs, researchers are on focusing on alternative source phytochemicals to discover new anticancer compounds. MTT assay is based on the Dehydrogenase enzyme produced by mitochondria and endoplasmic recticulam of viable cells. The enzyme converts yellow color MTT tetrazolium salt to purple insoluble formazan complex (Fotaxis and Timbrell 2006). Medicinal plant can be considered as a safer alternate for the synthetic drug but their safety cannot be guaranteed always. If the pharmacologically active compound which showed toxicity against tumor cells, might be toxic to normal cells to including other normal and immune cells. Hence, any drug or drug formulation has to go through the toxicity test, in vitro test, in vivo test and finally clinical trial. So, preliminary safety of extract under the study of toxicity against different cell lines to cancer cell line can be done (Rumana et al., 2015).

Tinospora cordifolia is known for treatment of different kinds of diseases. It helps in immune system modulator and it also shows in vitro cytotoxicity effect. Only few study have reported anticancer activity of this plant but cytotoxic effect of methanol extract is reported thus it also helps in breast cancer cells by inducing apoptosis and shows cytotoxic effect. The cytotoxic effect was higher in this plant rather than other Rosa species. The value of *Tinospora cordifolia* was 128.47±44.42 and the maximum value of *Rosa species* (white) was 110.76±21.54, which are the maximum concentration required to inhibit the cells by both of them. The *Rosa species* (purple) and *Rosa brunoii* have lower value of inhibition so that they required in lower concentration of extracts to inhibit the cells (Mohsin et al.,2017).

5.5 GC-MS Analysis

GC-MS analysis helps us to identify different types of compounds with maximum peak in retention time. There are major compounds identified with maximum peak in retention time are sugars, fatty acids, esters, sterol, phenolics and heterocylic compounds. Among four different medicinal plants, *Tinospora cordifolia* has highest different kinds of compounds characterized. 30 different types of compounds are identified.(Albinjose et al., 2015). The fatty acidsreported to exhibit antioxidant, hypocholesteric, anti-inflammatory, antibacterial, inhibitors activities etc (Sermakkani and Thangapandian 2012).

The GC-MS analysis helps the extract to identify with different types of compounds in different individual extracts. In Rosa species, the maximum 16 different compounds are identified in *Rosa species* (purple) and *Rosa brunonii* whereas *Rosa sp.* (white) has 15 components identified. The volatile oils are obtained by hydrolization of petals of *Rosa damiscena*. Variation in essential oil depends upon the genetic variation, climate, geography, stage of plant growth and environmental factors (Kamran et al., 2014).

6. CONCLUSION

In this study, traditionally and ornamentally important medicinal plants are taken for different types of analysis. Estimation of polyphenols, flavonoid was carried out by using standard concentration of compound and the plant extract concentration was calculated according to their mean weight and SD. Similarly, percentage of radical scavenging activity was also done using DPPH assay to find percentage inhibition of plant extract in IC50 value which was also expressed in mean weight and SD. Then, the respective reading helped to find estimation of compounds, qualitatively and percentage of inhibition of radicals. These plants have some good chemical compounds that can lead us for further analysis of different activities.

Further, the extract of different concentration on different microorganisms that are pathogenic to human health with positive control as relative antibiotics, was tested. It showed inhibition zone with certain length that concluded antibacterial effect by plant extracts. Plant extracts with different concentration was taken for cytotoxicity effect, it seemed to have good cytotoxic effect to VERO cell line. Finally, absorbance was taken with given range and the reading was analyzed.

Whole data interpretation, graphical illustrations was carried out with statistical analysis and calculation. All the data are taken triplicate and the mean value was calculated to determine the graph plot which helps to find the exact amount for the analysis of the mean value.

The determination of the chemical constituents can be done with the help of GC-MS analysis which helps to know the possible relative compound that are found on the plant extracts.

This research recommends the study of the more medicinal plants with extensive study using modern equipment and analytical tools. Different kinds of assay and comparative study can be done which helps to identify important compounds, oils, and other compounds that are noble. These types of plants extracts help in the treating of different kinds of diseases such as diabetes, urinary tract infections, atherosclerosis, cancer etc.

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APPENDIX

A list of Reagents and Culture media

1. Preparation of 1 M Na2Co3-100 ml

10.599 gram of Na2Co3 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 Glacial acetic acid was taken and mixed with ethanol. Finally the volume was adjusted to 200ml by adding ethanol.

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

10 gm of commercially supplied aluminuim chloride was weighted and dissolved in water. Finally volume was maintained to 100 ml .

4. Preparation of 1M Potassium acetate- 100ml

9.81 gm of potassium acetate was dissolved in water and the volume is finally maintained to 100 ml by adding water.

5. Preparation of 0.1 mM DPPH – 100ml

3.94 mg of DPPH was dissolved in methanol and volume was maintained to 100 ml. 1,1-diphenyl-2-picryhydrazyl (DPPH) has molecular weight 394.32 gm/mol .

6. Preparation of Follin-Ciocalteu phenol reagent (1:10 dilution)

6ml of commercially supplied Follin-Ciocalteu phenol reagent was taken and mixed with 54ml of distilled water to prepare 60ml of Follin-Ciocalteu phenol reagent (1:10 dilution).

7. Composition of Nutrient agar media (NA)

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 Luria Bertina broth(LB) Miller Media

Components	Gram/L
Casein enzyme hydrolysate	10
Yeast extract	5.0
Sodium chloride	10
pH	7.5±0.2

9. Composition of Mueller Hington Agar (MHA) media

Components	Gram/L
Beef infusion form	300
Casein enzyme hydrolysate	17.5

Starch	1.56
Agar	17
pH	7.3±0.2

10. RPMI (Roswell Park Memorial Institute) complete medium

RPMI powder	10 gm
NaHCO3	2gm
HEPES	1.4gm
L-Glutamine	2mM
TDW	1L
Gentamycin	20µg/ml
Streptomycin	100µg/ml
Penicillin	100U/ml
pH	7.4