PRELIMINARY PHYTOCHEMICAL ANALYSIS OF DIFFERENT SPECIES OF *SWERTIA* FROM NEPAL

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List of Abbreviations

^0C	Degree Celsius
µl	Microlitre
µg	Microgram
λ	Wavelength
ATCC	American Type Culture Collection
BFDI	Bioactivity Directed Fractionation and Isolation
DMSO	Dimethyl Sulfoxide
DPPH	1, 1- diphenyl-2 picryhydrazyl
DW	Dry Weight
g	Gram
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
IR	Infra Red
IUCN	International Union for Conservation of Nature
LB	Luria Bertani
LC	Liquid Chromatography
mg	Milligram
mL	Milliliter
MHA	Muller Hinton Agar
MS	Mass Spectrometry
NA	Nutrient Agar
NMR	Nuclear Magnetic Resonance

NTFP	Non-Timber Forest Products
OD	Optical Density
QE	Quercetin Equivalent
RSA	Radical Scavenging Activity
RT	Room Temperature
SD	Standard Deviation
Spp	Species
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
TPC	Total Phenolic Content
UV	Ultra Violet
Wt	Weight
WHO	World Health Organization
ZOI	Zone Of Inhibition

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Abstract

Gentians are reported to contain potent bitter compounds like iridoids, xanthones, mangiferin and c-glucoflavones that are known to heal many digestion disorders. Biochemical fingerprinting of the genus *Swertia* can be useful in screening elite populations within and between *Swertia* species. Only few of these *Swertia* species have been studied extensively for various bioassays. This study is attempted to estimate three important bioactive compounds: swertiamarin, amarogentin and mangiferin from different Nepalese *Swertia* species and their antibacterial and antifungal activities.

Total phenolic and flavonoid contents were quantified spectrophotometrically and *in vitro* DPPH free radical scavenging assay was measured. Agar well diffusion method was employed for antibacterial and antifungal assays. Thin Layer Chromatography was performed on TLC Aluminium plates pre-coated with silica gel 60 for identification and estimation of swertiamarin, amarogentin and mangiferin from *S. chirayita, S. angustifolia, S. paniculata, S. racemosa, S. nervosa, S. ciliata and S. dilatata* collected from different regions of Nepal Himalayas. Elution was done using ethyl acetate:methanol:water (77:15:8 v/v/v) and ethyl acetate:methanol:formic acid:water (67:17:8:8v/v/v). Semi quantitative estimation was done using *GelQuant.NET* software using the standard compounds.

Swertiamarin and amarogentin in *S. dilatata* (0.17, 0.24 mg/gm DW) was reported comparable to *S. chirayita* (0.12, 0.26 mg/gm DW) which is considered as an adulterant in chirayita trade. This estimation suggests that so called inferior species like *S. dilatata, S. paniculata* and *S. racemosa* can be a potential and promising source of swertiamarin/amarogentin in herbal and pharmaceutical industries.

Promising concentration of phenolics and flavonoids produced promising DPPH free radical scavenging values. Lowest IC_{50} value was seen in methanol extracts of *Swertia chirayita* (23.92±1.04 ug/mL) compared with other species of *Swertia*, even lower than ascorbic acid. Aqueous extracts exhibited poor anti oxidative capacity due to less TPC and flavonoids. Antibacterial activity against Gram negative bacteria was found to be sensitive to the methanol extract up to 6.25 mg/mL in many of the *Swertia* species that proves the use of chirayita by the local communities in digestive disorders. Various bioassays and quantification values indicated other species of *Swertia* might be good candidates against infectious and chronic diseases.

Key words: *Swertia* species, antioxidant, antibacterial, antifungal, amarogentin, swertiamarin, mangiferin.

CHAPTER I

INTRODUCTION

1.1 Background

Plant kingdom is a treasure house of food, feed and source of medicines that show various potent bioactivities against much illness. Potential compounds are extracted and used in pharmaceutical industries for drug formulations. Drugs from these plants are easily available, less expensive, safe and efficient and have fewer side effects (Dewick, 1996, Phillipson and Wright, 1996). The species of *Swertia* comprises the genus with higher therapeutic and pharmacological applications (Brahmachari *et al.,* 2004). Plants have contributed a lot to modern medicine; numerous drugs in use today are derived from plants, in one-way or another (Table 1.1). Himalayan plants have been said to have very high chemical diversity and thus offers a potential source for the discovery of new and useful natural products for use in medicines (Cragg *et al.,* 1997).

Drugs	**Plant sources**	**Therapeutic applications**
Morphine	*Papaver somniferum*	Analgesic
Quinine	*Cinchona succirubra*	Anti-malarial
Taxol	*Taxus brevifolia*	Anti-cancer
Lysergic acid diethylamide	*Claviceps purpurea*	Migraine and headaches
Vincristine	*Catharanthus roseus*	Anti-leukemic
Galantamine hydrobromide	*Narcissus spp.*	Alzheimer's disease
Cardiac glycoside	*Digitalis purpurea*	Heart Disease
Atropine	*Atropa belladonna*	Relaxing eye muscles
Acetyl Salicylic acid	*Salix spp.*	Analgesic/Pain killer
Canabinoids	*Cannabis sativa*	Autoimmune diseases

Table 1.1: Top 10 drugs obtained or derived from different plants and their therapeutic applications

The discovery of penicillin from *Penicillium notatum* by Fleming in 1928 promoted the intensive investigation of nature as a source of novel bioactive compounds. Since then plants and microorganisms have together served as a prolific source of structurally diverse and bioactive metabolites, yielding many important products in the pharmaceutical industry today (Cragg *et al.,* 1997). This has been made possible by the systematic investigations carried out on just 15% of the total terrestrial flora, mostly of higher plants (Balandrin *et al.,* 1993). Thus, a larger fraction still remains to be investigated.

Over 250,000 species of total flowering plants on this planet and of these, an estimated 155,000 could be found in the tropics (Cordell and Shin, 1991). Nepal harbors more than 7000 higher plants, 5% of which are endemic (Joshi and Joshi, 1991). It is estimated that various communities in Nepal use approximately 1000 species of wild plants in traditional medicinal practice (Chaudhary, 1998). Nepal has a natural gift of vascular plants with 1624 species of medicinal plants representing about 20% of the total flora (Tiwari, 1999). Tiwari and Shrestha (2000) published a list of 1630 species having medicinal values. This list has however, not mentioned any medicinal properties of these plants. Thus, screening these medicinally valuable plants for the detection and identification of different therapeutic compounds are very useful in developing countries like Nepal in terms of the economy and medicinal applications.

Swertia (family *Gentianaceae*) is a well-known medicinal herb that has been mentioned in Vedas and pharmacopeias (Joshi and Dhawan, 2005). It is indigenous to the temperate Himalayas from Kashmir to Nepal to Bhutan (WWF, 2008; Joshi and Dhawan, 2005) with high medicinal values. Nepal occupies a central segment of Himalayan mountain range, so, *Swertia* species diversity is huge. Nepal alone houses 31 species of *Swertia* (Joshi and Joshi 2008) among 150 species of *Swertia* distributed worldwide of which *Swertia acualis* is endemic to Nepal (Joshi, 2008). This higher distribution of *Swertia* allows its trade in 61 districts out of 75 districts in Nepal of which Eastern Nepal, Sankhuwasabha being its capital (Phoboo and Jha, 2010). Locally, *Swertia chirayita* is called as “Tito”, “Tite”, “Pothi chirayita” and “Dakle chirayita” in Nepali language. In other language it is called as “Tento” in Gurung;“Timda” in Tamang; “Suingkhangwa” in Limbu; “Rauka” in Magar; “Ghyatig” in Tibetan languages (WWF, 2008).

The higher content of its important secondary metabolites make it a therapeutically powerful medicinal plant. According to Joshi and Dhawan, 2005 and Khanal *et al.,* 2014, the genus *Swertia* contains xanthones and their derivatives, alkaloids, secoiridoid glycosides, flavonoids and terpenoids followed by more than 30 other compounds and their derivatives. So, the observed biological effects of a medicinal plant are the consequence of

the interplay of a certain concentration of the numerous biologically active constituents produced by the plant. Thus, intensive phytochemical screening enables to demystify the phytoconstituents and reveal its bioactive compounds.

Swertia is a large genus of herbs that are taxonomically, morphologically and biologically diverse. *Swertia chirayita* is one of the most important species with high ethno-pharmacological significance and high trade value. Other species are reported to be substitutes and alternatives to *S. chirayita*. However, almost all of the species are known to have some role in traditional medicine healing mostly enteric disorders and fever. The use of these species varies according to the localities and countries (Brahmachari *et al.,* 2004; Joshi 2008; Khanal *et al.,* 2014; Negi *et al*., 2011). The ethnobotanical knowledge and indigenous use of these *Swertia* species in Nepalese locals are reviewed by Joshi 2008 and Joshi and Joshi 2008.

1.2 Phytochemical Analysis: A Natural tool in Drug Discovery

Phytochemical analysis is a primary tool needed for all compounds being evaluated in a plant in order to establish their nature, and hence the fundamental validity and reproducibility in bioassays and hence therapeutic methods (Cordell and Shin, 1997). Screening of phytochemicals has been a major stake of pharmaceutical companies in seeking new antibiotics/compound scaffolds. Over time, chemical and biological aspects were revealed using primitive techniques evolved, and eventually integrated (Cordell and Shin, 1999) into a high performance liquid chromatography–mass spectrometry system where the samples for chemical and biological analysis could be collected and analyzed. Now this technique is used to search for novel anticancer agents in plant extracts. The further development of such techniques could prove critical in providing a correlation between specific active constituents in the extract and their biological activity (Cordell and Shin, 1997).

The discovery of drugs from plants was, for many years, conducted on the basis of the ‘‘magic bullet’’ theory. However, in practice, a plant may contain several, closely-related metabolites with quite similar activities (Silva *et al.,* 2001). These factors (compound variation and metabolite complexity) are important in bioactivity guided drug discovery. Now, a completely new perspective on secondary metabolite profiling has emerged with the introduction of metabolomics (Verpoorte *et al.,* 2005, 2007; Heinrich, 2008; Okada *et al., 2*010), defined as ‘‘both the qualitative and quantitative analysis of all the metabolites in an organism’’. The technique typically employs multivariate analysis of the proton NMR

spectra of crude extracts, and the principal component analysis of the low molecular weight secondary metabolites (Verpoorte *et al.,* 2007).

The long run for the search for bioactive compounds is essential to combat the emerging diseases. Plants act as a rich source of those potential compounds. The choice and availability of plants, their traditional uses or ethno pharmacology, chemotaxonomy and plant ecological observations are important in determining the plant therapeutic aspects, the usual strategy being the observation of its biological assay or panel of assays. The plants may undergo bioassay-guided isolation of active constituents or rigorous phytochemical isolation of as many compounds as possible (Heinrich, 2008). The complete phytochemical screening provides a detail map of its constituents and possible therapeutic candidates can be selected from those compounds. The compound with marked bioactivity is targeted, isolated and purified, followed by the structural elucidation and comprehensive biological testing of these isolated compounds (Silva *et al.,* 2001; Heinrich, 2008).

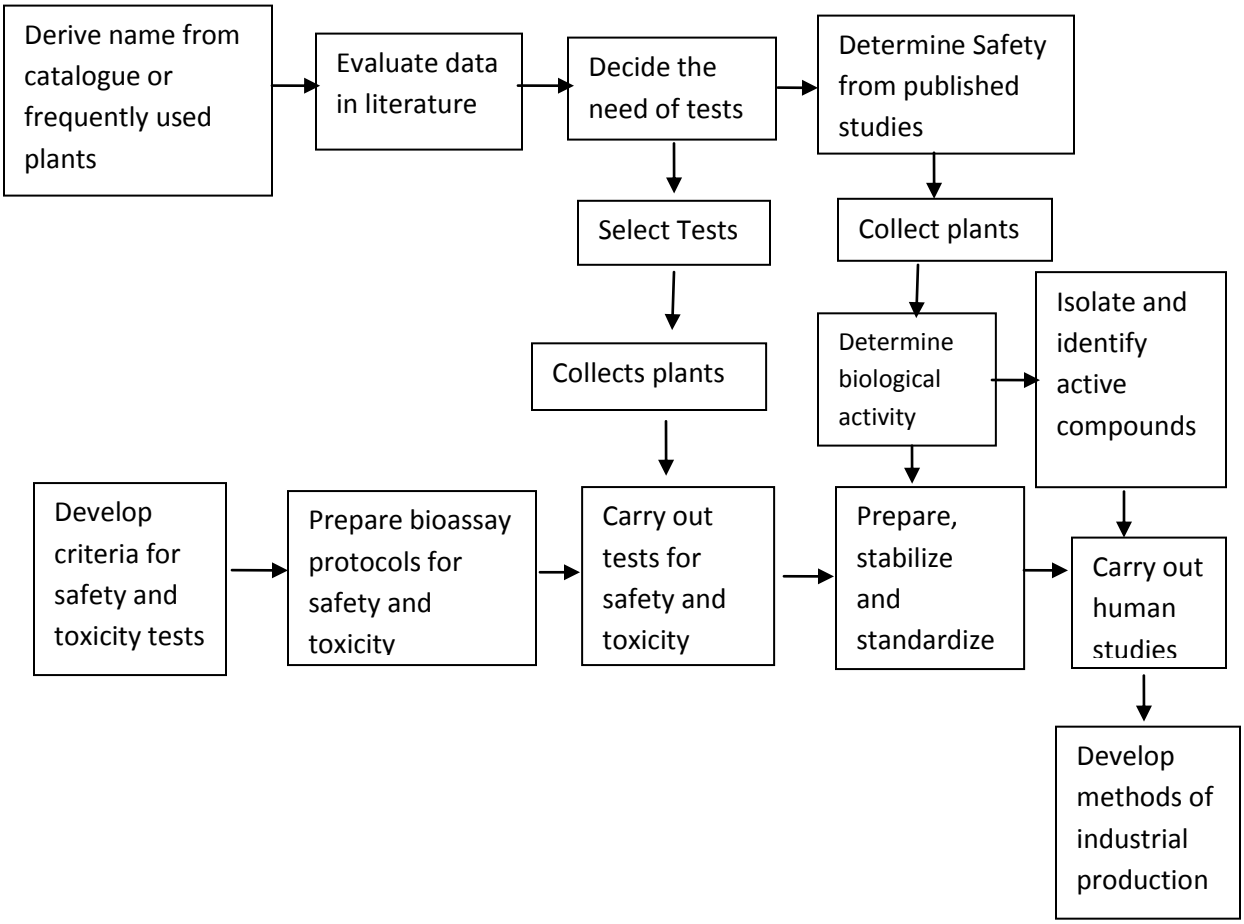

Figure 1.1 Flowchart for the study of plants used in traditional medicine to industrial production of therapeutic compounds (Modified from Farnsworth *et al.,* 1985)

1.3 Aims and objectives

The **hypothesis** proposed here is that species of *Swertia* contains active constituent(s) that are rich in antioxidants, flavanoids, xanthones and other important compounds. Their overall quantification helps to reveal their bioactive properties.

The main objective of the study is semi-quantitative estimation of major compounds in different species of *Swertia*, accessing their bioactivity and study adulteration of other species against *Swertia chirayita* through phytochemical analysis. This objective is achieved through following activities:

I. To collect *Swertia* species from their wild habitat from various parts of Nepal.
II. To obtain their methanol and aqueous extracts.
III. To perform qualitative phytochemical screening on those plant samples.
IV. To estimate total phenolics and flavonoids in their methanol and aqueous extracts.
V. To evaluate their *in vitro* antioxidant properties.
VI. To evaluate their antibacterial and antifungal activity from the plant extracts.
VII. To estimate the major phytoconstituents with TLC system with reference to amarogentin, swertiamarin and mangiferin.

1.4 Rationale

The medicinal plants are the reservoir of drugs for many diseases. It is important to screen their components to evaluate different types of pharmacological effects. Medicinal plants are the best sources for antioxidant, antibacterial and other bioactive compounds that are the major components of traditional healing system in rural communities. As *Swertia* species have varied importance in human therapeutic and preventive medicine, their study reveals new medicinal prospects and pharmaceutical applications. The present study aims to compare the major phytochemicals in different *Swertia* species from Nepal and to study of adulteration of various traded species by the comparison of their reference compounds.

In addition to dominant *Swertia chirayita,* other species are exported from Nepal as an adulterant. Their detailed phytochemical analysis and bioactivity of the other species has not been in extensively studied. The present study focuses the preliminary phytochemical analysis and their bioassays. Particularly, bitter compound amarogentin is known for the anti-leishmanial, antipyretic, analgesic as well as anticancer property. Besides, mangiferin

and Swertiamarin are the other important compounds present in *Swertia* species known for their hypoglycemic activity and other bioactive properties. Finally, *Swertia* species is known traditionally for the multi-purpose therapeutic agent.

The present study aims to differentiate *S. chirayita* from *Swertia angustifolia, S. nervosa, S. paniculata, S. dilatata and S. ciliata* that form an important part of *Swertia* trade, where these species are routinely mixed with the potent *Swertia chirayita*. This research aims to differentiate these species on the basis of their phytoconstituents; amarogentin, swertiamarin and magniferin content. So, this study is aimed at the detailed qualitative and semi-quantitative analysis of specific compounds like mangiferin, amarogentin and swertiamarinin those species. Also, this helps to grade the quality of different species of *Swertia* in nearness to *S. chirayita.* The qualitative grading of the species of *Swertia* adds the knowledge and so forth the species can be used on accordance to the need and presence of important compounds. Other species also can have bitter compounds that can substitute endangered *S. chirayita.* The present work is preliminary and is of high importance in revealing the properties of other *Swertia* species.

1.5 Scope of the Study/ Justification

Nepal houses 31 different species of *Swertia* that provides an ample traditional and pharmacognostic importance and the species of *Swertia* form an important NTFP to be traded from Nepal. In the international trade, Nepal enjoys around 50% of the total trade. In this regard, *S. chirayita* is routinely mixed with its nearby species like *S. paniculata, S. nervosa, S. angustifolia, S. racemosa, S. ciliata and S. dilatata.* This adulteration of various inferior species of *Swertia* with *S. chirayita* decreases the therapeutic effectiveness in terms of their bioactive constituents and bitterness.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Over the past decade, there has been a resurgence of interest in the investigation of natural materials as a source of potential drug substance. The World Health Organization (WHO) reported that 25 - 50 % of modern medicines are made from plants first used traditionally (WHO, 2002). Indeed, increasingly more pharmacognostic investigations of plants are being carried out to find novel drugs or templates for the development of new therapeutic agents (Beringer, 1999). The World Health Organization lists 11% of 252 basic drugs produced from flowering plants and one fourth of all prescribed drugs are directly or indirectly derived from plant sources (Patterson *et al.,* 2005). As the number of useful bioactive compounds from natural products is currently in use and many are being investigated the medicinal plants serve an essential part of novel medicines against human ailments (Balanus and Kinghorn, 2005). Butler (2004) has focused on the need to develop faster and better techniques for plant collection, crude extract preparation, bioassay screening, compound(s) isolation and drug development to keep pace with the increasing drug discovery efforts. The development of more drugs from medicinal plants continues to increase due to the diversity of plant species, much of which have not been pharmacologically and phytochemically characterized (Kohen *et al.,* 2005). Bioactivity Directed Fractionation and Isolation enables the isolation of natural products according to the results from bioactivity assays. Further modification of these active compounds lead to enhanced biological profiles and generates innovative compounds (Butler, 2004; Kohen *et al.,* 2005).

Secondary metabolites are chemically and taxonomically diverse compounds with obscure functions. They are widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas (Mann, 1978). Plant products can be derived from roots, barks, leaves, flowers, fruits or seeds. Knowledge of the chemical constituents of plants assist for synthesis of complex chemical substances (Cragg and David, 2001) and can be used as pharmacological drugs or modified accordingly against various infections. Besides, they add up to the chemical library such that much more new drugs can be derived using bioinformatics and combinatorial tools (Newman *et al*., 2000; Xie *et al.,* 2008).

2.2 Medicinal Plants of Nepal

Nepal is situated on the southern slopes of the central Himalayas ranging from lowlands in the Terai to the Himalayan highlands in the north covering all of the climatic conditions. This topographical diversity of life-zones houses a large variety of flora and fauna (WWF 2008). Nepal occupies an area of 1,47,181 Km^2 with the latitudinal range from 26°22' to 30°27' N and longitudinal ranges from 80°14' to 88°12' E; covering only 0.3% of the total land area of the world but has huge altitudinal variation. Nepal claims over 2.2 per cent of the biological wealth of the world's natural flowering plants. Nepal ranks 25th in biodiversity richness globally and ninth-richest flowering plant diversity in Asia (BPP, 1995). Nepal has 6653 species of flowering plants; among which about 34 % are medicinal. The number of people relying on use of medicinal plants for their primary healthcare is fairly large (Manandhar, 2002). Such plants of medicinal value have been routinely used for their home remedies, traditional therapies and daily livelihood in the rural communities (WHO 2005). About 1000 species of medicinal plants are reported to have been used traditionally by various communities in Nepal for their primary healthcare system (Chaudhary, 1998). Shrestha *et al,* 2000 have enlisted 1630 species of medicinal plants of Nepal.

2.3 The Family Gentianeceae

The family *Gentianaceae* is a family of flowering plants of 87 genera and about 1600 species containing a wide range of colors and floral patterns. Members of the family have leaves that are opposite each other on the stem. The leaves often lack leaf stalks and have smooth margins. The flowers have four or five united petals, which may be deeply divided. Gentians have distinctive, bell-shaped blossoms. They are usually bisexual and regular with 4 or 5 separate sepals, 4 or 5 united petals and 4 or 5 stamens. The stamens are inserted on the corolla tube and alternate with the lobes. The ovary is positioned superior. It consists of 2 united carpels forming a single chamber. It matures as a capsule with many seeds. Seeds are small with oily endosperms and a straight embryo (Pant *et al.,* 2000).

Most members of the Gentian family are reported to contain potent bitter compounds that stimulate the digestive system and are known to heal many digestion disorders. Volatile oils are also present in the Gentian family that proves them to be anti-helmintic (Struwe and Albert, 2002). They are known for the bitter taste and their use in traditional remedies against many human ailments. The bitter taste is attributed to the presence of

pharmacologically important group of compounds like iridoids, xanthones, mangiferin and c-glucoflavones. The iridoids (mainly seco-iridoid glycosides) appear to be present in all species investigated, with a predominance of swertiamarian and/or gentiopicroside; with 90 different compounds have been reported from 127 species in 24 genera (Negi *et al.,* 2011; Sharma *et al.,* 2011). Xanthones are not universally present in gentianaceae, but about 100 different compounds have been reported from 121 species in 21 genera. Mangiferin has been recorded from 42 species in seven genera (Negi *et al.,* 2011; Jensen and Schripsema, 2002). The important genus of this family includes *Swertia, Gentiana, Blackstonia, Sabbatia, Halenia, Gentianella, Eustoma, Centaurium, Voyriella and Schenkia* among others (Struwe and Albert, 2002).

2.4 The genus *Swertia*

Swertia L (*Gentianaceae – Gentianeae – Swertiinae*) is a morphologically diverse but taxonomically distinct genus containing 150 species. The plant is annual, biennial or perennial herb ranging from 2-4 cm. to over 1.5 m in height with tetra or pentamerous flowers, 1 or 2 nectaries at the base of characteristically rotate corolla lobes. The genus mostly occurs in alpine or temperate habitats in Asia, Africa and North America. The circumscription of the genus has often been debated, resulting disagreement amongst taxonomists due to the morphological similarities (nectariferous and rotate corolla lobes) among the species of *Swertia* and the related genera (Joshi and Joshi, 2008)

2.4.1 Distribution of *Swertia* in Nepal

Swertia is a native Himalayan species mostly found on the higher altitudes from 1500 to 4600 m. There are many species of *Swertia* but *Swertia chirayita* forms a major species on this genus. So, these species extend from Kashmir to Bhutan at an altitude of 1200-3000m and Khasi hills at 1200-1500m and can also be grown in sub-temperate regions between 1500-2100m altitude (Phoboo and Jha, 2010; Sharma *et al.,* 2011; Khanal *et al.,* 2014) However, the plant growth is more favorable on north facing slopes and the altitude ranges between 1500 to 3000m (Sharma *et al*., 2011; Joshi and Dhawan, 2005). Whereas, on a southwest slope, the growth is better under conifer mixed broad leaved forests that provides the better microsite (Bhatt *et al*., 2006). In Nepal, the plant is reported in the subtropical (1200m) to alpine zone (3000m) through western, central and eastern regions (Shrestha *et al.,* 2010; Phoboo and Jha, 2010).

Due to its diverse topographical distribution, Nepal houses 31 species including five varieties of *Swertia* (Khanal *et al.,* 2014; Shrestha *et al.,* 2010) among, *Swertia acualis* is the endemic one (Joshi, 2008). This plant is native and indigenous to Himalayan landscape. Nepal occupies a prominent percentage of *Swertia* distribution and has a majority share in its trade (Joshi and Joshi, 2008). *Swertia* ranks high in terms of medicinal importance and drug value among the local Nepalese folklore. They use it as primary medicines in fever and enteric diseases mostly as infusion, decoction, paste and juice. This high ethno-medicinal value has made *Swertia* one of the largest exports on medicinal plants and NTFPs from Nepal. So far, *Swertia* species has been reported from 54 districts of Nepal (Barakoti *et al.,* 1999; Phoboo *et al.,* 2010; Shrestha *et al.,* 2010, Joshi and Joshi, 2008).

Pant (2008) mentions some 104 NTFPs commonly traded in Nepal. Among the high value NTFPs, *Swertia* species occupy one of the major positions in the trade (Edwards, 1996). Among the total 31 species of *Swertia*, only the following 9 species are commonly traded:

I. *Swertia chirayita* (Roxb. ex Fleming) H. Karst
II. *Swertia angustifolia* Buch.-Ham. ex D. Don
III. *Swertia tetragona* Edgew
IV. *Swertia racemosa* (Griseb.) C.B. Clarke
V. *Swertia ciliata* (D. Don ex G. Don) B.L. Burtt
VI. *Swertia dilatata* C.B. Clarke
VII. *Swertia multicaulis* D. Don
VIII. *Swertia alata* (Royale ex D. Don) C.B. Clarke
IX. *Swertia nervosa* (G. Don) C.B. Clarke

S. chirayita plays a dominant role covering about 80% of total traded volume of chiretta and is superior in quality to other species. Most of the chiretta (80-90%) are exported as a crude drug to India, occasionally to China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Sri Lanka, Bangladesh, Pakistan, or USA. Nepal trades about 50 % of the world's total volume of chiretta **(**http://gentian.rutgers.edu/overview.htm).

The market demand of *Swertia* is one of the highest revenue earning export for Nepal. The major bulk is exported to India and other countries, owing to about 45 % of the total traded *Swertia* from Nepal (Joshi and Dhawan, 2005). Weight loss during storage and adulteration

with the nearby species of *S. chirayita* is a major constraint in 'chirayito' trade. The adulteration of *S. chirayita* with other low value species of *Swertia* (*S. angustifolia, S. alata, S. ciliata, S. bimaculata, S. dilatata, S. paniculata, S. petiolata and S. tetragona*); all commonly known as "Bhale chirayito", is a common practice in chirayito trade (Joshi and Dhawan 2005; Khanal *et al.,* 2014). Some of these species are also used as substitutes of *S. chirayita* in medicine, but most of them are considered inferior in terms of their bitterness (Joshi and Dhawan 2005). Up to 5 % adulteration has been reported common but excess adulteration reduces the price of chirayito (Bhattarai and Ghimire, 2006). Besides *Swertia* species, common other adulterants include *Andrographis paniculata* (green chirayita), *Exaccum spp.* and *Slevolgia orientalis* (Joshi and Dhawan 2005). As stated by Bhatt *et al.,* 2006, the true chirayito can be distinguished by its intense bitterness, brownish purple stem, continuous yellowish pith and petals with double nectaries.

2.5 Chemical and pharmacological aspects of *Swertia*

Plants belonging to *Gentianacece* are well known for their pharmacological properties. They have bitter compounds called glycosides and hence are best remedies for digestive disorders (Hottestmann Kalad *et al.,* 1981; Khanal *et al.,* 2014). Some of the most promising and high potential drugs like amarogentin, swertiamarin and swechirin have been studied for drug development (Brahmachari *et al.,* 2004) due to the occurrence of iridoids, xanthones, mangiferin and c-glucoflavones. The iridoids (mainly secoiridoid glucosides) appear to be present in all species investigated, with a predominance of Swertiamarin and/or gentiopicroside. Xanthones are not universally present in *Gentianaceae*, but about 100 different compounds have been reported from 121 species in 21 genera (Sharma *et al.,* 2011; Negi *et al.,* 2011).

Struwe and Albert (2002) mention the biosynthesis of xanthones to categorize four major groups of species. Group 1 (*Anthocleista*, *Blackstonia*, *Gentianopsis*, *Macrocarpaea* and *Orphium*) includes the taxa containing only few and biosynthetically primitive xanthones. Group 2 (*Comastoma*, *Gentiana*, *Gentianella*, *Lomatogonium*, *Swertia*, and *Tripterospermum*) contains xanthones with an intermediate degree of biosynthetic advancement. Group 3 (*Frasera*, *Halenia* and *Veratrilla*) has the most advanced compounds, with the xanthones found in group 2 being the biosynthetic precursors. Group 4 (*Canscora*, *Centaurium*, *Chironia*, *Eustoma*, *Hoppea*, *Ixanthus* and *Schultesia*) contains another set of biosynthetically advanced compounds. The comparison of the above groups is also done on

the basis of strict consensus trees based on molecular data (*trn* L intron and *mat* K sequences).

Swertia is an important medicinal genus that has been mentioned in pharmaceutical codex, traditional medic systems and many pharmacopoeias (Joshi and Dhawan, 2005; Tabassum *et al.,* 2012; Negi *et al.,* 2011). Ayurveda also focuses its uses as antipyretic, anthelminthic, antiperiodic, laxative, in asthma and leukemia conditions (Negi *et al.,* 2011). This feature necessitates exploring its phytoconstituents that enables for such a huge spectrum of pharmacology. Brahmachari *et al.,* 2004 and Negi *et al*., 2011 have stated the pharmacological bioactivity of various compounds isolated from *Swertia* species, mostly attributing to its seco-irridoids and xanthones. These bioactive compounds are responsible for the therapeutic effects and pharmacological activities (Joshi and Dhawan, 2005; Khanal *et al.,* 2014).

Swertia chirayita is reputed for its medicinal and pharmaceutical value as it is a rich source of alkaloids and flavonoids that exhibit broad spectrum bioactivities. Its roots are reported to possess significant antipyretic, analgesic properties and a high therapeutic index (Kumar *et al.,* 2003). Various previous studies on its isolation and structural elucidation of active constituents of *Swertia chirayita* have showed the compounds responsible for pharmacological and biological activity. More than twenty polyhydroxylated xanthones have been characterized, and some of these are Swertinin, Swerchirin, Mangiferin, Decussatin and Isobellidifolin (Bhattacharya *et al.,* 1976). Of the bitter secoiridoids, important ones are the bioactive Amarogentin and Swertiamarin. The herb contains swertanone, swertenol, episwertinol, gammacer-16-en-3ß-ol, 21-a-H-hop-22(29)-en-3ß-ol, taraxerol, oleanolic acid, ursolic acid, swerta-7, 9(11)-dien-3ß-ol and pichierenol. This plant also contains a range of pentacyclic triterpenoids (e.g. ß-amyrin, friedlin, lupeol, taxaxerol, chiratenol, kairatenol, oleanolic acid and ursolic acid). Other classes of compounds identified include a lignan (syringaresinol), and the ubiquitous ß-sitosterol (Rastogi *et al.,* 1991). Karan *et al.,* 2012 tried to establish the chemotaxonomic relationship between different *Swertia* species on the basis of thin layer chromatography fingerprint profile. The maximum number of xanthones was observed in methanol extract of *S. chirayita* followed by *S. cordata, S. lurida* and *S. purpurascens.* And *S. angustifolia* showed the minimum number of xanthones.

The intensive research on its phytochemical investigation of the genus *Swertia*, as carried out so far, has delivered some 200 compounds with varying structural patterns. Among these constituents; xanthonoids, terpenoids, flavonoids, and alkaloids from the major classes of compounds with many modifications in these compounds (Sharma *et al.,*2011).

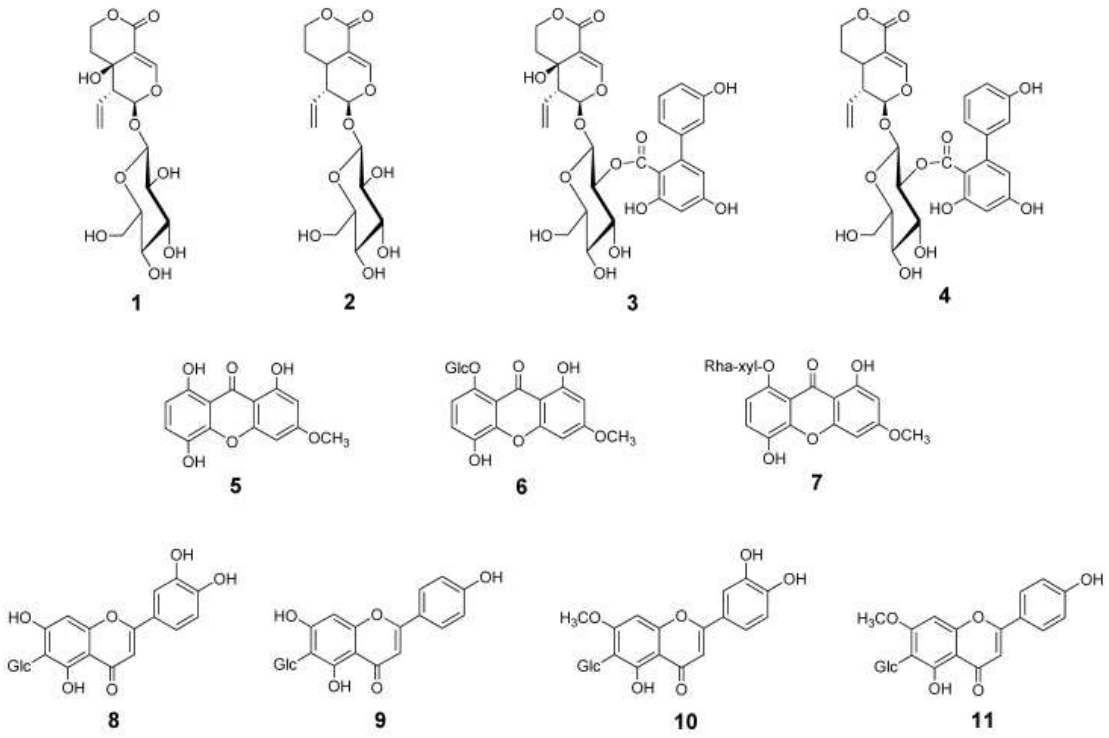

Figure 2.1: Structures of *Swertia* components (Adapted from Wang, 2008)

(1) Swertiamarin, (2) Sweroside, (3) Amaroswerin, (4) Amarogentin, (5) Bellidifolin, (6) Swertianolin, (7) Pseudonolin, (8) Isoorientin, (9) Isovitexin, (10) Swertiajaponin and (11) Swertisin.

Swertia herbs contain iridoid glucosides, xanthones and flavonoids. Of these, swertiamarin is the most abundant active constituent and other active compounds reported are sweroside, amaroswerin, amarogentin, bellidifolin, Swertianolin, pseudonolin, isoorientin, isovitexin, swertiajaponin and swertisin (Wang, 2008). Among these, amarogentin (Inoue *et al.,* 1966) is the most bitter principle and it has anti-proliferative and pro-apoptotic actions (Saha *et al.,* 2006).

The metabolites in *Swertia* herbs significantly varies according to geographic, climatic, environmental and other factors. As the application of *Swertia* herbs becomes more extensive, a quality standard is urgently required to identify the raw materials. Quality control and evaluation of *Swertia* herbs have generally targeted Swertiamarin, due to the high content of this compound (Takei *et al.,* 2001). The content of amarogentin, mangiferin and Swertiamarin has been determined using thin-layer chromatography and high-performance liquid chromatography (HPLC). However, a single compound alone could not

be responsible for the overall pharmacological actions of *Swertia* herbs and synergistic effects among the various constituents probably play significant roles. Among these compounds, the different species of *Swertia* contain various iridoid glycosides like amarogentin, amaroswerin and mangiferinin varying amounts (Demizu *et al.,* 1986).

Amarogentin: It is a seco-irriod glycoside with one of the bitterest compound known and the bitter taste persists even at a dilution of 1g in 14000 liters of water (Anonymous, 1976). Its bitterness can be tasted even at a dilution of 1: 58,000,000 (Singh 2008). It is known for its topo-ismerase inhibition (Ray *et. al.* 1996), chemopreventive, anti-leishmanial and gastroprotective properties (Negi *et al.,* 2011. Of the studies species of *Swertia*, the highest percentage of amarogentin is found in S*. chirayita*; however the nearby species like *S. bimaculata, S. dilatata* and *S. paniculata* are known to have this bitter compound in lesser amount (Negi *et al.,* 2011; Bhramachari *et al.,*2004).

Mangiferin: It is c-glucoxanthone that exhibits diverse pharmacological activities that is used in the treatment of arthritis, diabetes, hepatitis, cardiac and mental disorders (Sanchez *et al.,* 2000). It is a good antioxidant agent with anti-tumor, antiviral, anti-atherogenic, immunodilatory, anti-proliferative and diuretic properties (Pandey *et. al.,* 2012).It is also reported to have significant anti-diabetic activity similar to the clinical drug **Glibenclamide.** Mangiferin significantly increased heart tissue phospholipids in isoproterenol induced cardio-toxic rats suggesting cardioprotective and hypolipidemic effects (Andreu *et al.,* 2005). As per the HPTLC analysis by Pandey *et al.,* 2012, this compound is found in *S. chirayita and S. nervosa*, while it is absent in *S. bimaculata, S. dilatata and S. paniculata.*

Swertiamarin: It is a secoirirdoid that has been reported to possess hepatoprotective, anti-inflammatory, anti-bacterial, anticholinergic, free radical scavenging and antispastic (Vaijanathappa and Badami, 2009). Swertiamarin has also been reported as anti-lipidimic agent comparable to the clinical drug **Atorvastatin** which may also contribute to its cardio-protective and anti-atherosclerotic role (Vaidya *et al.,* 2009).

2.6 Phytochemical extraction, Identification and quantification of compounds

Before doing any purification and isolation work, specific compounds have to be extracted from the plant biomass. The first aim is the isolation of a known metabolite or as many compounds as possible for an organized phytochemical profiling. An initial extraction is done on a small quantity of material to obtain a primary solvent extract. Once definite metabolites have been recognized in the initial extract, then we can go for its isolation in bulk quantities. As natural products are so diverse and show distinct physicochemical properties, the question arise is how can these compounds being extracted proficiently from our target plant material. Solvent-extraction methods can be used for laboratory scale research for isolation of specific metabolites (Salminen, 2003; Katerere *et al.,* 2004).

2.6.1 Phytochemical Extraction

The preliminary procedure to obtain organic constituents from plants is extraction in a soxhlet apparatus with a range of solvents, starting with non-polar solvent and then polar solvents for more polar compounds. The extract obtained is filtered and concentrated in a rotary evaporator (Katz *et al.,* 1966; Houtman *et al.,* 2007). This concentrated extract may also crystallize on standing that is collected by filtration and its composition is observed with available chromatographic techniques in many solvents. Once a specific compound is separated, then we go for its crystallization. The obtained crystals are purified by recrystallisation with intermittent analysis and check. With mixture of substances, the crystals are redissolved in a suitable solvent and chromatographic methods are employed for their separation (Houtman *et al.,* 2007).

The total extraction process lies in the interplay between the polarities of the solvents used. Different compounds of varying polarity dissolve in corresponding polar or non-polar solvents used during the extraction procedure. The extracts obtained need to be concentrated using a rotary vacuum evaporator. Generally, the more polar the solvent, the more heat is required to evaporate it. Thus, the most volatile solvent that will be effective is chosen. However, mixtures of them are often used during isolation of specific compounds (Wagner *et al.,* 1983, Sewell and Clarke, 1978). With diverse chromatographic methods, many fractions of compounds can be obtained and tested for biological activity. The successive bioassays will identify which fractions possess the most bioactivity and that specific compound can undergo further separation Once the

biologically active fractions are obtained, further purification can be done to identify the constituents to explore where the biological activity actually lies(Williamson *et al.,*1996). For example, in a protocol stated by Koul *et al.,* 2009, the chemical analysis of the components of *S. chirayita* was done. They have stated the fractionation and purification of amarogentin, amaroswerin and mangiferin from the extracts of *S. chirayita* using a gradient of polar and non-polar solvent systems leading to crystallization and structure validation using IR and NMR spectral data.

Baral *et al.,* 2012 studied preliminary phytochemical screening of four species of *Swertia* (*S. chirayita, S. angustifolia, S. ciliata* and *S. dilatata*) from Nepalese Himalayas with acetone and methanol solvents. They reported the presence of volatile oils, carotenoids, sterols and triterpenes, fatty acids, caumarins, quinones, polyphenols and glycosides in both solvent extracts. Similarly, Bhattarai (2014) studied phytochemicals present in different populations of *Swertia chirayita* from different places of Nepal, where semi-quantification of major compounds of *Swertia chirayita* (amarogentin, Swertiamarin and mangiferin) was done and reported the bioactivity of those *Swertia chirayita* samples against different bacterial strains and found them inhibiting major Gram negative bacteria.

Neupane *et al.,* 2013 performed genetic diversity assessment and DNA barcoding of various populations of *Swertia chirayita* from Nepal. Out of the total 479 amplified bands by 27 ISSR primers for 42 accessions of *Swertia chirayita*, eleven primers revealed 16 ISSR markers that were specific to various *S. chirayita* populations. The study has produced insights for the conservation and sustainable utilization of *S. chirayita* of Nepal. Another similar work conducted by Shrestha *et al.,* 2013 investigated the level of genetic diversity in five *S. chirayita* populations of Nepal from various places using Polymerase Chain Reaction based Random amplified polymorphic DNA technique. The result showed high genetic polymorphism in *S. chirayita* populations, good survival potentiality and adaptability in changing environmental scenario that might be helpful to plant breeders for elite cultivar development and generate insights for the formulation of conservation strategy of this vulnerable species together with its phytochemical distinctiveness.

2.6.2 Identification of Major Compounds

Once extraction is fully carried out, the second step is the separation and purification of the compounds where chromatography techniques come into play (Wagman and Cooper 1983; Björnstad *et al.,* 2009). Chromatography involves a sample or extracts being dissolved in a mobile phase that is forced through an immobile, immiscible stationary phase. The phases are chosen such that components of the sample have varying solubility in each phase. As a result of these differences in mobility, sample components will become separated from each other as they travel through the stationary phase (Wagman and Cooper 1983; Björnstad *et al.,* 2009).

Chromatography is an analytical method that is widely used for the separation, isolation, identification, and quantification of components in a mixture. Components of the mixture are carried through the stationary phase by the flow of a mobile phase. Separations are based on differences in migration rates among the sample components (Fried and Sherma, 1994). The most important of these methods are: Thin layer chromatography, Column Chromatography, Gas Chromatography and High Pressure/Performance liquid chromatography.

Among the various separation and identification techniques, Thin Layer Chromatography is a simple, quick, and inexpensive procedure that gives a quick idea the number of chemical components in a mixture (Synder, 2009). TLC is also used to identify a compound in a mixture when the RF of a compound is compared with the RF of a known compound and compare a corresponding compound with its respective standard (Smith *et al.,* 1965; Zullich *et al.,* 1975; Kovac-Besovic and Duric 2003).

A TLC plate is usually made of sheet of glass, metal, or plastic coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is placed near its bottom, and then placed in desired pool of solvent in a developing chamber. This eluent is the mobile phase that slowly rises up the TLC plate by capillary action. As the solvent moves upfront different components are separated on the basis of their mobility. When the solvent has travelled more than half of the plate, the plate is removed, dried, and the separated components of the mixture are visualized (Smith *et al.,* 1965; Zullich *et al.,* 1975). Plates can be visualized, depending on the chemical structure of the compounds at visible light, UV (254 nm and 365 nm) or by using spray reagents (Wagman and Cooper 1983). The effectiveness of the separation depends on the mixture to be separated, the choice of the mobile phase and the adsorption layer (Fritz and Schenk, 1987). The term

retention factor R_f, is used to describe the chromatographic behavior of solutes. The R_f value is the ratio of the distance moved by the solute and solvent front. Comparison of these R_f values makes it possible to resolve complex mixtures qualitatively (Fritz and Schenk, 1987).

2.6.3 Quantification of major compounds

Quantification of the compounds present in the plant samples can be achieved by HPLC, HPTLC and commercial software programs. High-performance liquid chromatography (HPLC) is a type of liquid column chromatography technique mostly used to identify, separate and quantify the active compounds in a solution (Martin *et al.,* 2005). HPTLC is the alternative to the HPLC technique and enhanced form of thin layer chromatography. Various enhancements can be made to the TLC to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements. This may be using a UV scanner to identify the various compounds that are present in the samples used (Morlock *et al.,* 2010).

2.6.4 Semi-quantificationby GelQuant.NETsoftware

Nowadays, the quick and rapid semi-quantification or estimation of organic compounds can be done with various software tools that are provided by various companies in gel systems and pictures. The GelQuant.NET software is one such a kind of gel imaging software provided by Biochemlab Solutions that computes the respective pixels for each standard JPEG, BMP, PNG, TIFF images. It makes the background correction and presents a quantitative data in terms of pixels or a ratio of the pixels of the band against its background. Then these pixels can be changed to its corresponding concentration by using standard curve of a standard compound. By the use of a standard calibration curve, the unknown concentration of the compound can be found. GelQuant.NET reads .GEL files natively and allows correct quantification. GelQuant.NET software allows intuitive and fast operation due to extensive implementation of mouse and Drag-Drop functions. It is easy to Magnify, Zoom, Rotate by any angle, Flip, Evaluate overexposure, adjust Brightness, Contrast, Invert and Save images. Intensity of a band or an image segment is quantified by drawing a selection rectangle and pressing the middle button of a mouse. Absolute intensity, local average corrected intensity and % of the signal over total signal across several areas are computed (http://biochemlabsolutions.com/GelQuantNET.html).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

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

3.1.2 Instruments: All the instruments that were used during this thesis were in good working condition.

3.1.3 Plant Samples: All the plant samples were collected by the investigator from various districts (Kathmandu, Lalitpur, Rasuwa, Dolakha and Palpa) of Nepal. Whole plants were collected and identified by a taxonomist from Central Herbarium and Plant Laboratory, Lalitpur.

3.1.4 Bacterial Strains: ATCC cultures of *Escherichia coli* (25922), *Salmonella typhii* (14028), *Klebsiella pneumonia* (700603), *Pseudomonas aeruginosa* (27853), *Staphylococcus aureus* (25923) and *Enterococcus faecalis* (25912).

3.1.5 Fungal Strains: *Candida albicans*, *Sacharomyces cerevasiae* and *Pischia pastoris.*

3.2 Selection of the plant samples

The plant samples were chosen according to higher ethno medicinal value among Nepalese folklore, potential drug formulation (high content of amarogentin) and less studies being done in other species of *Swertia* found in Nepalese Himalayas.

3.3 Collection and identification of the plant samples

Whole plants were collected from different places of Nepal during their flowering periods of August to October, 2013. The selected samples collected were photographed and altitude of the collection sites was taken using GPS. Identification of the plants material was done by a taxonomist from Central Herbarium and Plant Laboratory, Godawari. Herbarium specimens were prepared and voucher specimens were submitted for deposition at National Herbarium, Godawari, Lalitpur for the future references. Following table 3.1 summarizes the different species of *Swertia* that were collected from the different places of Nepal.

Table 3.1 Collection of different *Swertia* species from various places of Nepal

S.N.	Name of the plant	Place of collection	Altitude	Part of the plant collected
1	*Swertia chirayita*	Phulchowki, Lalitpur Deurali, Rasuwa	1976 m 2094 m	Whole plant
2	*Swertia angustifolia*	Palpa Daman, Makwanpur	1625 m 2310 m	Whole plant
3	*Swertia paniculata*	Kalinchowk Temple, Dolakha	3740 m	Whole plant
4	*Swertia racemosa*	Way to Cholangpati, Rasuwa	3265 m	Whole plant
5	*Swertia nervosa*	Kuri to Kalinchowk Temple, Dolakha	3178 m	Whole plant
6	*Swertia ciliata*	Above Deurali, Dolakha	1984 m	Whole plant
7	*Swertia dilatata*	Kuri, Dolakha	2364 m	Whole plant

3.4 Preparation of the plant material

The collected plant material were cleaned off mud, fungi and any unwanted materials, then air/shade dried at 32-35^{o}C for 6 days to remove all their moisture. The dried plant material was powdered with the help of grinder, passed through a wire sieve (porosity 220 microns) and the fine powder was collected on sterile and dry polyethylene bag for extraction**.**

3.5 Preparation of plant extracts and extract dilution

50 grams of fine powder of each plant sample was taken separately and dissolved in 500 ml of 100 % methanol and left to percolate for 24 hours. After percolation, these samples were subjected to ultra-sonication for 2 hours and centrifuged in falcon tubes. The solvent was filtered and methanol was evaporated on the rotatory evaporator under the vacuum at the room temperature till the solid mass was obtained. Obtained solid mass was weighed carefully to express the gram of extract extracted per 50 grams of the plant powder. For each sample extract were prepared individually. The extracts were kept at 4^{0}C for further analyses.

Each 100 mg of crude plant extract was weighed accurately and dissolved on 1 ml methanol and 1mL of distilled water. This 100 mg/ml stock of each plant extract was used for antimicrobial tests, antioxidant activity, quantification of the total phenol and total flavonoids. Other dilutions were also prepared from this stock solution.

3.6 Qualitative phytochemical analysis

The methanol and aqueous extracts were used to screen for the presence of various secondary metabolites by using the following protocols suggested by Harborne, 1973 and Trease and Evans, 1989.

Test for flavonoids: 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: Crude extract was mixed with each of 2ml of chloroform and 2ml of acetic acid. The mixture was cooled in ice and concentrated H_2SO_4 was added carefully. 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 greyish color indicated the presence of terpenoids.

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.

3.7 Quantitative phytochemical analysis

3.7.1 Total polyphenol content (Chang *et al*., 2002)

The total polyphenol content of seven *Swertia* species was determined using the Folin–Ciocalteu phenol reagent (Chang *et. al.,* 2002). For this 0.1 ml of each extract (2.5 mg/ml) was separately mixed with the 1 ml of Folin–Ciocalteu phenol reagent (Merck Ltd, India) (1:10 dilution with the distilled water) and 0.8ml of aqueous 1 M Na_2CO_3 solution. The reaction mixture was allowed to stand for about 15 minutes and then absorbance was measured at 765 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). A calibration curve was obtained using Gallic acid (Moly Chem, Mumbai) in methanol using the concentration ranging from 25-250µg/ml as standard. Based on this standard graph, the concentration of the individual samples was calculated. Total polyphenol content was expressed in terms of the milligrams of the Gallic acid equivalent per gram of the dry mass (mg GAE/g). The test was triplicated for the reproducibility of results.

3.7.2 Total flavonoid content (Begum, 2011)

The total flavonoid content in the plant extract was estimated using the Aluminium chloride ($AlCl_3$) colorimetric method. 0.25 ml of extract (10 mg/ml) was separately mixed with the 0.75 ml of ethanol, 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium

acetate (CH_3COOK) and 1.4 ml of the distilled water. The reaction mixture was incubated for 30 minutes at room temperature. Then absorbance of the mixture was measured at 415 nm using the UV-spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The calibration curve was obtained with the help of the quercetin (Sigma Co. Ltd) as standard dissolved in methanol with the concentration from 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). The test was triplicated for the reproducibility of results.

3.7.3 Total Antioxidant activity via DPPH free radical scavenging assay

The antioxidant activity of extract of seven *Swertia* species 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). Different concentration of plant extracts and ascorbic acid (10-100µg /ml) were prepared in methanol on clean test tubes. 0.5ml of samples of plant extract as well as ascorbic acid of each concentration was taken separately in clean test tubes. To this sample 0.5 ml of the 0.2mM DPPH solution was added. The tubes were shaken uniformly for proper mixing and incubated in dark for half an hour. The control was prepared as above but without the plant extract or ascorbic acid and methanol was taken as blank. The absorbance was taken on spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5) at 517 nm.

The radical scavenging activity was calculated using the following formula.

$$\%\ Radical\ Scavenging\ Activity\ =\ [(Control - sample)/Control)]\ x100\%$$

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

3.8 Determination of Antibacterial activity

3.8.1 Preparation of Nutrient agar (NA)

NA plates and NA slants were prepared for the antibacterial tests. About 28 gram of NA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and poured in distilled water. The contents were dissolved on the water completely and the final volume was maintained to 1000 ml followed by boiling for uniform mixing. This media was sterilized by autoclaving at 15lbs pressure at 121°C for 15 minutes. The autoclave tape was used as an indicator for the completeness of sterilization. The media was allowed to cool to 45-50°C, the media was poured on sterilized and properly labeled petridishes. About 25 ml of the media was poured on each petridish of 9 cm diameter in sterile conditions under a laminar flow hood. All of the plates were left for the solidification of media. For the preparation of the NA slants, screw tight bottles were filled with autoclaved NA media and placed in an inclined position. These bottles were left for solidification.

3.8.2 Preparation of Luria Bertani Miller (LB) broth and Mueller Hinton Agar (MHA)

The LB broth was required to culture and subculture the microorganisms prior to antimicrobial tests. About 25 gram (Hi Media Laboratories Pvt. Ltd, Mumbai, India) from the powder supplied was carefully weighed and transferred on a conical flask. The content was dissolved in distilled water and final volume was maintained to 1000 ml. This media was transferred to the screw bottles and sterilized on autoclave at 15 lbs pressure and 121°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 dispensed in sterile and dry culture tubes.

38 grams (Hi Media Laboratories Pvt. Ltd, Mumbai, India) of the powder was weighed and final volume was maintained to 1000 ml. The media was sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes. The media was mixed carefully before pouring. The media was poured on sterile and dry petriplates under aseptic conditions.

3.8.3 Preparation of the standard bacterial culture Inoculums

The individual pure ATCC culture of *Escherichia coli*, *Salmonella typhii*, *Staphylococcus aureus, Enterococcus feacalis and Klebsiella pneumonia* was obtained from CDBT, TU and were streaked on LB broth with the help of the sterilized inoculating loop. The inoculated culture bottles were kept on the shaking incubator at 37°C and 120 rpm for overnight. The turbidity of the sub-cultured bacterial suspension was adjusted at the 0.5 McFarland standards (freshly prepared on the other day for the antibacterial tests. These bacterial inoculums were used for the swabbing on the MHA plates to test the antimicrobial effects of the plant extracts.

3.8.4 Antibacterial test

First, all the sterile and dry plates were properly labeled with name of bacteria, name of the plant species and the concentration of the plant extract to be added. The MHA plates were inoculated with the appropriate bacterial culture by a sterile cotton swab aseptically. The culture plates were allowed to dry for about 15 to 20 minutes.

The antimicrobial tests were performed by modified agar well diffusion method as per Lindequist *et. al*., (2006) with slight modifications. On the above prepared MHA plates five wells were prepared on the solid MHA media with the help of the sterile cork borer (4 mm diameter). Five different concentrations (100 mg/ml, 50 mg /ml, 25 mg /ml, 12.5 mg /ml and 6.25 mg /ml) of the plant sample were prepared on DMSO. With the help of the sterile pipette the 30 µl of the each individual plant extract were poured in the above prepared wells. The DMSO was taken as negative control while the Streptomycin at the concentration of the 50, 25, 12.5 and 6.25 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 for individual plant extract for individual bacteria at different concentrations.

3.9 Determination of Antifungal activity

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

About 25 gram of PDA powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed, dissolved in distilled water and final volume was maintained to 1000 ml. This media was autoclaved at 15 pounds pressure and 121°C for 15 minutes, was cooled in

laminar airflow and dispensed in sterile petriplates. Likewise, PD broth was prepared in culture tubes excluding agar.

3.9.2 Preparation of the standard fungal cultures

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

3.9.3 Antifungal test

Properly labeled PDA pertiplates were taken and test cultures were inoculated as per Lindequist *et. al*., 2006 by modified agar well diffusion method. The petriplates were allowed to dry for about 15 to 20 minutes.

On the above prepared PDA plates, six wells were prepared with the help of the sterile cork borer (3 mm diameter). Five different concentrations (100 mg/ml, 50 mg /ml, 25 mg /ml, 12.5 mg /ml and 6.25 mg /ml) of the plant extracts were prepared on DMSO. With the help of the sterile pipette, 30 µl of the each individual plant extract was poured in the above prepared wells. The DMSO was taken as negative control. Streptomycin at the concentration of the 50, 25, 12.5 and 6.25 mg/mL was taken as the positive control. The plates were incubated on the microbial incubator overnight at 28°C and the zone of inhibition was observed for individual plant extract for individual bacteria at different concentrations.

3.10 Identification of major phytochemicals by Thin Layer Chromatography

Thin layer chromatography (TLC) was employed to observe the main components of different species of *Swertia*: Amarogentin, Mangiferin and Swertiamarin present in the methanolic extracts. TLC analysis was done on both Silica glass plate and Almunium TLC plates as per the Wagner and Bladt (1983) and TLC Atlas (2004).

Silica TLC plates were prepared for initial detection and analysis. To prepare a 1.0 mm thick SilicaTLC plate, 30 grams of silica gel (0.063-0.200 mm mesh) were suspended in 60 mL of deionized water, shaken uniformly for 2-3 minutes. The resultant slurry was poured on a 15 x 15 cm glass plate and spread evenly with a glass rod until the surface was even. The plate was allowed to dry for half an hour until the transparency of the layer disappeared and activated by oven drying at 110◦C for 30 minutes and then stored in a dry atmosphere until use. Plastic coated prepared TLC plates were used for subsequent optimization and visualization of extracts.

3.11.1 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 in each standard was prepared.

3.11.2 TLC of plant extracts

Plant extracts of various concentrations were used to visualize and optimize the required concentration of those extracts. 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. For this, 5 ul of methanolic extracts (30 mg/ml) of the samples were loaded carefully and run in a suitable solvent system on the TLC plate making a spot 1 cm above the bottom as following as suggested by TLC Atlas, 2004. After sample application the plates were placed vertically into a solvent vapor saturated TLC chamber. Different solvent systems for the three compounds were used. The spotting line was about 0.5 cm from the developing solution.

After the mobile phase had moved about 80% from the spotting line, the plate was removed from the developing chamber and dried in a hot air oven (Wagner and Bladt, 1983). All the TLC separations were performed at room temperature, i.e. 18-23°C. Detection was carried out by UV light at 254 and 365 nm by observing a particular fluorescence of the compound and by spraying 10% sulphuric acid over the run TLC plates. The Silica TLC plate was dried 5-10 minutes in a hot air oven at 100°C for 3-5 min and observed.

3.11 Semi-quantitative estimation of major compounds using GelQuant.NET software

The compounds that were visualized using the TLC were quantified by the GelQuant.NET software provided by Biochemlab Solutions Co. using the reference compounds; amarogentin, swertiamarin and mangiferin.

Aluminium TLC plates of 250 microns thickness (Whatman Ltd, Kent, UK) were used. 3 to 5uL of each methanol extracts (30 mg/mL) was run on TLC plates and compared with the respective standard compounds. All the run TLC plates were visualized in the UV spectrophotometer at 354nm. The various samples showed different intensities of the respective compounds inferring the presence of these compounds in varying amounts in those samples.

3.11.1 Preparation of standard curve

For the preparation of standard calibration curve and to assess linearity different concentration of the marker stock solutions (500 ug/mL, 250ug/mL, 125ug/mL and 62.5ug/mL) were applied in different tracks as bands to furnish in the amounts in the range of 0.5-0.0625 ug per band. The peak areas in terms of pixel ratio were plotted against the corresponding concentrations and regression analysis was done to obtain the calibration equation.

3.11.2 Semi-quantitative estimation of major phytochemicals

All the samples were visualized for the respective marker compounds from the TLC plates and pixels for each band was calculated from the software and quantified from the linear equation obtained from the standard curves.

3.12 Statistical Analysis

All the experiments were performed in triplicates for each sample and the values were reported as mean ± SD. The obtained data were also subjected to the analysis of variance and mean values were compared. All the statistical analysis was done using Graphpad Prism5 and Excel software (Microsoft Word 10). One way ANOVA was done for different species of *Swertia* (within the group) and paired t test was employed between the methanol and aqueous extracts (between the groups).

CHAPTER IV

4. RESULTS

4.1 Percentage yield of plant extracts

Seven different species of *Swertia* were subjected to methanol and aqueous extraction. The total amount of methanol and aqueous plant extract isolated from 50 grams of each finely powdered whole plant material is shown below. The highest and lowest yield of extract was found on *S. chirayita* (10.48%) and *S. dilatata* (6.98%) respectively in case of methanol extracts; and highest and lowest percentage yields in *S. chirayita* (12.68%) *and S. racemosa* (7.25%) respectively in aqueous extracts.

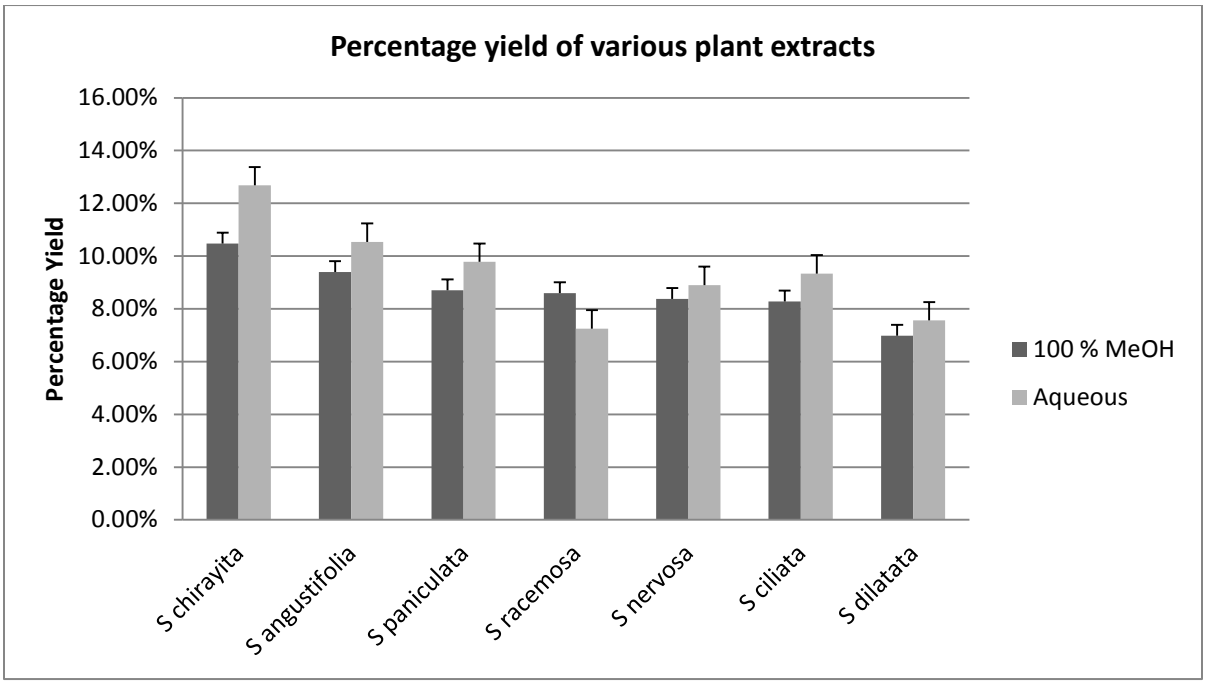

Figure 4.1 Total percentage yields of different species of *Swertia*

All of these extracts were sticky and greasy in nature. All the methanol plant extracts were greenish except *S. nervosa* and *S. paniculata* that showed yellowish green and grayish coloration respectively. However, all of the aqueous extracts were slightly green in color.

4.2 Qualitative Phytochemical Analysis

Summary of different tests performed on aqueous and methanolic extracts of different species of *Swertia* is tabulated in table 4.1.

Table 4.1 Qualitative phytochemical analysis of different species of *Swertia*

Plant Extracts	**Alkaloids**	**Resins**	**Phenols**	**Flavonoids**	**Glycosides**	**Diterpenes**	**Tannins**	**Phytosterol**
	Mayer's Test	**Acetone Water Test**	**Ferric Chloride Test**	**Alkaline Reagent Test**	**Modified Brontrager's Test**	**Copper Acetate Test**	**Gelatin Test**	**Salkowski's Test**
SCH-MET	+++	++	+++	+++	++	++	+	++
SCH-AQ	+	-	+	-	+	-	-	+
SAG-MET	+++	+	++	++	+	++	++	+
SAG-AQ	+	-	++	-	++	+	+	+
SPA-MET	++	++	++	+++	++	++	-	++
SPA-AQ	+	+	+	+	+	-	-	+
SRA-MET	+++	-	++	++	+	+	+	++
SRA-AQ	++	-	-	-	-	-	-	+
SNE-MET	++	++	++	+	++	++	-	-
SNE-AQ	-	-	+	-	-	+	-	+
SCI-MET	+++	++	+	++	-	-	++	-
SCI-AQ	+	-	-	-	-	-	+	-
SDI-MET	+++	+	+++	++	+++	++	+	++
SDI-AQ	++	+	-	+	+	-	-	+

+++ Highly Positive; ++ Moderately Positive; + Positive; - Negative or Not Detectable

MET- Methanol; SCH- *Swertia chirayita*; SAG- *Swertia angustifolia*; SPA- *Swertia paniculata*;
AQ- Aqueous; SRA- *Swertia racemosa*; SNE- *Swertia nervosa*; SCI- *Swertia ciliata*; SDI- *Swertia dilatata.*

Methanol extracts showed the presence of more secondary metabolites than the aqueous extracts. Methanol extracts depicted the presence of alkaloids, phenols, resins, flavonoids, glycosides, terpenes and phytosterols; whereas, aqueous extracts were found to show alkaloids, glycosides and phenols. Alkaloids were reported in all of the extracts except aqueous extract of *S. nervosa*. Similarly, flavonoids, phenols and sterols were reported in most of the species in both methanol and aqueous extracts.

4.3 Total Flavonoid Content

Standard graph of quercetin obtained by plotting the absorbance at 415 nm against the concentration from the 10 µg/ml to 100 µg/ml is shown in figure 4.2.

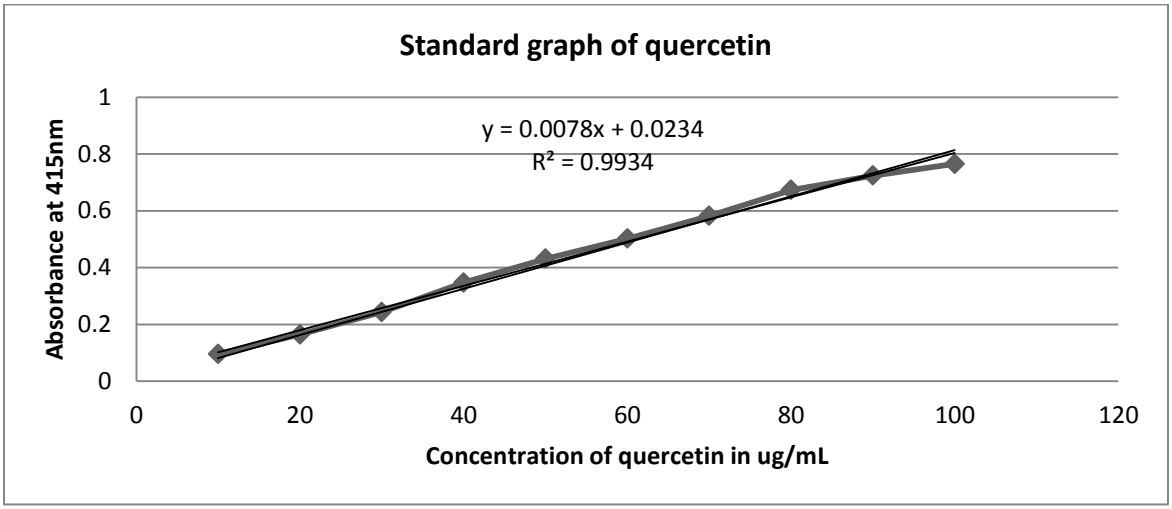

Figure 4.2 Standard graph of quercetin

Total amount of the flavonoid present in methanolic and aqueous extracts of different species of *Swertia* (expressed in mg QE /g ± SD) is shown in table 4.2. For the methanolic extracts, the highest amount of flavonoid 26.24±0.2 mg QE/g was found on *S. chirayita* while the lowest amount of flavonoid 18.53±0.15 mg QE/g was recorded for *S. angustifolia.* The flavonoid content of other species remained in between these two extremes. The total flavonoid content in methanolic extracts of *S. angustifolia* was significantly lower than that of rest of the species. The difference in TFC content of methanolic extracts of other species was not statistically significant ($p<0.05$).

Table 4.2 Total flavonoid content of different species of *Swertia*

***Swertia* species**	**100% MeOH**	**Aqueous**
S. chirayita	26.24±0.2[a]	23.08±0.25[a]
S. angustifolia	18.53±0.15[b]	6.46±0.19[b]*
S. paniculata	24.92±0.7[a]	9.54±0.85[c]*
S. racemosa	26.2±1.03[a]	16.62±1.26[d]*
S. nervosa	24.79±0.15[a]	12.39±0.19[e]*
S. ciliata	23.44±0.39[a]	10.32±0.48[d]
S. dilatata	22.58±1.02[a]	19.59±1.28[a]*

Note: In each column values with different letters are significantly different (P<0.05) within the group.
* represents statistically significant difference (P<0.05) compared to methanol extract in each row.

In case of aqueous extracts, the highest amount of flavonoid 23.08±0.25 mg QE/g was found on *S. chirayita* while the lowest amount of flavonoid 6.46±0.19mg QE/g was recorded for *S. angustifolia.* The flavonoid content of other species remained in between these two extremes. The total flavonoid content in aqueous extracts of *S. angustifolia, S. paniculata, and S. nervosa* was significantly lower than that of rest of the species. There was no statistically significant difference ($p<0.05$) in TFC content of aqueous extracts of other species. The total flavonoid content of aqueous extracts of different species of *Swertia* except *S. chirayita* were significantly lower ($p<0.05$) than respective methanolic extracts.

4.4 Total Polyphenol content

Standard graph of gallic acid obtained by plotting the absorbance at 765 nm against concentration ranging from 25µg/ml to 250 µg/ml is shown in figure 4.3.

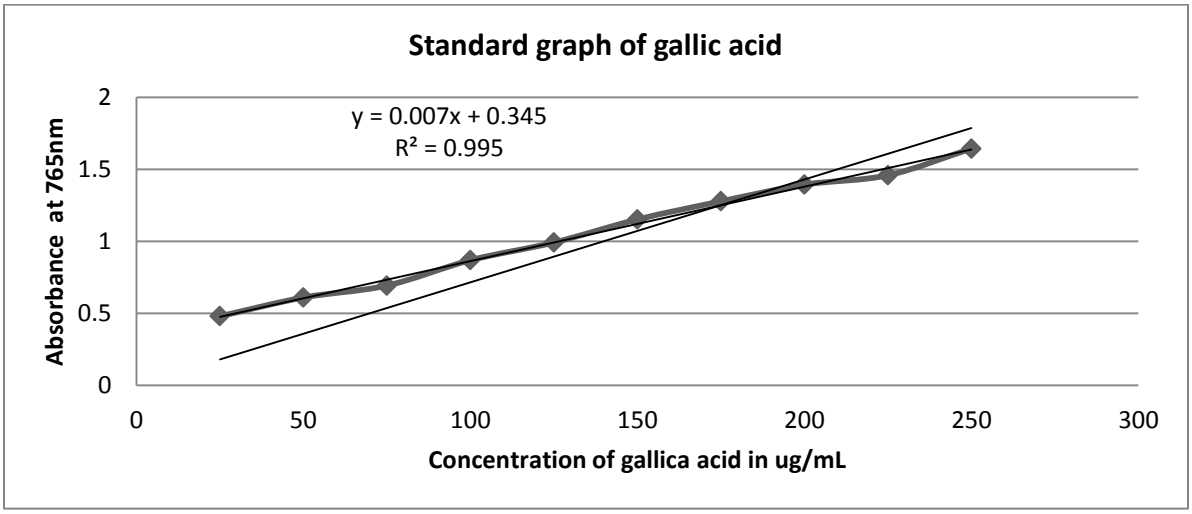

Figure 4.3 Standard graph of gallic acid

The total polyphenol content present in methanolic and aqueous extracts of different species of *Swertia* is presented in table 4.3. For the methanol extracts, the highest amount of polyphenol content 70.19±1.02 mg GAE/g was found on *S. dilatata* while the lowest amount of polyphenol 22.02±2.08 mg GAE/g was recorded for *S. angustifolia.* The polyphenol content of other species remained in between these two extremes. The total polyphenol content in methanolic extracts of *S. angustifolia, S. paniculata, S. nervosa and S. ciliata* was significantly lower ($p<0.05$) than that of rest of the species.

Table 4.3 Total polyphenol content of different species of *Swertia*

***Swertia* species**	**100% MeOH**	**Aqueous**
S. chirayita	67.49±2.07[a]	39.09±2.07[a]
S. angustifolia	22.02±2.08[b]	17.52±2.08[b]*
S. paniculata	33.35±0.7[c]	27.32±3.78[b]*
S. racemosa	67.32±1.03[b]	43.54±3.16[e]*
S. nervosa	53.63±0.15[d]	44.73±1.25[e]*
S. ciliata	41.8±0.39[e]	40.45±1.16[a]*
S. dilatata	70.19±1.02[c]	49.23±1.01[d]*

Note: In each column values with different letters are significantly different (P<0.05) within the group.
* represents statistically significant difference (P<0.05) compared to methanol extract in each row.

In case of aqueous extracts, the highest amount of polyphenol 49.23±1.01 mg GAE/g was found on *S. dilatata* while the lowest amount of polyphenol 17.52±2.08 mg GAE/g was recorded for *S. angustifolia.* The polyphenol content of other species remained in between these two extremes. The total polyphenol content in aqueous extracts of *S. angustifolia, S. nervosa and S. dilatata* was significantly different (p<0.05) than that of rest of the species. Also the polyphenol content of aqueous extracts of *S. angustifolia, S. racemosa, S. nervosa, S. paniculata, S. ciliata* and *S. dilatata* were statistically significant (p<0.05) from their methanol extracts.

4.5 Total DPPH Antioxidant activity

The graph of standard ascorbic acid was drawn using its various concentrations from 5 ug/ml to 100 ug/ml against the DPPH radical scavenging percentage. IC 50 value was calculated for ascorbic acid and samples were compared to this standard graph.

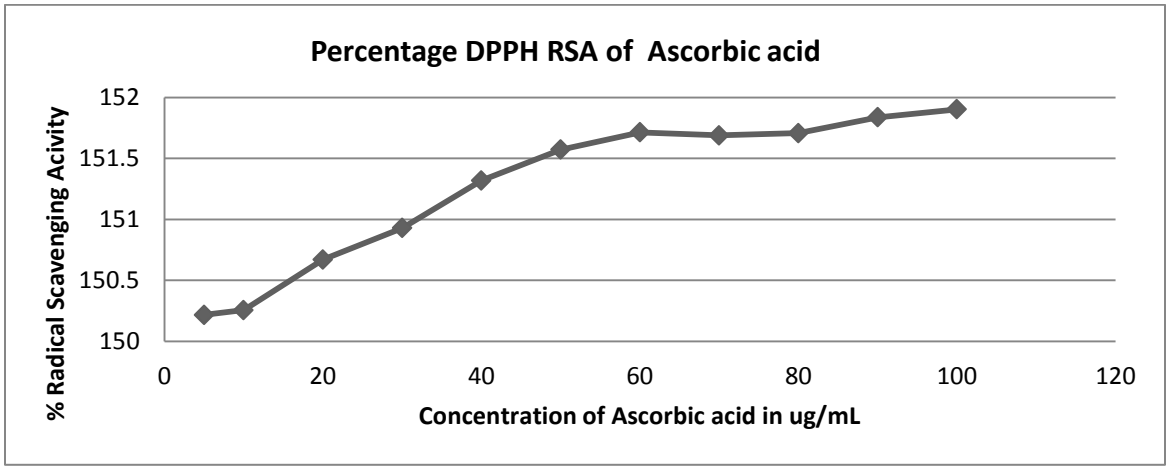

Figure 4.4 Percentage Radical Scavenging Activity of Ascorbic Acid

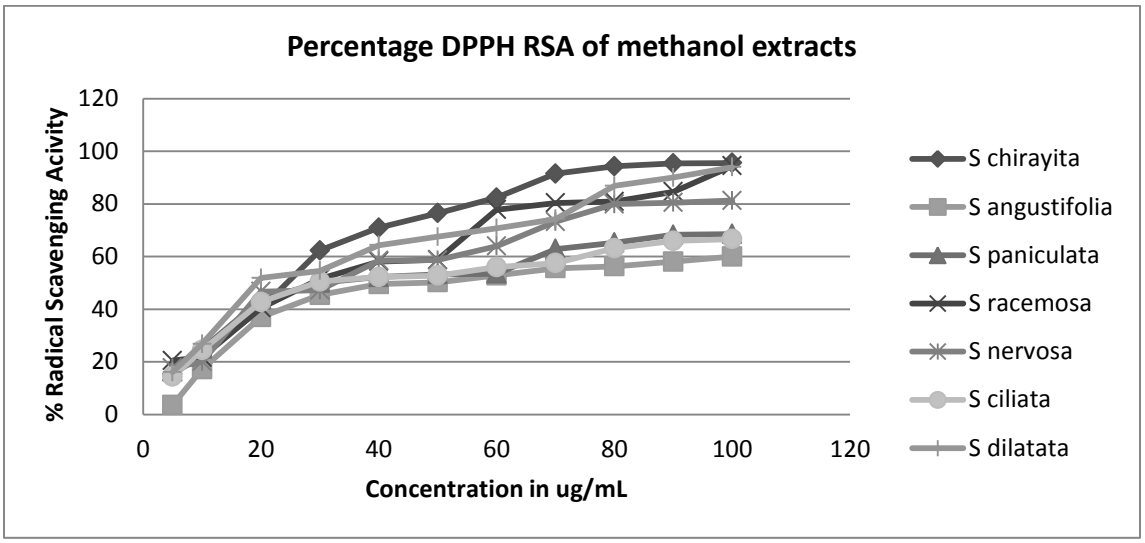

Figure 4.5 Percentage DPPH RSA of methanol extracts of different species of *Swertia*

For the methanol extracts, highest scavenging activity was seen in *S. chirayita* accounting 95.56% and lowest was observed 60.01% in *S. angustifolia* at 100 ug/ml. Other species the percentage RSA was found between these two values. Higher concentration of plant extracts showed an increasing percentage of radical scavenging activity in DPPH, revealing concentration dependent scavenging property.

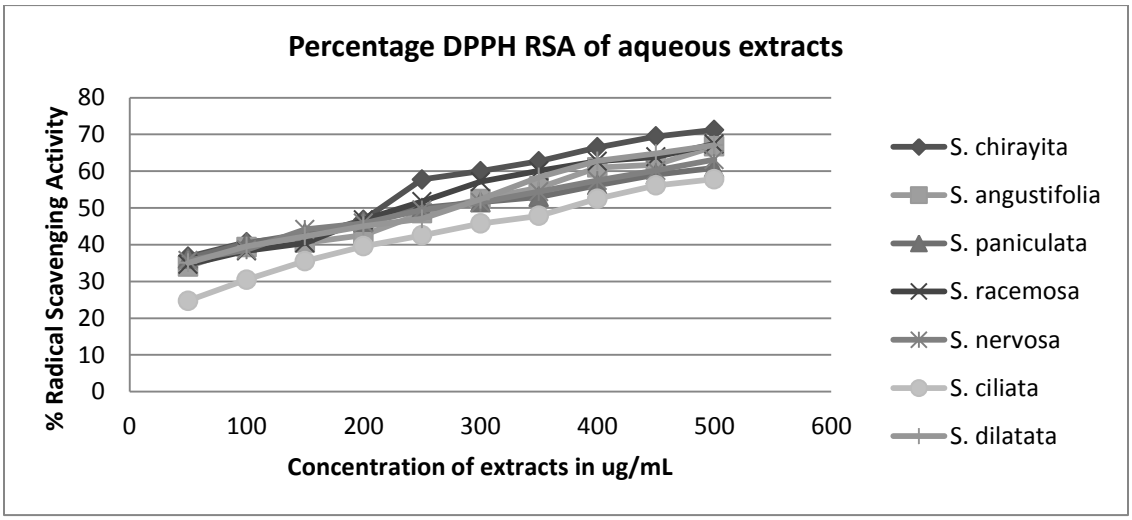

Figure 4.6 Percentage DPPH Radical Scavenging Activity of aqueous extracts of different species of *Swertia*

Among the aqueous extracts, highest scavenging activity was seen in *S. chirayita* accounting 71.21% and lowest was observed 57.8% in *S. angustifolia*. For the other species, the percentage RSA was found between these two values. Higher concentration of plant extracts showed an increasing percentage of radical scavenging activity in DPPH, revealing concentration dependent scavenging property.

Table 4.4 IC_{50} values different species of *Swertia*

***Swertia* species**	**100% MeOH**	**Aqueous**
S. chirayita	23.92±1.01[a]	213.62±4.07[a]*
S. angustifolia	46.57±1.74[b]	267.85±4.21[b]
S. paniculata	29.94±0.89[c]	250.97±2.75[c]*
S. racemosa	28.39±0.41[d]	231.26±2.33[d]*
S. nervosa	32.2±0.91[c]	262.46±2.12[b]*
S. ciliata	29.14±1.5[d]	372.26±2.18[e]*
S. dilatata	29.06±0.71[e]	276.97±3.03[f]

Note: In each column values with different letters are significantly different (P<0.05) within the group.
* represents statistically significant difference (P<0.05) compared to methanol extract in each row.

The IC_{50} value for ascorbic acid was found to be 26.73±2.13 ug/mL. For methanol extracts, the lowest and highest IC_{50} values were observed in *Swertia chirayita* (23.92±1.01 ug/mL) and *S. angustifolia* (46.57±1.74 ug/ mL). The species with lower IC_{50} are considered as better antioxidants. So, accordingly, *S. chirayita* has the best antioxidant activity (even better than ascorbic acid) among its different species. The IC_{50}value of the methanol extracts of other species of *Swertia* species was observed in between these two extremes. The IC_{50} values in methanolic extracts of *S. angustifolia, S. paniculata, S. nervosa, S. ciliata* and *S. dilatata* were significantly different ($p<0.05$) than that of rest of the species.

In contrast to the methanol extracts, aqueous extracts reported poor anti oxidative capacity. Their IC_{50} values were more than 200 ug/mL. Among the aqueous extracts, highest IC_{50} value of 213.62±4.07 ug/mL was estimated for *S. chirayita* and lowest IC_{50} value of 372.26 ± 2.18 ug/mL was seen in *S. ciliata*. The IC_{50} values of aqueous extracts of other species were observed n between these two extremes. The IC50 values in aqueous extracts of *S. angustifolia, S. paniculata, S. racemosa, S. ciliata and S. dilatata* were significantly different ($p<0.05$) than that of rest of the species. Also the IC_{50} values of aqueous extracts of *S. chirayita*, *S. racemosa, S. nervosa, S. paniculata* and *S. ciliata* were significantly different ($p<0.05$) from their methanol extracts.

4.6 Correlation between TPC and % DPPH Inhibition

The quantity of total phenolic content is directly related to the inhibition percentage of DPPH radicals. The higher the polyphenols, the greater is the radical scavenging capacity of the plant extracts. A significant correlation of 0.977 between TPC and antioxidant activity for methanol extracts of different species was found that is shown in the figure 4.7.

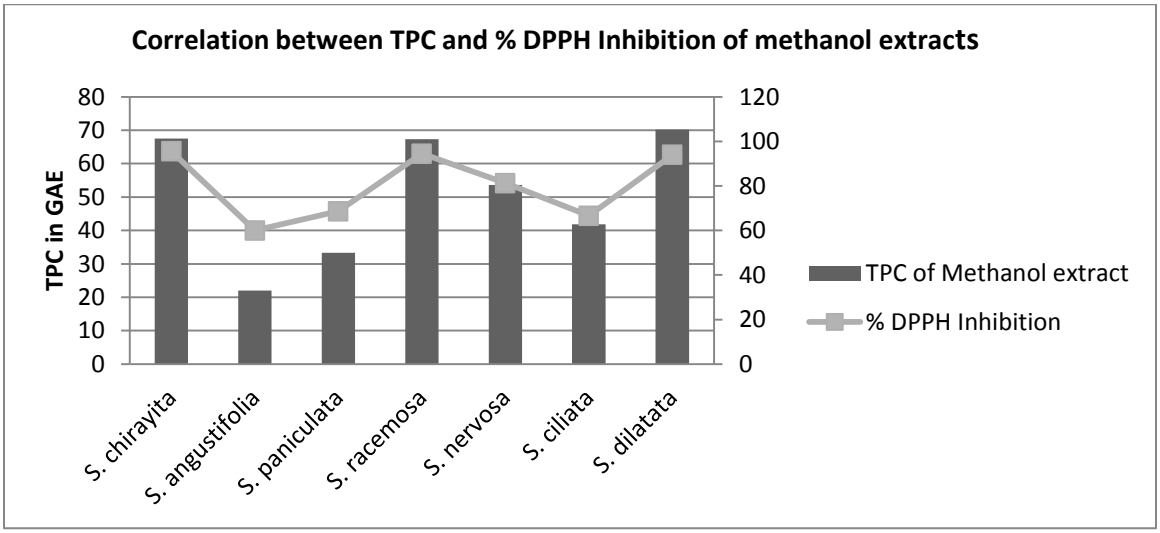

Figure 4.7 Correlation between TPC and DPPH RSA of methanol extracts.

In case of aqueous extracts, the correlation was only 0.068 at 95% confidence interval that is represented by the figure 4.8. The higher correlation between antioxidant activity and TPC was found in methanol extracts compared to their respective aqueous extracts.

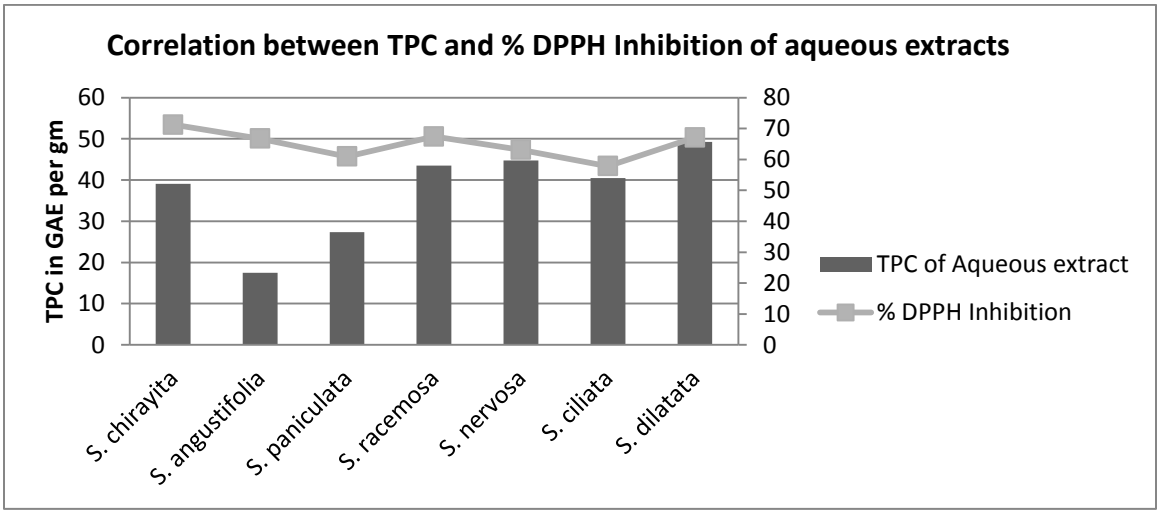

Figure 4.8 Correlation between TPC and DPPH RSA of aqueous extracts.

4.7 Antibacterial Assay

The antibacterial activity of both methanol and aqueous extracts of *Swertia* species was tested against ATCC cultures of *Escherichia coli* (25922), *Salmonella typhii* (14028), *Klebsiella pneumonia* (700603), *Pseudomonas aeruginosa* (27853), *Staphylococcus aureus* (25923) and *Enterococcus faecalis* (25912). Antibiotic drug Streptomycin was taken as a positive control and DMSO (the solvent of the plant extract) was taken as a negative control against six bacterial strains and compared with the standard Streptomycin. The results were expressed with zone of inhibition on mm and shown on following tables.

Table 4.5 Zone of Inhibition (in mm) of different bacterial strains in Streptomycin

Bacterial Cultures	**Zone of Inhibition (in mm) in Streptomycin**			
	50 mg/mL	**25mg/mL**	**10 mg/mL**	**5 mg/mL**
Escherichia coli	25	24	21	16
Staphyloccous aureus	29	21	18	15
Salmonella typhii	30	26	24	23
Klebsiella pneumonia	41	38	37	34
Enterococcus feacalis	28	25	23	21
Pseudomonas aeruginosa	31	27	25	22

For the standard drug Streptomycin, all the six bacterial strains gave zone of inhibition that is represented in the above table 4.5. The zone of inhibition for *Klebsiella pneumonia* was comparatively higher than the other strains.

Table 4.6 Zone of Inhibition of different methanol extracts in Gram negative organisms

Plant Extracts	**Bacterial Culture**	**Zone of Inhibition (in mm)**						
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	+ve control	-ve control
SCH-MET	*E. coli*	13	12	11	8	7	25	-
	S. typhii	11	10	10	9	9	30	-
	K. pneumoniae	12	9	9	8	6	41	-
SAG-MET	*E. coli*	9	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	22	11	7	-	-	41	-
SPA-MET	*E. coli*	8	-	-	-	-	25	-
	S. typhii	9	8	7	6	6	30	-
	K. pneumoniae	18	12	6	-	-	41	-

SRA-MET	*E. coli*	7	-	-	-	-	25	-
	S. typhii	13	10	6	-	-	30	-
	K. pneumoniae	12	-	-	-	-	41	-
SNE-MET	*E. coli*	-	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-	-	41	-
SCI-MET	*E. coli*	-	-	-	-	-	25	-
	S. typhii	6	-	-	-	-	30	-
	K. pneumoniae	8	-	-	-	-	41	-
SDI-MET	*E. coli*	10	8	-	-	-	25	-
	S. typhii	9	7	6	-	-	30	-
	K. pneumoniae	8	7	-	-	-	41	-

The methanol extract of *S. chirayita* inhibited all the five Gram negative bacterial cultures at all concentrations. *S. typhii and K. pneumonia* were strongly inhibited up to the concentration of 6.25 mg/ml by methanol extracts of *S. chirayita* and *S. paniculata*. The other species reported moderate bioactivity that is presented in the above table 4.6. However, *S. nervosa* was ineffective against all of the bacterial cultures tested.

Table 4.7 Zone of Inhibition of aqueous extracts of different species of *Swertia* in Gram negative organisms

Plant Extracts	**Bacterial Culture**	**Zone of Inhibition (in mm)**						
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	+ve control	-ve control
SCH-AQ	*E. coli*	7	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	8	-	-	-	-	41	-
SAG- AQ	*E. coli*	-	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-	-	41	-
SPA- AQ	*E. coli*	-	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-	-	41	-
SRA- AQ	*E. coli*	-	-	-	-	-	25	-
	S. typhii	8	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-		41	-

SNE- AQ	*E. coli*	-	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-		41	-
SCI- AQ	*E. coli*	-	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-	-	41	-
SDI- AQ	*E. coli*	6	-	-	-	-	25	-
	S. typhii	-	-	-	-	-	30	-
	K. pneumoniae	-	-	-	-	-	41	-

As compared to their methanolic extracts, aqueous were ineffective to inhibit the Gram negative bacteria. *E. coli*was inhibited by *S. chirayita* and *S. dilatata*, *K. pneumonia* by *S. chirayita* and *S. typhiii* by *S. racemosa*, all at 100 mg/ml concentration.

Table 4.8 Zone of Inhibition of different methanol extracts in Gram positive organisms

Plant Extracts	**Bacterial Culture**	**Zone of inhibition in mm (in mm)**						
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	+ve control	-ve control
SCH-MET	*S. aureus*	16	14	11	-	-	29	-
	E. feacalis	12	8	7	6	-	28	-
SAG-MET	*S. aureus*	10	9	-	-	-	29	-
	E. feacalis	-	- -	-	-	-	28	-
SPA-MET	*S. aureus*	10	-	-	-	-	29	-
	E. feacalis	7	-	-	-	-	28	-
SRA-MET	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SNE-MET	*S. aureus*	9	7	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SCI-MET	*S. aureus*	8	-	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SDI-MET	*S. aureus*	13	10	7	-	-	29	-
	E. feacalis	10	7	-	-	-	28	-

For gram positive bacteria, the methanol extract of *S. chirayita* and *S. dilatata* gave zone of inhibition upto 25 mg/ml in *S. aureus*. *E. feacalis* was inhibited up to 12.5 mg/ml by *S. chirayita* methanol extract. Other species of *Swertia* methanolic extracts showed less antibacterial activity (up to 100 mg/ml) that is shown in the above table 4.8.

Table 4.9 Zone of Inhibition of different aqueous extracts in Gram positive organisms

Plant Extracts	**Bacterial Culture**	**Zone of inhibition in mm (in mm)**						
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	+ve control	-ve control
SCH-AQ	*S. aureus*	8	6	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SAG- AQ	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SPA- AQ	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	7	-	-	-	-	28	-
SRA- AQ	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SNE- AQ	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SCI- AQ	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	-	-	-	-	-	28	-
SDI- AQ	*S. aureus*	-	-	-	-	-	29	-
	E. feacalis	9	-	-	-	-	28	-

As compared to their methanolic extracts, aqueous were ineffective to inhibit the Gram positive bacteria. *S. aureus* was inhibited by *S. chirayita* up to 50 mg/ml, *E. feacalis* by *S. dilatata* and *S. paniculata* (both at 100 mg/ml). All of them reported very less zone of inhibition as compared to the standard Streptomycin.

None of the plant extracts showed antibacterial activity against *Pseudomonas aurigenosa*. Comparison of Gram negative and Gram positive inhibition zones shows the higher zones of inhibition in former than the latter. But the range of inhibition shown by methanol extracts is definitely large and even 5 mg/ml of extracts concentration has depicted inhibition. Comparing the zone of inhibition of plant extracts with Streptomycin methanol extracts of *S. chirayita, S. paniculata and S. dilatata* were found to be more inhibitory compared to *S. angustifolia, S. ciliata, S. racemosa* and *S. nervosa.* All the extracts showed the concentration dependent bioactivity.

4.8 Antifungal Assay

Both the methanol and aqueous extracts were tested for their antifungal activity. Three characterized fungal strains of *Pichia pastoris*, *Candida albicans* and *S. cerevisiae* were tested taking Sterptomycin as the standard drug.

Table 4.10 Zone of Inhibition (in mm) of different fungal strains in Streptomycin

Fungal Cultures	Zone of Inhibition (in mm) in Streptomycin			
	50 mg/mL	**25mg/mL**	**10 mg/mL**	**5 mg/mL**
Pichia pastoris	16	13	10	8
C. albicans	18	11	7	-
S. cerevisiae	19	14	9	9

In positive control, three fungal cultures showed varied inhibition zones at different concentrations, however, *C. albicans* did not show ZOI at 5 mg/ml concentration.

Table 4.11 Zone of Inhibition of different methanol extracts in fungal cultures

Plant Extracts	Fungal Culture	Zone of Inhibition (in mm)						
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.25 mg/ml	+ve control	-ve control
SCH-MET	*Pichia pastoris*	13	12	11	8	-	15	-
	C. albicans	-	-	-	-	-	12	-
	S. cerevisiae	8	-	-	-	-	17	-
SAG-MET	*Pichiapastoris*	12	8	7	-	-	15	-
	C. albicans	9	8	7	7	-	12	-
	S. cerevisiae	12	10	7	7	-	17	-
SPA-MET	*Pichia pastoris*	-	-	-	-	-	15	-
	C. albicans	-	-	-	-	-	12	-
	S. cerevisiae	-	-	-	-	-	17	-
SRA-MET	*Pichiapastoris*	-	-	-	-	-	15	-
	C. albicans	-	-	-	-	-	12	-
	S. cerevisiae	-	-	-	-		17	-
SNE-MET	*Pichia pastoris*	-	-	-	-	-	15	-
	C. albicans	-	-	-	-	-	12	-
	S. cerevisiae	-	-	-	-		17	-

SCI-MET	*Pichia pastoris*	-	-	-	-	-	15	-
	C. albicans	-	-	-	-	-	12	-
	S. cerevisiae	-	-	-	-	-	17	-
SDI-MET	*Pichia pastoris*	-	-	-	-	-	15	-
	C. albicans	-	-	-	-	-	12	-
	S. cerevisiae	-	-	-	-	-	17	-

None of the aqueous extracts showed antifungal activity. Only the methanol extracts of *S. chirayita and S. angustifolia* showed antifungal activity, up to 6.25 mg/mL. Other plant extracts did not report any zone of inhibition. *S. angustifolia* showed antifungal inhibition against all the tested organisms; *S. cerevisiae, Candida albicans and Pichia pastoris*. *S. chirayita* showed moderate inhibition against *Pichia pastoris* up to 12.5 mg/mL. However, *S. angustifolia* was found to be actively inhibitory against all fungal strains. All the extracts showed the concentration dependent bioactivity. No significant difference in zone of inhibition was seen with respect to the concentration of the plant extract used.

4.9 Semi-quantitative estimation of compounds by TLC

First different standard curves were prepared by using various concentrations of the respective standard compounds and then semi quantitative estimation was done using the software. Separate semi quantification was done for each of the compounds in various species of *Swertia* only in methanol extracts, as the aqueous extracts were not completely dissolved.

4.9.1 Semi-quantitative estimation of swertiamarin

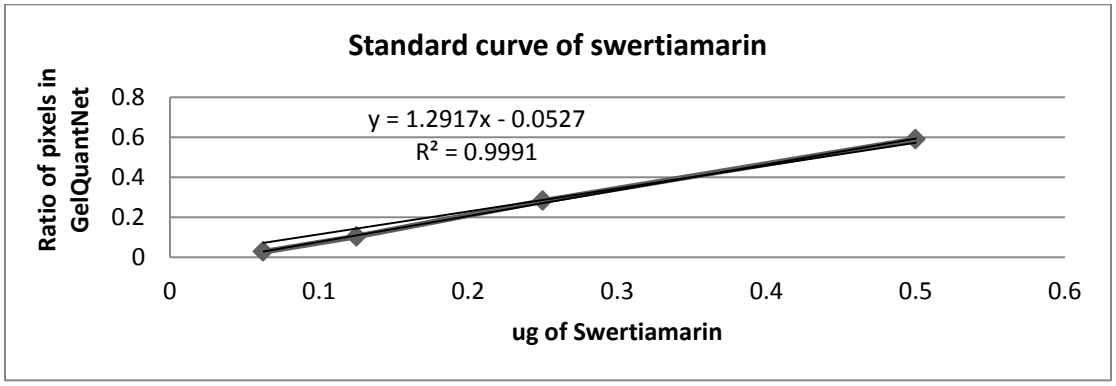

Figure 4.9 Standard curve of Swertiamarin

Standard curve of swertiamarin was prepared by taking various concentrations of standard swertiamarin compound against respective pixel ratio is shown in the figure 4.10.

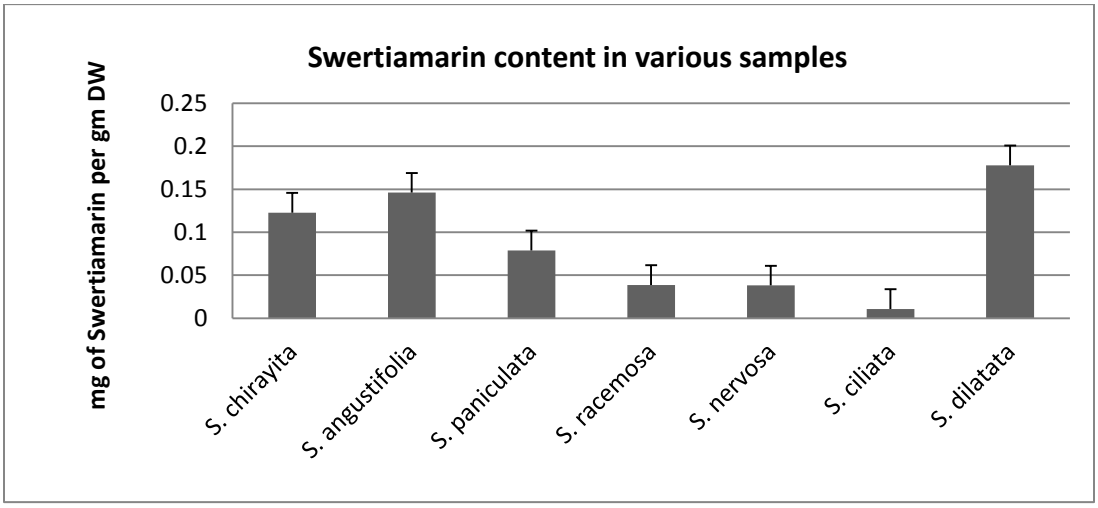

Figure 4.10 Semi-quantitative estimation of swertiamarin in different species of *Swertia.*

The highest content (0.15±0.023 mg/gm DW) of swertiamarin was reported in *S. dilatata* and lowest (0.011±0.023 mg/gm DW) in *S. ciliata*. The content of swertiamarin in other species was in between these two extremes and is shown in the figure 4.10. The swertiamarin content of *S. ciliata*, *S. nervosa* and *S. racemosa* were significantly lower than the swertiamarin content of rest of the species.

4.9.2 Semi-quantitative estimation of amarogentin

Standard curve of amarogentin prepared by taking various concentrations of standard swertiamarin compound against respective pixel ratio is shown in fig 4.11.

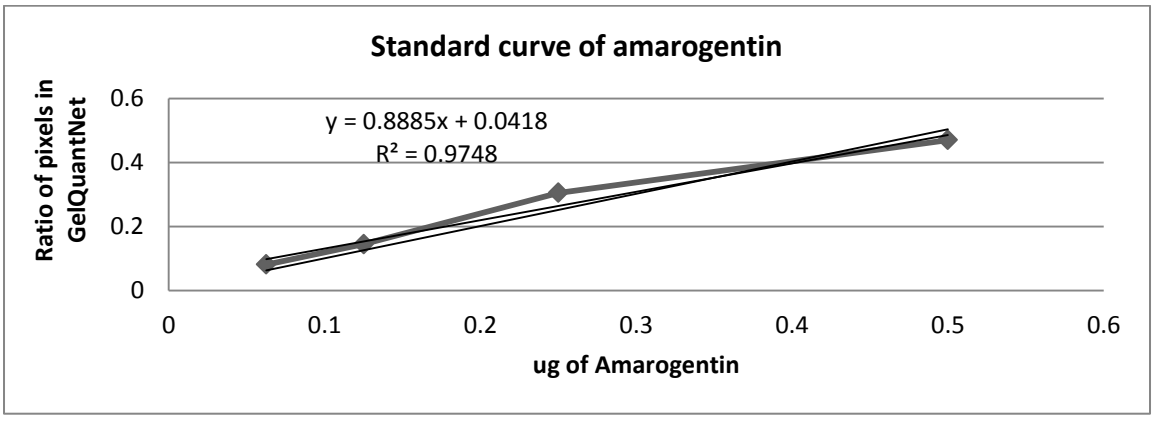

Figure 4.11 Standard curve of amarogentin

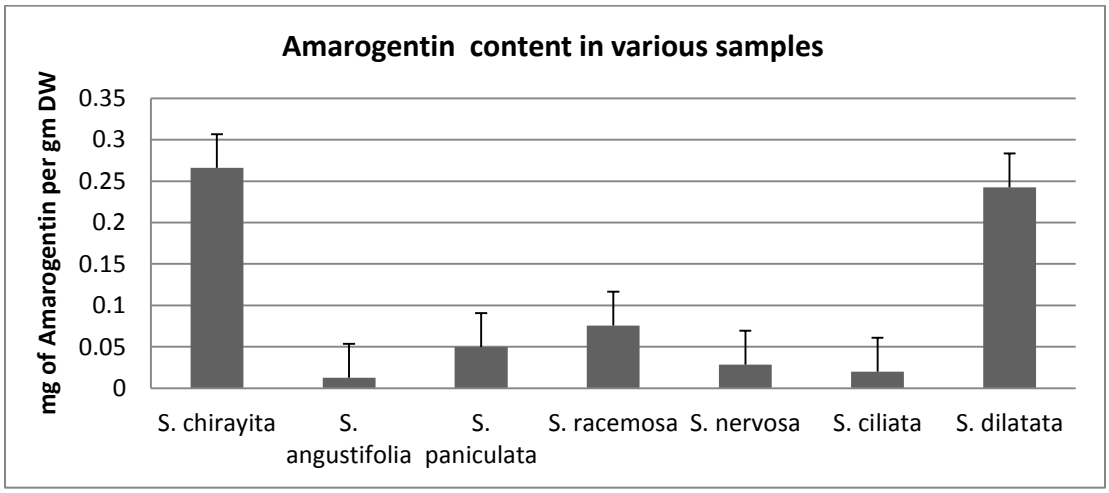

Figure 4.12 Semi-quantitative estimation of amarogentin in different species of *Swertia.*

The highest content of amarogentin (0.27±0.04 mg/gm DW) was reported in *S. chirayita* and lowest (0.012±0.04 mg/gm DW) in *S. angustifolia* methanolic extracts. The content of amarogentinin other species was in between these two extremes and is shown in the figure 4.12. The amarogentin content of *S. angustifolia, S. ciliata*, *S. nervosa* and *S. paniculata* were significantly lower than the amarogentin content of rest of the species.

4.9.3 Semi-quantitative estimation of mangiferin

Standard curve of mangiferin prepared by taking various concentrations of standard swertiamarin compound against respective pixel ratio is shown in fig 4.13.

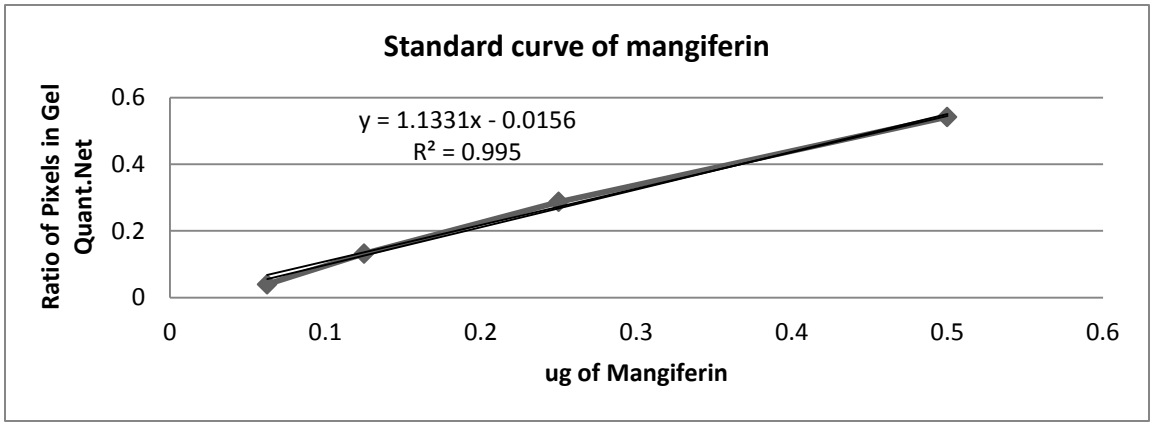

Figure 4.13 Standard curve of mangiferin

The highest content of mangiferin (0.22±0.007 mg/gm DW) was reported in *S. dilatata* and lowest (0.012±0.007 mg/gm DW) in *S. chirayita* methanolic extracts. The content of mangiferin in other species was in between these two extremes (figure 4.14). The mangiferin content of seven different species of Swertia is not significantly different from each other.

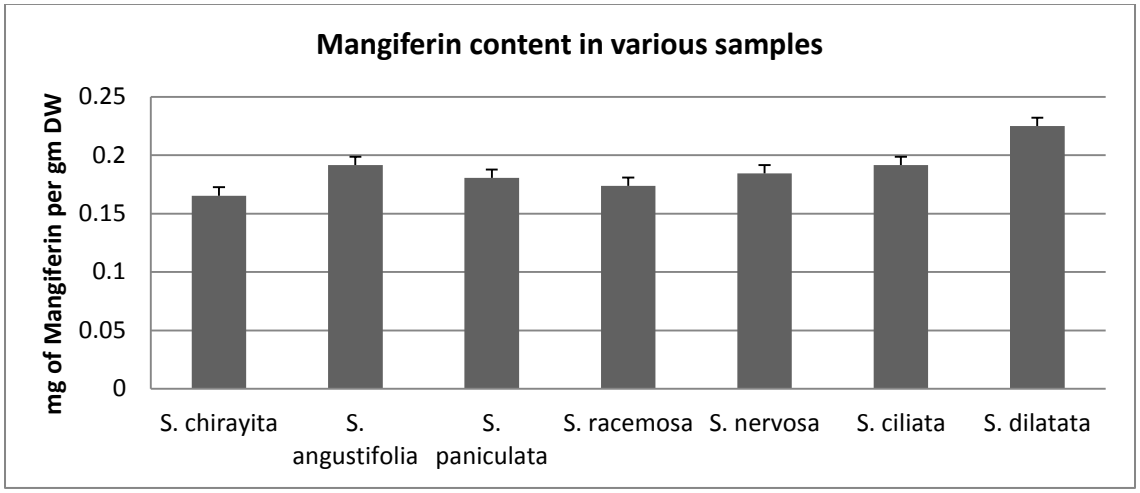

Figure 4.14 Semi-quantitative estimation of mangiferin in different species of *Swertia*

CHAPTER V

DISCUSSION

5.1 Percentage yield of plant extracts

The primary step of phytochemical investigation is the extract preparation from plants. The percentage yield of the extracts depends on the polarity and type of solvent system, parts of the plant used and method of extraction procedure. The percentage yield has been reported from 5% (Ghimire *et al.,* 2012) to 15.6% (Bhargava *et al.,* 2009) on the basis of the type of extraction process and solvent polarity. The present results also suggest and accord with the variation in percentage yield of different plant extracts. This variation may be attributed to genotypic differences and their collection stages.

Previous works in *S. chirayita* have reported use of different solvents for extraction process. In most of the cases, methanol has been used (Wang *et al.*, 2011; Kweera *et al.,* 2011) followed by 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). Aqueous extracts has shown higher extractive yield than methanol extracts because of the high polarity of water than methanol that results for the solubility of both polar and less polar compounds (Katerere and Eloff, 2008). The solubility of the compounds in different *Swertia* species may vary according to the nature and their polarity. The variation in percentage yield may also be due to overnight percolation and ultrasonication procedures for extraction used here. Alcohols, especially methanol and ethanol, are the major solvents in the extraction of Gentianeceae plants as the compounds like amarogentin, mangiferin and swertiamarin are particularly more soluble in alcohols. Furthermore, higher biological activities have been reported in methanol than other solvents like ethanol, petroleum ether, chloroform, acetone and water (Kweera *et al.,* 2011).

5.2 Qualitative Phytochemical Analysis

The preliminary phytochemical identification provides a rough outline for the presence of major secondary metabolites in the plant species. The qualitative screening of different species of *Swertia* revealed the presence of major phytochemicals. Higher solubility of compounds in alcohols (Kweera *et al.,* 2011) results in depiction of more secondary metabolites in methanol extracts than the aqueous extracts.

The qualitative phytochemical screening of *Swertia* species showed the presence of alkaloids, flavonoids, polyphenols and steroids in both type of extracts. However, the intensity of coloration was comparatively higher in methanol extracts than their aqueous extracts. The screening of major secondary metabolites was found in accordance with the phytochemical screening done by Laxmi *et al.,* 2011, Bhattarai (2014), Wang *et al.,* 2011 for *S. chirayita* and Baral *et al.,* 2012 for *S. angustifolia, S. ciliata* and *S. nervosa*.

5.3 Total Flavonoid Content

Several flavonoids have been identified as potential inhibitors of oxidative enzymes in inflammatory processes that inhibit the metabolic disorders such as inflammation, heart diseases and cancers (Middleton, 2000). The flavonoid content in many plants probably contributed to their observed interesting cytochrome oxidase enzyme inhibitory activity. In addition, different classes of flavonoids have also been reported to possess other pharmacological activities such as antimicrobial and anti-parasitic activity. Most of these flavonoids have low toxicity to animal cells (Havsteen, 2002).

Quantification of flavonoid by 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 DW and 3.17±0.19 catechin equivalents g/100 g DW respectively. According to Chen *et al.,* 2011, the total flavonoid content was estimated to be 4.98±0.40 mgrutin equivalents/g extract in *S. chirayita* DW. Kim *et al.,* 2002 mentions the vitamin C equivalent antioxidant capacity in mg/100 g dry weight in DPPH scavenging assays in the order of quercetin > epicatechin > catechin > Trolox > rutin > chlorogenic acid. By this comparison the present results of flavonoid content is found to be higher than previously stated by Naqvi *et al.,* (2013) and Chen *et al.,* 2011. However, Tripathi *et. al.,* 2005 reported total flavonoid content of 50 µg of aqueous extract of *Swertia chirayita*, being 10.6µg equivalents of quercetin that is very higher compared to the present results. The aqueous extracts have lower flavonoid content than their methanol extracts. This may be due to the use of soxhlet extraction technique by the previous workers.

Similarly, Bhattarai (2014) documented a range of 20.57 to 6.8 mgQE/gm DW of flavonoid content from methanolic extracts of *Swertia chirayita* populations from Nepal that is quite lower than the present findings. All the species of *Swertia* have higher flavonoid content than the flavonoid content of *S. chirayita* populations as stated by Bhattarai (2014).

Comparatively, methanol extracts showed higher flavonoid content than their aqueous extracts. *S. chirayita* had the highest flavonoid content in both methanol and aqueous extracts. This high content results its least IC50 value, making it the best antioxidant species among the seven studied *Swertia* species. This higher quantitative value also justifies its use in traditional medicines (Joshi, 2008). The lowest value was reported in *S. angustifolia*, justifying being less valuable and being used an adulterant in the trade of *S. chirayita* (Joshi and Dhawan, 2006).

5.4 Total Polyphenol Content

Phenolic components are potential antioxidants that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. Phenolic compounds by their hydroxyl groups scavenge radicals such as singlet oxygen, superoxide and hydroxyl radicals (Huang 2005). It has been recognized that the phenolic-linked anti-oxidant property in medicinal plants are good antioxidants since the generation of reactive oxygen species has been linked to majority of the systemic diseases including cancer, cardiovascular diseases, and type 2 diabetes (Dembinska-Kiec *et al.,* 2008).

Comparatively, higher total phenolic content was found in the methanol extracts than their aqueous extracts. Phoboo *et al.,* 2007 also reported TPC value of alcoholic extracts have more than aqueous extracts. Among the methanol extracts, the highest and lowest content was seen in *S. dilatata* and *S. angustifolia* respectively. The value of TPC of *S. chirayita* was found to be near to *S. dilatata*. In a similar study, Wijekoon *et al.,* 2011 also found water to be the least effective solvent for the extraction of phenolic content. The phenolics better dissolve in methanol than water that corresponds to the current findings that has reported decrease of TPC in aqueous extracts than their methanolic extracts in most of the species of *Swertia*. Chen *et al.,* (2011) quantified TPC much greater than the present values in the ethanolic extract of *S. chirayita*. But, the quantification of total phenols by Dutta *et al.,* 2012, has reported lesser TPC values in water and 100% ethanol than the current results. This variation in TPC may be due to the use of ethanol, altitudinal variations, genotypic differences, species diversity, time of collecting samples and the procedures of extraction employed.

5.5 DPPH Radical Scavenging Inhibition Assay

Antioxidants are important compounds which possess the ability to protect the body from damages caused by free radical-induced oxidative stress. Free radicals like super oxide, hydroxyl radicals, nitric oxide and other reactive species 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 that accepts electron radical or hydrogen radical to become a stable diamagnetic radical which is scavenged by proton donating substrate to reduce the absorbance. The decrease in absorbance is a measurement of the radical scavenging. It has been reported that the decrease in the absorbance of DPPH radical caused by the phenol compounds is due to the reaction between antioxidant molecules and radicals resulting in the scavenging of the radical by hydrogen donation that discolors from purple to yellow (Chan, 2007).

Antioxidant property can be inferred on the basis of percentage RSA and IC_{50} value. Antioxidant activity DPPH inhibition of the plant extract is expressed as percentage of inhibition of stable radical or inhibition concentration fifty (IC_{50}) in reference to a standard compound. The plant with higher %RSA has the lower IC_{50}. The plant 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.

Comparatively, higher scavenging property was seen in the methanol extracts than their aqueous counterparts in all of the *Swertia* species and a significant variation in their IC_{50} values. *S. chirayita* that gave the lowest IC_{50} value than all of the species of *Swertia*, even better than the standard compound ascorbic acid. *S. angustifolia* reported the highest IC_{50} value among the studied species of *Swertia.* This lower IC_{50} value can be justified by the lower total flavonoid and phenolic content. And comparing with the IC_{50} value of ascorbic acid, *S. chirayita* proves to be the best antioxidant among the species studied. The percentage scavenging activity increased with increasing concentration of the extracts.

Phoboo *et al.,* 2007 has reported the higher antioxidant activity via DPPH RSA in ethanol solution of *S. chirayita* than the aqueous extracts. They reported a significant difference between antioxidant activity of ethanol and aqueous extracts ($p<0.05$), similar to the present study where the total antioxidant activity was significantly different ($p<0.05$) in aqueous and methanol extracts of different species of *Swertia.* Aqueous extracts had lower antioxidant activity, or higher IC_{50} value than methanol extracts. Similar paper authored by Chen *et al.,* 2011 in ethanol extract of *Swertia chirayita* exhibited a steady increase in

inhibition percentage with increasing concentration of ethanol extracts. Farhan *et al.,* (2012) also reported higher antioxidant activity of ethanol extracts than the aqueous extracts in *Malva parviflora.* Antioxidant activities of methanol extracts demonstrated by Hajimehdipoor (2013) showed that *S. longifolia* aerial parts and roots had considerable radical scavenging activity. Among various extracts, IC_{50} value was reported least in methanol extracts of *S. longifolia* followed by methanol, ethyl acetate, chloroform, hexane and water. Hexane and water extracts which contain lipophilic and hydrophilic compounds, respectively, showed the least activity (Schwartz, 1996).

Antioxidants are substances that delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions (Schwartz J.L. 1996). It has been proposed that the health beneficial effects of polyphenols could result from either their antioxidant functions and/or independently from these properties; by acting as modulators of cellular signaling processes (Rammal *et al.,* 2012).

5.6 Correlation between TPC and DPPH Antioxidant Activity

Most antioxidant activities from plant sources are correlated with phenolic-type compounds (Chen *et al*., 2011). A significant correlation between TPC and antioxidant activity for both aqueous and ethanol solution extracts of different plant parts was found after the analysis of the TPC and DPPH RSA. The higher correlation between antioxidant activity and TPC was found in methanol extracts than the aqueous extracts. The reason may be due to the higher quantity of polyphenols in methanol extracts, which exhibit anti-oxidant capacity to scavenge the DPPH free radicals.

Aerobic metabolism involves the production of reactive oxygen species which are responsible for many cell disorders. Oxidative stress causes oxidation of biological molecules and alteration of their structures (Han *et al.,* 2007). Phenolics are considered as one of the most important and widely found antioxidants in foods and medicinal plants. *Swertia* species extracts contain many naturally occurring polyhydroxy xanthones and flavonoids that have been associated with a wide range of biological and pharmacological properties including antioxidant, analgesic and hepatoprotective activities (Nagalekshmi, 2011; Alam *et al.,* 2010). The results of the present study indicate that both aqueous and methanol extracts of *S. chirayita* possesses significant phenolic-linked antioxidant activity. This also justifies the ethno-parmacological uses of *Swertia* species in the local communities.

5.7 Antibacterial Assay

Most of the methanol extracts showed significant activity against Gram negative organisms like *S. typhii, K. pnumoniae* and *E. coli.* No antibacterial activity against *P. aeruginosa w*as observed. Comparatively, aqueous extracts showed less activity against *E. coli* and *S. typhii* and were inactive against *K. pnumoniae, S. aureus* and *E. feacalis*. Both type of extracts showed concentration dependent activity. Methanol extracts of *S. chirayita* exhibited the highest inhibition on all of the tested bacteria even at the extract concentration of 6.25 mg/mL. This may be due to the presence of higher amount of flavonoids and phenolics. Among the tested organisms, *Staphylococcus aureus and Salmonella typhii* were comparatively more inhibited. *S. angustifolia* reported the least antibacterial bioactivity as it contains the minimum number of xanthones (Karan *et al.,* 2012).

In similar experiments conducted by Laxmi *et al.,* (2011), the methanol extracts of *S. chirayita* showed more antibacterial activity than their aqueous extracts. They showed higher inhibition of methanol extracts against *S. typhii, E. coli and S. aureus* which are in accordance to the present work. Likewise Ramesh *et al.,* (2002) have reported antimicrobial activity of the *S. corymbosa* that exhibited concentration dependent bioactivity on *S. aureus, E. coli, K. pneumonia and S. typhii.* Alam *et al.,* 2009 stated antibacterial activity of ethanol extract of *S. chirayita* against *S. aureus, E. coli, and S. typhii.* Highest antibacterial inhibition was reported against *S. aureus* but in the present study, the higher inhibition was seen against *S. typhii* (6.25 mg/mL) and *K. pnumoniae* (up to 6.25 mg/mL). Moreover, Samaddar *et. al.* (2013) reported antimicrobial activity of swertiamarin and amarogentin against *Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa*. These compounds present in the different species of *Swertia* are the major compounds to show bioactivity. So, other species of *Swertia* like *S. paniculata, S. racemosa, S. dilatata* and *S. nervosa* revealed antibacterial activity due to the presence of these potent compounds that were estimated later during this experiment.

Alam *et al.,* (2009) tested the antimicrobial activity of Swertiamarin against 14 gram positive and gram negative and reported antibacterial activity against *Bacillus cereus, Bacillus subtilis, Citrobacter freundii, Escherichia coli and Proteus mirabilis* but no activity against *Staphylococcus aureus.* The bioactivity may be the indicative of the presence of broad spectrum antibiotic compounds. There are several modes of action through which phytoconstituents show antimicrobial activity such as disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Cowan *et al.,* 1999).

The methanol extract contains tannins, flavonoids, alkaloids and glycosides. Tannins and flavonoids are known to possess antimicrobial potential against bacteria and fungi. The antimicrobial potential of the plant extract may be due to the presence of these phytochemicals (Hasalam, 2006; Scalbert, 1991).

5.8 Antifungal Assay

Only the methanol extracts of different species of *Swertia* gave inhibitory effect against tested fungal inoculums. The methanol extracts of *S. angustifolia* showed significant activity up to 12.5 mg/mL and *S. chirayita* only gave a zone of inhibition in *Pichia pastoris* up to 12.5 mg/mL. Both the extracts showed concentration dependent activity. Tannins and flavonoids present in the plant extracts are known to possess antimicrobial potential against bacteria and fungi (Hasalam, 2006 and Scalbert, 1991).

In similar experiment conducted by Laxmi *et al.,* 2011, the methanol extract of *Swertia chirayita* showed antifungal activity against *Cladosporium oxysporum and Aspergillus niger*; and aqueous extract showed significant activity against *Cladosporium oxysporum and Aspergillus flavus.* According to Ramesh *et al.,* 2002, the antifungal activity of methanol and aqueous extracts of *Swertia corymbosa* yielded no activity against *Aspergillus flavus, Aspergillus fumigates, Aspergillus niger and Candida albicans.* Alam *et al.,* 2009 stated antifungal activity of ethanol extract of *S. chirayita* against *Aspergillus niger and Candida albicans*. However, *Sacharomyces cerevesiae* was not inhibited by any methanol and aqueous extracts that is in accordance to the present findings. Particularly, the extracts of *S. angustifolia* were found to be antifungal that may be due to the higher swertiamarin content in these samples.

5.9 Qualitative and Semi-quantitative estimation

The swertiamarin content of *S. ciliata*, *S. nervosa* and *S. racemosa* were significantly lower than the swertiamarin content of rest of the species. Studies on different species of *Swertia* by Wang *et al.,* 2011 reported swertiamarin in the range of 3.8 to 4.05 mg per gram dry mass for *S. japonica,* 1.08 mg/gm DW for *S. pseudochinesis* and 6.37 mg/gm DW for *S. binchuangensis*, that is higher content than the present study. HPLC quantification carried out by Phoboo *et al.,* 2010 noted 0.25 and 0.29 mg/g DW of swertiamarin in aqueous and ethanol root extracts that is higher than the present findings. The lower content of

swertiamarin in this experiment may be attributed to its semi-quantitative estimation procedure, experimental errors, climatic variations and other factors.

HPLC quantification done by Phoboo *et al.,* 2010 found 0.28 and 0.23 mg/g DW in aqueous and 12% ethanol extracts. This result corroborates with the present findings of having around 0.24 mg/gm of Amarogentin in *Swertia chirayita* and *S. dilatata* methanol extracts. From a similar study of Wang *et al.*, 2011, *S. japonica* contained 0.9 to 3.4 mg/gm DW of amarogentin, S. punicea 0.17 mg/gm DW and *S. binchuangensis* contained 0.22 mg/gm DW. The present study also reports their approximate results, especially in *S. chirayita and S. dilatata*, showing little variations. Other species of *Swertia* were found to be containing less amount of amarogentin than the S. chirayita methanol extracts. This result justifies the mixing of those inferior species with *S. chirayita*, especially in chiretta trade.

Semi-quantitative estimation of mangiferin reported no significant difference between the mangiferin content of different species of *Swertia.* However, the amount of mangiferin was slightly higher in *S. dilatata* in comparison to other species methanol extracts. A similar study by Phoboo *et al.,* 2010 reported higher amount of 0.46 and 0.4 mg/gDW of mangiferin in aqueous and ethanolic root extracts from *S. chirayita.* This study corresponds to our findings indicating a little higher content of mangiferin. This may be due to their use of optimized HPLC system for quantification. Another compound profiling of Himalayan *Swertia* species by Pandey (2012) reported 0.789% mangiferin in *S. nervosa*, 1.236 to 3.46% in *S. chirayita*, but mangiferin was not detected in *S. paniculata* and *S. dilatata*. However, these latter species gave mangiferin levels equivalent to *S. chirayita* in the present study. This might be due to the fresh specimens collected from the Nepalese Himalayan landscape, species differences and genotypic variations.

Three biomarkers: amarogentin, swertiamarin and mangiferin can be used to screen the elite populations of *Swertia* species in accordance to the compounds quantified. *S. chirayita* contained the highest amount of amarogentin. The so called inferior species can be an alternative source of these seco-iridoids in light of alarming degradation of *S. chirayita* populations as the content of amarogentin, mangiferin and swertiamarin is appreciative. *S. angustifolia* can be a better source of swertiamarin; and moreover *S. dilatata*, *S. paniculata* and *S. nervosa* can be used as alternatives for *S. chirayita* that has an alarming status.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This present investigation deals the preliminary phytochemical analysis on different *Swertia* species from Nepalese Himalaya. The antibacterial results showed that methanol extracts exhibited better inhibition in than aqueous extracts. It is also found that the Gram-negative microorganisms were more sensitive to the plant extracts than the Gram-positive microorganisms. This property to inhibit Gram negative organisms is quite rare and was seen in *Swertia* species. This could be the reason of its ethnomedicinal and therapeutic use in enteric disorders (Hottestmann Kalad *et. al.*, 1981; Brahmachari *et. al.*, 2004) that are caused by the Gram negative organisms such as *Klebseilla, Salmonella and Enterococus*. Particularly, the extracts of *S. angustifolia* are antifungal. This in vitro study demonstrated that folk medicine can be linked as effective as modern medicine to combat pathogenic microorganisms, especially in entero-pathogens.

The methanol extract of *S. chirayita* was highly anti-oxidant even better than the standard ascorbic acid. So, this DPPH assay further proves the importance of *Swertia chirayita* in modern pharmaceutics and folklore medicines. *S. chirayita* contained the highest of phenolics and flavonoids thus, proving to be superior among the studied species; and *S. angustifolia* contains the least phytoconstituents that have been referred as the adulterant in *Swertia* trade in Nepal. *S. dilatata* and *S. paniculata* can be better alternatives than other species of *Swertia* (S*. angustifolia, S. nevosa and S. ciliata*) that form a major part of *Swertia* trade from Nepal. So, the adulteration of *S. chirayita* with *S. angustifolia* and other species is justified as it contains less phytohemicals and lower antioxidant property.

Qualitative and semi-quantitative estimation showed the presence of amarogentin, mangiferin and sweriamarin present in all *Swertia* samples. *S. chirayita* showed the highest amarogentin content among the studied *Swertia* species. So, it can be concluded that other species that are routinely mixed with *Swertia chirayita* are adulterants that contain less amounts of major bioactive amarogentin. Comparatively, Swertiamarin was found higher in *S. angustifolia* that is considered as the major adulterant in *Swertia* trade. Thus, *S. angustifolia* can be a major source of swertiamarin.

6.2 Recommendations

In regard to the present preliminary study, following recommendations can been made.

I. This study is novice that has covered only a small number of *Swertia* species from Nepalese Himalaya, so the remaining species of *Swertia* should also be studied and explored for their bioactivities; especially of *S. dilatata* that has a comparable amarogentin and swertiamarin content with *S. chirayita.*

II. With the availability of primary information on the studied plant extracts, further studies should be carried out like phytopharmacology, their standardization, identification and isolation of active and pharmacological compounds. This may be followed by development of lead molecules for many diseases that can be validated/tested in in-vivo models.

III. The species of *Swertia* contain bioactive amarogentin, swertiamarin and mangiferin, so screening of these species is important in terms of phytopharmacological studies; particularly amarogentin. So, screening of indigenous *Swertia* species and isolation of other novel/important compounds should be the primary goal in future research.

IV. The HPLC validated method should be used as a marker of different *Swertia* species that are exported from Nepal. This species profiling on the basis of bioactive compounds can be helpful in monitoring and establishing fair *Swertia* trade from Nepal. This will contribute to intensive pharmaceutical comprehension and also helps to boost the economy of our country.

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APPENDIX - A (List of Reagents and Culture media)

1. Preparation of 1 M Na2CO3 -100 ml

10.599 gram of the Na2CO3 (Merk 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 adding ethanol.

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

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

4. Preparation of 1M potassium acetate (CH3COOK) – 100 ml

Weigh 9.814 gram of the potassium acetate Merk Pvt. Ltd, Mumbai, India) and dissolved in water. Finally maintain the volume to 100 ml by adding water.

5. Preparation of 0.2mM DPPH solution - 100 ml

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

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

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

7. Composition of Nutrient agar media

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

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

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

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

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

9. 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

10. Composition of Yeast Extract Peptone Dextrose (YEPD) broth

The composition of YEPD broth (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follows:

Components	**gram/L**
Yeast Extract	10
Peptone	20
Dextrose/Glucose	20

11. Composition of Potato Dextrose Agar (PDA)

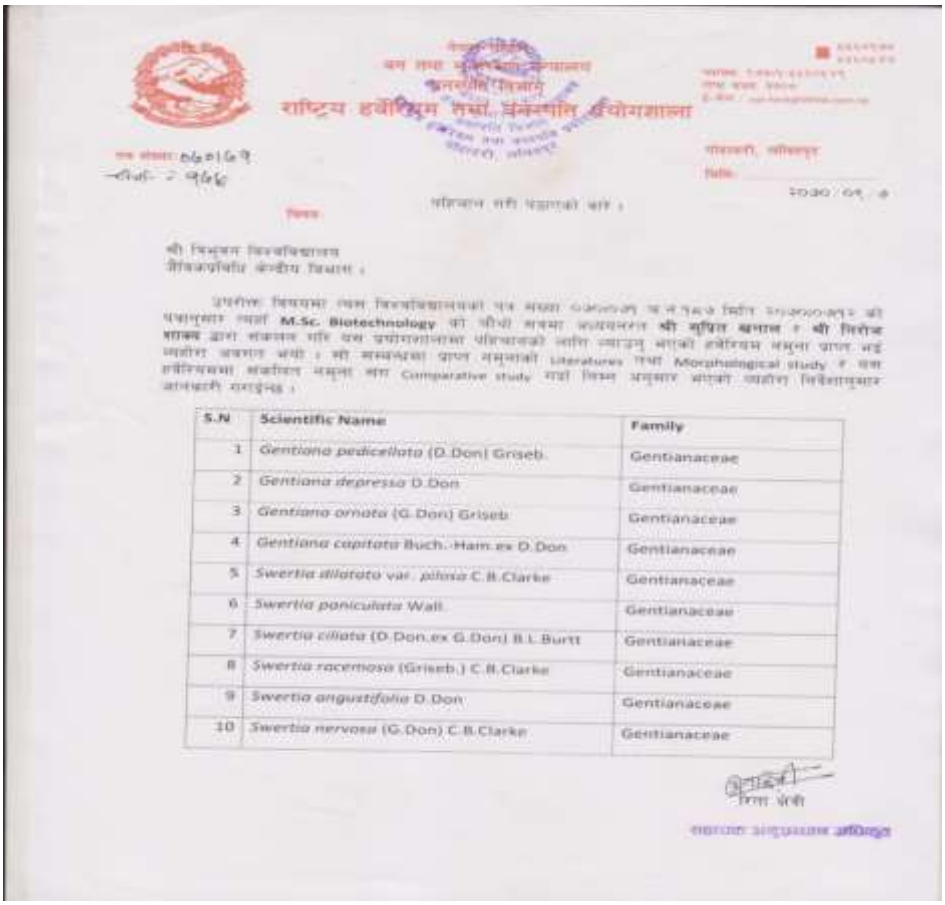
The composition of PDA broth (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follows:

Components	**gram/L**
Potato	200
Agar	2
Dextrose/Glucose	20

12. Solvent Systems for Amarogentin, Swertiamarin and Mangiferin

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

APPENDIX – B (LIST OF PHOTOGRAPHS)



राष्ट्रिय हर्बेरियम तथा वनस्पति अध्ययनशाला

श्री त्रिभुवन विश्वविद्यालय
जीवविज्ञानविधि केन्द्रीय विभाग ।

S.N	Scientific Name	Family
1	*Gentiana pedicellata* (D.Don) Griseb.	Gentianaceae
2	*Gentiana depressa* D.Don	Gentianaceae
3	*Gentiana ornata* (G.Don) Griseb	Gentianaceae
4	*Gentiana capitata* Buch.-Ham.ex D.Don	Gentianaceae
5	*Swertia dilatata* var. *pilosa* C.B.Clarke	Gentianaceae
6	*Swertia paniculata* Wall.	Gentianaceae
7	*Swertia ciliata* (D.Don.ex G.Don) B.L.Burtt	Gentianaceae
8	*Swertia racemosa* (Griseb.) C.B.Clarke	Gentianaceae
9	*Swertia angustifolia* D.Don	Gentianaceae
10	*Swertia nervosa* (G.Don) C.B.Clarke	Gentianaceae

Letter of plant Identification

Swertia chirayita (Roxb. ex Fleming) H. Karst

Swertia angustifolia Buch. Ham. ex D. Don

Swertia paniculata Wall
(http://forwildlife.wordpress.com)

Swertia racemosa (Griseb.) C.B. Clarke

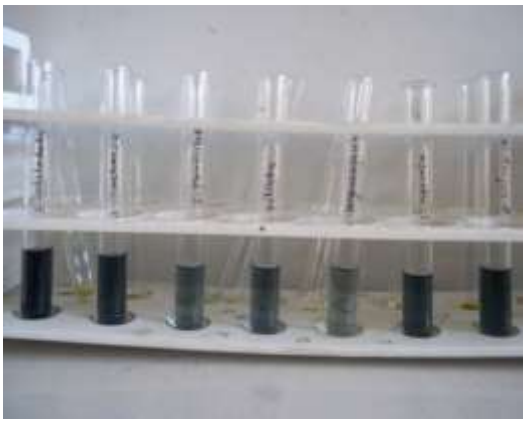
Swertia nervosa (G. Don) C.B. Clarke

Swertia ciliata (D. Don ex G. Don) B.L. Burtt

Swertia dilatata C.B. Clarke

Plant extract preparation

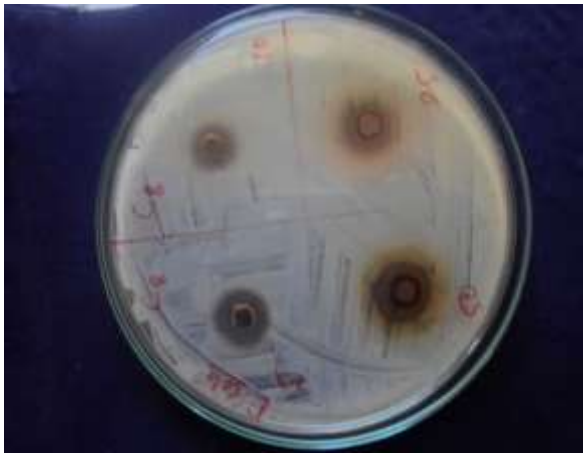
Quantification of flavonoids



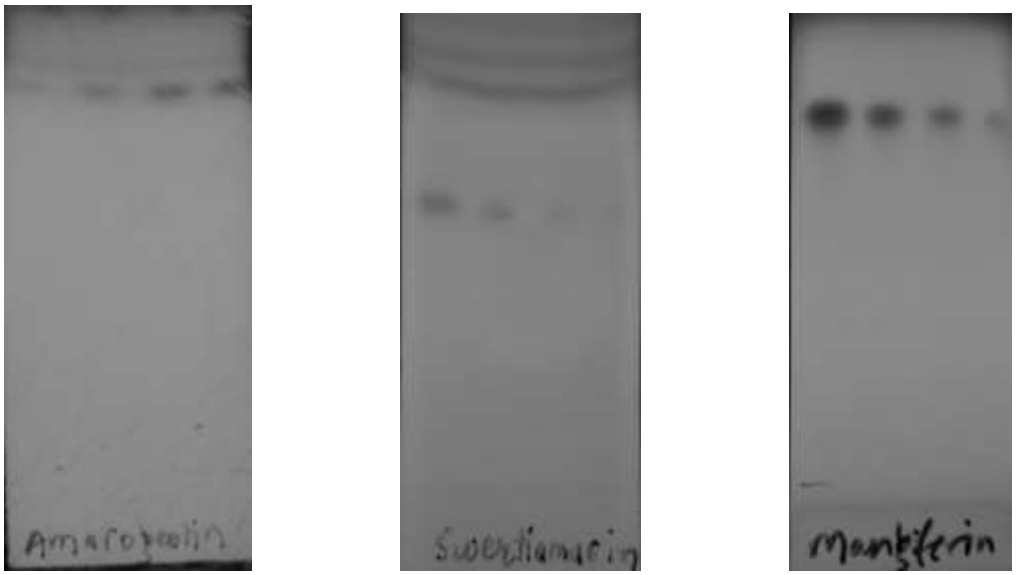
Quantification of polyphenols

Activity of Streptomycin on *S. typhii*

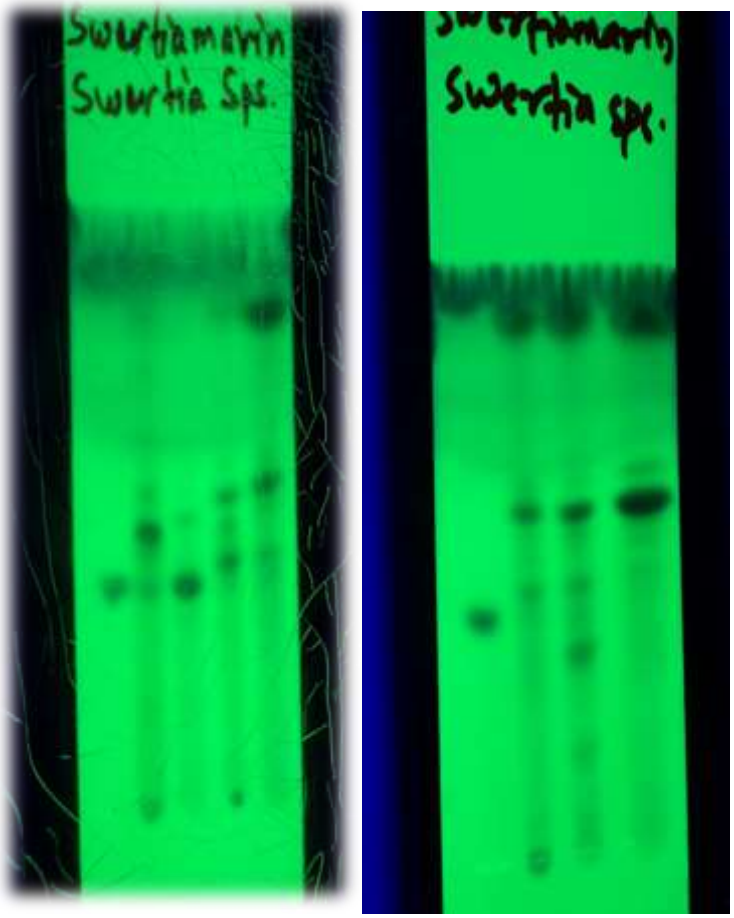
Activity of *S. dilatata* on *K. pumoniae*



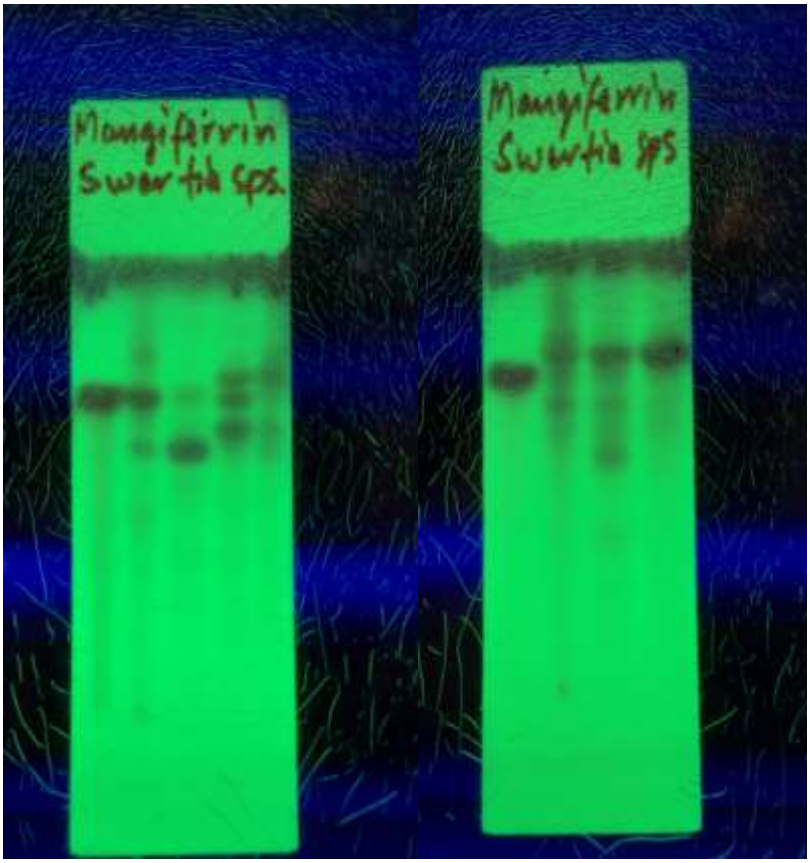
Antibacterial activity of *S. chirayita* methanol extract on *E. coli*



Detection and semi-quantification of amarogentin, swertiamarin and mangiferin standard (From left: Amarogentin, Swertiamarin and Mangiferin at 500, 250, 125 and 62.5 ug/mL)



Detection and semi-quantification of amarogentin and swertiamarin in different species of *Swertia* methanol extract (From left: Standard*, S chirayita, S. angustifolia, S. paniculata, S. racemosa, S. nervosa, S. ciliata, S. dilatata*).



Detection and semi-quantification of Mangiferin in different species of *Swertia* methanol extract (From left: Standard, *S chirayita, S. angustifolia, S. paniculata, S. racemosa, S. nervosa, S. ciliata and S. dilatata*)