VARIATION IN BIOLOGICAL ACTVITIES OF METHANOLIC EXTRACT OF NEPALESE *SWERTIA CHIRAYITA* POPULATION

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Namita Bhattarai

Acronyms

ATCC	American Type Culture Collection
D/W	Distilled water
DW	Dry Weight
DMSO	Dimethyl sulfoxide
DPPH	1, 1- diphenyl-2 picryhydrazyl
GAE	Gallic Acid Equivalent
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectroscopy
HPLC	High performance liquid chromatography
HPTLC	High performance TLC
IC_{50}	Inhibitory Concentration 50
IL	Inflorescence and leaf mixture.
IL	Interleukin
IR	Infra-Red
ISSR	Inter Simple Sequence Repeat
ITS	International Transcribed Spacers of the nuclear rDNA Repeat
IUCN	International Union for Conservation of Nature
LB	Luria Bertani
LC	Liquid Chromatography
MHA	Muller Hinton Agar
NA	Nutrient Agar
NAST	Nepal Academy of Science and Technology
PCR	Polymerase Chain Reaction
POPGENE	Population Genetic Analysis (Statistical package)
QE	Quercitin Equivalent
RAPD	Random Amplified Polymorphic DNA
RECAST	Research Centre for Applied Science and Technology
RSA	Radical Scavanging Activity
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
TPC	Total Phenolic content
UV	Ultra Voilet
WHO	World Health Organisation
ZOI	Zone Of Inhibition

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Abstract

Swertia chirayita is a medicinal plant indigenous to temperate Himalaya. It is known to contain many bioactive compounds having pharmacological activities. An assessment of phenolic content, flavonoid content, antioxidant property, and antimicrobial property of methanolic extracts from different populations of *S. chirayita* was done in the present work. Furthremore, semiquantitative estimation of three marker chemicals namely Amarogentin, Swertiamarin and Mangiferin was also done. Preliminary phytochemical screening showed the presence of saponin, alkaloid, phenols, flavonoids, glycoside, tannin, terpenoid and phytosterol in methanolic extracts from all populations. Evaluation of antimicrobial acitivity of methanolic extracts of different populatios of *S. chirayita* showed the antimicrobial activity against *Escherichia coli, Salmonella typhii, Klebsiella pneumoniae, Staphylococcus aureus* and *Enterococcus faecalis* but no activity against *Pseudomonas aeuregenosa.* Similarly, antioxidant activity was shown by the extracts of all populations of chirayita. However the extracts from the populations collected from Makawanpur showed the lowest IC_{50} value 42.07±3.39. Thin layer chromatography (TLC) showed the presence of all three marker compound in all samples. Semiquantitative estimation of three marker compounds was done using GelQuant.Net software. Highest Amarogentin content (0.299 mg/gm DW) was found in wild populations from Lalitpur (Phulchowki) and lowest Amarogentin content in wild populations from Jumla (0.005 mg/gm DW). The content of Swertiamarin was highest (0.15 mg/gm DW) in wild populations of Lalitpur (Phulchowki) and lowest (0.017) in cultivated populations from Ilam. Similarly, highest Mangiferin content (8.83 mg/gm DW) was found in wild populations from Tehrathum and the lowest content (0.23 mg/gm DW) in wild samples from Lamjung.

Key words: medicinal plant, *Swertia chirayita*, Methanolic extracts, TLC, antioxidant activity, antimicrobial activity and marker chemical compound.

Chapter 1-Introduction

1.1 Introduction

The central Himalaya is a huge repository of medicinal plants. Nepal for being located at this portion of the Himalaya, known for its disproportionately rich biodiversity and has always remained a place of great interest to the botanists and phytochemists involved in researching medicinal herbs (Watanabe *et al*., 2005).

Medicinal plants always played an important role in the health development of mankind. In developing countries, 80% of populations are totally dependent on plants for their primary health care (Tabassum *et al*., 2012). Over 25% of prescribed medicines in industrialized countries derive directly or indirectly from medicinal plants. A multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques led to drug discovery from plant (Newman *et al.*, 2000). Plants provide us new lead molecules for the development of drugs against various pharmacological targets (Tabassum *et al*., 2012).

It has been estimated that our world is inhabited by 250,000 higher plant species and more than 80,000 plant species are reported to have at least some medicinal value and around 5000 species are known to have specific therapeutic values (Tabussum *et al*., 2012).

Nepal is rich in biodiversity due to its various geographical distributions. Traditional plant based medicine has been practiced long time before and still in use today. Number of medicinal and aromatic plants reported in Nepal varies according to various sources from 571 to 1950. In 2008, compilation of Medicinal and Aromatic Plant Database of Nepal (MAPDON) was published, which revealed 1950 species of medicinal plant in Nepal. Out of which 1906 species are identified under vascular group comprising 1614 native, 192 introduced and cultivated and 100 naturalized taxa. Whereas recent research showed that there are more than 2300 medicinal plant which are already had been identified till date (DPR, 1984; Manandhar, 2002; Sharma, 2004; Gewali, 2008; Luitel and Pathak, 2013).

Family Gentianaceae contains 87 genera and nearly 1,700 species of annual and perennial herbs and rarely shrubs, native primarily to northern temperate areas of the world (Struwe and Albert, 2002; Encyclopedia Britanica). In Nepal 17 genera and 116 species of the Gentianaceae family has been reported (Press *et al*., 2000). *Swertia* is one of the medicinally important genera of the family which has been listed to contain 150 species world wide and 31 species in our country of which one species (*S. acualis*) being endemic and distributed through 600m to 5500m (Joshi, K. 2008; Joshi and Joshi, 2008).

Swertia chirayita is an erect biennial or pluri-annual herb that have the high medicinal value demand in national and international market (Joshi and Dhawan, 2005; Ghimire *et*

al., 2008) and is an important factor for the economy of Nepal. About 45% of chirayita in the Himalayan region is collected from Nepal (Shah *et al*., 1999). This plant is widely used in Ayurvedic, Unani and Siddha systems of Medicines (Williamson, 2002). It is bitter tonic effective in indigestion, bloating, and nausea and also said to be effective in protecting the liver. An herbal antiseptic and antifungal veterinary ointment Melicon V is prepared from the herb (Kumar *et al*., 2010).

This species alone comprises about 3% of the total trade of medicinal plants (Phoboo and Jha, 2010) to be exported from the country. These days nine species of *Swertia* including *S. chirayita* and *S. angustifolia* are traded (Pant, 2005). Among the different species of the genus, *S. chirayita* is considered the most important for its medicinal properties (Karan *et al*., 1996).

Literatures mention *Swertia chirayita* (Roxb. ex Fleming) H. Karsten as *Swertia chirata* Buch-Ham.; *Ophelia chirata* Grisebach; *Agathotes chirayita* Don; *Gentiana chirayita* Roxburgh and *Gentiana floribunda* Don (Joshi and Dhawan, 2005; WWF, 2008). Locally *Swertia chirayita* is called as “Tito”, “Tite”, “Pothichirayita”, and “Daklechirayita” in Nepali language. In other languages it is called as “Tento” in Gurung; “Timda” in Tamang; “Suingkhangwa” in Limbu; “Rauka” in Magar; “Khupli” in Rai; “Ghyatig” in Tibetan languages (WWF, 2008).

The compilation of the chemical constituents on *Swertia* genus has been done from time to time by various authors as Wang *et al*., 1992; Pant *et al*., 2000; Negi *et al*., 2011b. Metabolite profiling of *S. chirayita* has been reported to contain xanthones, terpenoids, glycosides, flavonoids, alkaloids, phenolics acids and many more compounds of which the xanthones and secoiridoids glycosides are pharmacologically most important (Tabassum *et al*., 2012; Negi *et al*.,2011a; Brahmachari *et al*., 2004). Major compounds that have been screened for the pharmacologic effect includes xanthones like swerchirin, Mangiferin and the secoiridoid glycosides like Amarogentin, amaroswerin, Swertiamarin, etc. Their property on hypoglycemic effect, anti-inflammatory, antimalarial, antileishmanial, antiperiodic, analgesic, antimicrobial, hepatoprotective, antihemlinthic, and anticolinergic property were reported (Brahmachari *et al*., 2004; Negi *et al*., 2011a; Tabassum *et al*., 2012; Phoboo *et al*., 2012; Rafatullah *et al*., 1993).

Mangiferin is the first xanthone investigated pharmacologically. It shows broad spectrum biological activities. It is reported to possess strong anti- inflammatory activity in arthritic mice, and accounted for lowering down TNF- alpha, IL-1beta, IL-6, and IFN-gamma and up regulation of IL-10 in the joint homogenates of mice. (Tabassum *et al*., 2012).

Amarogentin is the most acerbic compound found till date which shows Topoisomerase inhibition, chemo-preventive, and antileishmanial effects (Ray *et al*., 1996; Tabassum *et al*., 2012).

Swertiamarin is one of the secoiridoid glycosides present in the Chirayita which has anticolinergic property (Negi *et al*., 2011b).

Various chemical profiling papers of chirayita has been published. The use of sophisticated instruments including HPLC, HPTLC, GC-MS, and other has been used to screen the level of major phytoconstituents (Phoboo *et al*., 2010; Bhandari *et al*., 2006; Negi *et al*., 2011b).

1.2 Rationale and scope of the study

Swertia chirayita is known for various medicinal properties like antimalarial, hepatoprotective, anti-inflammatory activities etc. Due to its widespread use in various types of alternative medical practices like Ayurveda, Unnani, Siddha, Tibetan and Chinese traditional medicine, this plant is constantly being exported from Nepal in large quantities (Phoboo and Jha, 2010). The large scale export of this plant has made it vulnerable in Nepal and there is an immediate need for its conservation. This has led to a significant threat to the natural populations of this species (Phoboo and Jha, 2010).

Considering this threat from overexploitation of the species from wild habitat several strategies have been used for sustainable harvesting and conservation of this species. One of the strategies that have been taken is through the cultivation of this species in community forests and farmers' fields (Phoboo and Jha, 2010). But there have relatively few studies on quality of the products collected from natural habitats and those from domesticated sources in terms of quantity of marker chemical compounds. Due to geographical variation and different habitats of *Swertia chirayita* there exist different populations within different environment. Since the medicinal value of the medicinal plants is due to the presence of different secondary metabolites, populations with high content of these chemicals will help to increase the quality of the herbs as well as the income of the rural people through the cultivation and trade of these herbs. Furthermore, cultivation of high yielding lines in farmer's fields and in community forests will reduce pressure on natural populations so that the species presence would be assured.

There is a local belief that plants from East Nepal are biologically more active (Phoboo *et al*., 2010). So the selection of best line in terms of their biological activity as well as quantity of the biologically active chemical is required for the cultivation, conservation and trading. Metabolic profiling reveals the presence of active phytochemicals and studies on the variation of these chemical among different populations/species guide us to select the best line. Therefore the present study aims to screen the different Nepalese populations of *S. chirayita* in terms of the quantity of Amarogentin, Swertiamarin and Mangiferin present in their methanolic extracts.

1.3 Objectives

1.3.1 General objective:

Screening the existing natural populations and cultivated populations of *Swertia chirayita* in terms of contents of Amarogentin, Swertiamarin and Mangiferin and evaluation of crude extract for their biological activities in terms of antimicrobial and antioxidant properties.

1.3.2 Specific objective:

- To evaluate the antimicrobial and antioxidant property of methanolic extracts of collected samples.
- To carry out quantitative estimation of the major marker compounds present in methanolic extracts
- To analyze the relationship between the biological activity and quantity of phytochemicals present
- Selection of best populations for further study

Chapter 2 – Literature review

2.1 Medicinal plant of Nepal- an overview

Plants are the source of medicine and produce an amazing diversity of low molecular weight compounds. They contain hundreds of thousands of such compounds. Among these some are part of 'primary' metabolic pathways (those common to all organisms) and the rest are termed as 'secondary' metabolites; this term is historical and was initially associated with inessentiality but, here, a 'secondary' metabolite is defined as a compound whose biosynthesis is restricted to selected plant groups (Pichersky and Gang, 2000). These non-nutrient plant chemical compounds or bioactive components are often referred to as phytochemicals that are responsible for plant defense (Abo *et al.,* 1991; Liu, 2004).

The Use of herbal medicine has been mentioned since prehistoric times. The oldest treaties Rigveda have recorded 67 medicinal plants while Yajurveda has mentioned 81 species and Atharvaveda 290 species. Medicines based on plants were 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 (Joy *et al*., 1998).

WHO described medicinal plant as any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo- pharmaceutical semi synthesis (Doughari, 2012). Due to low toxicity of medicinal plant and increasing inefficacy of many modern drugs, resistance possessed by several bacteria to various antibiotics, medicinal plants are increasingly gaining acceptance even among the literates in urban areas (Voravuthikuchai and Kitpipit, 2003).

Several studies on medicinal plants have revealed that medicinal plants contain bioactive compounds which can be classified as alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones, essential oils and steroids (Doughari, 2012). Different methods can be employed to extract phyotochemicals.

Nepal covers the total area of 1,47,181 sq.Km with the latitudinal range from 26°22' to 30°27' N and longitudinal ranges from 80°14' to 88°12' E, i.e. only 0.03% of the total land area of the world but has huge altitudinal variation. Within the width of 130 Km the altitudinal range is from 68m, Mahottari to 8,848m, Mt. Everest. Such huge coverage of altitude variation has made our country rich in natural resources. It is reported that more than 80% of the rural Nepalese people depend on traditional remedies that involve the use of local plants in various forms and combinations (Rajbhandary and

Bajracharya, 1994). There are 1792 medicinal plants in Nepal (Baral and Kurmi, 2006) out of which 49% are herbs, 29% trees, 14% shrubs, 8% climbers (Bhattarai and Ghimire, 2006). Traditionally, there is the use of about 1000 species of medicinal plants by various communities in Nepal for their primary healthcare system (Chaudhary, 1998).

Earlier plants as medicines were 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. Discovery of drugs from plants has traditionally been time-consuming, so faster methods for plant collection, bioassay screening, isolation and development of compound must be adopted (Karan *et al*., 1996).

2.2 Family Gentianaceae

Plant belonging to this family is cosmopolitan in distribution. Gentianaeae contains flowering plants of 84 genera and nearly 1,700 species of annual and perennial herbs and, rarely, shrubs. Plants belonging to this family are native to the northern temperate areas of the world (Singh, 2008; Strew and Albert, 2002). They are known for the bitter taste and used in traditional remedies against loss of appetite, fever, and are still included in many "tonic" formulations (Negi *et al*., 2011a; Jensen and Schripsema, 2002). In Nepal 17 genera and 116 species of the Gentianaceae family has been reported (Press *et al*., 2000).

2.2.1 Genus *Swertia*

Swertia is named in honor of Emanuel Sweert (1552-1612), a Dutch gardener. Member of the genus are annual, biennial or perennial herbs. The plants species of genus *Swertia* (family Gentianaceae) is diverse and large genus populated with 170 species (Brahmachari *et al*., 2004; Hajimehdipoor *et al*., 2008) and distributed at the mountainous region of tropical Asia, Europe, America and Africa, like most other member of the gentian family. Its original home is the area around Himalayas (Negi *et al*., 2011a; Dutt *et al*., 1996). The genus mostly occurs in alpine or temperate habitats in Asia, Africa and North America (Sharma *et al*., 2011). There are 31 species distributed in Nepal (Joshi and Joshi, 2008), 79 species in China (Hajimehdipoor *et al*., 2008), 40 species in India (Dutt *et al*., 1996). *Swertia* is a large genus of herbs distributed in the mountainous regions of tropical area at an altitude of 1200-3600m (Negi *et al*., 2011a).
Nepal is endowed with 31 species including five varieties of *Swertia* (Joshi and Joshi, 2008; Shrestha *et al*., 2010) among, *Swertia acualis* is the endemic one (Joshi, 2008). *Swertia* species has been reported to be distributed on 54 districts of Nepal (Barakoti *et*

al., 1999; Phoboo *et al*., 2010; Shrestha *et al*., 2010). Species of *Swertia* are reported to have ethnobotanical value in various Nepalese communities and most popular medicinal preparation includes infusion, decoction, paste and juice (Joshi K, 2008). Some 104 non-timber forest products (NTFPs) are collected and commonly traded in Nepal. Among them *Swertia* species occupy one of the major positions in the trade. A total of nine species are traded in Nepal and these includes, (1) *Swertia chirayita* (Roxb. ex Fleming) H. Karst., (2) *Swertia angustifolia* Buch. Ham.Ex D.Don (3) *Swertia tetragona* Edgew. (4) *Swertia racemosa* (Griseb.) C.B. Clarke (5) *Swertia ciliata* (D. Don ex G. Don) B.L. Burtt, (6) *Swertia dilatata* C.B. Clarke, (7) *Swertia multicaulis* D. Don, (8) *Swertia alata* (Royle ex D. Don) C.B. Clarke, and (9) *Swertia nervosa* (G.Don) C. B. Clarke (Shrestha *et al.,* 2010). Among different species of *Swertia*, *Swertia chirayita* is considered the most important for its medicinal properties (Joshi and Dhawan, 2005).

2.2.2 *Swertia chirayita* (Roxb. ex Fleming) H. Karsten

Swertia chirayita is an erect, binneal or pluri-annual herb, 60-150 cm (Pradhan and Badola; 2012, Ghimire *et al*., 2008; Joshi and Dhawan, 2005) with robust, branched stem. The stems are orange brown or purplish in color and contain large continuous yellowish pith; Leaf nearly rough but glabrous, three to five nerved, dark greenish brown to blackish in colour; petioles almost absent. The Roots are yellowish brown and simple, tapering and stout, short, almost 7 cm long and usually half an inch thick (Sharma *et al.,* 2011). Both self-pollination and cross-pollination has been reported in the plant (Pradhan and Badola, 2012).

Swertia chirayita is used in various systems like American and British pharmacopoeias, Indian Pharmaceutical codex and in different conventional systems of medicines like Ayurvedic, Unani and Sidha (Tabassum *et al*., 2012). Concoction of Chirayita with cardamom, turmeric and kutki is given for gastrointestinal infections, and along with ginger. It is considered good for fever when given along with neem, manjishta and gotu kola, it serves as a cure for various skin problems. It is used in combination with other drugs in cases of scorpion bite (Kirtikar and Basu, 1984; Nandkarni, 1976). Thus the widespread use of plant in traditional medicine reflects the pharmacological importance and has high demand in national and international market. The demand rate is also increasing 10% annually. Nepal is the main exporter of the *Swertia chirayita* which is the important factor for the economy of Nepal. About 45% of the Chirayita in the Himalaya region is collected from Nepal (Joshi and Dhawan, 2005; Shah 1999). *S. chirayita* is traded in 61 out of the 75 districts of Nepal (Phoboo and Jha, 2010) and chief supplying districts are Shankhuwasabha, Tehrathum, Dhankuta, Ilam, Panchthar, Rasuwa,

Solukhumbu, Taplejung, Ramechhap, Dolakha, Sindhupalchowk, Gorkha, Dolpa, Rolpa, Salyan, SIndhuli, Accham, Doti and Makwanpur (Bhattarai and Acharya, 1998). To meet national and international demand chirayita is collected from wild habitat recklessly which has been resulted in decrease in the exisiting population of Chirayita and IUCN has been categorized it as critically endangered. This reflects the need of conservation of plant. The plant is recognized for the economic development and many conservation and cultivation programmes has come into existence (Joshi and Dhawan, 2005; Joshi, 2008; Phoboo and Jha, 2010). In India National Medicinal plant Board has prioritized for conservation and cultivation in Uttranchal (Joshi and Dhawan, 2005).

In Nepal *Swertia chirayita* has been kept in the list of vulnerable species and is one of the species prioritized for commercial cultivation (Bhattarai *et al*., 2001; Singh *et al*., 2006). Low germination percentage and viability of seeds, long gestation periods and delicate field handling are some of the factors which discourage commercial cultivation of the plant (Joshi and Dhawan, 2005). Hence to promote the sustainable use and conservation of the wild variety of plant various in situ and ex- situ exercise along with *in vitro* propagation technique play a vital role. Shrestha P. (2013) successfully carried out *In vitro* propagation of *S. chirayita* taking nodal explant and found 30% rate of acclimatization on the study for six weeks period at Central Department of Biotechnology (CDBT).

2.3 Chemical composition and pharmaceutical aspects

Swertia chirayita is highly demanded medicinal herbs and it's medicinal usage is declared in American and British pharmacopoeias, Indian Pharmaceutical codex and in different conventional systems of medicines like Ayurvedic, Unani and Sidha (Tabassum *et al*., 2012). In Indian medical system chiretta is used as remedy for bronchial asthma, liver disorders, chronic fever, anemia, stomachic and diarrhoea. Chiretta is also used in dying cotton cloth and in liquor industry as bitter ingredients. In Ayurveda, *S. chirayita* is used as antipyretic, antihelminitic, antiperiodic, laxative and in asthma and leucorrhoea. In Yunani system the plant is used as astringent, tonic, stomachic, lessens inflammation, sedative to pregnant uterus and chronic fevers (Kirtikar and Basu, 1984). *S. chirayita* is also used in British and American pharmacopoeias as tincture and infusions (Joshi and Dhawan, 2005). Biological activity is attributed to the various secondary metabolites present in the plant.

The plants of the *Swertia* genus are rich sources of xanthones, flavonoids, irridoid and seco-irridoid glycosides, terpenoids, and alkaloids. The major bioactives of *Swertia* are

xanthones. Pant *et al.* (2000) has enlisted a total of 43 compounds whereas, Brahmachari *et al.* (2004) has enlisted a total of 48 compounds including 13 tetraoxygenated xanthones, 6 xanthone glycosides and derivatives, 18 terpenoids, 3 alkaloids, and 4 secoiridoid glycosides, whereas Joshi and Dhawan (2005) has tabulated a total of 40 compounds. It has now been observed that a number of plant products which are in regular use as chemotherapeutic agents contain xanthones as active constituents. Simple polyoxygenated xanthones have been isolated from most of them. Some specific activities have been reported for xanthones and iridoids from Gentianaceae (Negi *et al*., 2011a).

Systemic study of Hostettmann-Kaldas *et al.* (1981) documented that unlike iridoids, xanthones are apparently not present in all plant species investigated in the family Gentianaceae (Negi *et al*., 2011a). More than twenty polyhydroxylated xanthones have been characterised, and some of these are **swertinin**, **swerchirin**, **Mangiferin**, **decussatin** and **isobellidifolin** (Bhattacharya *et al*., 1976).

2.3.1 Mangiferin

Mangiferin was the first xanthone to be investigated pharmacologically and has been found to exhibit a broad spectrum of biological activities (Negi *et al*., 2011b). It is reported to possess considerable hypoglycemic property and also reported to have significant anti-diabetic activity similar to the clinical drug glibenclamide and acts by stimulation of insulin production from the pancreas, extra-pancreatic action and enhancement of glycolytic enzymes (Sellamuthu *et al*., 2009). It is a good antioxidant (Phoboo *et al*., 2012), and also possess antitumor (Guha *et al*., 1996), antiviral (Zheng and Lu 1990), antiatherogenic (Murugananadan *et al*., 2005), immunodilatory (Guha *et al*., 1996; Andreu *et al*., 2005), antiproliferative, cardiotonic and diuretic properties (Andreu *et al*., 2005). Antimicrobial activity is also reported (Singh *et al*., 2009).Pronounced anti-inflammatory activity has also been observed in Mangiferin. Oral and topical compounds containing Mangiferin are useful for the treatment of diseases caused by herpes virus (Negi *et al*., 2011a). In Cuba, Mangiferin is traditionally used as an antiinflammatory, analgesic and also as an antioxidant under brand name Vimang®. In Sri Lanka, Mangiferin is used in the obesity treatment and particularly for diabetes type II under brand name Salaretin® (Singh *et al*., 2009).

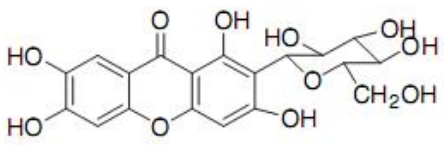

Fig Mangiferin

2.3.2 Swertiamarin

Important secoiridoid glycoside present in the *Swertia* species has been reported to have very low toxicity and is antibacterial (Kumarasamay *et al*., 2003), anticholinergic (Suparna *et al*., 1998) and antinociceptive (Jaishree *et al*., 2009). It also possess hepatoprotective and antiedematogenic/antiinflammatory, free radical scavenging activity (Vaijanathappa and Badami, 2009). Swertiamarin has also been reported as a potent lipid lowering agent comparable to the clinical drug atorvastatin which may also contribute to its cardio-protective and anti-atherosclerotic role (Vaidya *et al*., 2009).

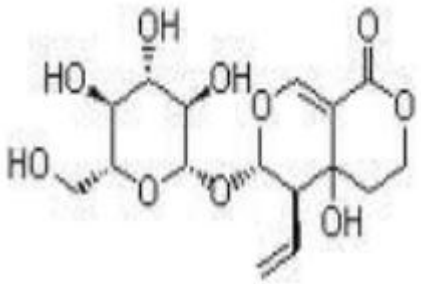

Fig Swertiamarin

2.3.3 Amarogentin

The most bitter principle compound present in *Swertia chirayita* whose bitterness persists even at a dilution of 1: 58,000,000 (Singh 2008). It is a known topoisomerase inhibitor (Ray *et al*., 1996), chemopreventive and is reported to have antileishmanial (Medda *et al*., 1999) and gastroprotective properties (Niiho *et al*., 2006). Antimicrobial activity is also shown by Amarogentin (Samaddar *et al.*, 2013).

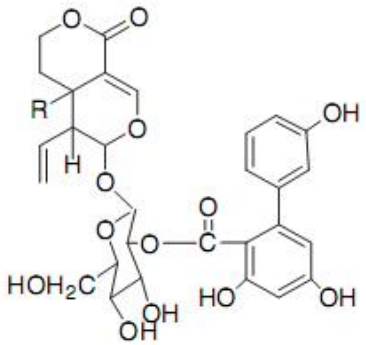

Fig Amarogentin (R=H)/Amaroswerin (R=OH)

Such plant derived biomolecules make up a significant segment of natural product-based pharmaceuticals. Particularly, Swerchirin and Amarogentin have been processed for the drug design (Tabassum *et al*., 2012; Bhardwaj *et al*., 2011). Natural products can be used

directly as pharmaceutical compounds or as lead chemical for drug enforcement to produce compound with high activity and/or low toxicity (Doughari, 2012).

2.4 Chromatographic technique

The term, “chromatography" was coined by the Russian botanist, Tswett. **Chromatography"** represents a separation technique; whereas a **"chromatograph"**is a system for performing chromatography. Although originally intended to separate and recover (isolate and purify) the components of a sample, today, complete chromatography systems are often used to both separate and quantify sample components.

There are two main classification of chromatography system based on mobile phase and stationary phase as shown in Table: (hitech.com)

Mobile phase	Stationary phase	Analysis	Sample Types
Gas	Solid/Liquid	Gas chromatography (GC)	Samples that are gaseous at ordinary temperatures and samples that vaporize when heated ·Odorous samples such as petrochemicals, perfumes, and thinner are easier to analyze by GC. ·High molecular weight compounds are measured after pyrolysis.
Liquid	Solid/Liquid	Liquid chromatography (LC)	Liquid samples and solvent-soluble solid samples ·Compared to GC, LC has a wide range of measurement subjects. ·High molecular weight compounds can be analyzed, if soluble in solvent.

2.4.1 Thin layer chromatography

TLC is a principal separation technique in plant chemistry research which gives chemist a quick idea the number of chemical components in a mixture. It can be used for identification of known and unknown compounds on the basis of RF (Retention factor) value.

There is commercial availability of TLC and high performance TLC (HPTLC) precoated plates, e.g., those with the inorganic adsorbent layers (silica or silica gel and alumina); organic layers (polyamide, cellulose); organic, polar covalently bonded modifications of the silica gel matrix (diol, cyanopropyl, and aminopropyl); and organic, nonpolar bonded stationary phases (RP2, RP8, RP18) with different densities of coverage of the silica matrix (starting from that denoted as W, for the lowest density of coverage and thus wettable with water) (Waksmundzka-Hajnos et al., 2008).

TLC enables separation of a crude plant extract without an earlier purification and is widely used for qualitative analysis of organic compounds, isolation of the individual compounds from multicomopnent mixtures, quantitative analysis, and preparative-scale isolation.

2.5 Variation analysis among *Swertia chirayita* populations

Nepal is affluent in breed variance of *Swertia*, by virtue of enormous assortment of geomorphological aspect and plenty of contrasting environs (Tabassum *et al*.,2012). Considering the range of different niches occupied by the plant, there is a possibility that many ecotypes and/or chemotypes of *S. chirayita* exist so the study of morphological, molecular and biochemical variations among different populations for *S. chirayita* make significant in terms of the amount of diversity they possess. Thus populations with maximum diversity can be identified and isolated for conservation without any duplication within the conserved germplasm (Joshi and Dhawan 2005; Rao and Hodgkin 2002).

Study on variation of the medicinally important phytochemical among *Swertia chirayita* population can make an effort to cultivate and conserve the best population. Phoboo *et al*. (2012) carried out similar study and evaluated the amount of important phytochemicals i.e., Amarogentin, Swertiamarin and Mangiferin present in the *Swertia chirayita* samples collected from cultivated and wild habitats of nine district as Bajura, Mugu, Dolpa, Rasuwa, Sindhupalchok, Dolakha, Taplejung, Dhankuta and Panchthar. HPLC profiling of the 12% ethanolic and aqueous extract was carried out to analyze the variation exist between population. They have reported that there was no difference in the amounts of all the three phytochemicals between extracts from wild and cultivated plants. So the cultivation of *Swertia chirayita* could be a sustainable strategy for its conservation and trade.

Pandey *et al.* (2012) carried out HPTLC fingerprinting method using Mangiferin as biomarker to screen different species and population of *Swertia* and *Swertia chirayita*. Different species and populations of *Swertia* were collected from three states of India viz, Darjeeling District of West Bengal, Sikkim and Arunachal Pradesh. Mangiferin was not detected in *S. bimaculata, S.dialata. S. paniculata*. Mangiferin was detected in *S. nervosa* sample collected from Darjeeling both in vegetative and flowering stage as 0.789% and 1.12% respectively. In *S. chirayita* Mangiferin was detected in all populations in both vegetative as well as flowering stage leaf sample and the value ranges from 1.236% (in vegetative leaves from Sikkim) to 4.37% Mangiferin (in flowering stage leaf from Arunachal pradesh). The results also emphasizes the fact that High Performance TLC using Mangiferin as a biomarker can be utilized for quality screening among different species and population of *Swertia*. Diversity in Mangiferin content exist between and within the species. The elite population needs *in vitro* as well as *in vivo* conservation.

Morphological, biochemical and Genetic variance among and between *Swertia chirayita* has been reported by various authors (Rijal, 2009; Pant *et al*., 2004; Chassot *et al.* 2001). In context of Nepal, initiation of the molecular characterization of *Swertia* spp. Have been reported with the phylogeny and molecular differentiation of 11 Nepalese *Sweria* species: *S. angustifolia, S. chirayita, S. ciliate, S. dilatata, S. lurida, S. macrosperma, S. multicaulis, S. nervosa, S. paniculata, S. pedicilleta* and *S. racemosa* (Joshi K., 2008, 2011) using standard molecular technique (PCR and DNA sequencing). The data obtained from both ITS and Chloroplast (trnl-F) regions were analyzed together with Distance, Parsimony and Bayesian analyses illustrating in phylogenetic trees. The study indicated that *Swertia* is highly paraphyletic. Both ITS and trnl-F data support the close relationship of *S. lurida* with *S. chirayita*.

Shrestha J. (2012) studied genetic diversity within and between various *Swertia chirayita* populations using specific RAPD markers. Thirty four accessions of *S. chirayita* along with 6 allied species were analyzed using 26 RAPD primers. The genetic interrelationship between the populations were clarified by assessment of Nei's genetic identity and distance using POPGENE 1.32 that revealed *S. chirayita* populations of Sankhuwashabha and Terathum as closest (0.9489, identity) and Kaski and Sankhuwashabha as most distant (0.7078, identity). Assessment of genetic variation within populations estimated with percent polymorphism, Shannon's diversity index and Nei's gene diversity reflected the highest within diversity for population of Nagarjun > Phulchowki > Tehrathum > Shankhuwashabha > Kaski.

Similarly, Neupane S, (2013) studied genetic diversity within and between various *Swertia chirayita* populations using specific ISSR markers. 42 accessions of *S. chirayita* were analyzed using 27 ISSR primers. The genetic interrelationship between the populations were clarified by assessment of Nei's genetic identity and distance using POPGENE 1.32 that revealed *S. chirayita* populations of Sankhuwashabha and Terathum as closest (0.9318, identity) and Kaski and Ilam as most distant (0.7052, identity). Assessment of genetic variation within populations estimated with percent polymorphism, Shannon's diversity index and Nei's gene diversity reflected the highest within diversity for population of Sankhuwashabha > Terathum > Phulchowki > Ilam > Nagarjun > Kaski.

Chapter 3 – Materials and Methods

3.1 Setting of laboratory

This thesis work was conducted in the laboratory of the central department of biotechnology, Tribhuvan University.

3.2 Sample material preparation

3.2.1 Collection of plant material

The samples of *Swertia chirayita* were collected from different localities as presented in Table 3.1.

Table 3.1 Details of samples collected from different locations of Nepal

S.N.	Location	Geographical location	Site of Collection	Parts of plant used
1	Ilam (Cultivated)	Eastern	Maipokhari	Whole plant
2	Ilam (wild)	Eastern	Mangalbare	Whole plant
3	Makwanpur (wild)	Central	Tistung	Whole plant
4	Shankhuwashabha (cultivated)	Eastern	Tamaphok-09	Whole plant
5	Shankhuwashabha (wild)	Eastern	Kalika ban upabhokta samiti Tamaphok-09	Whole plant
6	Tehrathum (cultivated)	Eastern	Basantapur-03	Whole plant
7	Tehrathum (wild)	Eastern	Chaite samudayik ban upabhokta samiti	Whole plant
8	Rasuwa (wild)	Central	Thulosyabru	Whole plant
9	Phulchowki (wild)	Central	Phulchowki, Lalitpur	Whole plant
10	Jumla (cultivated)	Western		Whole plant
11	Jumla (wild)	Western		Whole plant
12	Lamjung (wild)	Central		Whole plant
13	Mugu (wild)	Western		Whole plant

3.2.3 Grinding

Shed dried plant material was grinded to fine power using grinder. The fine power was passed through a wire sieve and the fine powder was collected on the polyethylene bag for further use.

3.2.4 Extract preparation

3.2.4.1 Methanolic extract preparation

About 10 gram of fine powder from each sample was taken separately. The extraction technique followed was percolation with intermittent sonication. For this purpose, the sample to solvent ratio was maintained at 1:10 (w/v) in a beaker. The resulting system was then allowed to stand for overnight at room temperature. Then the solution was subjected to intermittent sonication for two hours, i.e. continuous cycle of sonication at 30 kHz for 30 min. It was then filtered through Whatman no.1 filter paper (Whatman Ltd, Kent, and UK). Extraction was carried out for two times and filtrates were mixed (1^{st} and 2^{nd} filtrate). The mixed filtrates were then subjected to evaporation at reduced pressure in a rotary vacuum evaporator at Research Centre for Applied Science and Technology (RECAST), Tribhuvan University. The condensed extract thus obtained was transferred to clean weighed glass vial and allowed to dry at room temperature. Percentage yield of crude extract of the respective plant sample was calculated by following formula.

Percentage yield (%) = (Dry weight of extract/dry weight of plant material)*100

The resulting dry extract was then sealed and stored at 4 ◦C until use.

3.2.5 Extract dilution

20 mg of Crude extract of each sample was weighed and dissolved in 1ml methanol. This prepared 20mg/ml stock solution was used for quantification of the total phenolics and total flavonoids, to quantify antioxidant activity and TLC detection of the marker compound.

100mg of the crude extract of each sample was weighed and dissolved in DMSO and finally the volume is maintained to 1ml. This stock solution was used for antimicrobial screening and was stored at 4◦c until use.

3.3. Qualitative phytochemical analysis

The various extracts and their respective dilutions obtained from methanolic solvents were used to screen for the presence of bioactive compounds by using the protocols following Harborne and Baxter, 1995 and Todkar *et al.,* 2010.

Test for flavonoids

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

Test for glycosides

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

Test for steroids

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

Test for terpenoids

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

Test for saponins

Crude extract was mixed with 5ml of D/W in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for alkaloids

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

Test for phenols and tannins

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

3.4. Quantitative phytochemical analysis

3.4.1 Total phenolic content determination

The total phenolic content of the *Swertia chirayita* was determined using the Folin–Ciocalteu phenol reagent (Chang *et al*., 2002; Roy *et al.,* 2011) with slight modification.

0.1 ml of each extract (2.5 mg/ml) was separately mixed with the 1 ml of Folin–Ciocalteu phenol reagent (Merck Specialities pvt Ltd, India (1:10 dilution with the distilled water) and 0.8ml of aqueous 1 M Na_2CO_3 solution. The reaction mixture was allowed to stand for about 15 minutes and the absorbance of the reactants was measured at 765 nm using the UV- visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained using the solution of gallic acid (Moly Chem, Mumbai, India) as standard in methanol and water (50:50 v/v) using the concentration ranging from 25-250µg/ml. Based on this standard graph, the concentration of the individual samples were calculated. The total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE g^{-1}). For each extract, three replicates were performed for the reproducibility of results.

3.4.2 Total flavonoid content determination (Khanal S, 2011)

The total flavonoid content in the plant extract was estimated using the Aluminium chloride ($AlCl_3$) colorimetric method (Chang *et al.*, 2002; Roy *et al.,* 2011) with slight modifications. 0.25 ml of extract (10 mg/ml) was separately mixed with the 0.75 ml of methanol, 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium acetate (CH_3COOK) and 1.4 ml of the distilled water. The reaction mixture was allowed to stand for about 30 minutes in room temperature. The absorbance of the mixture was measured at 415 nm using the UV – visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained with the help of the quercetin (Sigma) standard solutions in methanol with the concentration ranging from the 10-100µg /ml. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass (mg QE/g). For each experiment three replicates were used for the accuracy and reproducibility of results.

3.5 Antioxidant activity

3.5.1: Preparation of the 0.2mM DPPH solution

1, 1- diphenyl-2 picryhydrazyl (DPPH) has the molecular weight of 394.32 gm/mol. Thus, 100 ml of 0.2mM solution of DPPH was prepared weighing the 7.886 mg of the DPPH carefully and dissolving it in ethanol and finally maintain the volume to 100 ml.

3.5.2 Measurement of DPPH free radical scavenging activity

The antioxidant activity of extract of different populations of *Swertia chirayita* and standard (Ascorbic acid) was assessed on the basis of the radical scavenging effect of the stable 1, 1- diphenyl-2 picryhydrazyl (DPPH) - free radical activity following the protocol of Singh *et al*., (2002) with slight modification. Different concentration of plant extract (30-150µg/ml) and ascorbic acid (30-400µg /ml) were prepared in methanol on the clean and clear test tubes. The sample volume was taken 0.5 ml. To this sample 0.5 ml of the

0.2mM DPPH solution was added. The tubes were shaken vigorously for the uniform mixing. These tubes were allowed to stand for half an hour in dark. The control was prepared as above but without the plant extract or ascorbic acid. Methanol was taken to collect the baseline on the spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The absorbance was taken on spectrophotometer at 517 nm.

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

$$\%\ Radical\ scavenging\ activity = [(Control\ abs - sample\ abs)/Control\ abs)] * 100$$

Standard graph was plotted taking the concentration on the X-axis and percentage scavenging activity on the Y-axis. Based on this graph, IC_{50} value of each sample was calculated based on the formula $IC50 = EXP\ (LN\ (conc. > 50\%) - ((pi > 50\% - 50)/\ (pi > 50\% - pi < 50\%) * LN\ (conc. > 50\%/conc. < 50\%)))$. The IC50 is the concentration of an inhibitor where the response is reduced by half. The IC_{50} value of the different species was compared. The populations having the lowest IC_{50} is considered to have the best antioxidant property.

3.6 Antimicrobial activity

3.6.1 Preparation of Culture media

3.6.1.1 Nutrient agar (NA)

About 28 gram of the powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and poured in distilled water. The contents were dissolved in water completely and the final volume was maintained to 1000 ml followed by boiling for uniform mixing. This media was sterilized in an autoclave at 15lbs pressure and 121°C for 15 minutes. The autoclave tape was used as an indicator for the completeness of sterilization. After this the media was taken out of the autoclave and cooled to about 45-50°C and poured on sterilized and properly labeled petridishes. About 20 ml of the media was poured on each petridishes of 9 cm diameter. The plates were then left for the solidification. The pouring process was carried out on the sterile cabinet. For the preparation of the slant media screw tight bottles were filled with the media followed by autoclaving in the condition as mentioned above. The bottles were then placed in an inclined position and left for solidification of the medium.

3.6.1.2 Luria Bertani broth, (LB) Miller media

The Luria Bertani broth, (LB) Miller medium is a liquid medium. About 25 gram of LB powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and transferred to a conical flask. The content was dissolved in distilled water and final volume was maintained to 1000 ml. This media was transferred to the screw bottles and sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes. Autoclave tape was used for the indication of the completeness of the sterilization. Finally this media was cooled in laminar airflow and was used for the suspension type of bacterial culture.

3.6.1.3 Preparation of Mueller Hinton Agar (MHA)

38 grams of MHA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was weighed and suspended in distilled water. The final volume was maintained 1000 ml. The content was heated to boiling to dissolve the medium completely. 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 poured on sterile petridishes under aseptic conditions for further proposes.

3.6.2 Preparation of the standard culture Inoculums

The individual pure ATCC culture of bacteria *Escherichia coli*, *Salmonella typhii*, *Shigella, Pseudomonas aeruginosa*, *Staphylococcus aureus, Enterococcus faecalis and Klebsiella pneumoniae* were streaked on the different nutrient agar plates. Those plates were incubated on the incubator at 37°C for about 24 hours and pure and isolated colonies were obtained. Each distant colony was aseptically transferred to the Luria Bertani (LB 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 120 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 of the plant extracts.

3.6.3 Transfer of the 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 swab was used for one bacterium. The culture plates were allowed to dry for about 30 minutes.

3.6.4 Antibacterial test

The antimicrobial test was performed by modified agar diffusion method. Six wells were prepared on the solid MHA media with the help of the sterile cork borer of 4 mm

diameter. Three different concentrations (100 mg/ml, 50 mg/ml and 25 mg/ml) of the plant sample were prepared in the DMSO. With the help of the sterile pipette the 30 µl of the each individual plant extract were poured in the above prepared well. The DMSO was taken as negative control while the streptomycin at the concentration of the 10 mg/ml was taken as the positive control. The plates were incubated on the microbial incubator overnight at 37°C and the zone of inhibition was observed and noted for individual plant extract of individual bacteria for different concentration for further analysis.

3.7 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was performed to detect the marker chemical compounds i.e. Amarogentin, Mangiferin and Swertiamarin in the methanolic extract of different population of Swertia chirayta using TLC Atlas, 2004 as reference. TLC analysis was done on both Silica glass plate and Almunium TLC plates.

3.7.1 Preparation of standard

Standard compounds Amarogentin was purchased from Chromadex USA while that of Mangiferin and Swertiamarin were purchased from ZeLang Pharma, Nanjing, China. 2mg/ml stock of each standard was prepared.

3.7.2 TLC of plant sample

A thin layer chromatography of the crude methanolic extract of each sample was carried out to detect the marker chemicals i.e. Amarogentin, Swertiamarin and Mangiferin.

3.7.3 Preparation of TLC plates, sample preparation and application

TLC analysis was done on both Silica glass plate and Almunium TLC plates. Silica TLC plates were prepared for initial detection and analysis.

For preparation of TLC plates 30 grams of silica gel was homogenized with 60 ml of distilled water and this suspension was well distributed over clean and dried 15 x 15 cm TLC glass plates. The plate was then air dried until the transparency of the layer disappeared and then oven dried at 110◦C for 30 minutes and then stored in a dry atmosphere until use. Plastic coated prepared TLC plate was also used.

20 mg/mL methanolic extract was used for TLC detection. 1 µl of each sample was applied on the TLC plate making a spot 1 cm above the bottom.

3.7.3 Development of the chromatogram

TLC chamber was saturated for about 30 min with respective solvent system. After application of the sample, TLC plates were kept in the solvent in TLC chamber and allowed the mobile phase to move through the adsorbent phase up to 3/4th of the plate. According to literature same solvent system was used for Amarogentin and Swertiamarin detection but different solvent system was used for Mangiferin detection as suggested by TLC Atlas, 2005. The plate was air dried and visualize under UV chamber at 254nm. Presence of the reference compound in each the sample was confirmed by comparison with standard compound.

Different solvent system used are listed in table 3.2

Table 3.2 Solvent systems used for separation of different phytochemicals present in *Swertia chirayita*

Compound	**Solvent System**	**Ratio of the solvents**
Amarogentin	Ethyl Acetate: Methanol: Water	7.7 : 1.5 : 0.8
Swertiamarin	Ethyl Acetate: Methanol: Water	7.7 : 1.5 : 0.8
Mangiferin	Ethyl Acetate: Methanol: Formic acid: Water	6.7 : 1.7 : 0.8 : 0.8

3.8 Semi Quantification of marker compound

TLC gives the preliminary determination of the marker compound and its tentative quantification was carried out to study the variation of marker chemical among the collected samples of *Swertia chirayita.* The chromatograms were photographed by a digital camera and the image was analyzed by using GelQuant.net software. Estimation of marker compounds was done semi-quantitatively by comparing the intensities of fluoresecence/quenching of bands in the sample and the reference standards.

3.9 Statistical analysis

All the experiments were performed in triplicates for each sample. Statistical analysis were done using Excel software and GraphPad Prism Version 5 software.

Chapter 4 – Results

Results

Here all the 13 samples of different population of *Swertia chirayita* were subjected to methanolic extraction and the extracts were used for various experiments. The results of these experiments are given below.

4.1 Yield of plant extract

The plant extract collected from various region of Nepal were subjected for methanolic extraction using ultrasonic extraction. The amount of methanolic plant extract extracted from 10 gram of the finely powered plant material of the *Swertia chirayita* collected from different region of Nepal is shown in following Fig 4.1. The highest percentage yield of the extract was found in Tehrathum cultivated (TRC) sample i.e. 16.5% and lowest yield was found from lamjung (LMW) sample i.e. 3.8%. The amount of extract per unit biomass was found to be higher in sample from cultivated populations compared to those from wild populations. There is no significant correlation ($P<0.05$) between wild and cultivated samples' yield.

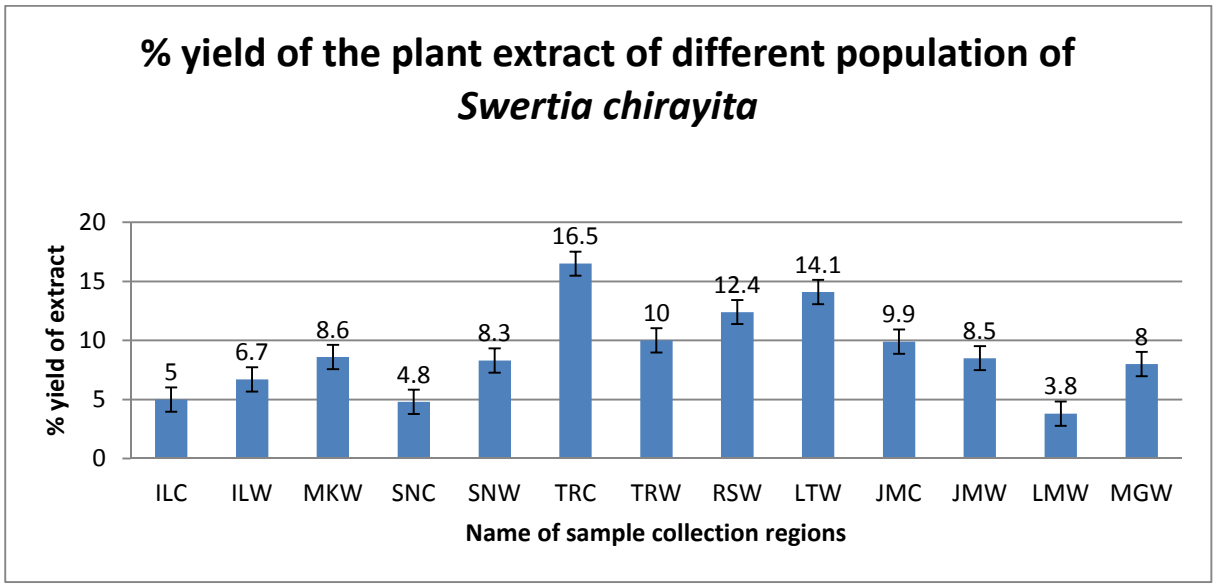

Fig 4.1 % yield of extract of *Swertia chirayita* sample

(IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

4.2 Preliminary detection of phytochemicals

Methanolic extracts of *Swertia chirayita* were subjected to preliminary phytochemical detection and the results were presented in table 4.1.

Table 4.1 Preliminary test for the presence of phytochemical in different population of *S. chirayita*

Plant extraxt	Saponin	Alkaloids	Phenols	Flavonoids	Glycosides	Diterpenes	Tannins	Phytosterol
	Froth test	Mayer's test	Ferric chloride test	Alkaline reagent test	Modified Brontrager's test	Copper acetate test	Gelatin test	Salkowski's test
ILC	++	++	++	++	++	++	+	++
ILW	++	++	++	++	++	++	+	++
MKW	+++	+++	++	++	++	++	+	++
SNC	++	++	++	++	++	++	+	++
SNW	++	++	+	++	++	++	+	++
TRC	++	++	++	++	++	++	+	++
TRW	++	++	++	++	++	++	+	++
RSW	++	++	++	++	++	++	+	++
LTW	+++	+++	++	++	++	++	+	++
JMC	+++	+++	++	++	++	++	+	++
JMW	++	+++	++	++	++	++	+	++
LMW	+	+	+	+	++	+	+	+
MGW	+++	++	++	++	++	++	+	+

+= Presence; ++= moderately presence; +++= highly presence;

(IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

4.3 Total flavonoid content determination

To determine total flavonoid content in the sample standard graph was plotted using standard solution of the quercetin ranging in concentration from 10µg/ml to 100 µg/ml (Fig 4.2). Based on this standard graph and equation i.e. y=0.0105x, total amount of the flavonoid present in the 13 different samples of *Swertia chirayita* was determined. The results were expressed in mgQE/g dry plant ±SEM (Fig 4.3).

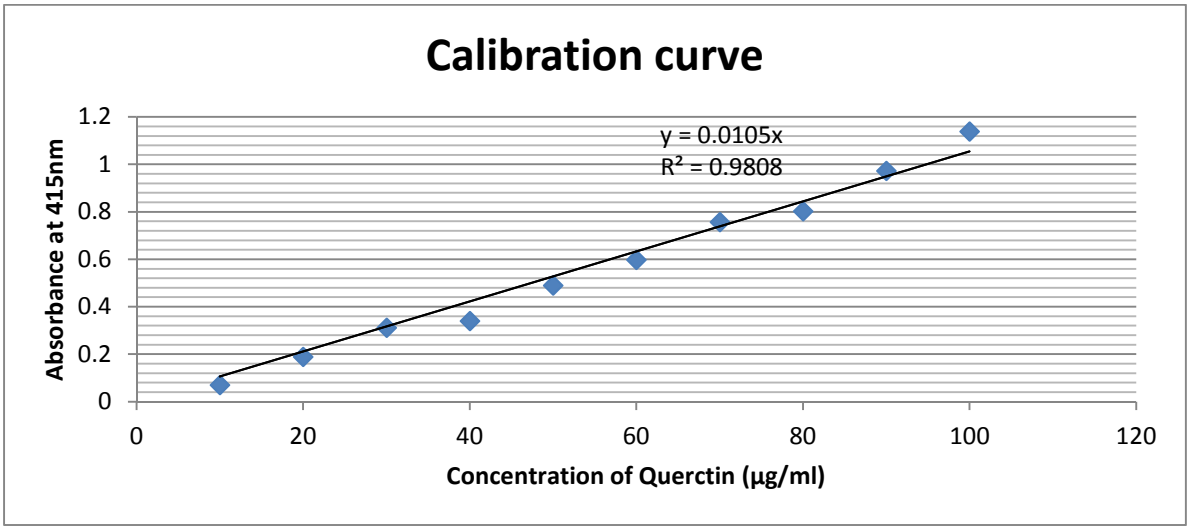

Fig 4.2 Standard curve for calibration of total Flavanoid

The highest amont of flavonoid 20.57±0.021 mgQE/gm dry plant was determined in the extracts from wild populations from Makwanpur district while the lowest amount of flavonoid 6.48±0.29 mgQE/gm dry plant was recorded from the methanolic extracts of wild populations from Lamjung district (Fig 4.3). There was no significant difference (P<0.05) in total flavonoid content between wild and cultivated samples.

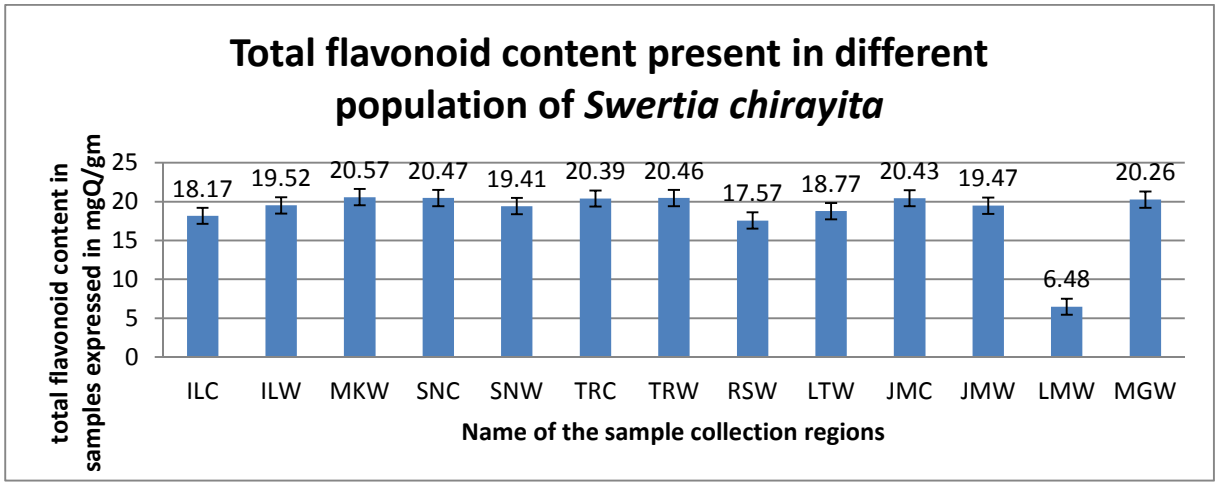

Fig 4.3 Total flavonoid content present in methanolic extract of *Swertia chirayita*.

(IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

4.4 Determination of total phenolic content

Standard solution of Gallic acid ranging from the concentration of 25µg/ml to 250 µg/ml was used to obtain standard graph and equation as shown on Fig 4.4. Based on this equation, the concentration of the total phenol content present in methanolic extract of 13 different sample of *Swertia chirayita* was determined. The results were expressed as mgGAE/g ±SEM (Fig 4.5).

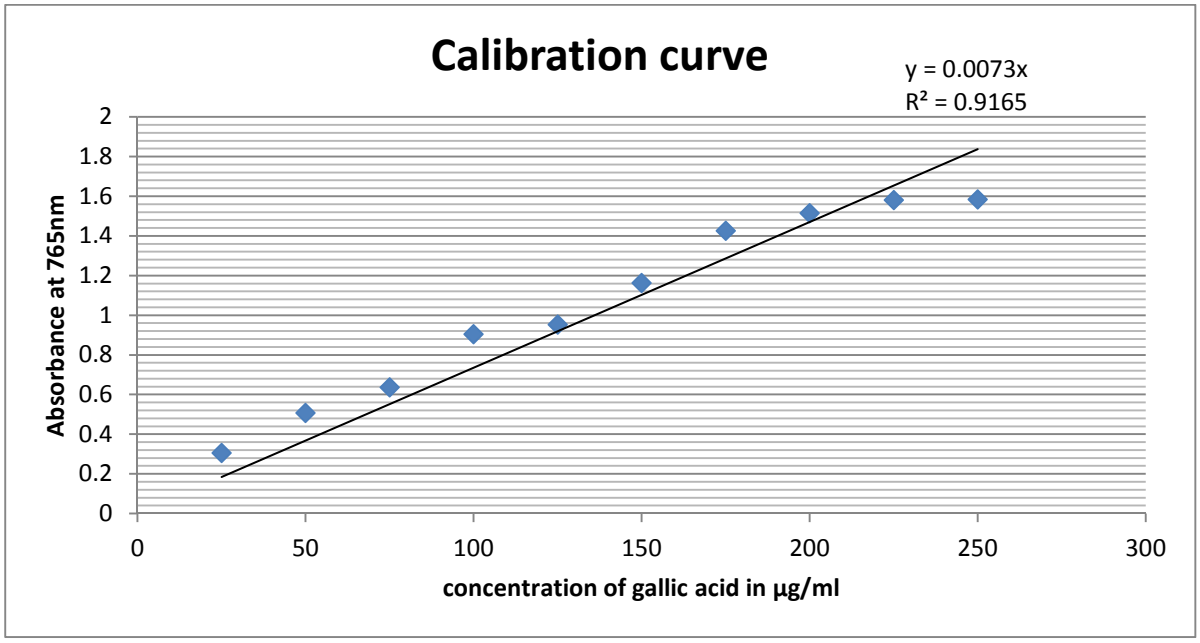

Fig 4.4 Standard curve for calibration of total phenol content

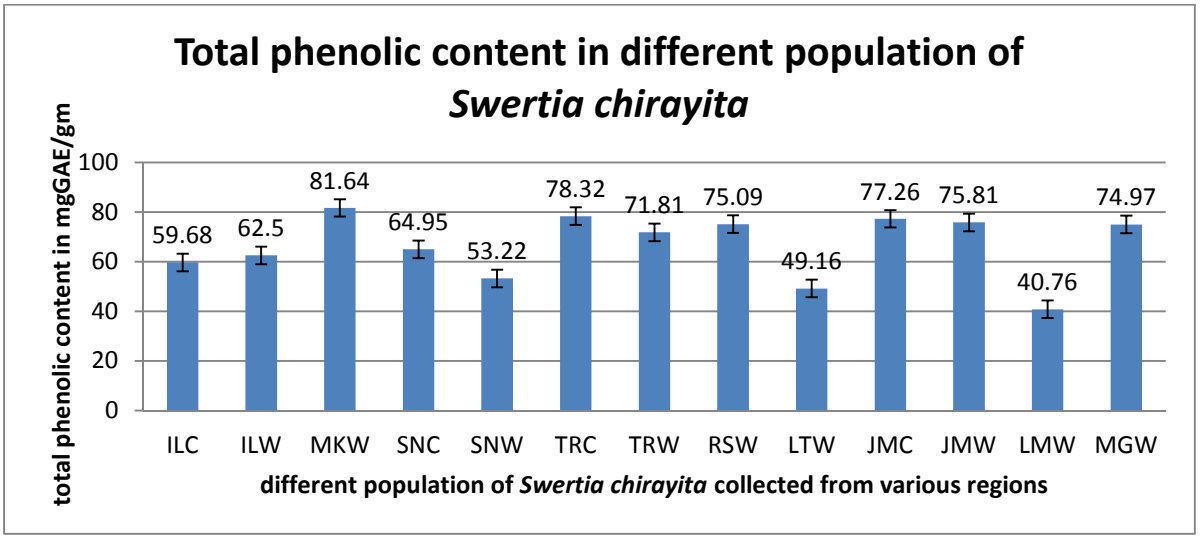

Fig 4.5 Total phenol content present in methanolic extract of *Swertia chirayita*.

(IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

Highest concentration (81.64 ± 0.83 GAE) of Phenol content was found in methnolic extract of wild samples from Makwanpur while lowest concentration (40.76 ± 2.15.GAE)

of phenol content was found in methanolic extract of Lamjung. There is no significant difference ($P<0.05$) of total phenolic content between wild and cultivated samples from the same locality.

4.5 Antioxidant activity of the plant extract

Antioxidant activity of methanolic extract of *Swertia chirayita* collected from various region of Nepal was determined using the solution of DPPH (0.2mM) and taking Ascorbic acid as the pure antioxidant reference compound. IC_{50} value was calculated for each sample taking the concentration vs. % radical scavenging activity. There was gradual increase in % radical scavenging activity as the concentration of the extract increased (Fig 4.6, Fig 4.7, and Fig 4.8). The IC_{50} value for Ascorbic acid was found to be 34.65 (Fig 4.9).Maximum IC_{50} value (193.73 ± 3.67) was found in the sample collected from Lamjung and minimum (42.07 ± 3.39) was found in the methanolic extracts of wild populations from Makwanpur (Fig 4.9). The samples with lower IC_{50} are considered as the best antioxidants and vice versa. The lower IC_{50} is possessed by the *Swertia chirayita* collected from Makwanpur and hence considered as the best antioxidant among other samples of *Swertia chirayita* collected from different region of Nepal. There is no significant difference ($P<0.05$) of IC_{50} value between wild and cultivated samples.

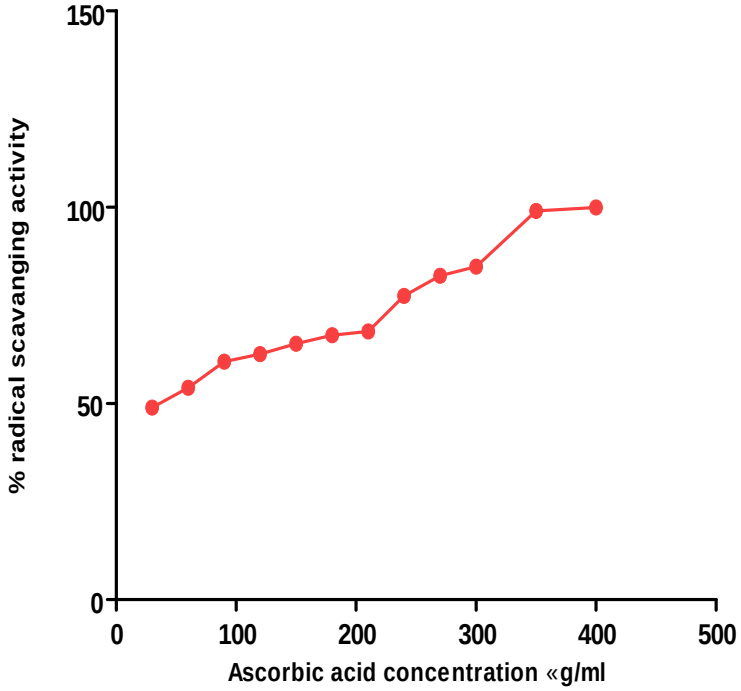

Fig 4.6 standard graph of Ascorbic acid

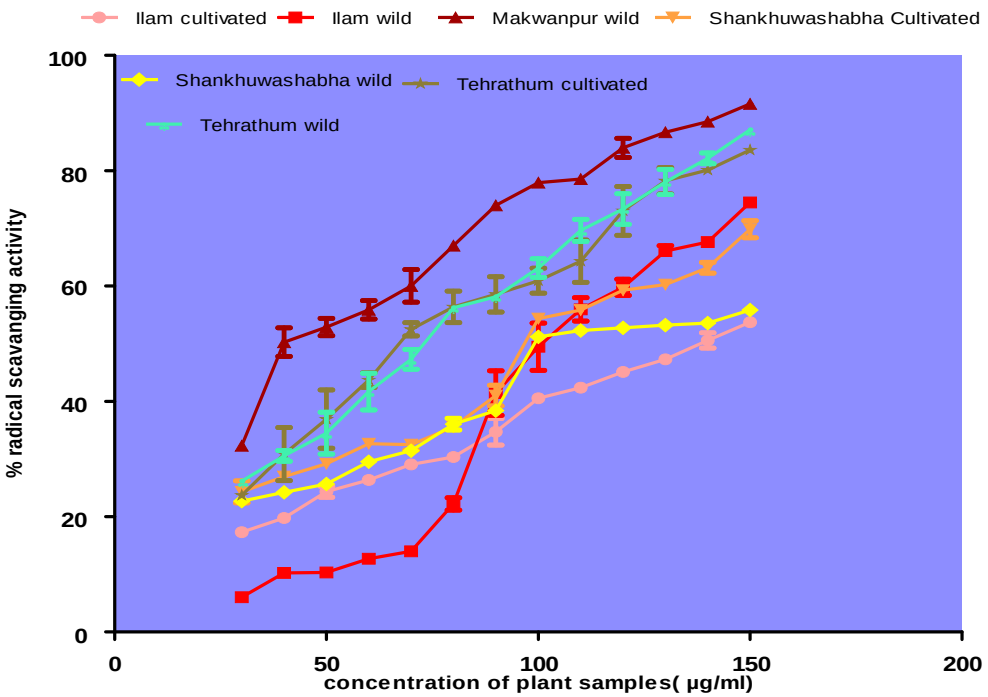

Fig 4.7 % radical scavenging activity of plant samples vs. Concentration of plant extract

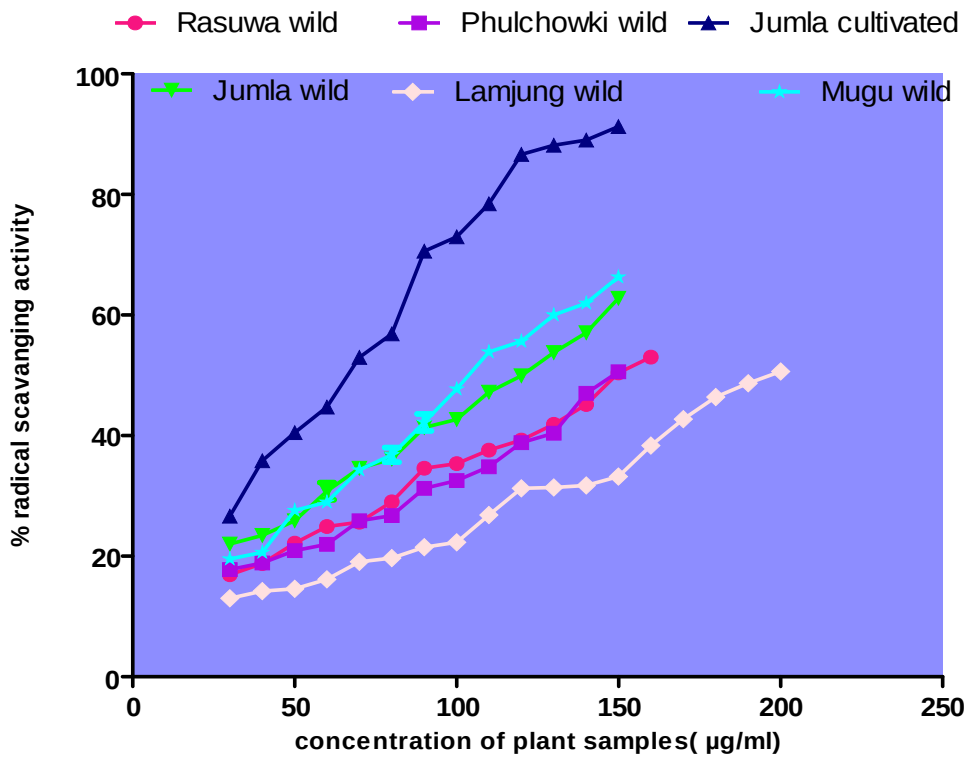

Fig 4.8 % radical scavenging activity of plant samples vs. Concentration of plant extract

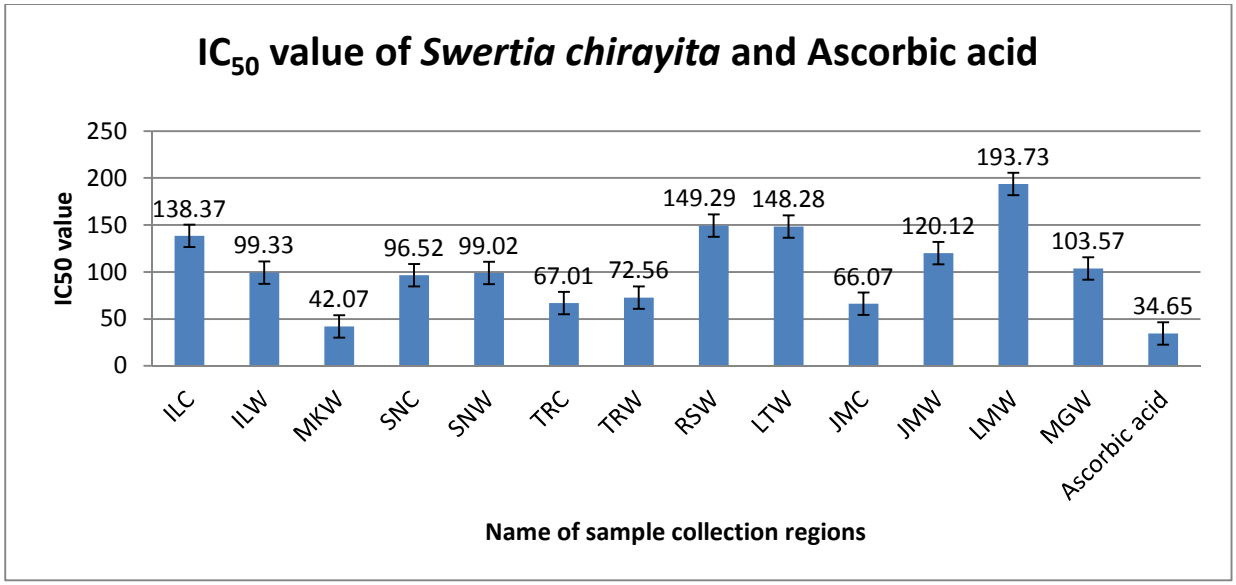

Fig 4.9 IC_{50} value of standard compound (ascorbic acid) along with samples

4.6 Antimicrobial activity

The antibacterial activity of *Swertia chirayita* collected from 13 different region of Nepal was tested against ATCC culture of following bacteria:

Gram negative:

1) *Escherichia coli* (25922) 2) *Salmonella typhii* (14028) 3) *Klebsiella pneumonia* (700603) 4) *Pseudomonas aeruginosa* (27853)

Gram positive:

1) *Staphylococcus aureus* (25923) 2) *Enterococcus faecalis* (25912)

The zone of inhibition was measured for gram negative and gram positive bacteria and the results were expressed on mm including 4mm diameter of well and shown on following table from 4.1 to 4.2

Table 4.2 Antimicrobial activity of the different population of *Swertia chirayita* against gram negative bacteria

S.N.	Samples	Tested Organism	Zone of inhibition in mm (with diameter of well 4 mm)					
			100 mg/ml	**50 mg/ml**	**25 mg/ml**	**12.5 mg/ml**	**+ve control**	**-ve control**
1	**ILC**	*E. coli*	12	10	8	-	22	-
		Salmonella typhiii	9	8	6	-	25	-
		Klebsiella pneumoniaee	11	9	8	6	30	-
2	**ILW**	*E. coli*	13	10	9	6	22	-
		Salmonella typhii	11	9	7	-	25	-
		Klebsiella pneumoniae	13	12	9	7	30	-
3	**MKW**	*E. coli*	**15**	**14**	**10**	**8**	22	-
		Salmonella typhii	**13**	**10**	**9**	**7**	25	-
		Klebsiella pneumoniae	14	12	19	7	30	-
4	**SNC**	*E. coli*	14	13	10	7	22	-
		Salmonella typhii	12	9	8	6	25	-
		Klebsiella pneumoniae	14	12	9	7	30	-
5	**SNW**	*E. coli*	**15**	**14**	**10**	**8**	22	-
		Salmonella typhii	11	9	8	7	25	-
		Klebsiella pneumoniae	14	13	10	8	30	-
6	**TRC**	*E. coli*	**15**	**13**	**10**	**8**	22	-
		Salmonella typhii	12	10	7	6	25	-
		Klebsiella pneumoniae	**15**	**13**	**9**	**8**	30	-
7	**TRW**	*E. coli*	14	13	9	6	22	-
		Salmonella typhii	11	8	6	-	25	-
		Klebsiella pneumoniae	14	12	8	7	30	-
8	**RSW**	*E. coli*	12	10	8	-	22	-
		Salmonella typhii	9	8	5	-	25	-

		Klebsiella pneumoniae	12	9	7	6	30	-
9	**LTW**	*E. coli*	**15**	**13**	**11**	**9**	22	-
		Salmonella typhii	11	10	7	6	25	-
		Klebsiella pneumoniae	13	11	9	8	30	-
10	**JMC**	*E. coli*	**15**	**13**	**10**	**8**	22	-
		Salmonella typhii	11	10	8	7	25	-
		Klebsiella pneumoniae	**15**	**13**	**9**	**8**	30	-
11	**JMW**	*E. coli*	14	13	9	8	22	-
		Salmonella typhii	10	9	7	-	25	-
		Klebsiella pneumoniae	13	11	8	-	30	-
12	**LMW**	*E. coli*	10	8	7	-	22	-
		Salmonella typhii	8	6	-	-	25	-
		Klebsiella pneumoniae	11	9	7	-	30	-
13	**MGW**	*E. coli*	14	13	10	7	22	-
		Salmonella typhii	10	7	6	-	25	-
		Klebsiella pneumoniae	14	12	9	8	30	-

(IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

Above table shows that all the samples of *Swertia chirayita* shows activity against *E.coli*, *Salmonella typhi, Klebsiella pneumoniae.* Increased zone of inhibition has been measured as the concentration of extract increases. Activity against *E. coli* was observed at the concentration of 100mg/ml, 50 mg/ml and 25mg/ml for all samples. No zone of inhibition was observed for samples ILC, RSW and LMW at 12.5mg/ml concentration. Highest zone of inhibition is measured for sample MKW, SNW, TRC, LTW, JMC i.e. 15mm. at 100mg/ml concentration. And lowest zone of inhibition is shown by the sample LMW i.e. 10mm at 100mg/ml concentration. Activity against *Salmonella typhii* was observed at the concentration of 100mg/ml, 50 mg/ml and 25mg/ml for all samples. No zone of inhibition was observed for samples ILC, ILW, TRW, RSW, JMW, LMW and MGW at 12.5mg/ml concentration. Highest zone of inhibition is measured for sample MKW, 13mm. at 100mg/ml concentration. And lowest zone of inhibition is shown by the sample LMW i.e. 8mm at 100mg/ml concentration. Activity against *Klebsiella*

pneumoniae was observed at the concentration of 100mg/ml, 50 mg/ml and 25mg/ml for all samples. No zone of inhibition was observed for samples JMW and LMW at 12.5mg/ml concentration. Highest zone of inhibition is measured for sample TRC and JMC i.e., 15mm. at 100mg/ml concentration. And lowest zone of inhibition is shown by the sample ILC and LMW i.e. 11mm at 100mg/ml concentration.

Table: 4.3 Antimicrobial activity of the different population of *Swertia chirayita* against gram positive bacteria

S.N.	Samples	Tested Organism	Zone of inhibition in mm (with diameter of well 4 mm)					
			100 mg/ml	50 mg/ml	25 mg/mL	12.5 mg/ml	+ve control	-ve control
1	**ILC**	*Staphylococcus aureus*	9	7	-	-	16	-
		Enterococcus faecalis	11	8	7	-	23	-
2	**ILW**	*Staphylococcus aureus*	10	9	-	-	16	-
		Enterococcus faecalis	12	8	7	5	23	-
3	**MKW**	*Staphylococcus aureus*	**12**	**10**	**6**	-	16	-
		Enterococcus faecalis	**15**	**14**	**11**	**9**	23	-
4	**SNC**	*Staphylococcus aureus*	11	8	-	-	16	-
		Enterococcus faecalis	12	11	9	8	23	-
5	**SNW**	*Staphylococcus aureus*	10	8	-	-	16	-
		Enterococcus faecalis	13	11	8	6	23	-
6	**TRC**	*Staphylococcus aureus*	11	9	-	-	16	-
		Enterococcus faecalis	14	12	11	7	23	-
7	**TRW**	*Staphylococcus aureus*	10	7	-	-	16	-
		Enterococcus faecalis	12	10	7	6	23	-
8	**RSW**	*Staphylococcus aureus*	8	7	-	-	16	-
		Enterococcus faecalis	12	8	6	-	23	-
9	**LTW**	*Staphylococcus aureus*	10	9	-	-	16	-
		Enterococcus faecalis	14	12	9	7	23	-

10	**JMC**	*Staphylococcus aureus*	10	9	-	-	16	-
		Enterococcus faecalis	14	11	8	7	23	-
11	**JMW**	*Staphylococcus aureus*	9	7	-	-	16	-
		Enterococcus faecalis	13	10	7	-	23	-
12	**LMW**	*Staphylococcus aureus*	8	7	-	-	16	-
		Enterococcus faecalis	10	7	-	-	23	-
13	**MGW**	*Staphylococcus aureus*	9	8	-	-	16	-
		Enterococcus faecalis	11	8	7	5	23	-

Above table shows that all the samples of *Swertia chirayita* showed activity against *Staphylococcus aureus* and *Enterococcus faecalis*. Activity against *Staphylococcus aureus* was observed at the concentration of 100mg/ml, 50 mg/ml and only sample MKW shows zone of inhibition at 25mg/ml. No zone of inhibition was observed for all the samples at 12.5 mg/ml concentration. Highest zone of inhibition is measured for sample MKW i.e., 12mm at 100mg/ml concentration. And lowest zone of inhibition is shown by the sample LMW and RSW i.e.8mm at 100mg/ml concentration. Activity against *Enterococcus faecalis* was observed at the concentration of 100mg/ml, 50 mg/ml, and 25 mg/ml for all samples except LMW sample. No zone of inhibition was observed for ILC, RSW, JMW, and LMW samples at 12.5 mg/ml concentration. Highest zone of inhibition is measured for sample MKW i.e. 15mm at 100mg/ml concentration. And lowest zone of inhibition is shown by the samples RSW and LMW i.e. 10mm at 100mg/ml concentration.

4.7 TLC detection of marker compound

After preliminary detection of marker compound in Glass plate, its confirmatory and final detection and quantification was done in Aluminium coated TLC plate and plastic coated plate using 100% methanolic extraxct of samples. Amarogentin, Swertiamarin and Mangiferin were detected in all samples. Photographs are included in appendix.

4.8 Semi Quantitative estimation of marker compound

Calibration curve was obtained for Amarogentin, Swertiamarin and Mangiferin and quantification of these marker compounds in samples were carried out using standard curve and equation. Standard calibration curves were included in appendix.

4.8.1 Quantification of Amarogentin

The Amarogentin content in different populations collected from different localities is shown in Fig 4.10. The highest value was obtained for samples from Lalitpur (Phulchowki) (0.299 ±0.005 mg/gm DW) and lowest for that from jumla (0.006 ±0.001 mg/gm DW). The values of Amarogentin content in other samples from other populations were between these two extremes.

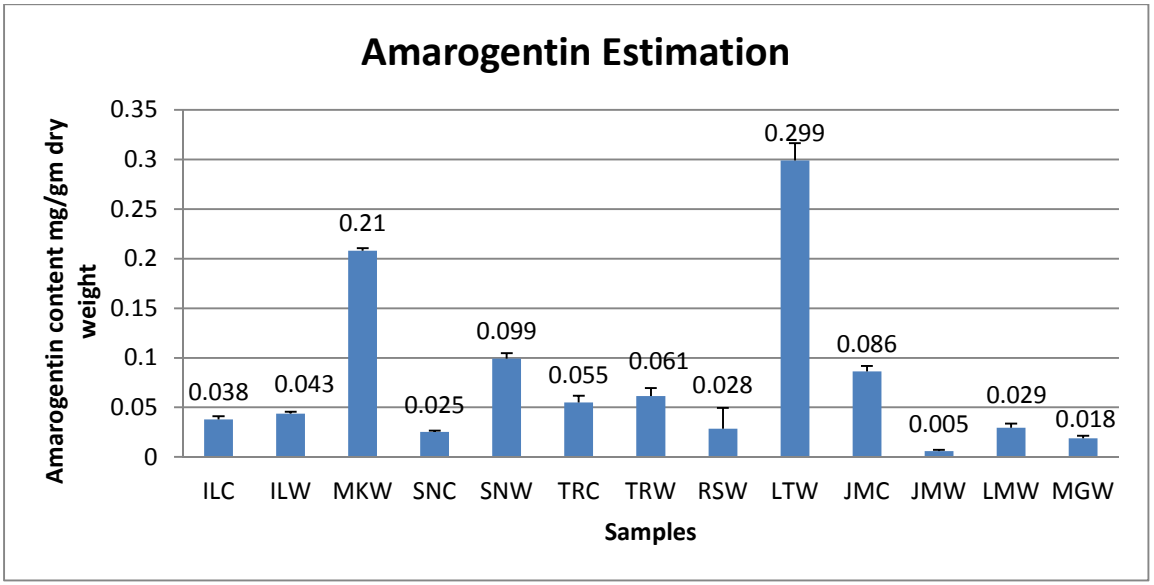

Fig 4.10 Amarogentin content in methanolic extracts of different population of *S. chirayita* (IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

The amount of Amarogentin was found to be higher in samples from wild populations in all the localities except Jumla. The differences in the mean values were statistically significant to varying degrees of p values ($p < 0.05$ for samples from Ilam, 0.01 for samples from Tehrathum and 0.001 for samples from Sankhuwasabha and Jumla).

4.8.2 Quantification of Swertiamarin

Swertiamarin content in different populations is presented in Fig 4.11. The highest Swertiamarin content (0.15±0.010) was found in samples from Phulchowki (Lalitpur) and lowest (0.017± 0.001) from that in Ilam cultivated sample. The values in the Swertiamarin content in samples from other localities were between these two extremes.

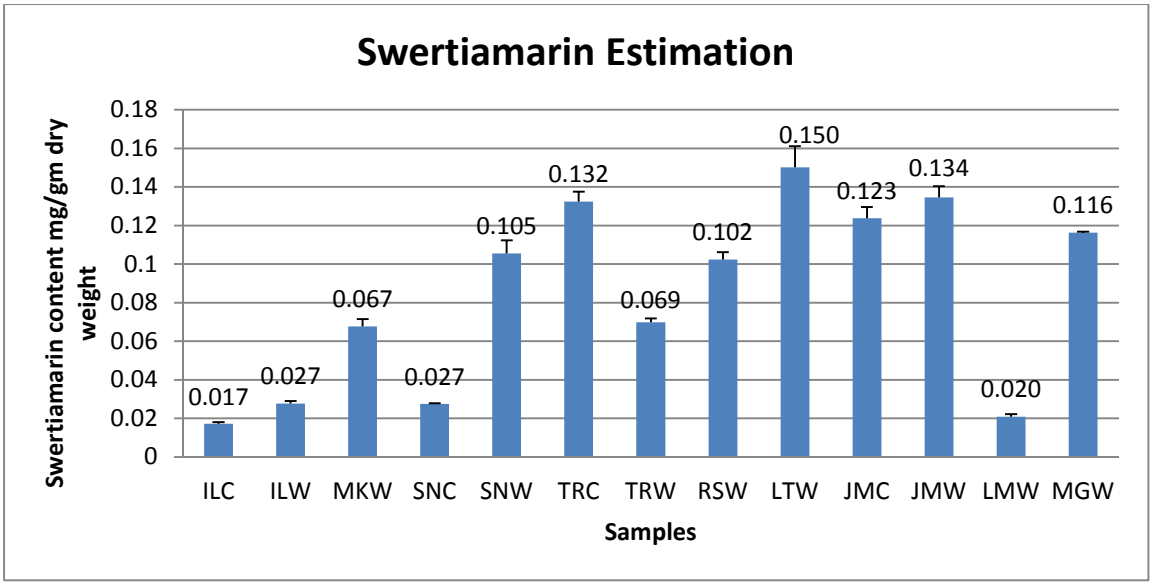

Fig 4.11 Swertiamarin content in methanolic extracts of different population of *S. chirayita* (IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TR- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

The differences in Swertiamarin content between different populations were statistically significant in most of the cases. With the exception of Tehrathum, wild populations in all localities had higher Swertiamarin content than cultivated populations. Except for the samples from Jumla, The differences in the Swertiamarin content between wild and cultivated populations were statistically significant ($P<0.001$).

4.8.3 Quantification of Mangiferin

The Mangiferin content in different populations of *S. chirayita* is presented in Fig 4.12. The highest Mangiferin content (8.83±1.59 mg/gm DW) was found in wild populations from Tehrathum and the lowest (0.23±0.03 mg/gm DW) in wild populations from Lamjung.

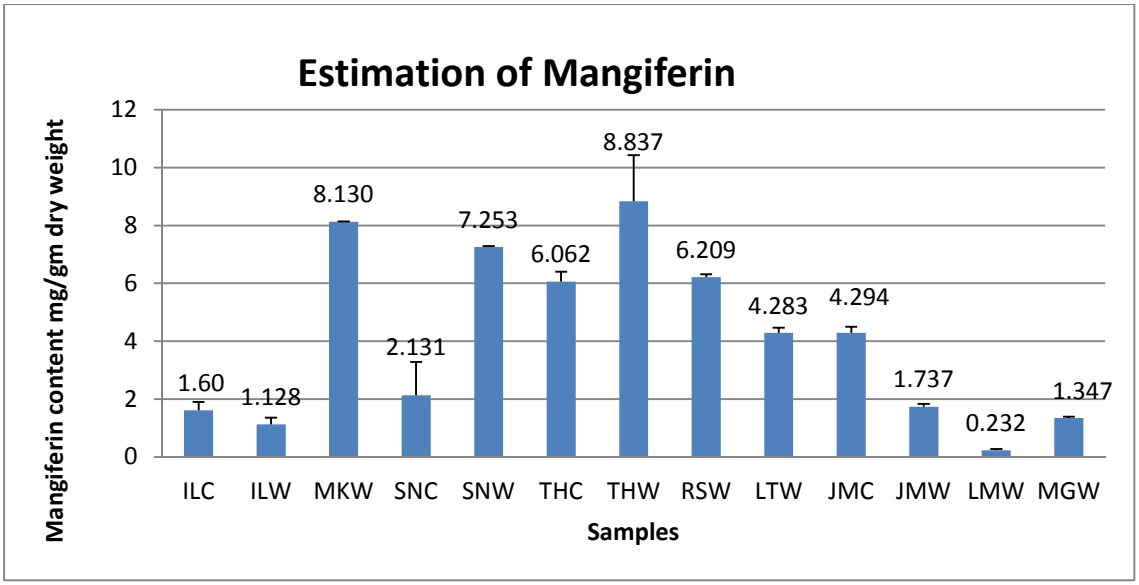

Fig 4.12 Mangiferin content in methanolic extracts of different population of *S. chirayita* (IL- Ilam, MK- Makwanpur, SN- Shankhuwasabha, TH- Terethum, RS- Rasuwa, LT- Lalitpur, JM- Jumla, LM- Lamjung, MG- Mugu; C- Cultivated, W- Wild)

The Mangiferin content in wild populations from Lamjung were significantly lower ($p<0.001$) than that in all other samples. Out of four localities samples from two localities showed high Mangiferin content in cultivated populations while those from remaining two localities showed high Mangiferin content in wild populations. The differences however were significant only in samples from Sankhuwasabha and Jumla.

4.8 Correlation analysis

Correlation analysis between biological activities and phytochemical constituents is presented in table 4.3.

Table 4.3 Correlation analysis

S.N.	*In vitro* assay	Pearson's r value	Level of Significance at $\alpha<0.05$
1.	DPPH (IC_{50}) and TPC	-0.7060**	Yes
2.	TPC and Flavonoid	0.6726*	Yes
3.	DPPH (IC_{50}) and Mangiferin content	-0.5612*	Yes
4.	DPPH (IC_{50}) and Amarogentin content	-0.1374^{ns}	No
5.	DPPH (IC_{50}) and Swertiamarin content	-0.2988^{ns}	No

Correlation analysis shows that there is significant negative correlation between IC_{50} value and total phenolic content means highest the TPC lowest will be the IC_{50} value and vice versa. There is also significant negative correlation between IC_{50} and Mangiferin content but there is no significant correlation between IC_{50} value and other two marker compounds. There is also positive correlation between TPC and Flavonoid content.

Chapter 5- Discussion

5.1 Yield of plant extract

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. Percentage yield of the plant extract depends on the parts of plant used, nature of solvent used and method of extraction employed. Bhargava *et al.* (2009) reported 15.6% yield of *Swertia chirayita* from cold maceration aqueous extraction. Das *et al.* (2011) reported 13% yield from ethanolic extraction of *Swertia chirayita*. In the resent investigation the percentage yield in plant extract was found to vary among population of *Swertia chirayita*. The maximum percentage yield of extract in present investigation was found to be higher than that reported earlier. There is variations in percentage yields among different populations of *S. chirayita*. This variation may have been caused due to difference in the degree of maturity of the samples, differences in the duration of storage period, differences in the genotype and environmental conditions, etc.

Various solvents have been used for extraction of active compounds from *S. chirayita*. In most of the cases the extraction medium has been methanol (Wang *et al*., 2008; Kweera *et al.,* 2011). However other extraction media like petroleum ether (Kweera *et al.,* 2011), Chloroform (Kweera *et al.,* 2011), acetone (Kweera *et al.,* 2011), ethanol (Alam *et al*., 2009; Nagalekshmi *et al.,* 2011; Phoboo *et al.,* 2010; Rehman 2012) and water (Phoboo *et al.,* 2010; Rehman 2012) have also been used. The main reason for use of alcohols, and methanol in particular seems to be high solubility of Mangiferin and Swertiamarin in these extraction media. Furthermore, it has also been shown that some of the biological properties of *S. chirayita* extracts like antibacterial property can best be seen in methanol compared to other solvents like ethanol, petroleum ether, chloroform and acetone (Kweera *et al.,* 2011).

5.2 Preliminary phytochemical analysis

The secondary metabolites existing in the plant extract play a key role in the pharmacological actions of any plant or plant parts. Qualitative phytochemical analysis is done to reveal the presence of bioactive phytoconstituents. The qualitative screening of *Swertia* species showed the presence of major phytochemicals in methanolic extract. Alkaloids, flavonoids, terpenoids, steroids, tannins, saponins and glycosides were present in the studied extract. These results are consistent with the previously conducted studies by Kweera *et al.* (2011) and Laxmi *et al.* (2011). The presence of wide

range of phytochemical constituent indicates that the plant could be used in a multitude of ways which may be beneficiary to the population.

5.3 Total flavonoid content determination

Flavonoids are a group of poly phenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity. Naqvi *et al.* (2013) reported total flavonoid content in methanolic and ethanolic extract of *Swertia chirayita* as 3.38±0.23 catechin equivalents g/100 g of dry matter and 3.17±0.19 catechin equivalents g/100 g of dry matter respectively. Tripathi *et al.* (2005) reported total flavonoid content of 50 µg of aqueous extract of *Swertia chirayita*, being 10.6µg equivalents of quercetin.

With the exception of the samples from Lamjung, the flavoinoid content in samples from all other localities were higher than that reported by Tripathi *et al.* (2005). The data obtained in the present investigation cannot be compared with the data of Naqvi *et al.* (2013) because of the different standard equivalent. The differences in the amount of flavonoids in present investigation and that reported earlier may be due to differences in extraction techniques employed i.e. aqueous extraction in literature and methanolic extract in present investigation used. Lower amount of flavonoid content in samples from Lamjung may be due to long storage duration of the samples than the others samples.

5.4 Total phenolic content determination

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. Plant phenolic constitutes one of the major groups of compounds acting as primary antioxidants or free terminators. Phenolic compounds are commonly found in both edible and inedible plants, and have been reported to have multiple biological effects, including antioxidant activity. Phenolics are able to scavenge reactive oxygen species due to their electron donating properties. (Jothy *et al.,* 2011).

Phoboo *et al.* (2012) reported total phenolic compounds (TPC) of the chirayita collected from nine different district of Nepal and evaluated TPC for inflorescence and leaf (IL) mixture, stem and root part for each sample and highest TPC was reported for IL mixture for all samples and lowest in root part. Highest phenolic content was found in IL mixture of Dolakha (central Nepal) sample and was found to be 19.9 mg GAE/g (DW) and 24 mg GAE/g (DW) for aqueous and 12% ethanolic extract. The result of present investigation shows higher amount of TPC than the reported earlier by Phoboo. Higher amount of TPC in present investigated sample may be due to difference in extraction medium and method i.e. Phoboo *et al.* (2012) carried out overnight percolation of the sample for aqueous and 12% ethanolic extraction where as in present investigation overnight percolation in methanol along with intermittent sonication for 2 hour was employed for the extraction. There is no significant difference between TPC of plant extract collected from wild and cultivated habitat.

5.5 Antioxidant activity

Antioxidants are vital substances which possess the ability to protect the body from damages caused by free radical-induced oxidative stress. Free radicals (super oxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypochloric acid and proxynitrite) produced during aerobic metabolism in the body, can cause oxidative damage of amino acids, lipids, proteins and DNA and has been linked to majority of the systemic diseases including cancer, cardiovascular diseases, and type 2 diabetes (Phoboo *et al.*, 2012). DPPH is a stable free radical, due to the delocalization of the spare electron on whole molecule. Thus DPPH does not dimerize. The delocalization of the electron determines the purple colour with an absorbance band with a maximum 517. When DPPH reacts with the hydrogen donors, the reduced form (molecular) DPPH is generated, accompanied by the disappearance of the purple colour and appearance of the yellow colour. Therefore, absorbance reduction depends on linearly on the antioxidant concentration (Thaipong *et al.*, 2006) Antioxidant property can be inferred on the basis of % radical scavenging activity (RSA) and IC_{50} value. Antioxidant activity DPPH inhibition of the plant extract is expressed as % inhibition of stable radical or inhibition concentration fifty (IC_{50}) in reference to a standard compound. The plant with higher % RSA has the lower IC_{50}. The plant extract with lowest IC_{50} value is considered having better antioxidant properties. A significant difference on IC_{50} value of the studied plant species has been found.

Phoboo *et al.* (2012) has reported the highest antioxidant activity in aqueous and ethanol solution of *S. chirayita* from Dolakha with 85.3% and 85.8% DPPH inhibition,

respectively, and the lowest in aqueous root extracts of *S. chirayita* with 3.9% DPPH inhibition. There was no significant difference between antioxidant activity of ethanol solution and aqueous extracts ($p<0.05$). However, a significant correlation between total phenolic content and antioxidant activity for both aqueous and ethanol solution extracts of different plant parts was found. The results of the present investigation are similar to those reported by Phoboo *et al*. (2012).

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function: lower the IC_{50} value higher is the antioxidant property. Lowest IC_{50} value is reported for Makwanpur i.e. 42.07 and highest IC_{50} value is for Lamjung sample i.e. 193.73.

Correlation analysis shows that there is significant negative correlation between TPC and IC_{50} value, this shows that phenolic compounds present in the extract are responsible for antioxidant activity. There is also significant negative correlation between IC_{50} value and Mangiferin content but no significant correlation between IC_{50} value and Amarogentin and Swertiamarin content. Literature also mentions the antioxidant property of Mangiferin but no activity of other two marker compounds (Phoboo *et al.*, 2012). Free hydroxyl groups and catechol moiety of Mangiferin is responsible for its good antioxidative property (Wei ZQ *et al.*, 2011). There is no significant difference between IC_{50} value of the plant extract collected from wild and cultivated habitat.

5.6 Antimicrobial activity

Plants are rich sources of important phytoconstituents which was already identified by preliminary identification test. Each constituent has its own effect against microorganisms, tannins and flavonoids were known to possess antimicrobial potential against bacteria and fungi (Laxmi *et al.*, 2011).

Antimicrobial screening of *Swertia chirayita* was conducted by various authors and their activity was reported against various organisms. Activity of methanolic extract, crude rectified spirit extract and ethanolic extract of *S. chirayita* against E. coli was reported by Laxmi *et al.* (2011); Sultana *et al.* (2007); and Rehman, (2012) respectively. Similarly antimicrobial activity against *Salmonella typhii* is also reported in methanolic and crude rectified spirit extract of *S. chirayita*. (Laxmi *et al.*, 2011; Sultana *et al.*, 2007). Ethanolic extract of *S. chirayita* was found to be active against *K. pneuomoniae*. (Sultana *et al.,* 2007; and Rehman, 2012). Methanolic extract and ethanolic extract of *S. chirayita* was reported to be active against *Staphylococcus aureus* (Kweera *et al.*, 2011; Sultana *et al.*,

2007). Methanolic Extract of *S. chirayita* reported by Laxmi *et al.* (2011) was found to be inactive against *P. aeruginosa* but ethanolic extract was found to be active against *P. aeruginosa* (Rehman, 2012). In present investigation methanolic extract of all population of *S. chirayita* was found to be active against E. coli, *Salmonella typhii*, *K. pneuomoniae and Staphylococcus aureus* but no activity against *P. aeruginosa*. This result is supported by earlier findings. Present investigation also reported activity of methanolic extract against *Enterococcus faecalis* which was not reported earlier. In all cases ZOI of standard was found to be higher than that of samples. This may be due to crude form of sample.
Variation in antibacterial activity among different population may be due to altitudinal variation of the sample that causes variation in important phytoconstituent and marker chemical compounds that are respossible for antimicrobial activity. Various authors reported antimicrobial property of these marker compounds i.e. Amarogentin, Swertiamarin and Mangiferin. Samaddar *et al.* (2013) reported Antibacterial activity against selected human clinical pathogens was tested by the disc diffusion method and reported antimicrobial activity of Swertiamarin and Amarogentin against *Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa.*

Kumarasamy *et al.*, 2003 carried out antimicrobial test against 14 gram positive and gram negative and reported antimicrobial activity of Swertiamarin against *Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Escherichia coli and Proteus mirabilis* but no activity against *Staphylococcus aureus.*

Antimicrobial activity of Mangiferin is also reported against some gram negative and gram positive bacteria. Mangiferin and its analogue showed moderate to mild activity against *Staphylococcus aureus,* and *Escherichia coli*.

The activity may be the indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins. There are several modes of action through which phytoconstituents show antimicrobial activity such as through interference with the phospholipids bilayer of the cell, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction or inactivation of genetic material. In general, the mechanism of antimicrobial action is considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou *et al.*, 2008).

5.7 Semi Quantitative estimation of major phytochemical and Variation analysis

Amarogentin which is the bitterest compound till date has relatively few studies about their content and quantification in *Swertia chirayita*. Wang *et al.,* (2008) studied 11 different phytochemicals including Amarogentin from different species of *Swertia*. They also studied the population wise variation of this compound in *S. japonica* and reported Amarogentin values in the range of 0.9 to 3.4 mg per gram dry mass for *S. japonica* populations. The ranges of values of Amarogentin in other species of *Swertia* were between 0.17 mg/gm DW for *S. punicea* and 0.22 mg/gm DW for *S. binchuangensis*. Phoboo *et al.* (2010) also studied the variation in Amarogentin content in Nepalese population of *Swertia chirayita* using HPLC and reported highest Amarogentin content in aqueous and 12% ethanolic extracts of IL (Inflorescence and leaf) samples (0.28 and 0.23mg/gm DW, respectively) from Bajura and lowest content in root extract from Dolakha (0.006 and 0.002 mg/g DW, respectively). The values of Amarogentin content found in our present investigation 0.005 mg/gm DW for wild populations from Jumla and 0.299 mg/gm DW for wild populations of Lalitpur (Phulchowki) are in agreement with the findings of Phoboo *et al.* (2010).

There has been very few study about important seco-iridoid i.e. Swertiamarin. Wang *et al.* (2008) studied 11 different phytochemicals including Swertiamarin from different species of *Swertia*. They also studied the population wise variation of this compound in *S. japonica* and reported Swertiamarin values in the range of 3.8 to 4.05 mg per gram dry mass for *S. japonica* populations.The range of values of Swertiamarin in other species of *Swertia* were between 1.08 mg/gm DW for *S. pseudochinesis* and 6.37 mg/gm DW for *S. binchuangensis*. Phoboo *et al.* (2010), carried out variation study among different population of Nepalese *Swertia chirayita* and reported that highest Swertiamarin content (1.28 mg/gm DW) in ethanolic extract of IL mixture from Sindhupalchwok and lowest (0.01 mg/gm DW) for ethanolic extracts of stem samples from Dhankuta. Similarly they found high Swertiamarin content in wild populations and low in cultivated populations. In present investigation the values of Swertiamarin ranged from 0.017 mg/gm DW for cultivated populations from Ilam to 0.15 mg/gm DW for wild populations from Lalitpur (phulchwoki) are within the range as those reported by Phoboo *et al.* (2010).

Mangiferin is one of the most important xanthones present in various species including *Swertia chirayita*. Variation study of the Mangiferin content in different population of Swertia chirayita and different Species of Chirayita has been already carried out by

Phoboo *et al.* (2010) and Pandey *et al.* (2012), respectively. Pandey *et al.* (2012) reported Mangiferin content in leaf as high as 3.46% during vegetative stage and 4.37% during flowering stage in the methanolic extracts of *S. chirayita* collected from Arunanchal Pradesh. Similarly Phoboo *et al.* (2010) reported highest Mangiferin content i.e., 0.6 mg/gm dry from ethanolic extract of root collected from Taplejung. In present investigation Mangiferin content was lower than that reported by Pandey *et al.* (2012) and higher than that reported by phoboo *et al.* (2010). The differences in the quantification may be due to difference in extraction medium and sensitivity of the method. The method used for quantification in present investigation gives tentative quantification. Kim *et al.* (2010) reported that solubility of Mangiferin in methanol (23.4) is higher than that in either water (15.1) or ethanol (7.4). Therefore, high level of Mangiferin per unit dry weight of *Swertia* populations in present investigations can therefore be attributed to its higher solubility in methanol than in 12% aqueous ethanol.

With the exception of Amarogentin and Mangiferin content in plants collected from cultivated populations from Jumla, the levels of all three phytochemicals taken in present investigation were relatively higher in plants from wild populations compared to those from cultivation. Lower level of nitrogen (Fritz *et al.*, 2006) and exposure to more harsh stress conditions (Dixon and Paiva, 1995) in wild habitats might have contributed to high levels of these phytochemicals in these plant.

As mentioned in literature, variations have been found in *Swertia chirayita* populations in terms of genetic makeup as well as in the amount of different phytochemicals. Since the studies on genetic variation as well as phytochemical variations have not been carried out involving the same samples from different populations, it is difficult to ascertain as to whether the observed variations in phytochemical content in present investigation are caused by genetic factors or other factors. Therefore, future studies on phytochemical profiling of *Swertia chirayita* populations should be accompanied by studies on genetic variations involving the same sample.

Chapter 6 – Conclusion

Conclusion

The present study illustrate the antioxidant and antimicrobial property of different population of *S. chirayita*, one of the most important medicinal plants of Nepal, along with the content of the important chemical constituents i.e., Amarogentin, Swertiamarin and Mangiferin. This study suggest that the *S. chirayita* collected from all regions are biologically active but sample collected from Lamjung showed least activity compared to others samples and also had the low content of all three marker compound.

Semi quantitative estimation of the marker compound using TLC revealed the variations in the amount of the marker compounds in wild and cultivated populations. The variations however, were not statistically significant ($p<0.05$) in most of the samples studied. The values of Amarogentin and Swertiamarin content per unit biomass were in the range reported earlier for Nepalese populations of *S. chirayita*. The amounts of Mangiferin however were much higher than that reported earlier by Phoboo *et al*. (2010).

This is the preliminary study to explore the different population of *S. chirayita* for their biological activity as well as content of pharmacologically important constituents. There is still a wide scope for exploring different aspects of *S. chirayita* especially for setting up a system of quality assurance for the products collected from Nepal.

Recommendation

Based on the technical expertise developed and practical difficulties observed in carrying out the present work, following recommendations are made regarding the future work in such areas.

1. The method used in present investigation is simple and cheap, and should therefore be validated so that it can be applied to develop some kind of quality assurance system for Nepalese medicinal plants so that Nepalese people can get maximum benefit from the trade of medicinal plants. The system should be worked further and quantification validated by using sophisticated tools like HPLC.

2. The studies conducted so far in phytochemical screening are based on samples from relatively few localities. So it was not possible to select the best lines for commercial cultivation. Future works should focus on taking samples from as many localities as possible.

3. Since there are variations in the amount of marker compounds in samples from different localities, and the samples from some localities are far superior to those from other localities, future works in *S. chirayita* should be focused on the cause of such variations.

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Appendix

1. Preparation of 1 M 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 Glacial acetic acid (20%) - 200 ml

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

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

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

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

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

5. Preparation of 0.2mM DPPH solution - 100 ml

100 ml of 0.2mM solution of 1, 1- diphenyl-2 picryhydrazyl (DPPH) was prepared by weighing 7.886 mg of the DPPH and dissolving it in ethanol and finally maintaining the volume to 100 ml by addition of ethanol.

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

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

7. Mayer's reagent

To 1.358 gram of mercuric chloride, 60 ml of water and 5 gram of potassium iodide was added and dissolved in 10 ml water. The two solutions were mixed up and final volume was made 100 ml.

8. Composition of Nutrient agar media

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

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

9. Composition of Luria Bertani broth, (LB) Miller media

The composition of Luria Bertani broth, (LB) Miller media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

Components	**gram/L**
Casein enzyme hydrolysate	10
Yeast extract	5.0
Sodium chloride	10.0
Final pH	7.5± 0.2

10. Composition of Mueller Hinton Agar (MHA)

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

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

11. Preparation of 0.5 McFarland standards- 100 ml

The components that were used for the preparation of the 0.5 McFarland standard is as follows.

Components	**Amount in ml**
Sulfuric acid, 0.18 M	99.5
Barium chloride, 0.048 M	0.5

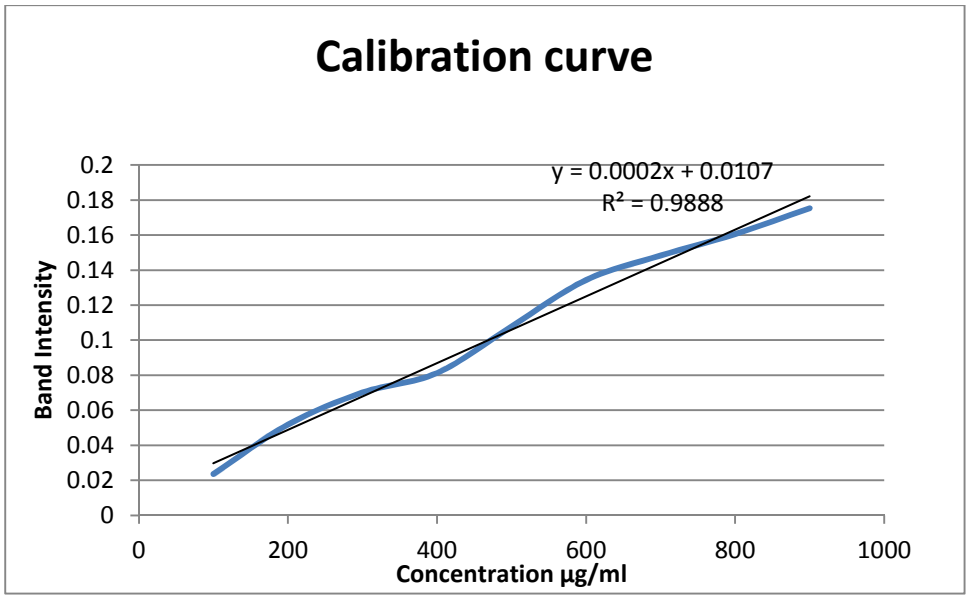

Figure Standard curve for Estimation of Amarogentin.

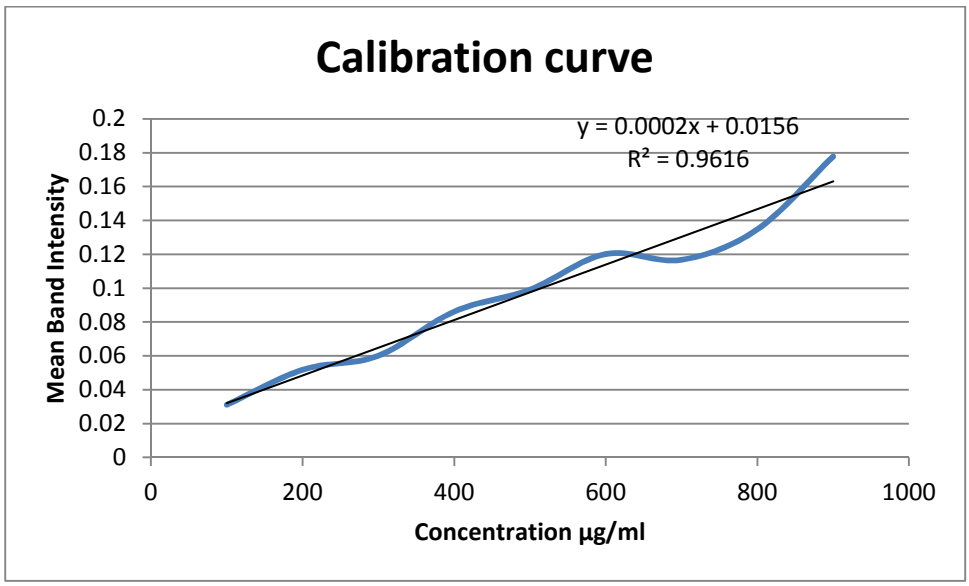

Figure Standard curve for Estimation of Swertiamarin.

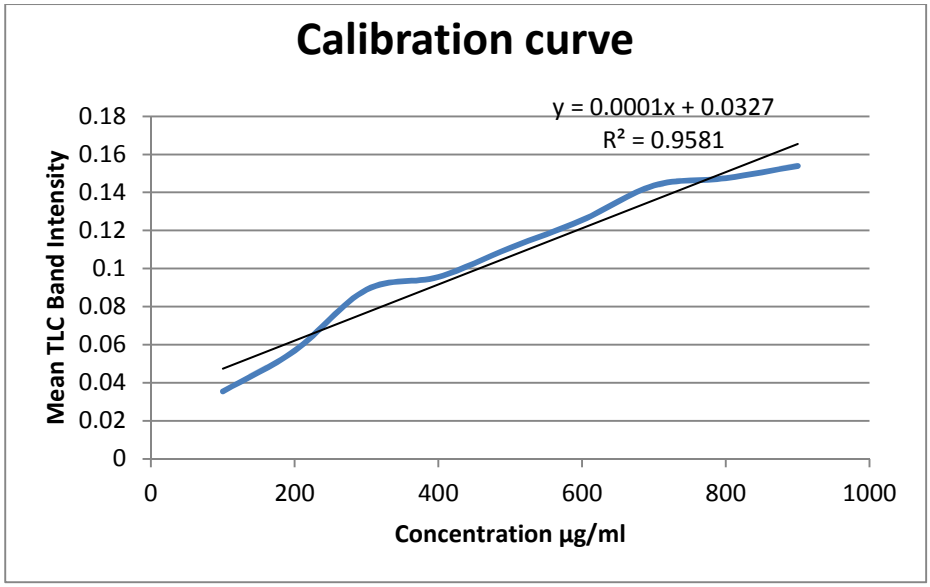

Figure Standard curve for Estimation of Mangiferin.

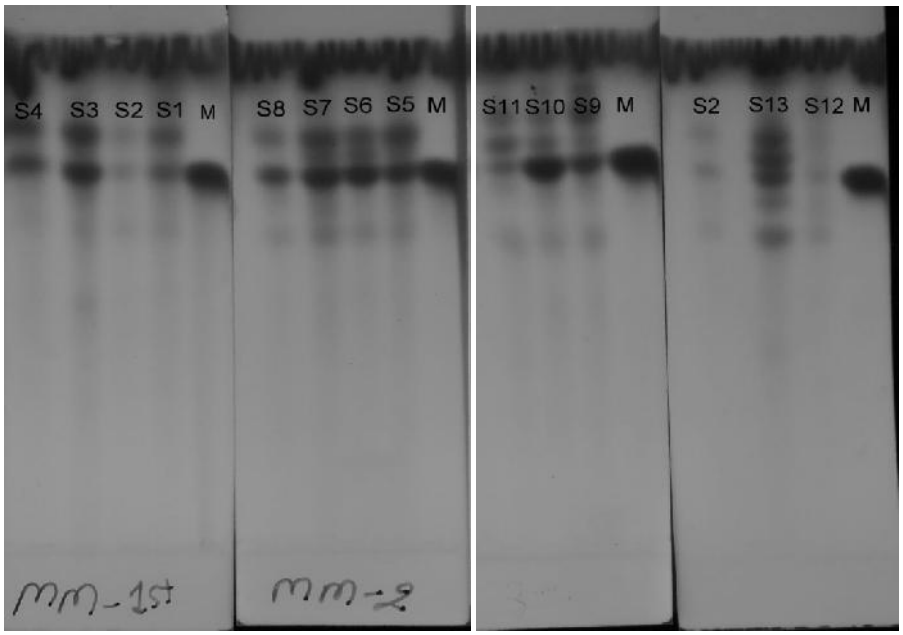

Fig TLC of samples for detection of Mangiferin

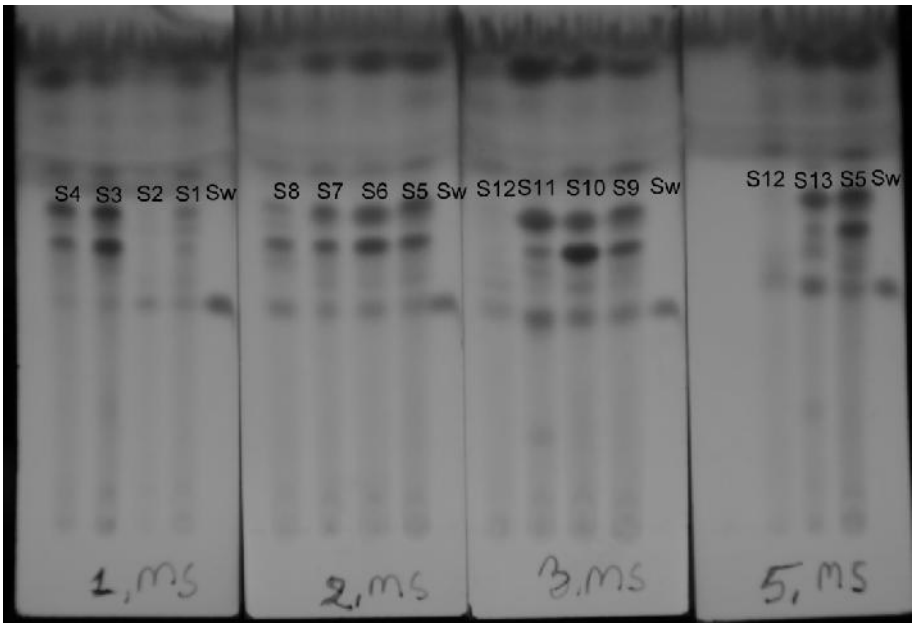

Fig TLC of samples for detection of Swertiamarin

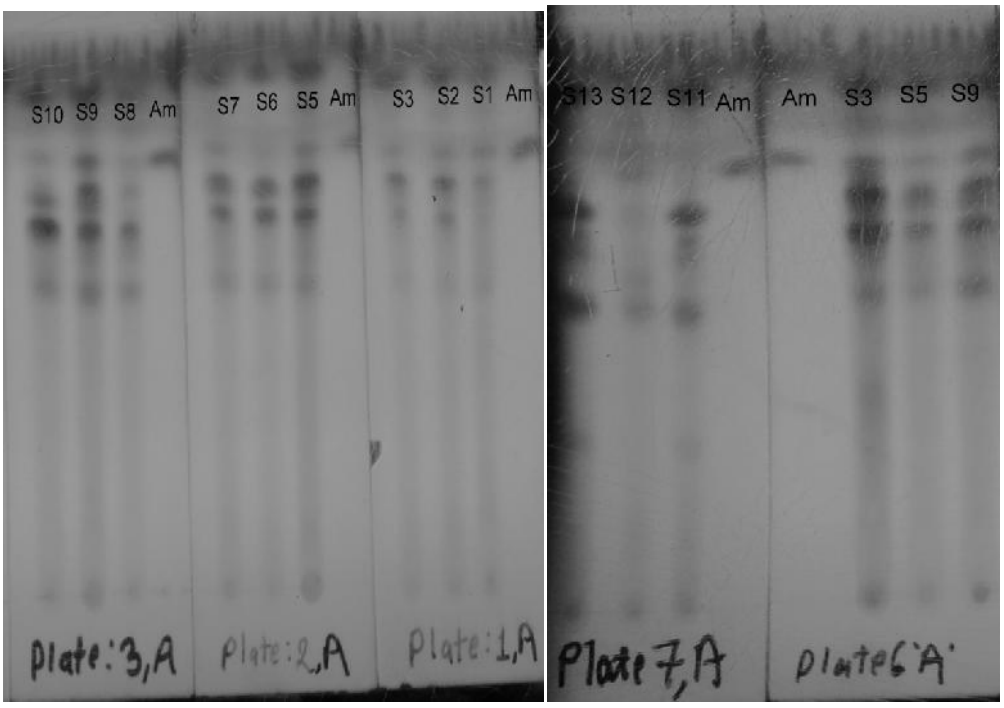

Fig TLC of samples for detection of Amarogentin

(S1= Ilam cultivated, S2=Ilam wild, S3=Makwanpur wild, S4= Shankhuwashabha cultivated, S5= Shankhuwashabha wild, S6= Tehrathum cultivated, S7= Tehrathum wild, S8=Rasuwa wild, S9= Phulchwoki wild(lalitpur), S10= Jumla cultivated, S11= Jumla wild, S12=Lamjung Wild, S13= Mugu wild: M= mangiferin standard, Sw= swertiamarin standard, Am= Amarogentin standard).

Fig Plant sample material (*S. chirayita*)

Fig Concentrating plant extract in rotavapour

Fig showing ZOI against *Klebsiella pneumania*

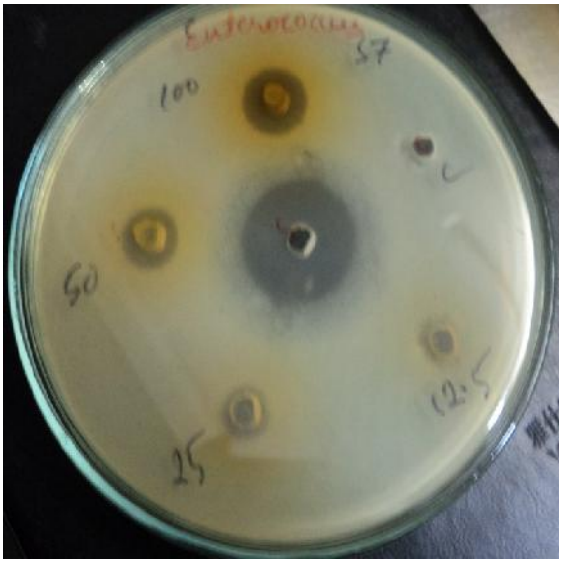

Fig showing ZOI against *Enterococcus faecali*