CHAPTER ONE: INTRODUCTION

1.1 Background

Medicinal plant can be defined as any plant with one or more of its organ, containing substances that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs (Sofowora, 1982). Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. Nearly 80% of the world's population usedtraditional medicines for primary health care, most of which involve the use of plant extracts (WHO, 2008). Almost 90% of the prescriptions were plant based in the traditional systems of Siddha, Unani, Ayurveda and Homeopathy (Patwardhan *et al.*, 2004). In rural areas of the developing countries, medicinal plants are used as the primary source of medicine (Chitme *et al.*, 2004). Additionally, medicinal plants are the richest bio-resource of drugs of traditional medicine, modern medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al.*, 2008). Nepalese medicinal plants have a long history since Vedic period when Nepal Himalaya was referred as sacred heaven of the potent medicinal and aromatic plants (Watanabe *et al.*, 2013; Baral and Kurmi, 2006). However, scientific documentation of medicinal plants has been started from the end of 19th centrury. In this regard, Shrestha *et al.*, (2000) listed 1,630 species, Baral and Kurmi (2006) listed 1,792 species and Ghimire (2008) reported 1,950 species of medicinal plants distributed in different parts of Nepal. Recent study suggested that the number of medicinal plants will be more than documented and roughly estimated around 20-28% expecting 7,000 species of flowering plants in Nepal (Watanabe *et al.*, 2013). Among the available species of medicinal plants, 1,463 species are used by the rural people in Nepal (MoFSC, 2006). These medicinal plants with curative properties are used to treat various diseases.

1.2 Mistletoes and their medicinal value

Mistletoes are highly specialized flowering plant of sandalwood family (Santalaes), that exploit and (or) parasitize a wide range of host plants (Mathiasen *et al.*, 2008;

Devkota *et al*., 2011). Some mistletoes parasitize on very large number of hosts in different families whereas some dwarf mistletoes parasitizes on one host species. Despite their harmful effects on the host plants, mistletoes have been considered as important components of plant diversity and forest ecosystems throughout the world (Kujit, 1964; Calder, 1983).

Mistletoes are found all over the world with wide range of habitat and their distribution is governed by climatic factors, forest edge, and forest disturbance, availability of suitable host species and behavior of avian dispersers (Devkota *et al*., 2011). However, Kujit (1964) reported that the occurrence of some mistletoes at higher elevation is limited by environmental factors and is not related to host preference. Similarly, Abulfatih and Emara (1988) recorded mistletoes restricted to its specific host between 2000-2400m. Unlike these observations, Kujit (1969) suggested the important of bird's behavior and concluded that distribution of mistletoes entirely depends upon the habits of the birds that disseminate the seeds.

The medicinal effects of mistletoes are often attributed to the antioxidant activity of the phytochemical constituents, mostly the phenolics (Osawa, 1994). Mistletoes are occasionally used for food or beverage and for a variety of medicinal purposes for humans and animals around the world (Mathiasen *et al.,* 2008). These are extensively applied for curing muscular swelling, sprains, fractures, dislocations etc. (Kunwar *et al.,* 2005). Some literature has accrued on the medicinal values of mistletoes (Hawksworth and Scharpf, 1987; Yang e*t al*., 1987; Obatomi *et al.,* 1994). Mistletoes are medicinally important and are used in different countries for different purposes. In Africa, mistletoes are used in treatment of various stomach troubles of children including diarrhea. The people of ancient Greek and Argentina uses these plants in spleen diseases and for problems related to menstruation, respectively. However, Indian people used mistletoes as a tea against diabetes (Bussing, 2003).

1.3 Phytochemicals

Many of the higher plant constituents which are listed can be categorized as flavor substances, plant volatiles, natural sweeteners, plant poisons, carcinogens, coloring matters, oestrogenic agents, natural hallucinogens, phytoalexins or allelochemicals (Harborne, 1998). Alkaloids are a structurally diverse group of over 12,000 cyclic nitrogen containing compounds that are found in over 20% of plant species. Although

no single classification exists, alkaloids are often distinguished on the basis of a structural similarity (e.g. indole alkaloids) or a common precursor (e.g. benzylisoquinoline, tropane, pyrrolizidine or purine alkaloids) (Zulak *et al.*, 2006).

Plant produces secondary metabolites within themselves as a defense mechanism to protect their existence by their own. Such secondary metabolites produced by plants are equally important for the human beings because of their medicinal values. The plant products like terpenoids, phenolics and flavonoids are of great importance. The terpenoids perhaps are the most structurally varied class of plant natural products. The terpenoid derives from the fact that the first members of the class were isolated from turpentine. All terpenoids are derived by repetitive fusion of branched five carbon units based on isopentane skeleton (Croteau *et al.,* 2000). Similarly, plant phenolics are generally characterized as aromatic metabolites that possess or formerly possessed, one or more acidic hydroxyl groups attached to the aromatic (phenyl) ring. Most plant phenolics are derived from the phenylpropanoid and phenylpropanoid acetate pathways. Numerous phenolics also play defensive roles or impact characteristic taste and odors to plant material (Croteau *et al.,* 2000). However, the flavonoids comprise an astonishingly diverse group of more than 4500 compounds. Among their subclasses are the anthocyanins (pigments), proanthocyanidins or condensed tanins (feeding deterrents and wood protectants), and isoflavonoids (defensive products and signaling molecules). The coumarins, furanocoumarins, and stilbenes protect against bacterial and fungal pathogens, discourage herbivore and inhibit seed germination (Croteau *et al.,* 2000). Flavonoids have been shown to exhibit the antioxidative, antiviral, antimicrobial, antiplatelet and antitoxic activities (Middleton and Kandaswami, 1994). Lignin is a class of dimeric phenypropanoid (C_6C_3) metabolites linked by way of their 8-8′ bonds (Croteau *et al.,* 2000). The biological activities of these polyphenols in different systems are believed due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen's, or decomposing peroxides (Osawa, 1994). Mistletoes contains phytochemicals like anthraquinones, saponins, tannins and alkaloids whose antimicrobial properties has been documented (Deeni and Sadiq, 2002). The most distinctive constituents of most European and Asian mistletoes are proteins, the viscotoxins, lectins and carbohydrates. The small molecular weight compounds include flavonoids and phenylpropanoids if varying structural types, and

they contribute to the anti-oxidative properties of the various Loranthaceae extracts examined (Adesina *et al.,* 2013).

1.4 Antioxidant properties

Antioxidant is a compound that has ability to inhibit oxidation rate or to neutralize a free radicals. Rapid production of free radicals can lead to oxidative damage to biomolecules (e.g. lipids, proteins and DNA) and may cause aging and diseases, such as atherosclerosis, diabetes, cancer, cirrhosis, inflammatory diseases, asthma, cardiovascular diseases, neurodegenerative disease and premature aging (Halliwell and Gutteridge, 1984; Addis and Warner, 1991; Halliwell, 1994; Niki, 1997; Poulson *et al.,* 1998; Young and Woodside, 2001). Preventive antioxidants attempt to stop the formation of reactive oxygen species (Sies, 1997). Both reactive nitrogen species (RNS) and reactive oxygen species (ROS) are common forms of free radicals. Examples of ROS are hydroxyl radical (OH•), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) as well as the peroxyl radical (HO_2•), whereas nitrogen dioxide radical (NO_2) and nitric oxide radical (NO) are some examples of RNS. However, antioxidants neutralize the effect of free radicals through different ways and may prevent the body from various diseases (Gupta and Sharma, 2006). Imbalance between the intensity of oxidative processes (that induce the formation of reactive oxygen species) and counteracting antioxidant system is called oxidative stress. Most of the pathological changes in living organisms is associated with the processes of carcinogenesis induced by free radicals. State of equilibrium is maintained due to the presence of antioxidant enzymes (e.g. superoxide, dismutase, and peroxidase) and other biologically active substances such as glutathione, ascorbic acid and beta-carotene. These compounds enable the removal of reactive oxygen species in cells (Barbasz *et al.,* 2012).

Antioxidant activity has measured using DPPH free radical scavenging assay (Yen and Chen, 1995). Antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage (Fang *et al.,* 2002). Antioxidant substances block the action of free radicals which have been implicated in the pathogenesis of many diseases including atherosclerosis, ischemic disease and in the aging process (Aruoma, 2003).

1.5 Antibacterial properties

Biodiversity leading to many different phytoconstituents in plant species, a wide variety of pharmacological activities have been the basis for herbal medicines. This includes plant derived drugs for the treatment of different ailments and diseases as a significant portion of modern medicine (Wang *et al.,* 2012). Antimicrobial analysis of various medicinal plants have shown that the phenolic, alkaloids, steroids, glycosides, saponins, flavoinds, essential oils and other resins presents in these plants contains effective bioactive compounds against multiple pathogens. Plant inhibits microorganisms, interfere with some metabolic processes or may modulate gene expression and signal transduction pathways (Manson 2003; Surh, 2003). The effect of plant extracts on different bacteria have been done by huge number of researcher in world but very few work has been done in Nepal (Panthi and Chaudhary, 2006). Many plants have been used because of their antimicrobial characters, which are due to compound synthesized in the secondary metabolism of the plants. For example, phenolic compound which are part of the essential oil as well as in tannin (Janssen *et al.,* 1987; Saxena *et al.,* 1994).

Plants are being used in treatment and management of number of disease, both in traditional and in complementary medicine, in several cultures (Pterus, 2011). The lectinic and mitogenic properties of the Loranthaceae family and the capacity of many plants producing antibiotics to produce mitogens are known (Lewis and Elvin-Lewis, 1977). Both gram positive and gram negative bacterial activity showed by the organic solvent fraction of mistletoe from its twigs and leaves (Hussain *et al.,* 2011; Oguntoye *et al.,* 2008).

1.6 Hypothesis

Different species of mistletoes contains active constituents like flavonoids, phenolics, antioxidants and other important compounds.

1.7 Aims and Objectives

The main objective of the study is semi-quantitative estimation of major compounds in seven species of mistletoes and accessing their bioactivities. This objective is achieved through following specific objectives:

- To explore the distribution of mistletoes in Nepal (using secondary data).
- To perform qualitative phytochemical screening of those plant species.
- To estimate total flavonoids and phenolics in their methanol extracts.
- To evaluate *in vitro* antioxidant properties.
- To evaluate antibacterial properties from the plant extracts.

1.8 Rationale of the study

Plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased.In Nepal, mistletoes are being used traditionally since ancient time (HMGN, 1970). Therefore, such plants seek the proper characterization of their useful compounds to validate their traditional uses and further exploration of essential constituents can open their prospect of greater pharmaceutical uses. Being plant group of different kind with their amusing life cycle or origin, when get phytochemically characterized can come up with the findings of newer biochemical constituents to adapt themselves in unique habitat and habit. The utilities of such plant based chemicals will be enormous and sure to relish the life of an organism. But, most of mistletoes species are not studied up to pharmaceutical level in Nepal.

There are countable records of phytochemical screening of mistletoes from other countries whereas, Nepalese mistletoes lag behind in this respect though it behold rich pocket in terms of species number. Most of phytochemically characterized mistletoes species are being used in curing number of diseases. Similar, possibilities of greater use for our species do exist but is uncovered or has remained in its very juvenile stage which, needs to step forward by phytochemically characterizing the maximum number of species. The research is conducted with this entire motive. The approach of their in- vitro antioxidant and antibacterial assay attempted is destined to further illustrate the necessity of mistletoes to the level of intensive research as there is a wide spectrum of antimicrobial activity of mistletoes against multiple drug resistant bacteria.

Hence, present study will be an initial study towards the characterization of bioactive compounds of indigenously favored group of plant species i.e. mistletoes along with study of their antioxidant and antibacterial properties.

1.9 Limitation of the study

- Only methanol solvent was used for the extraction.
- Due to the unavailability of chemicals qualitative phytochemical screening was done for only few compounds.
- Antioxidant activity was determined by only DPPH free radical scavenging assay.

CHAPTER TWO: LITERATURE REVIEW

2.1 Mistletoes diversity and their medicinal value

About 1500 species of mistletoes occur in the world and 19 species has been reported from Nepal (Nickrent, 2011; Devkota, 2005). There is an amazing gap of knowledge on the misteltoes of Nepal Himalayas, a biogeographically interesting transition zone between Eastern and Western Himalayan flora (Stearn, 1960).

The approximately 1300 species of mistletoes are distributed among four plant families and range in size of their aerial shoots from small (e.g., *Arceuthobium minutissimum* Hook. F., < 4 mm) to large (e.g., *Viscum album* L. and many *loranths*> 1 m) (Combes, 2001).

Eighty nine species of mistletoe are native to Australia (Barlow, 1996; Downey and Wilson, 2004). Thirteen species belonging to the Loranthaceae, commonly parasitizes eucalypts, most with a wide geographic distribution and host range (Reid and Yan, 2000).

After adding five new, previously unrecorded, mistletoes species to the flora of Nepal by Devkota and Glatzel (2005), Devkota and Koirala (2005), the inventory has been enriched from fifteen to twenty species. Devkota and Acharya (1996) reported 46 host species of mistletoes belonging to 25 families in Kathmandu valley; Devkota and Glatzel (2005) documented 95 host species belonging to 45 families in the Annapurna Region and 69 host species of 38 families were recorded by Devkota and Kunwar (2006) from Godawari-Pulchowki area of Kathmandu Valley in Central Nepal.

In Nepal mistletoes have traditionally been used by indigenous people since long but they have remained unused by the modern pharmacological practice. Mistletoes like *Dandrophthoe falacata, Viscum album* and *V. articulatum* was first documented in Nepal which are used by indigenous people (HMGN, 1970). Out of 19 species, 11 species are being used by indigenous people for different purpose (Manandhar, 1991, 1993, 2002; IUCN, 2004; Devkota, 1997; Kattel and Kurmi, 2004). O'Neill & Rana (2016) reported that among 15 collected species of mistletoes from Nepal central Himalaya, among them 10 species have medicinal value.

2.2 Phytochemical of Mistletoes

The methanolic extract of the aerial part especially leaves of *Viscum album* Linn. showed major biologically active phytoconstituents such as alkaloids, glycosides, saponins, polyphenols, flavonoids, tanins, terpenoids and cardiac glycosides (Kusi *et al.,* 2015).

Fukunaga *et al.,* (1989) worked on three different species of Japanese mistletoes i.e. *Taxillus yadoriki* Danser, *Taxillus kaempferi* Danser and *Korthalsella japonica* Englar. They reported that *T. yadoriki* contain flavonoid, glycosides, hyperin and quercitrin together with fatty acids, phytosterol and phytosterol-glucoside. Similarly, *T. kaempferi* contains fatty acids, phytosterol, phytosterol-glucoside, quercetin, avicularin, taxillusin, quercitrin and hyperin. In case of *K. japonica,* flavone glycoside, chrysoeriol-4′-O-glucoside together with fatty acids, phytosterol, oleanolic acid, and phytosterol-glucoside has been reported.

Moghadamtousi *et al.,* (2014) have also isolated another family of chemicals known as coriaria lactones from the *Loranthus parasiticus.* According to Lim *et al*., (2016) *Scurrula* and *Viscum* species have anticancer, antimicrobial, antioxidant and antihypertensive properties and the genus *Scurrula* inhibit cancer growth due to presence of phytoconstituents such as quercetin and fatty acid chains. Amer*et al.,* (2012) found two novel amino-alkaloids namely 4,5,4'-trihydroxy-3,3'-iminodibenzoic acid and 4,5,4',5'-tetrahydroxy-3,3'-iminodiabenzoic acid have been isolated and characterized. The alkaloids hyoscine, anabasine, nicotine and isopelletierine have been reported from an Australian mistletoes (*Benthamina alyxifolia* (Benth.) Tieghem) growing on *Duboisia myoporoides* (Trautner, 1952; Mortimer, 1957), a small tree of the family Solanaceae that produces these alkaloids.

The presence of protein in ethyl acetate residue fractions of crude petroleum ether extract of *Loranthus micranthus* Linn. whereas flavonoids, steroids and terpenes were present in all the chloroform, ethyl acetate and ethyl acetate residue fractions but in a low quantity (Cemaluk *et al.,* 2012). Phytochemical screening of *Viscum album* showed that the presence of alkaloids, carbohydrates, tanins and flavonoids (Oguntoye *et al.,* 2008). Crude methanolic extract from leaves of *L. micranthus* harvested from *P. americana* was found to possess terpenoids, steroids,

oils, proteins, resins, flavonoids, tannins, saponins, alkaloids, reducing sugar, acidic compounds, glycosides, and carbohydrates (Moghadamtousi *et al.*, 2014).

2.3 Antioxidants properties of Mistletoes

The total phenolic content of stem, leaf and flower extracts of *Scurrula ferruginea* were assessed and the data suggested that the stem extract contained the highest level of phenolic compounds. Furthermore, the phenolic content of the respective plant parts correlates to their antioxidant properties: the highest antioxidant activity was found in the stem, followed by the leaf and flower extracts of *Scurrula ferruginea* (Marvibaigi *et al*., 2014).

The leaves and leafy twigs of the African mistletoes, *Loranthus micranthus* are enriched with polyphenols. Polyphenols and phenolic glycosides isolated from methanol extracts of *Loranthus micranthus* leafy twigs parasitic on the host *Hevea brasiliensis* demonstrated significant antioxidant activity through a 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay. Among the tested polyphenols, tutin and peltatoside in particular exhibited the most prominent radical scavenging activity (Agbo *et al.,* 2013).

The methanolic extract of *Viscum album* collected from *Malus dimestica* exhibited the highest antioxidant activity with 0.14±0.12 mg/l (Vicas *et al.,* 2009). According to Onay-Ucar *et al.,* (2006), antioxidant capacity of *Viscum album* differed according to the harvesting time as well as the host tree. *Lornathus parasiticus* have identified antioxidantive, antimutagenic, antihepatotoxic and antinephrotoxic activity (Moghadamtousi *et al.,* 2014). The methanolic plant extract of *Dendrophthoe pentandra* showed antioxidant activity (Artanti *et al*., 2012).

Katsarou *et al.*, (2012) stated that extracts of *Loranthus europaeus* twigs and stems exhibited higher antioxidant activity in comparison to that of fruits, leaves and flowers. The crude methanolic extract of *Loranthus regularis* has anti-inflammatory, antinociceptive, antipyretic and antioxidant activity (Mothana *et al*., 2012). The methanolic extract of *Loranthus micranthus* leaves parasitic on host *Kola acuminate*, -(-) catechin-7-O-rhamnoside, -(-) catechin-3-O-rhamnoside and 4'-methoxy-catechin-7-O-rhaamnoside are the most potent antioxidant polyphenols (Ogechukwa *et al*., 2012).

2.4 Antibacterial properties of Mistletoes

Marvibaigi *et al.,* (2014) reported that the leaf, flower and stem *Scurrula ferruginea* crude acetone extract had good anti-bacterial activities against a range of gram positive and negative bacteria. Notably, the leaves and stems *Scurrula ferruginea* extract has potent anti-microbial activity against the clinically relevant skin pathogen, *Staphyloccocous aureus*. According to Hussain *et al.,* (2011) and Oguntoye *et al.,* (2008) organic solvent fraction of mistletoe extract from its twigs and leaves showed good antimicrobial activity against both gram positive and gram negative bacteria.

Some studies have shown that direct ingestion of the plant parts especially berries might result in mild effects like nausea, vomiting, bloody diarrhea and shock induced hypertension (Bussing, 2003). Kusi *et al.,* (2015) found the antibacterial screening of methanolic extract of *Viscum album* showed highly effective against *Pseudomonous aeruginosa* with zone of inhibition 16 ± 1 mm compared to 17 ± 1 mm of chloramphenicla (50 mcg). Moghadamtousi *et al.,* (2014) reported their study support the traditional use of the *Loranthus parasiticus* as a neuroprotective, tranquilizing, anticancer, immunomodulatory, antiviral, and diuretic and hypotensive agent.

Osadebe and Ukwueze (2004) tested the *Loranthus micranthus* extract from *Kola acuminate* and *Persea americana* have lesser extent but from *Irvingia gabonensis* showed higher activities against bacteria. *Loranthus micranthus* in Nigeria and South Africa has been widely used as ethnomedicine for treatment of hypertension, diabetes, and schizophrenia and as an immune system booster (Zorofchian *et al.,* 2013). Artanti *et al.,* (2012) described *Dendrophthoe pentandra* methanolic as well as aqueous extracts have antidiabetic activity. Cemaluk *et al.,* (2012) described ethyl acetate fraction showed the highest susceptibility to *Bacillus subtilis*.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Preliminary study of herbarium specimen

Mistletoes are neglected group of plants, to know the distribution status specimens deposited at National herbarium and Plant Laboratories (KATH) were thoroughly observed. The description on specimen sheet was collected for further analysis.

3.2 Research Plan

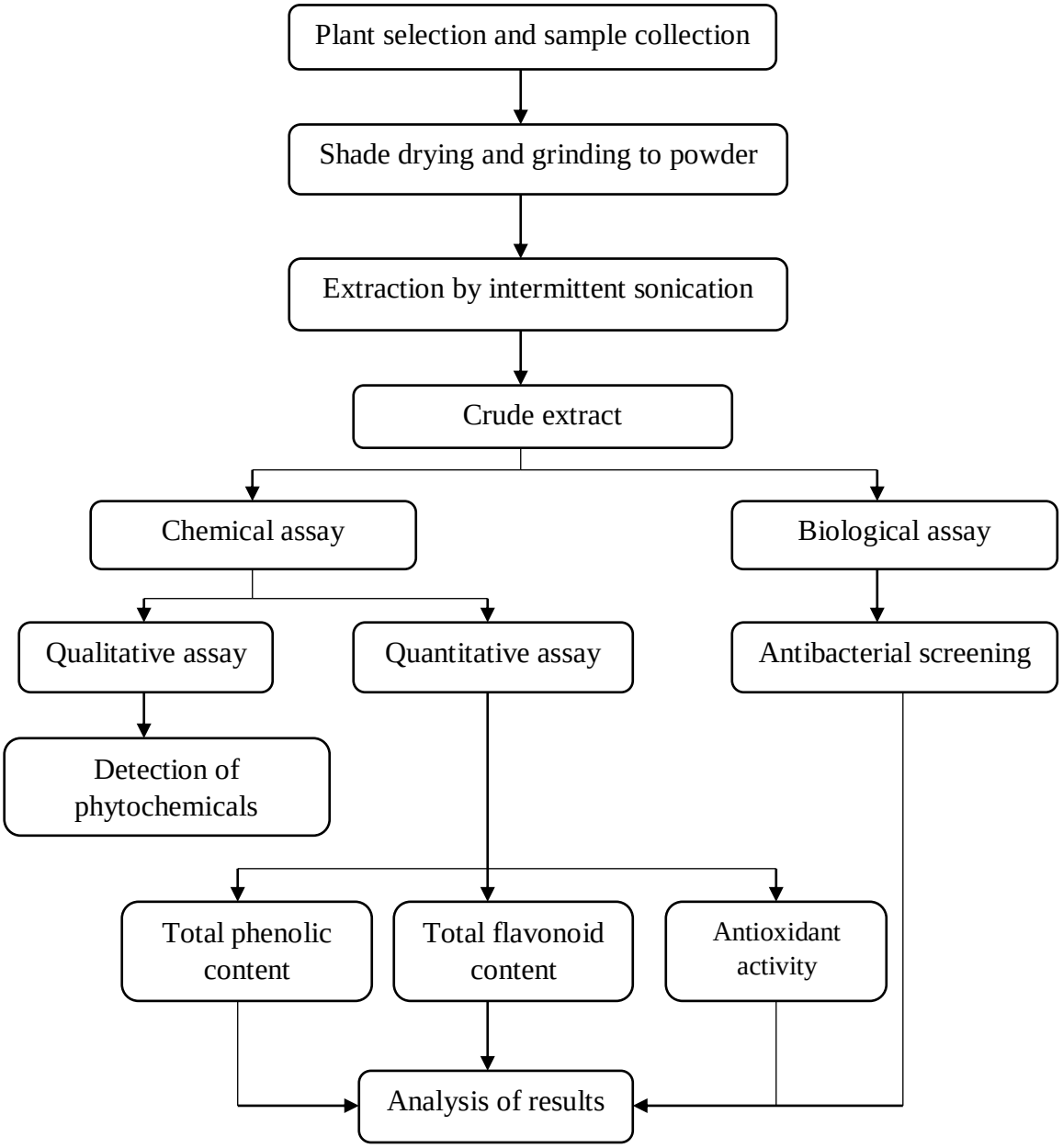

Figure 1: Flow chart showing the research plan

3.3 Collection and identification of Plant samples

Different species of Mistletoes were collected from surroundings of Kathmandu valley (Table 1). The specimens were identified by expert and cross checked with the specimen deposited at National Herbarium and Plant Laboratories (KATH), images of specimens deposited at herbarium of the Department of Botany, TROPICOS (www.tropicos.org), and concerning the book "Flora of Kathmandu Valley". Collected specimen were dried and fixed in herbarium sheet and deposited at Tribhuvan University Central Herbarium (TUCH).

Table 1: List of plants under study with elevation and parts used

S.N	Latin Name	Family	Elevation (m)	Host species	Location	Parts used
1	*Helixanthera ligustrina*	Loranthaceae	1690	*Pyrus pashia*	Below champadevi	Leaf
2	*Macrosolen cochinchinensis*	Loranthaceae	1667	*Schima wallichii*	Suryabinayak	Young Leaf
3	*Scurrula elata*	Loranthaceae	2000	*Myrica esculenta*	Between Godam to Chitlang Dhurali	Leaf
4	*Scurrula parasitica*	Loranthaceae	1933	*Pyrus pashia*	Between Godam to Chitlang Dhurali	Leaf
5	*V. articulatum var. liquidambaricolum*	Viscaceae	2300	*Carpinus sp.*	Between Godam to Chitlang Dhurali	Whole plant
6	*Viscum album*	Viscaceae	2102	*Prunus ceraseidos*	Chitlang	Whole plant
7	*Viscum articulatum*	Viscaceae	2484	*Quercus semecarpifolia*	Chitlang, Dhurali	Whole plant

3.4 Preparation of the plant material

The collected plant materials were air/shade dried at 32-35ºC for 6 days to remove all their moisture. The dried plant materials were powdered with the help of grinder, passed through a wire sieve (porosity 220 microns) and the fine powder collected on sterile and dry polyethylene bag was used for extraction**.**

3.5 Preparation of plant extracts and it′s dilution

Fifteen grams of fine powder of each plant sample was weighed separately and dissolve in 150 ml of methanol. Those mixtures were placed in a sonicator (UV-

Chromtech sonicator) for 2 hours. After 2 hours the solution was filter with the help of Whatmann No. 1 filter paper. Then added same volume of methanol to the residue for complete extraction and left it for another 1 hour 30 minutes for sonication. Again methanolic extract of each sample was filtered with the help of Whatmann No.1 filter paper. The filtrate was allowed to evaporate in sterilized laminar air flow until completely dry and formed solid mass (waxy). Obtained solid mass was weighed carefully to express the gram of extract extracted per 15 grams of the plant powder. For each sample, extract was prepared individually and kept at 4^0C for further use. Then each 100 mg of crude plant extract was weighed and dissolved on 1 ml methanol. This 100 mg/ml stock of each plant extract was used for antimicrobial tests, antioxidant activity, quantification of the total phenol and total flavonoids. The percentage yield of plant extract was calculated by using following formula:

$$\text{Percentage yield} = \frac{\text{Dry weight of Extract}}{\text{Dry weight of plant material}} \times 100$$

3.6 Phytochemical analysis

3.6.1 Qualitative phytochemical analysis

Crude methanolic extract of the plant samples were subjected to preliminary phytochemical screening to detect the major phytochemical constituents followed by Horborne (1973) and Trease and Evans (1989).

3.6.1.1 Test for saponins

Crude extract was mixed in 5ml of distilled water in a beaker and it was shaken vigorously. The formation of stable foam was indication of presence of saponins.

3.6.1.2 Test for terpenoids

Crude extract was dissolved in 2 ml of chloroform and left for evaporation till dryness. After this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish colour indicated the presence of terpenoids.

3.6.1.3 Test for flavonoid

Crude extract was mixed with 2 ml of 2% solution of NaOH which instantly give a yellow color. Addition of few drops of few drops of diluted acid turns the solution colorless indicates the presence of flavonoid.

3.6.1.4 Test for steroid

Crude extract was mixed with 2 ml of chloroform and then added 2 ml of concentrated H_2SO_4 and shaken gently. Appearance of reddish brown color indicates the presence of steroids.

3.6.1.5 Test for alkaloids

Crude extract was mixed with 2 ml of 1% HCL and heated gently. Few drops of Mayer's and Wagner reagent were added to the mixture. Turbidity of the resulting precipitate indicates presence of alkaloids.

3.6.1.6 Test for glycoside

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

3.6.1.7 Test for phenol

Crude extract was dissolved in 2 ml of 2% solution of $FeCl_3$. A bluish black coloration or dark green color indicates the presence of phenol.

3.6.2 Quantitative phytochemical analysis

3.6.2.1 Total Flavonoid Content

The total flavonoid content of mistletoes was determined by using the aluminium chloride colorimetric method with slight modification. 0.25 ml of extract (10 mg/ml) was separately mixed with the 0.75 ml of ethanol, 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium acetate and 1.4 ml of the distilled water. The solution mixture was shaken and allowed to stand at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm using UV-Visible spectrophotometer. Quercetin was used as standard solution in methanol with the concentration ranging from 10-100 µg/ml. and blank was prepared by adding all the reagents except the plant sample. The total flavonoid content was expressed in terms of milligram quercetin per gram of dry mass (mg QE/g). For each experiment the triplication were used for the accuracy and reproducibility of the result (Chang *et al.*, 2002).

3.6.2.2 Total Phenol Content

The total phenol content of mistletoes was determined by Folin-Ciocalteu Method (Ainsworth and Gillespie, 2007) with slight modification by mixing 0.1 ml of the sample extracts (2.5 mg/ml) with 1 ml Folin-Ciocalteu reagent (1:10 dilution with distilled water) and 0.8 ml of aqueous 1M sodium carbonate was subsequently added. The reaction mixture was left for 15 minutes and the absorbance was measured at 765 nm using UV-Visible spectrophotometer. A calibration curve was obtained using Gallic acid in methanol using the concentration ranging from 25-250 µg/ml as standard. Based on the standard curve, the concentration of each samples was calculated. Total polyphenol content was expressed in terms of the milligram of the Gallic acid equivalent per gram of the dry mass (mg GAE/g). For each extract, triplicate were used for the accuracy and reproducibility of result.

3.6.2.3 Antioxidant activity assay

2, 2- Diphenyl-1- picryhydrazyl (DPPH) was used to assay for the antioxidant activity. Antioxidant activity of the plant was determined through the DPPH free radical scavenging activity and ascorbic acid was used as standard, described by Singh *et al.,* (2002).

The different concentration of plant extract (25-200 µg/ml) and ascorbic acid (10-100 µg/ml) were prepared in methanol on the clean and dry ependroff tubes. The sample volume was taken 0.5 ml. To this 0.5 ml of the 0.2mM DPPH solution was added. The solution on the tube were shaken well and incubated in dark for 30 minutes. The control was prepared as above but without the plant extract or ascorbic acid. Methanol was taken as blank. And then absorbance of the solution in each tube were measured at 517 nm using spectrophotometer.

The free radical scavenging activity (RSA) of plant samples were calculated as follows and expressed in percentage:

$$\% \text{ Radical Scavenging activity (RSA)} = \frac{\text{Abs.control} - \text{Abs.sample}}{\text{Abs.control}} \times 100$$

The standard graph was plotted taking the concentration on the X- axis and percentage scavenging activity on the Y- axis.

IC_{50} value of each plant extract was calculated by using the following formula:

$$IC_{50} = \text{EXP}\left[\text{LN conc.} > 50\% - \frac{\text{pi}>50\% - 50}{\text{pi}>50\% - pi<50\%} \times \text{LN}\frac{\text{conc.}>50\%}{\text{conc.}<50\%}\right]$$

[EXP: Exponential function, LN: Natural log function, Signal > 50%: RSA value just above 50%, Signal < 50%: RSA value just below 50%, conc.> 50%: Concentration of extracts > 50%, conc< 50%: Concentration of extracts < 50%.]

3.7 Antibacterial activity

3.7.1 Microoraganism

The test microorganisms used for the antimicrobial screening was American Type Culture Collection (ATCC) gram negative bacteria *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATTC 27853)and gram positive bacteria *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228). These organisms were obtained from National Public Health Laboratory, Teku.

3.7.2 Preparation of culture media

3.7.2.1 Nutrient Borth (NB)

About 6.5 gram of NB powder was carefully weighed and dissolved in 500 ml distilled water. This media was sterilized on autoclave at 15 lbs pressure and 121^0 C for 15 minutes. The sterilized media was cooled in laminar airflow and was used for the suspension type of bacterial culture. Media was placed under aseptic conditions for further proposes.

3.7.2.2 Muller Hinton Agar (MHA)

Nineteen gram of MHA powder was weighed and dissolved in distilled water. The final volume was maintained of 500 ml. The media was sterilized by autoclaving at 15 lbs pressure and 121° C for 15 minutes. The media was mixed carefully before pouring. The media was transferred to the sterilized petriplates. About 20 ml of media was poured on each petriplates of 9 cm diameter. The plates were left for the solidification under aseptic conditions for further purposes.

3.7.2.3 Preparation of the standard culture Inoculums

The individual pure ATCC cultures*Echerichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* were streaked on the different MullerHinton agar plates. Those plates were incubated on the incubator at 37° C for about 24 hours and pure and isolated colonies were obtained. Each distinct colony was aseptically transferred to the nutrient broth for the suspension culture with the

help of the sterilized inoculating loop. The inoculated bottles were kept on the shaking incubator at 37° C and 127 rpm for overnight. The turbidity of the bacterial suspension was adjusted at the 0.5 McFarland standards for the antibacterial test. These inoculums were used for the swapping of the plates to test the antimicrobial effects to the plant extracts.

3.7.2.4 Transfer of bacteria on the petriplates

The test plates for the antimicrobial activity were first labeled with date, name of bacteria and name of the plant sample and the concentration of the plant extract to be added. The MHA plates were inoculated with the appropriate bacterial culture by a sterile cotton swab. One cotton swab was used for one bacterium. The culture plates were allowed to dry for minutes under aseptic condition.

3.7.2.5 Antibacterial screening

The antimicrobial test was performed by Agar Well Diffusion method of Perez and Bezevque, (1990) with slight modification. In MHA media with the help of sterile cork borer of 5 mm diameter and labeled properly with the sterile marker pen. Five different concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml) of the plant samples were prepared in the DMSO. In each well sterilized Whatmann filter paper disc was placed with the help of sterile forceps for the fast diffusion. With the help of sterile micropipette the 30 µl of the each individual plant extract was poured in the above prepared well. The DMSO was taken as the negative control while the gentamicin disc at concentration of 10 microgram was taken as positive control. The plates were incubated on the microbial incubator overnight at 37º C and the zone of inhibition was observed for individual plant extract for individual bacteria at different concentration for further analysis.

3.8 Statistical Analysis

All the experiments were performed in triplicates for each sample and values were reported as mean ± SD. The generated data was compiled using Microsoft Excel 2013 and analysis was done using SPSS Statistic 20.

CHAPTER FOUR: RESULTS

4.1 Distribution of mistletoes based on herbarium specimens

Altogether, 15 species of mistletoes has been observed in National Herbarium and Plant Laboratories (KATH) deposited after different scientific expeditions. Based on the detail information on herbarium sheet it was found that deposition was started from April 1949 and last deposition was on December 2004 (Table 2).*Scurrala elata* collected from 11 host species and 50 different locations including East (E), Center (C), West (W) and Far West (FW) region of Nepal suggested that it is widely distributed species in Nepal. Similarly, *Helixanthera ligustrina* is second largest species in terms of distribution and has been collected from 9 host species and 35 locations (C, W and FW). However, other species were collected from 1-8 host species and 5-29 different locations (Table 2). The elevation in which these species were collected ranged from 125m to 3500m. The oldest mistletoes specimen deposited at KATHwas *Scurrula elata,*collected from Eastern Bhutan at an elevation of 1800 mby Ludlow and Sherriff in January 1949.

Figure 2: First herbarium specimen of *Scurrula elata* deposited at KATH

Table 2: Distribution of mistletoes in different elevation on the basis of herbarium specimen deposited in KATH

S.N	Name of the species	Family	No. of Host tree	Locality	Total no. of location	Altitude(m)	Date of collection	First deposition
1	*Dendrophthoe falcata (L.f.) Etting.*	Loranthaceae	6	E*,W*,FW*	11	125 - 999	Dec. 1963-Feb. 1991	H. Hara, H. Kanai, S Kurosawa
4	*Helixenthera parasitica* Lour.	Loranthaceae	1	E,C	5	1050 - 1666	Apr. 1965-Sep. 1991	Dr. Banergi, A. V. Upadhya & B.B Baskota
5	*Helixenthera ligustrina* (Wall.) Danser	Loranthaceae	9	C,W,FW	35	390 - 2100	May 1954-July 1995	Stainton, Sykes and Williams
2	*Loranthus odoratus* (Wall.) Danser	Loranthaceae	8	C,W,FW	17	1300 - 2334	Mar. 1967-Nov.2000	NA*
3	*Macrosolen cochinchinensis* (Lour.) Tiegh.	Loranthaceae	6	E,C	14	250 - 1540	Nov. 1963-May 2004	H.Hara,H. Kanai,S. Kurosawa,G Murata,M. Togashi and T.Tuyama
6	*Scurrula elata* (Edgew.) Danser	Loranthaceae	11	E,C,W,FW	50	720 - 3500	Apr. 1949-Feb. 1997	Ludlow & Sheffif
7	*Scurrula graciliflora* (Roxb. ex Schult.) Danser	Loranthaceae	3	C, W	9	960 - 2334	Mar. 1962-Dec.2004	S.B Malla and S.B Rajbhandary
8	*Scurrula parasitica* L.	Loranthaceae	12	C,W,FW	29	200 - 2400	Mar. 1956-Aug. 2004	H Hara,H. Kanai, S. Kurosawa, G. Murata, M. Toganshi & T. Tuyama
9	*Scurrula pulverulenta* (Wall.) G. Don	Loranthaceae	7	E,C,W, FW	21	400 - 2200	Nov. 1967-Sep. 2001	NA
10	*Taxillus umbellifer* (Schult.) Danser	Loranthaceae	5	E,C	13	1166 - 2400	Jul.2 023-Jul. 1993	Dr. S.B Malla
11	*Taxillus vestitus* (Wall.) Danser	Loranthaceae	3	C,W,FW	10	1675 - 2600	Sep. 1969-Oct. 2002	T.B Shrestha & M.S Bista
12	*Viscum album* L.	Viscaceae	4	C,W,FW	21	1300 - 2650	Nov.1966-Nov. 2000	D.H Nicolson
13	*Viscum articulatum* Burm. F.	Viscaceae	7	E,C,W	19	400 - 2400	Feb. 1967-Dec. 2004	Mrs. P. Pradhan & Miss R. Thapa
14	*Viscum articulatum var. liquidambaricolum* (Hayata) S. Rao	Viscaceae	4	C,W	8	1450 - 2420	Sep.1963-Jun. 1995	Shrestha & Upadhaya
15	*Viscum monoicum* Roxb. ex Dc.	Viscaceae	2	E,C,W	5	333 - 1170	May 1978-May 1984	T.B shrestha & T.K Bhattacharya

E*= Eastern, C*= Central, W*= Western, FW*= Far-Western, NA= Not available

4.2 Percentage yield of plant extracts

Seven species of mistletoes as *Helixanthera ligustrina, Macrosolen cochinchinensis, Scurrula elata, Scurrula parasitica, Viscum articulatum* var. *liquidambaricolum, V. album and V. articulatum*were collected from Kathmandu valley. Plant samples were subjected for methanolic extract using ultrasonic extraction. The crude methanolic extract was obtained by the 15 gram of plant powder expressed in percentage (Figure 3). The methanolic extract was found greasy and sticky. The percentage yield was varied from 11.86% to 24.26%. *Viscum articulatum* showed highest yield percentage (24.26%) whereas *Scurrula parasitica* (11.86%) showed lowest.

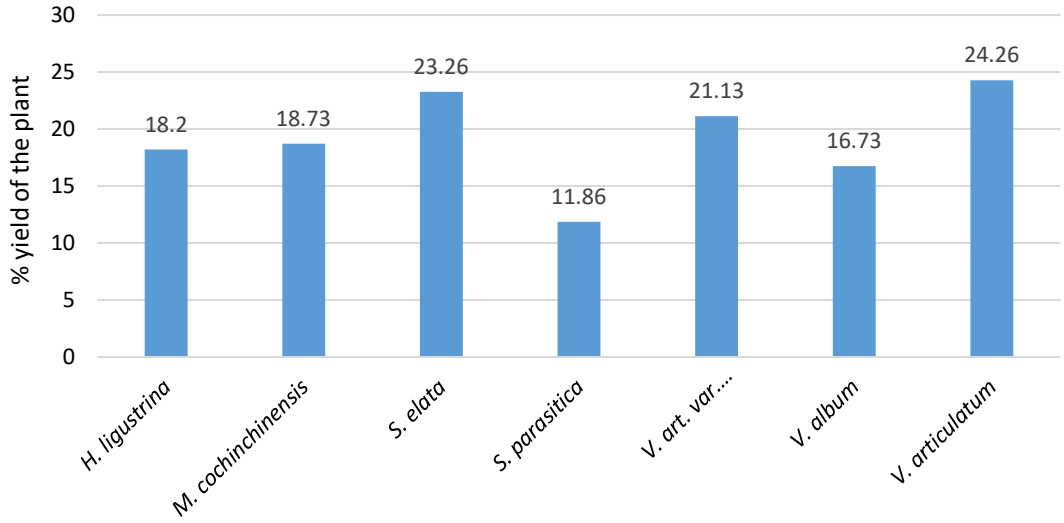

Figure 3: % yield of extract of different mistletoes species

4.3 Qualitative Estimation of Phytochemicals

Phytochemical test revealed the presence of terpenoids, flavonoids, steroids, glycoside and phenols in all of methanolic extracts of mistletoes species. Saponins were reported in all of the extracts except *Scurrula parasitica* and *Macrosolen cochinchinensis*. In contrast, alkaloids wereabsent in all extracts except in*Viscum articulatum* var. *liquidambaricolum* and *Macrosolen cochinchinensis*. Details of the phytochemical constituents have been given in Table 3.

Table 3: Preliminary test for the presence of phytochemical in mistletoes

Plant species	Saponin	Terpenoid	flavonoid	Steroid	Alkaloid	Glycoside	Phenol
Helixanthera ligustrina	+	+	+	+	-	+	+
Macrosolen cochinchinensis	-	+	+	+	+	+	+
Scurrula elata	+	+	+	+	-	+	+
Scurrula parasitica	-	+	+	+	-	+	+
V. articulatum var. liquidambaricolum	+	+	+	+	+	+	+
Viscum album	+	+	+	+	-	+	+
Viscum articulatum	+	+	+	+	-	+	+

('+' presence, '-'absence)

4.4 Total flavonoid content

Quercetin was used as standard to determine total flavonoid content in plant extracts. The total flavonoid content in crude methanolic extract was determined by standard graph with an equation y = 0.0014x + 0.0877 and R^2 = 0.9094 (Figure 4). The equation was obtained from graph of quercetin ranging in concentration from 10 µg/ml to 100 µg/ml. The results were expressed in mg QE/g ± SD.

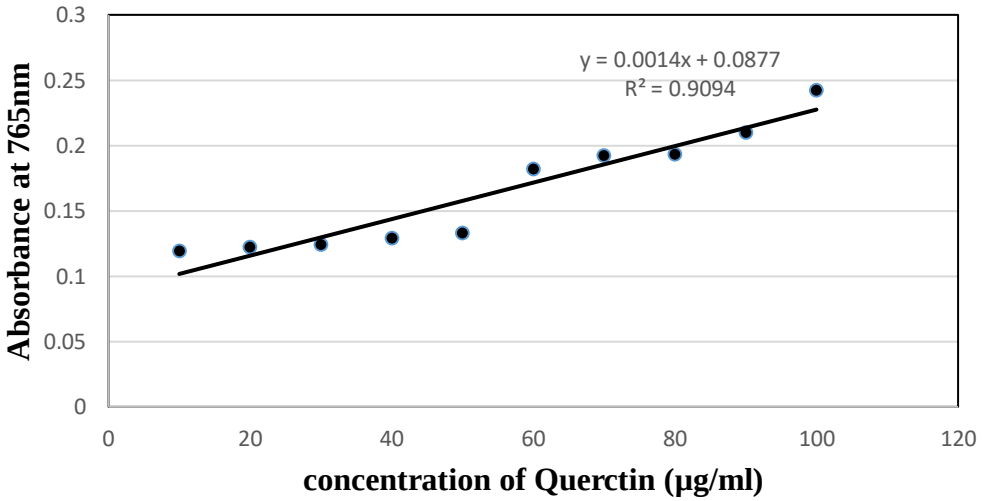

Figure 4: Standard curve for calibration of total flavonoid content

The highest amount of flavonoid content was estimated in *Viscum album* (31.45 ± 2.32 mg QE/g) while lowest in *Macrosolen cochinchinensis* (24.90 ± 2.26 mg QE/g) and rest of the species lies between these two values. The total flavonoid content in *Viscum album* was followed by *Helixanthera ligustrina* and *Scurrula parasitica* respectively. The obtained values of plant extracts showed in Figure 5.

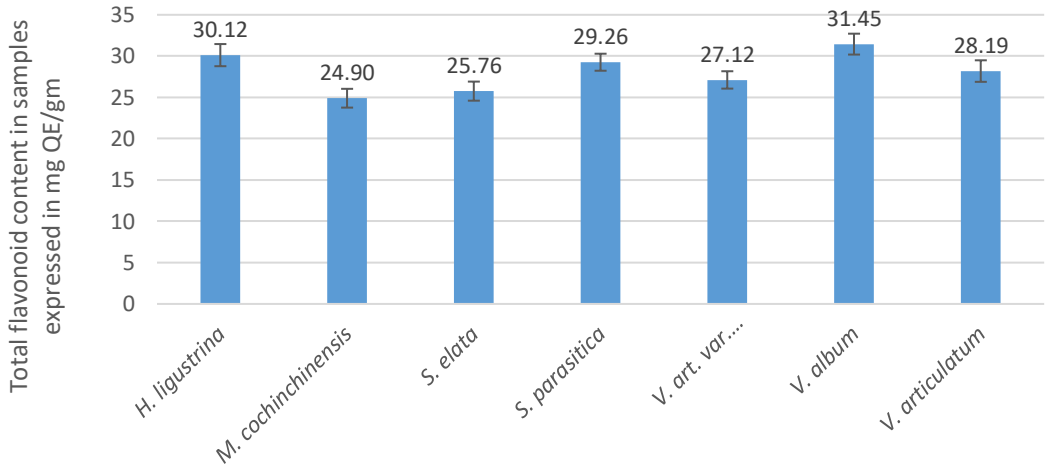

Figure 5: Total flavonoid content present in different mistletoes species

4.5 Total phenolic content

The total phenolic content in crude methanolic extract was determined by using calibration curve of gallic acid. Gallic acid ranging from the concentration of 25 µg/ml to 300 µg/ml was used to obtained standard graph and equation (Figure 6). Based on equation (y = 0.0016x + 0.2165 and R^2 = 0.846) the total phenolic content present in methanolic extract of seven different samples were determined. The result expressed in term of mg GAE/g ± SD.

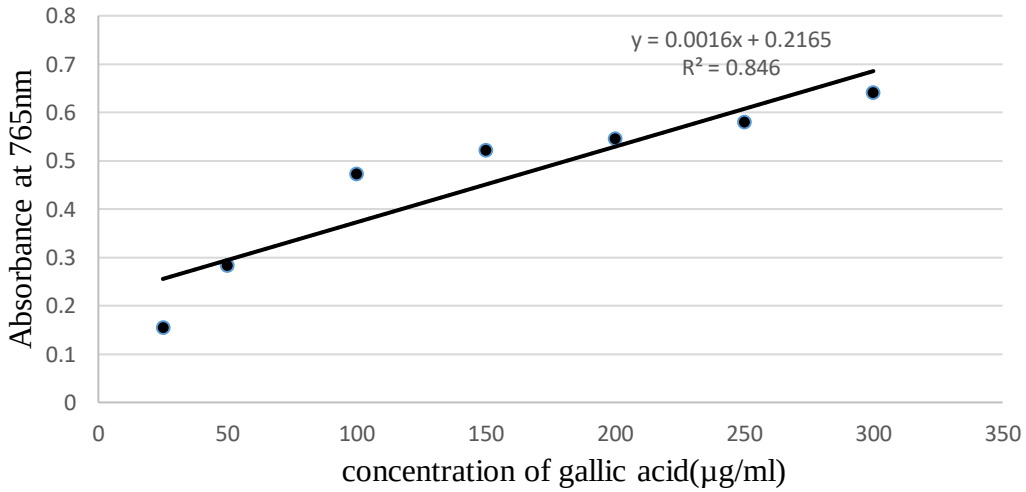

Figure 6: Standard curve for calibration of total phenolic content

The highest amount of polyphenol was found in *Scurrula parasitica* (32.90 ± 2.46 mg GAE/g) while lowest found in *Viscum album* (20.60 ± 2.06 mg GAE/g), rest of the species lies between these two values. *Scurrula parasitica* was followed by

Macrosolen cochinchinensis, *Helixanthera ligustrina*, *Viscum articulatum* and so on. The total polyphenol content present in plant extract showed in Figure 7.

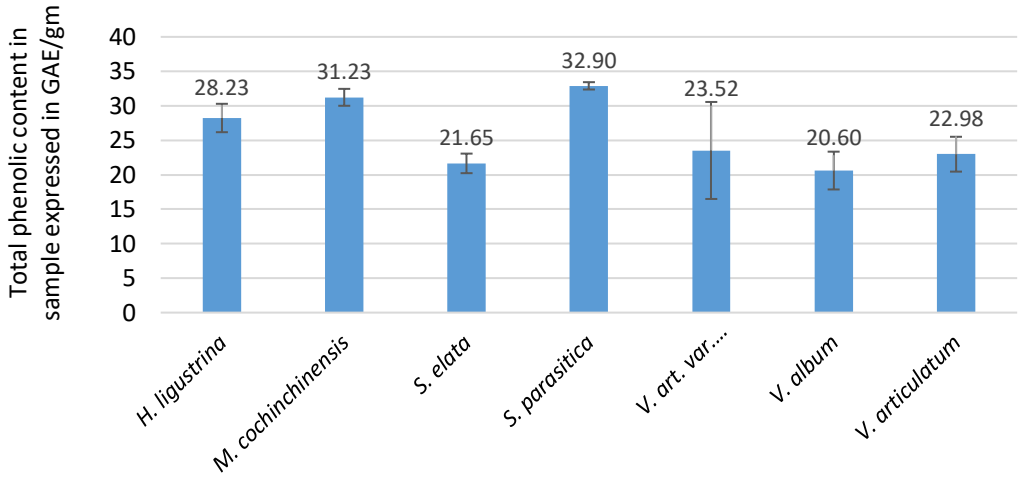

Figure 7: Total polyphenol content present in different mistletoes species

4.6 Antioxidant properties of Mistletoes

The antioxidant activity of the mistletoes was determined by using solution of stable free radical, 2, 2diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. DPPH was used as the source of free radical and ascorbic acid was used as a reference compound. Percentage of radical scavenging activity of ascorbic acid was determined by using different concentrations ranging from 10 µg/ml to 100 µg/ml (Figure 8).

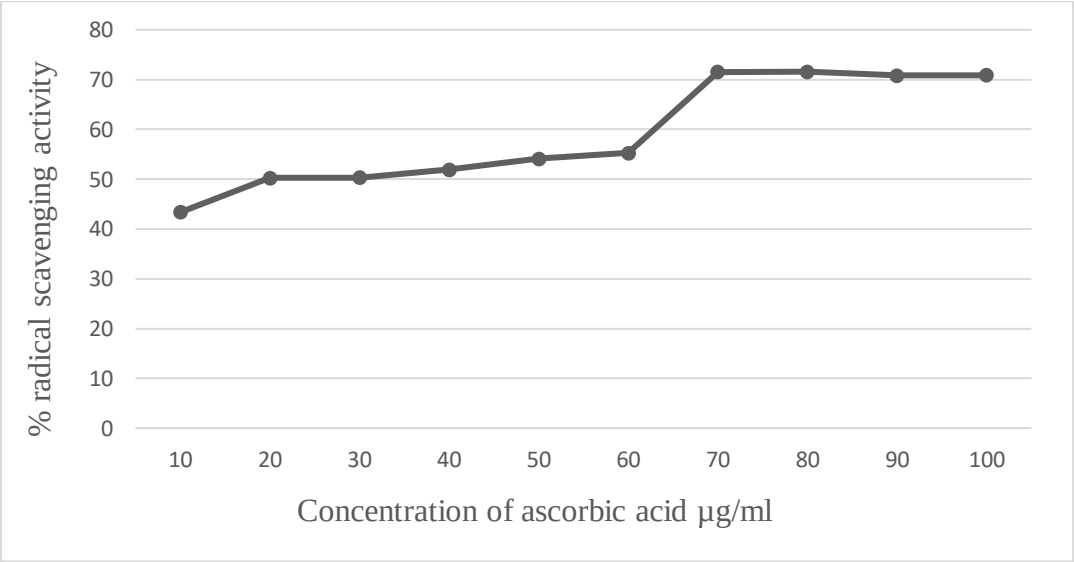

Figure 8: Standard graph of Ascorbic acid

The antioxidant activity of extract was determined by IC_{50} value. The radical scavenging activity (RSA) percentage of each sample was calculated by measured

absorbance of sample in spectrophotometer at 517 nm (Figure 9). IC_{50} value was calculated by using RSA % value for each sample. There was gradual increase in percentage radical scavenging activity as the concentration of extract increased.

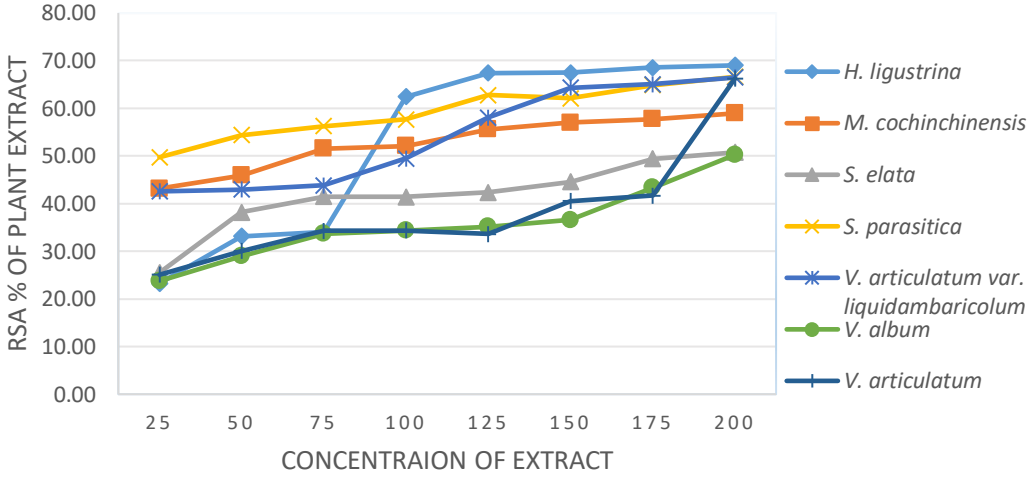

Figure 9: % radical scavenging activity and concentration of plant extracts (µg/ml)

The IC_{50} value for ascorbic acid was found to be 19.61 µg/ml. The highest IC_{50} value was found in *Viscum album* (199.04 ± 1.25 µg/ml) whereas *Scurrula parasitica* (26.04 ± 0.71 µg/ml) contained the lowest. The sample with lower IC_{50} value regarded having higher antioxidant property and vise-versa. The *Scurrula parasitica* showed lower IC_{50} value considered as the better antioxidant among other species. Two *Scurrula* species showed vast difference in IC_{50} value, *Scurrula parasitica* showed good antioxidant property as compared to *Scurrula elata*.Among three *Viscum* species *Viscum articulatum* var. *liquidambaricolum* showed better antioxidant property as compared to others.Similarly, *H. ligustrina and M. cochinchinensis* also showed lower IC_{50} value than *S. elata*, *V. album* and *V. articulatum*. The IC_{50} value has been shown in Figure 10.

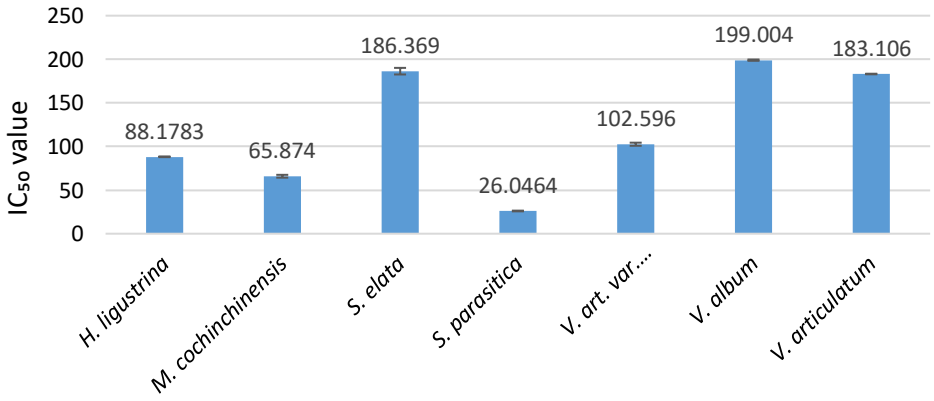

Figure 10: IC_{50} value of mistletoes plants

4.7 Relationship of antioxidant activity of the plant extract with total phenolic and flavonoid content

The IC_{50} value for DPPH radical scavenging activity of methanolic extract of selected mistletoes were statistically negatively correlated with the total phenolic content and positively correlated with the total flavonoid content in this study. When IC_{50} values were plotted against total phenolic content the linear equation y = -0.0673x + 34.056 and R^2 = 0.876 were obtained. The regression equation of IC_{50} value and total phenolic content showed in Figure 11. Again when IC_{50} value plotted against total flavonoid content the equation y = 0.0048x + 27.543 and R^2 = 0.0188 were obtained (Figure 11). The negative association of IC_{50} value of plant extracts with phenolic content and positive association with flavonoid content in this study. When increasing the IC_{50}value decreases the polyphenol content i.e increase in the antioxidant activity.

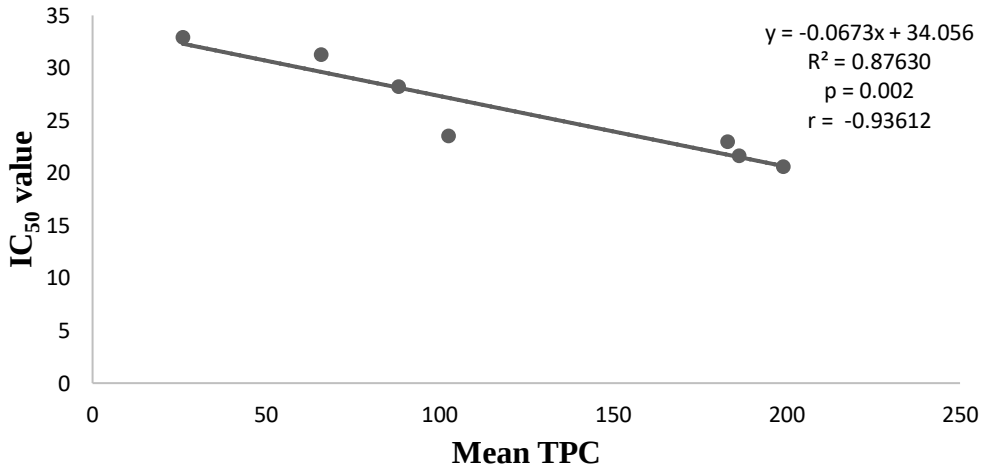

Figure 11: Relationship of antioxidant activity with total phenolic content

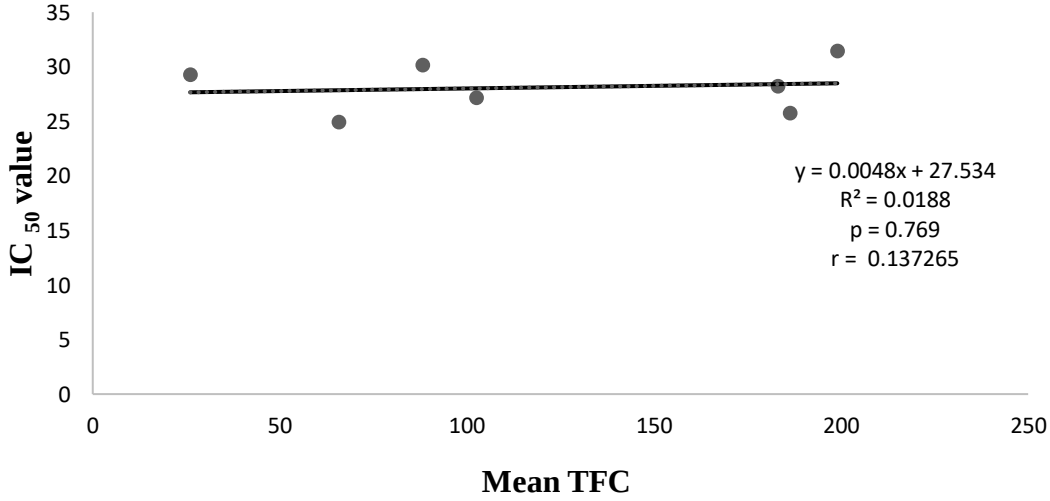

Figure 12: Relationship of antioxidant activity with total flavonoid content

4.8 Antibacterial properties of Mistletoes

The antibacterial activity of methanolic plant extracts were tested against four ATCC culture bacterial strain. Gentamycin was used as a positive control and Dimethyl Sulphoxide (DMSO) was used as negative control. Five different concentration of plant extract such as 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml were tested against four bacterial strains and compared with antibiotic gentamycin. Amongseven mistletoes species, only four species had antibacterial property. *Helixanthera ligustrina* and *Viscum articulatum* showed antibacterial activity against all tested organisms. *Scurrula parasitica* extract was not effective for *E. coli* and *P. aeroginosa* but it was effective against gram positive bacteria. *Macrosolen cochichinensis* inhibited all tested organisms except *Staphylococcus aureus*. It showed good antibacterial activity against *Pseudomonas aeroginosa* at all concentration as compared to other bacterial strain. *Scurrula elata*, *Viscum album* and *V. articulatum* var. *liquidambaricolum* extract showed negative result against all bacterial strain. The zone of inhibition was expressed in mm diameter (Table 4).

Table 4: Antibacterial activity of plant extract

Plants name	Oraganisms	Zone of inhibition in mm (with diameter of well 5 mm)					
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	Positive control
Helixanthera ligustrina	*Escherichia coli*	15.33 ± 3.05	13.66 ± 1.52	11.67 ± 0.57	10 ± 1	10.67 ± 0.57	22
	Pseudomonas aeroginosa	19 ± 3.6	15.67 ± 1.52	13.33 ± 1.52	10.67 ± 2.08	-	21
	Staphylococcus epidermidis	16 ± 1	13 ± 1	9.67 ± 0.57	9 ± 1	-	22
	Staphylococcus aureus	19 ± 1	16 ± 1	13.67 ± 0.57	10.67 ± 0.57	-	19
Macrosolen cochinchinensis	*Escherichia coli*	10 ± 1	9 ± 1	8.67 ± 2.08	6 ± 4.35	-	22
	Pseudomonas aeroginosa	21.33 ± 1.52	18 ± 1	13.67 ± 4.16	12.33 ± 3.05	11.67 ± 3.05	21
	Staphylococcus epidermidis	16.67 ± 1.52	11.67 ± 0.57	-	-	-	22
	Staphylococcus aureus	-	-	-	-	-	19
Scurrula elata	*Escherichia coli*	-	-	-	-	-	22
	Pseudomonas aeroginosa	-	-	-	-	-	21
	Staphylococcus epidermidis	-	-	-	-	-	22
	Staphylococcus aureus	-	-	-	-	-	19
Scurrula parasitica	*Escherichia coli*	-	-	-	-	-	22
	Pseudomonas aeroginosa	-	-	-	-	-	21
	Staphylococcus epidermidis	10.67 ± 0.57	8.67 ± 0.57	-	-	-	22
	Staphylococcus aureus	12.67 ± 0.57	11.33 ± 0.57	10.33 ± 0.57	-	-	19

Viscum album	*Escherichia coli*	-	-	-	-	-	22
	Pseudomonas aeroginosa	-	-	-	-	-	21
	Staphylococcus epidermidis	-	-	-	-	-	22
	Staphylococcus aureus	-	-	-	-	-	19
Viscum articulatum	*Escherichia coli*	14 ± 1	13 ± 1	12.67 ± 1.52	8.67 ± 0.57	-	22
	Pseudomonas aeroginosa	14.67 ± 0.57	12.67 ± 1.52	11.33 ± 1.52	10.33 ± 0.57	9.33 ± 0.57	21
	Staphylococcus epidermisdis	16.67 ± 2.08	13 ± 1	11.67 ± 0.57	10.67 ± 0.57	-	22
	Staphylococcus aureus	11.67 ± 0.57	9.67 ± 0.57	9.67 ± 0.57		-	19
V. articulatum var. liquidambaricolum	*Escherichia coli*	-	-	-	-	-	22
	Pseudomonas aeroginosa	-	-	-	-	-	21
	Staphylococcus epidermidis	-	-	-	-	-	22
	Staphylococcus aureus	-	-	-	-	-	19

CHAPTER FIVE: DISCUSSION

5.1 Distribution of Mistletoes

Mistletoes shows irregular and patchy distribution. Around the world about 1500 species are recorded, among them 19 species are documented from Nepal (Devkota, M.P, 2005; Nickrent, 2011, Devkota and Koirala, 2005; Devkota and Glatzel, 2005) but only 15 species were deposited in KATH with detail information. Distribution of mistletoes were abundant in 2000-3000 m elevation. It may be due to the availability of suitable host species in this elevation range (Devkota, 2003, Devkota and Glatzel, 2005). Few species of mistletoes were recorded above 3000 m elevation because of the scarcity of suitable hosts but also moist and very low temperature persist throughout the year (Devkota and Glatzel, 2005).

Occurrence and spread of mistletoe believed to be determined by host specificity, environmental conditions, host plant characteristics and movement of dispersal agents (Del *et al.*, 1996; Aukema Martinez del Rio, 2002). Devkota and Glatzel (2005) concluded that most of the Loranthaceae mistletoes have a wide range of host species than the Visscaceae mistletoes which is also observed in deposited herbarium specimen. Distribution of *Dendrophthoe falcate* was limited between 125 – 999 m (east, west, farwest) in deposited herbarium. Among 15 deposited mistletoes species, *Scurrula elata* was recorded from wide elevation range it may be due to the wide range of host specificity (Devkota*et al.,* 2010). Devkota and Acharya (1996) studied distribution of mistletoes in Kathmandu valley found that host trees and the behavior of avian visitors seem to be primary factors determining the distribution. Similarly, Kuijt (1969) concluded that mistletoes distribution depends entirely upon the habits of birds that spread the seeds.

5.2 Percentage yield of plant extract

The first essential step to investigate phytochemical and biological activity of plant is preparation of plant extract. An extraction technique is that which is able to obtain extracts with high yield and with minimal changes to the functional properties of the extract required (Quispe Candori *et al.*, 2008). However, percentage yield of the plant

extract depends on parts of plant used, type of solvent and process of extraction. Different types of solvent have been used for the extraction such as chloroform, ethyl acetate, crude petroleum, methanol, acetone, ethanol and water (Cemaluk *et al*., 2012; Vicas*et al*., 2009; Ucar *et al*., 2006; Gurer-Orhan *et al.*, 2004). Among the extraction processes, Ultrasonic assisted extraction process is one in which the ultrasound ranging from 20 kHz to 2000 kHz has been used (Handa *et al.*, 2008). In present study, sonication technique was used for plant extraction and methanol as solvent. The percentage yield of extracts observed in this study was ranging from 11.86% to 24.26%. Among the seven species *Viscum articulatum, S. elata* and *V. articulatum* var.*liquidambaricolum* showed highest (21.13-24.26 %), *Helixantheraligustrina*and *Macrosolen cochinchinesis* medium (18.2-18.73%) and *Scurrula parasitica* the lowest yield percentage (11.86%). The variation in yield percentage among the species might be due to the differences in plant part used (Table 1). However, the lowest yield for *Scurrula parasitica* may be due to use of mature leaves for extraction.

So far a very few research work has been done in this plant group except *Viscum album* for comparison. The percentage of yield for *V. album* reported by Kusi *et al.* (2015) was 26.67% which is higher than the present study (16.73%). This variation might be due to the differences in extraction process and plant parts used for the extraction (Table 1).

5.3 Phytochemical screening

Phytochemical screening provides a rough outline for the presence of major secondary metabolites. Secondary metabolites are the chemicals developed in plants for self-defense from environmental stress, animals and plants including microorganisms. Among the defense chemicals saponins are used as natural cleansers and cardiac glycosides are used for ulcer and diabetic treatment (Karunyadevi *et al.*, 2009). Likewise, flavonoids and terpenoids possess many useful activities like anti-inflammatory, anti-bacterial antioxidant and anti-diarrheal (Harborne *et al.*, 2000). Among the mistletoes, *Viscum album* is widely used for phytochemical studies and showed similar results for the presence of terpenoids in this study and was also in this study and was suggested by Wahab *et al.*, (2010). In contrast, presence of alkaloids reported by Kusi *et al.*, (2015) was not observed in this study. This may be due to the different parts used for extraction. The phytochemicals like alkaloids and saponins

were reported from African mistletoes i.e. *Tapinamthus dodoneifolius* (DC) Danser (Deeni and Sadiq, 2002) and flavonoids and glycosides from Japanese mistletoes i.e. *Taxillus yadoriki* Danser, *T. kaempferi* Denser and *Korthalsella japonica* Engler (Fukunaga *et al.,* 1989). In this study, saponin and alkaloid were absent in two and five mistletoes, respectively. Besides, all selected mistletoes showed positive results for the presence of terpenoids, flavonoids, steroid, glycoside and phenols. Presence of these secondary metabolites in different mistletoes of the present study suggested their medicinal potentialities, although, very few mistletoes were recognized for this purpose.

5.4 Total flavonoid Content

Flavonoids are secondary metabolites consisting of polyphenolic molecules containing 15 carbon atoms. Flavonoids are a class of secondary plant phenolics with significant antioxidant and chelating property (Heim *et al.*, 2002).

In this study, highest flavonoid content was found in *Viscum album* (31.45 ± 2.32 mg QE/gm). However, the total flavonoid content varies from 24.90 mg QE/g to 31.45 mg QE/g. Total flavonoid content in *Macrosolen cochinchinensis* was 24.90 mg QE/g which is more or less similar to the value reported by Lobo *et al.,* (2011) for*Macrosolen parasiticus.* This variation may be due to difference between the species of plant sample.Pattanayak *et al.,* (2012) investigated total flavonoid content in *Dendrophthoe falcataa* which was found to be 21.59 ± 1.09 mg QRT/g, the value was comparatively less than other mistletoes species in present study but in aqueous extract value was 33.42 ± 2.08 mg QRT/g which is close to *Viscum album* (31.45 mg QE/g). The flavonoid content in *Taxillus sutchuenensis* ethyl acetate fraction was found to be 94.11 ± 1.14 mg RE/g (Liu *et al.*, 2012). This value is far more than present study. This variation may be due to *Taxillus sutchuenensis* contain high bioactive compounds as compared to selected mistletoes.

5.5 Total phenolic content

Phenolic compounds are basically plant metabolites system and widely spread throughout the plant kingdom. Phenolic compounds have potential against oxidative damages diseases, so act as the good antioxidant (Kahkonen *et al.,* 1999).Antioxidant

action of phenolic compounds is because of their high tendency to chelate metals with heavy metals like iron and copper (Rice-Evans*et al.*, 1997).

In present study, *Scurrula parasitica* showed highest phenolic content (32.90 ± 2.46 mg GAE/g) among the selected mistletoes. Rahmawati *et al*., (2014) reported that total phenolic content in *Scurrula oortiana* was 280 mg GAE/g. The value of total phenolic found in Indian medicinal plants ranged from 28.66 – 169.67 mg/g, (Aqil *et al*., 2006). Methanolic extract of *Loranthus europaeus*showed highest value (158 ± 1.5 mg/g). Marbibaigi *et al.*, (2014) showed phenolic content in methanolic extract of *Scurrula ferruginea* leaf was 309.06 ± 1.15 mg GAE/g. Similarly, African investigators examined total phenolic content of *Viscum album* isolated from cocoa was 182 mg/100gm and cashew was 160 mg/100gm and using tannic acid as standard compounds (Oluwaseun and Ganiyu, 2008). The lower total phenolic content in seven species of present study may be due to the sample collection from different geographical region, collected from different host trees, collected in different season as suggested by Katsarou *et al*., (2012). In contrast, Ali *et al*., (2013) investigated methanolic extract of leaves of *Scurrula parasitica* was found to be 21.77 ± 1.41 mg GAE/g which is less than present study (32.90±2.46 mg GAE/g). The difference may be due to the collection of plant from different geographical location.

5.6 Antioxidant properties of Mistletoes

Damages caused by free radical induced oxidative stress, is the major causative agent of many disorders including cancer, tissue injury, neurodegenerative diseases, aging and rheumatoid arthritis (Halliwell and Gutteridge, 1984; Addis and Warner, 1991; Halliwell, 1994; Niki, 1997; Poulson *et al.*, 1998; Young and Woodside, 2001). In recent years, antioxidants derives from natural resources mainly from plants have been intensively used to prevent oxidative damages (Onay-Ucar *et al.*, 2006). So the antioxidant properties of plant extract are very important for investigation of their pharmaceutical uses.

DPPH free radical scavenging was used to measure the antioxidant capability of crude extract of mistletoes leaf. DPPH (2,2–diphenyl-1-picrylhydrazyl) is the major chemical used in this antioxidant assay which is able to be reduced to diphenylpicrylhydrazine after exposing to plant extracts having antioxidant compounds. IC_{50} value shows the amount of sample required to reduce 50% of the

initial amount of DPPH and it is the main parameter to determine the antioxidant activity (Khan *et al*., 2012). Higher antioxidant activity is related to lower IC_{50} value. Antioxidant property can be concluded on the basis of % radical scavenging activity (RSA) and IC_{50} value. Antioxidant activity DPPH inhibition of the plant extract is expressed as percent inhibition of stable radical or inhibition concentration fifty (IC_{50}) in reference to a standard compound. The plant with higher percentage RSA and corresponding lowest IC_{50} value is considered having better antioxidant properties.

In this study, methanolic extract of *Scurrula parasitica* showed the lowest IC_{50} value 26.04 µg/ml and considered better antioxidant property. The highest IC_{50} value found in *Viscum album* (199.04 µg/ml) andconsidered as least or less effective antioxidant plant among seven species. This result is contrast to Onay – Ucar *et al*., (2006) and Kusi *et al*., (2015) it may be due to the plant part used for extraction, plants collected from different host tree in different season and using different extraction process. The IC_{50} value for aerial part of *Dendrophthoe falcata* was reported 77.52 µg/ml by Pattanayak *et al.*,(2012). This value is ranging between the IC_{50} value of *Helixanthera ligustrina* and *Viscum articulatum* var. *liquidambaricolum* of the present study. Rahman *etal.*, (2012) reported that scavenging activity of *Macrosolencochinchinensis*found to be 39.47% at 500 µg/ml, this result is contrast to the present study (58.94% at 200 µg/ml). This difference may be due to plants collected from different environment and extraction process.

5.7 Relationship of antioxidant activity of the plant extract with total phenolic and flavonoid content

There is a fair correlation between antioxidant activities with total phenolic content. In several mistletoe species researcher reported a high potential relation between antioxidant capacity and total phenolic content (Kumar *et al.*, 2011; Aqil *et al.*, 2006; Oboh *et al*., 2008). Khanna *et al.*, (1968) reported that the higher antioxidant potential of hemiparasite is attributed to its own fIavonoid content and the transferred phenolic, hardly gives significant rise in antioxidant activity.

In this study, lower the IC_{50} value higher the DPPH scavenging activity and vice versa. The IC_{50} value of mistletoes was negatively correlated with total phenolic content i.e. increasing the IC_{50} value decreases polyphenol content and positive association with total flavonoid content. According to Pourmorad *et al.*, (2006)

presence of flavonoid which contain hydroxyls are responsible for radical scavenging effect, so high content flavonoid leads to increase in radical scavenging activity also. Chu *etal.,* (2000) observed that positive association of total flavonoid content with the percentage inhibition of DPPH radical and also supported by Ghimire *et al.,* (2011).

5.8 Antibacterialproperties of Mistletoes

Plant extracts can destroy bacteria or suppresses their growth ability to reproduce. The antibacterial activity of plant extract can be determined by measuring zone of inhibition. The inhibition zone produced by plant extracts usually contained antibacterial substances and able to suppress the growth of tested bacteria in the different concentrations (Matsen, 1979).

Plant extracts were screened for antibacterial test against four pathogenic strains. Different kind of diseases such as diarrhea and dysentery, skin diseases, respiratory diseases, pneumonia, meningitis, urinary tract infection, septic shock, gastrointestinal infection, staph skin infection, conjunctivitis, keratitis, blood infection, high fever etc. were caused by bacteria. The respiratory and urinary diseases were mainly caused by *Escherichia coli*, cough and tonsillitis may be caused by *Pseudomonas aeruginosa* and boils custs and wounds are affected by *Staphylococcus arueus* and *Escherichia coli*.

Seven plant extracts such as, *Helixanthera ligustrina, Macrosolen cochinchinensis, Scurrula elata, Scurrula parasitica, Viscum album, Viscum articulatum, Viscum articulatum* var. *liquidambaricolum and* only four species showed the positive effect against bacteria. Among four species *M. cochinchinensis* showed good result in *P. aeroginosa* followed by *S. epidermidis* and *E. coli.* In the present study *M. cochinchinensis* showed the significant inhibition in all bacteria except *S. aureus.* According to Tripathi *et al*., (2013) *M. cochinchinensis* showed no inhibition against *E. coli*. In case of *Viscum album* no inhibition zone against all tested organism and similar result was found in Tripathi *et al.,* (2013). But Kusi *et al*., (2015) reported the methanolic leaf extract of *Viscum album* had antibacterial activity, in regard to this finding whole plant used in present study. Deeni and Sadiq (2002) investigated antimicrobial activity of African mistletoes depends on the host physiological states of development, diurnal and seasonal variations. Genus *Scurrula* showed the antibacterial activity against *S. aureus* was supported by the previous study (Marvibaigi *etal*., 2014). Present study was also supported by Cemaluk and Nwankwo

(2012). In present study *Viscum album*, *Scurrula parasitica* and *Viscum articulatum* var. *liquidambaricolum* plants did not show antibacterial activity against tested organisms. These may be good antibacterial against other microbes.

Overall phytochemical screening of seven mistletoes species showed presence of different phytochemicals (flavonoids, phenolics and antioxidants) and bioactive properties as per stated hypothesis.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Mistletoes are hemiparasitic plants found in different host trees. Ethnic peoples have been used as medicine for curing different diseases so it has potentially importance for pharmaceuticals.

On the basis of observation of herbarium specimen deposited in National Herbarium and Plant laboratories (KATH), 15 species were deposited with detail information. The oldest mistletoe specimen deposited at KATH was*Scurrula elata*collected from eastern Bhutan at an elevation of 1800m, inApril 1949 by Ludlow and Sherriff(Table 2). The elevation in which these species were collected ranged from 125m to 3500m.

In the present study, evaluation of antioxidant activity along with the phytochemical constituents, the total polyphenol content and flavonoid content of methanolic extract of selected seven mistletoes species from surroundings of Kathmandu valley has been determined. The highest percentage yield was obtained from *Viscum articulatum* (24.26%) whereas lowest from *Scurrulaparasitica* (11.86 %) and in other species yield of plant extract lies between these two values. Phytochemical test revealed that five species of mistletoes are rich in terpenoids, flavonoid, steroid, glycoside and phenol. Total flavonoid content, polyphenol and antioxidant of methanolic extract were calculated. *Viscum album* showed highest flavonoid content whereas other *Viscum* species showed comparatively less. *Scurrula elata* showed lowest flavonoid content. The value of total flavonoid content found in two *Scurrula* species showed minor differences. The highest phenolic content found in *Scurrula parasitica* while *Viscum album* contains the lowest. *Scurrula parasitica* showed highest antioxidant activity with lowest IC_{50} value. Among seven species only four species showed antibacterial activity against tested bacteria. *Macrosolencochinchinensis* showed best antibacterial activity against *Pseudomonas aeroginosa*. *Helixanthera ligustrina* showed antibacterial activity against all gram positive and gram negative bacteria.

The phytochemical analysis of mistletoes revealed that it has potential in pharmaceutical level in Nepal. Many ethnic people used mistletoes as medicine since

long. Only few number of research work was done in Nepal, therefore, the number of research work should be increase towards isolation and estimation of bioactive compound of mistletoes.

Preliminary phytochemical screening of selected misltetoes showed presence of active constitutents and are found rich in flavonoids, phenolics, and antioxidants

6.2 Recommendations

Based on herbarium specimen of mistletoes in KATH, only 15 species were recorded, although 19 species were described in different literatures. Hence, required proper documentation of all mistletoes found in Nepal. Phytochemical analysis of seven species showed that presence of bioactive compounds. Similarly, there must be probability of such compound in other mistletoes, so need detail study of mistletoes and phytochemical compound can be used for pharmaceuticals. To evaluate anticancer, antidiabetic, anti-inflammatory, antifungal and cytotoxic effect of mistletoes should need further study.

In this study, only one solvent (methanol) has been used for extraction. In future, it should be using various solvents (e.g. hexane, chloroform, petroleum ether, ethyl acetate) to explore new bioactive compounds.

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Appendices

Appendix I: List of Materials used for the study

Apparatus and Equipment

Electric Balance	Electric grinder	Refrigerator
Hot air oven	Filter paper	Cotton swabs
Incubator	Laminar air flow	Forceps
Aluminium foils	Cotton Rolls	Autoclave
Camera	Gloves	Parafilm
Micropipettes	Ependrof tube	Measuring cylinder
Spirit Lamp	Petriplates	Beaker
Glass rod	Test tube	Conical flask

Appendix II: Preparation of Reagents

1. Preparation of 1M Na_2CO_3 - 100 ml

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

2. Preparation of 10% Aluminium chloride ($AlCl_3$) – 100 ml

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

3. Preparation of 1M Potassium acetate (CH_3COOK) – 100 ml

9.814 gram of the potassium acetate (Merk Specialities Pvt. Ltd., Mumbai, India) was weighed and dissolved in water. Finally the volume was maintained to 100 ml by addition of water.

4. Preparation of 0.2 mM DPPH Solution – 100 ml 100 ml of 0.2 mM solution of 2,2, diphenyl – 1 picryhydrazyl (DPPH) was prepared by weighing 7.886 mg of the

DPPH and dissolving it in methanol and finally maintaining the volume to 100 ml by addition of methanol.

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

10 ml of the commercially supplied Folin – Ciocalteu phenol (Thermo Fisher Scientific Pvt. Ltd., India) was taken and mixed it with 90 ml of the distilled water to prepare the 100 ml of 1:10 dilution of FC phenol reagent.

6. Preparation of 0.5 Mcfarland standards – 100 ml

100 ml of 0.5 Mcfarland standards was prepared by mixing carefully analysed 99.5 ml of Sulphuric acid (1%) and 0.5 ml of Barium chloride (1.175%).

7. Mayer's Reagent

1.358 g of mercuric chloride was dissolved in 60 ml of water. Similarly, 5.0 gram of potassium iodide was dissolved in 10 ml of water separately. The two solutions were mixed up to the final volume 100 ml.

Appendix III: Composition of media

1.Nutrient Broth (NB)

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

Compounds	**Gram/L**
Casein enzyme hydrolyses	10.0
Yeast extract	5.0
Sodium Chloride	10.0
Final pH	7.5 ± 0.2

2.Mueller Hinton Agar (MHA)

Compounds	**Gram/L**
Beef infusion form	300
Casein hydrolysate	17.5
Starch	1.56
Agar	17.0
Final pH	7.3 ± 0.2

Appendix IV: Total flavonoid content in methanolic extract

S.N	Plants name	Total Flavonoid Content mg QE/gm				S.D
		TFC 1	TFC 2	TFC 3	Mean TFC	
1	*Helixanthera ligustrina*	29.16	28.81	32.38	30.12	1.97
2	*Macrosolen cochinchinensis*	26.74	25.59	22.38	24.90	2.26
3	*Scurrula elata*	23.81	27.31	26.16	25.76	1.78
4	*Scurrula parasitica*	29.16	31.31	27.31	29.26	2.00
5	*V. articulatum var. liquidambaricolum*	24.59	28.52	28.24	27.12	2.19
6	*Viscum album*	33.74	29.09	31.52	31.45	2.32
7	*Viscum articulatum*	26.09	29.38	29.09	28.19	1.82

Appendix V: Total Phenolic content in methanolic extract

S.N	Plants name	Total Phenolic Content mg GAE/gm				S.D
		TPC 1	TPC 2	TPC 3	Mean TPC	
1	*Helixanthera ligustrina*	28.31	26.06	30.31	28.23	2.13
2	*Macrosolen cochinchinensis*	26.81	31.31	35.56	31.23	4.38
3	*Scurrula elata*	21.31	22.69	20.94	21.65	0.92
4	*Scurrula parasitica*	30.06	34.44	34.19	32.90	2.46
5	*V. articulatum* var. *liquidambaricolum*	23.69	18.69	28.19	23.52	4.75
6	*Viscum album*	16.56	23.31	21.94	20.60	3.57
7	*Viscum articulatum*	29.19	8.94	30.81	22.98	12.19

Appendix VI: IC50 values of Mistletoes

S.N	Plants name	IC_{50}(1st)	IC_{50}(2st)	IC_{50}(3st)	Mean IC_{50}	S.D
1	*Helixanthera ligustrina*	87.48	88.20	88.20	88.18	0.69
2	*Macrosolen cochinchinensis*	65.21	63.45	68.96	65.87	2.82
3	*Scurrula elata*	178.94	188.88	191.29	186.37	6.55
4	*Scurrula parasitica*	25.98	25.36	26.79	26.05	0.72
5	*V. articulatum var. liquidambaricolum*	106.06	101.44	100.29	102.60	3.06
6	*Viscum album*	199.42	197.59	200.00	199.00	1.26
7	*Viscum articulatum*	182.55	183.10	183.67	183.11	0.56

Appendix V: Percentage antioxidant activity of Mistletoes at different concentration

Conc. (µg/ml)	Mean % DPPH radical scavenging activity						
	H. ligustrina	*M. cochinchinensis*	*S. elata*	*S. parasitica*	*V. art. var. liq.*	*V. album*	*V. articulatum*
12	23.29	43.17	25.50	49.73	42.57	23.75	25.07
50	33.15	45.91	38.17	54.36	42.98	29.00	30.04
75	33.99	51.53	41.50	56.25	43.81	33.67	34.31
100	62.44	52.11	41.42	57.65	49.49	34.33	34.38
125	67.42	55.62	42.33	62.77	58.04	35.17	33.61
150	67.51	57.02	44.58	62.10	64.34	36.58	40.55
175	68.54	57.73	49.42	64.84	65.09	43.33	41.67
200	69.01	58.94	50.75	66.61	66.46	50.25	66.25

*H. ligustrina: Helixanthera ligustrina , M. cochinchinensis: Macrosolen cochinchinensis, S. elata: Scurrula elata, S. parasitica: Scurrula parasitica, V. art.*var. *liq: Viscum articulatum* var. *liquidambaricolum.*

Appendix VI. Photoplates

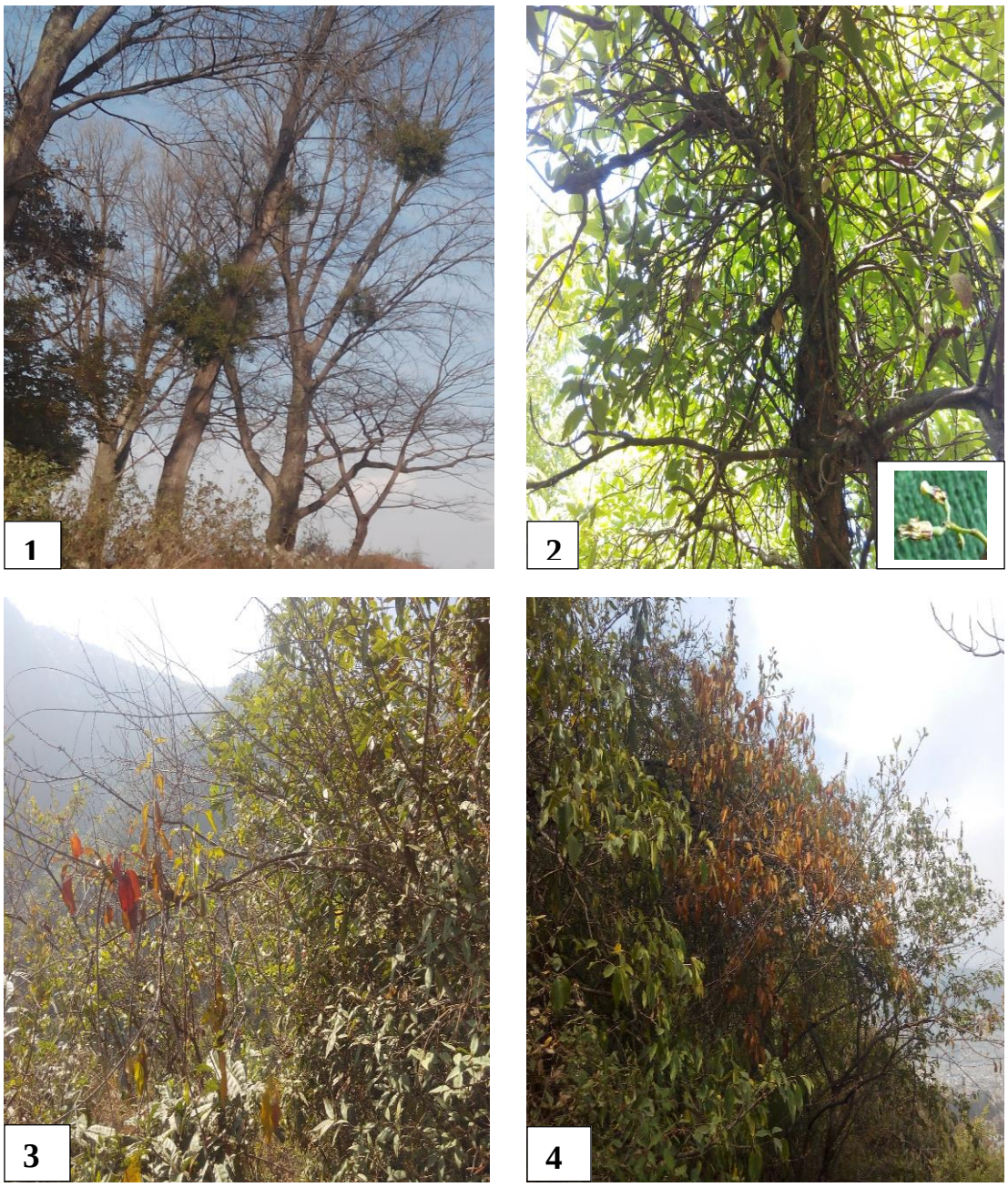

Plate 1: **1.***Helixenthera ligustrina* (Wall.) Danser**2.***Macrosolen cochinchinensis* (Lour.) Tiegh.**3.***Scurrula elata* (Edgew.) Danser**4.***Scurrula parasitica* Lour.

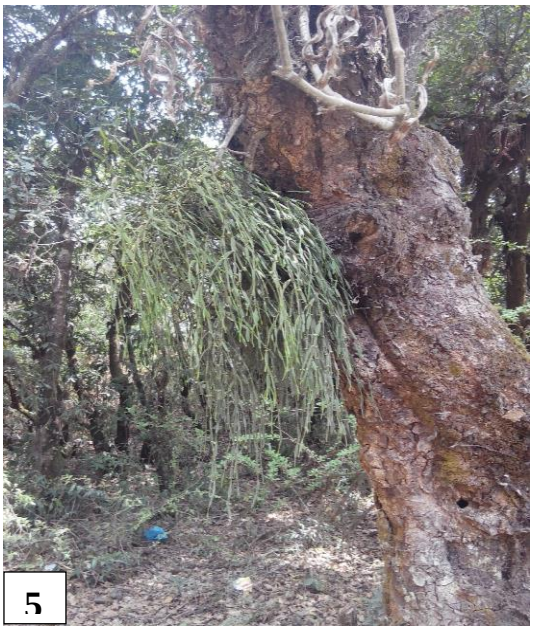

Plate 1: **5.***Viscum articulatum* Burm. f.,**6.***Viscum articulatum* var. *liquidambaricolum* (Hayata.) S. Rao, **7.***Viscum album* L.

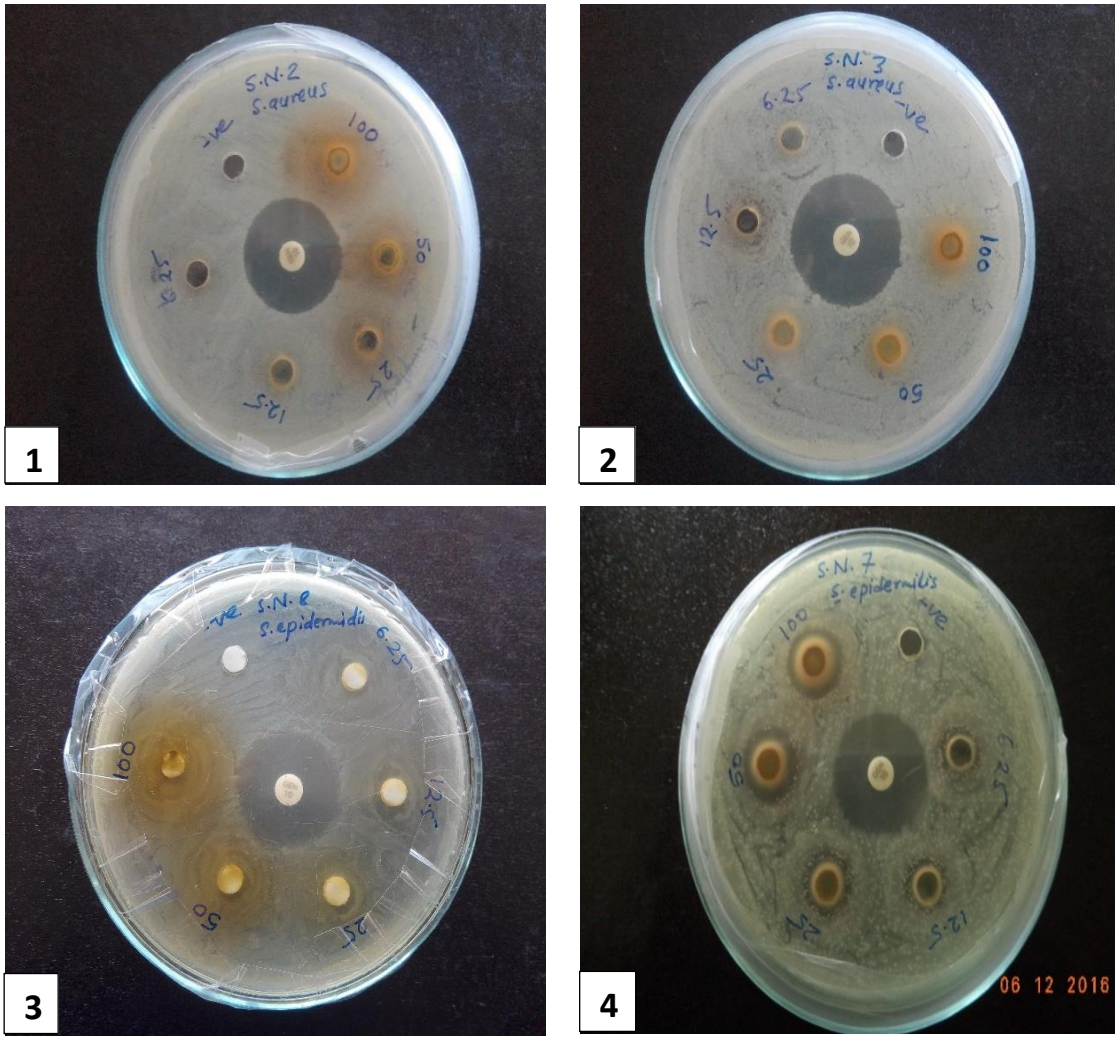

Plate 2: Antibacterial activities of, **1.***Helixanthera ligustrina*,**2.***Scurrula parasitica*

3. *Macrosolen cochinchinensis*, **4.***Viscum articulatum*

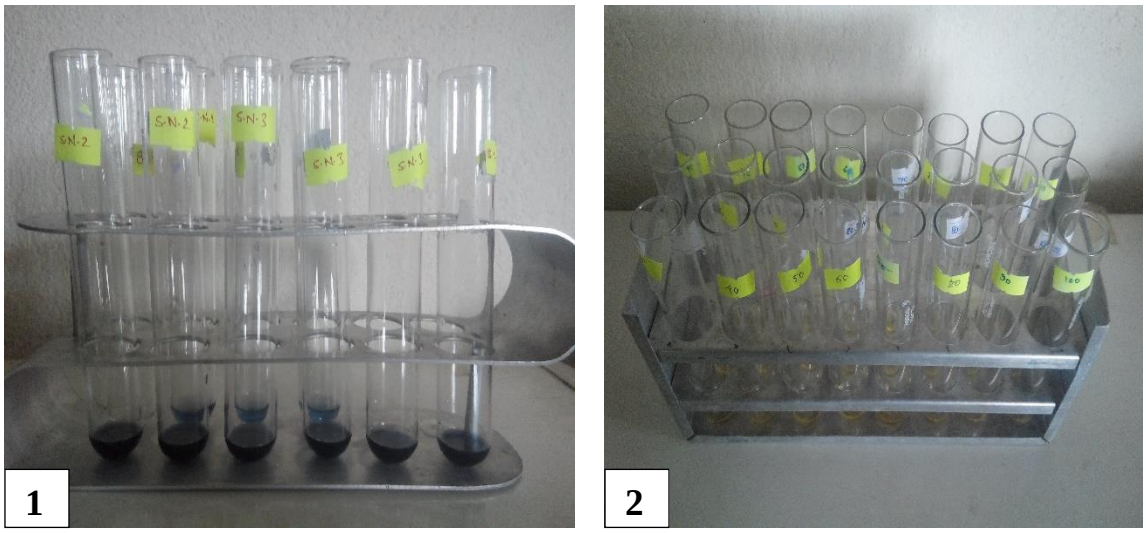

Plate 3: **1.** Quantitative estimation of Phenolic content, **2.** Determination of antioxidant activity