PHYTOCHEMICAL ANALYSIS OF DIFFERENT SPECIES OF *GENTIANA* FROM NEPAL

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List of Abbreviations

^0C	Degree Celsius
µl	Microlitre
µg	Microgram
λ	Wavelength
ATCC	American Type Culture Collection
BDFI	Bioactivity Directed Fractionation and Isolation
DMSO	Dimethyl Sulfoxide
DPPH	1, 1- diphenyl-2 picryhydrazyl
DW	Dry Weight
gm	Gram
GAE	Gallic Acid Equivalent
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectroscopy
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
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
TPC	Total Phenolic Content
UV	Ultra Violet
Wt	Weight
WHO	World Health Organization
ZOI	Zone Of Inhibition

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Abstract

Nepal houses 44 different species of *Gentiana.* Plants belonging to genus *Gentiana* are very well-known for their pharmacological properties. Ethnopharmacological data show that various species of *Gentiana* have been effectively used in herbal medicine to treat various ailments. They are known to possess potent therapeutic compounds like iridoids, xanthones and glucoflavones. This study attempts to evaluate phytochemical, antioxidant and antibacterial activities and estimate three bioactive compounds: swertiamarin, amarogentin and mangiferin in different species of *Gentiana* collected from different parts of Nepal.

Methods: 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 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. Semi-quantitative estimation was done using GelQuant.NET software using the standard compounds.

Results: The quantitative phytochemical analysis showed presence of higher amounts of polyphenol and flavonoid in methanol extracts as compared to the aqueous extract of various *Gentiana* species. Among methanol extracts highest concentration of polyphenol was observed in *G. depressa* (79.2±19.19mgGAE/gm) while *G. capitata* (44.6±3.97 mgGAE/gm) showed the lowest content. Similarly, *G. capitata* recorded the highest total flavonoid content (19.09±0.97mgQE/gm) and *G. ornata* (11.31±0.49 mgQE/gm) showed the least content. Likewise, the methanol extracts showed more promising antioxidant activity compared to the aqueous extracts. The methanol extract of *G. depressa* showed the best antioxidant activity among the different *Gentiana* sps with an IC_{50} value of 39.57±0.95 µg/ml. Semi-quantitative analysis showed that swertiamarin was present in higher quantities than amarogentin and mangiferin. Highest concentration of swertiamarin and mangiferin was identified in *G. ornata* (0.109±0.013 mg/gm DW and 0.018±0.001 mg/gm DW respectively) while *G. capitata* possessed highest concentration of amarogentin (0.075±0.005 mg/gm DW). Antibacterial assay showed varying degree of sensitivity to various pathogenic microorganisms. *G. ornata* was showed highest activity among all plant species against *K. pneumonia* and *E. feacalis* while *G. depressa* showed maximum sensitivity to *S. aureus* and moderate sensitivity to *K. pneumonia.* These results clearly show that crude methanol and aqueous extract of various *Gentiana* species possess potent pharmacological compounds, and justified the folkloric use of these plants in digestive and respiratory ailments.

Keywords: *Gentiana*, antioxidant, antibacterial, amarogentin, swertiamarin, mangiferi

CHAPTER 1: INTRODUCTION

1.1 Background

The relationship existing between plants and humans is as old as mankind, dating back to the origin of human civilization for food, clothing, shelter, fuel and medicine (Newman *et al*., 2000). Traditional medicine forms an integral part of Ethnobotany and Ethnopharmacology that use the medicinal plants against many human ailments; from small coughs, colds, parasitic infections and inflammation to many kinds of cancers and chronic diseases (Sumner, 2000; Newman *et al.,* 2000). The contribution of these plants to the therapeutic arsenal in the fight against disease dates back several centuries, and has, to a certain extent, been documented by the ancient communities (Taylor *et al.,* 2001). The World Health Organization (WHO) defines traditional medicine as the "diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose, or prevent illness". Traditional medicine utilizes biological resources and the indigenous knowledge of traditional plant groups, the latter being conveyed verbally from generation to generation. This is closely linked to the conservation of biodiversity and the related intellectual property rights of indigenous people (Timmermans, 2003).

The nature has embodied plants with many types of bioactive compounds, so the extraction of these compounds from plants using various kinds of isolation methods has been used. Although combinatorial chemistry, synthetic chemistry, and molecular modeling (Geysen *et al.,* 2003; Ley and Baxendale, 2002, and Lombardino and Lowe, 2004) has been used to screen new drugs of higher efficacy, natural products and particularly medicinal plants still remain a potent arena of drug discovery giving new chemical entities and new drug leads (Butler,2004; Newman *et al.,* 2000, 2003). Butler (2004) states that one quarter of the best-selling drugs in the world were derived natural products. It has also been reported that approximately 28% of new chemical entities between 1981 and 2002 were natural products or natural product-derived substances (Newman *et al.,* 2003). Furthermore it has been known that natural products also provide a starting point for laboratory syntheses with diverse structures and often with multiple stereo centers that can be challenging synthetically (Koehn and Carter, 2005; Peterson and Overman, 2004).

1.2 Ethnopharmacological research

Ethnopharmacology is the scientific study encompassing ethnic groups, their health in relation to their physical habits as well as the methodology in the creation and use of

medicines. This approach to drug discovery has been shown to be an effective and faster means of discovering new pharmaceuticals and bioactive compounds from higher plants (Farnsworth, 1994). Traditionally, many medicinal plants have been routinely used in various regions of the world by the local communities, making ethnopharmacology a reservoir of potential drug candidates and pharmaceutical compounds (Heinrich *et al.,* 2008). The rich biodiversity and variation in landscape in Nepal harbors a huge flora that has high medicinal properties, and have been practiced in the local communities.

The study of medicinal plants used in traditional medicine requires the effective integration of information on chemical composition of extracts, pharmacological activities of isolated compounds, as well as indigenous knowledge of traditional healers. The acquisition of ethnobotanical information remains an empirical aspect in any such study (Soejarto *et al*., 2005). The process of isolating and identifying lead compounds from a complex mixture requires a number of specific resources, including comprehensive knowledge, specialized equipment and skill (Paraskeva, 2007). The urgency of the discovery of new agents is a result of impenetrable factors that come into play, including the emergence of new killer diseases, known antimicrobial drug-resistance, the inefficiency of synthetic drug discovery and the high cost of bringing to market a single drug. A shift towards natural product research, which is further driven by remarkable advances in plant extraction technology, biotechnology and analytical chemistry, is therefore inevitable. There is a great need and ethical obligation to accurately document investigative findings on plants used for health purposes. This will additionally aid in the efficient preservation and conservation of traditional knowledge, thereby preventing the further disappearance of indigenous systems of medicine, which may potentially benefit society in general (Soejarto *et al*., 2005).

The collection, preservation, extraction and storage of the plant material are important steps in a phyotpharmacology study. Generally, abiotic and biotic factors such as light, temperature, moisture and microbes affect the chemical composition of plant material. For instance, the plant chemical composition changes under variable conditions when necessary precautions are not taken during collection and preparative stages of plant material harvesting for scientific study (Aremu, 2009). For most pharmacological and phytochemical analyses, extraction of dried plant material is preferred to using fresh plant material. The dried plant material is easier to handle and more stable because water content in fresh plant material changes over time causing a wide variation in plant chemical composition and properties (Aremu, 2009).

Secondary metabolites are diverse compounds found in plants that are reported to have many bioactive functions. The broad use of plant in human therapy, veterinary, agriculture, scientific research and countless other areas also states its prime

importance (Mann, 1978). Plant products can be derived from barks, leaves, flowers, roots, fruits, seeds. Knowledge of the chemical constituents of plants assist for synthesis of complex chemical substances (Cowan, 1999; Criagg 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; Tabassum *et al.*, 2012).

The extraction of plant material using different solvents is based on the biological concepts of permeability of the plant cells. Dried plant material extraction can be achieved using different solvent systems. These include the use of a single solvent or a series of solvents of increasing polarity either sequentially or non-sequentially. The dried plant material is extracted using different solvents to obtain the bioactive compound(s) present in it for pharmacological investigation. It is important to use a similar extraction method employed by the local people for traditional medicinal plant research (GuribFakim, 2006). This will enhance the extraction of the same natural bioactive product used by the people and probably help in validation of a medicinal plant.

Plants belonging to Gentianaceae are very well-known for their pharmacological properties. They have been widely used in traditional medicine and also as constituents in bitters and similar concoctions. The medicinal value is due to the presence of bitter glycosides (Hottestmann-Kaladas *et al.,* 1981). The genus *Gentiana* comprises over 400 species widely distributed in alpine habitats within the temperate regions of Asia, Europe, and North America (Wang *et al.,* 2014). They have been an integral part of traditional medicine systems such as Ayurveda, Amchi, Tibetan, Unani and have been mentioned in various pharmacopoeias around the world. The active constituents are bitter tasting secoiridoid glycosides which are applied in the treatment of gastrointestinal tract diseases (Skrzypczak *et al.*, 1993).

Secoiridoids (swertiamarin, gentiopicrine and sweroside) are the bitter principles in various gentians which are used for preparation of bitter tonics traditionally. In addition, secoiridoid glycosides have a variety of biological effects such as anti-tumor, anti fungal and hepatoprotective activities. According to the literature, xanthone compounds often exhibit a wide range of biological and pharmacological activities like antioxidative, hypoglycemic, antiviral, antibacterial and hepatoprotective. The methanol extract and its chloroform, ethyl acetate and n-butanol fractions of *G. asclepidea* roots by HPLC-DAD analyses suggested iridoid and secoiridoid glycosides as the primary components of members of the genus *Gentiana* (Mihailović *et al.*, 2011).

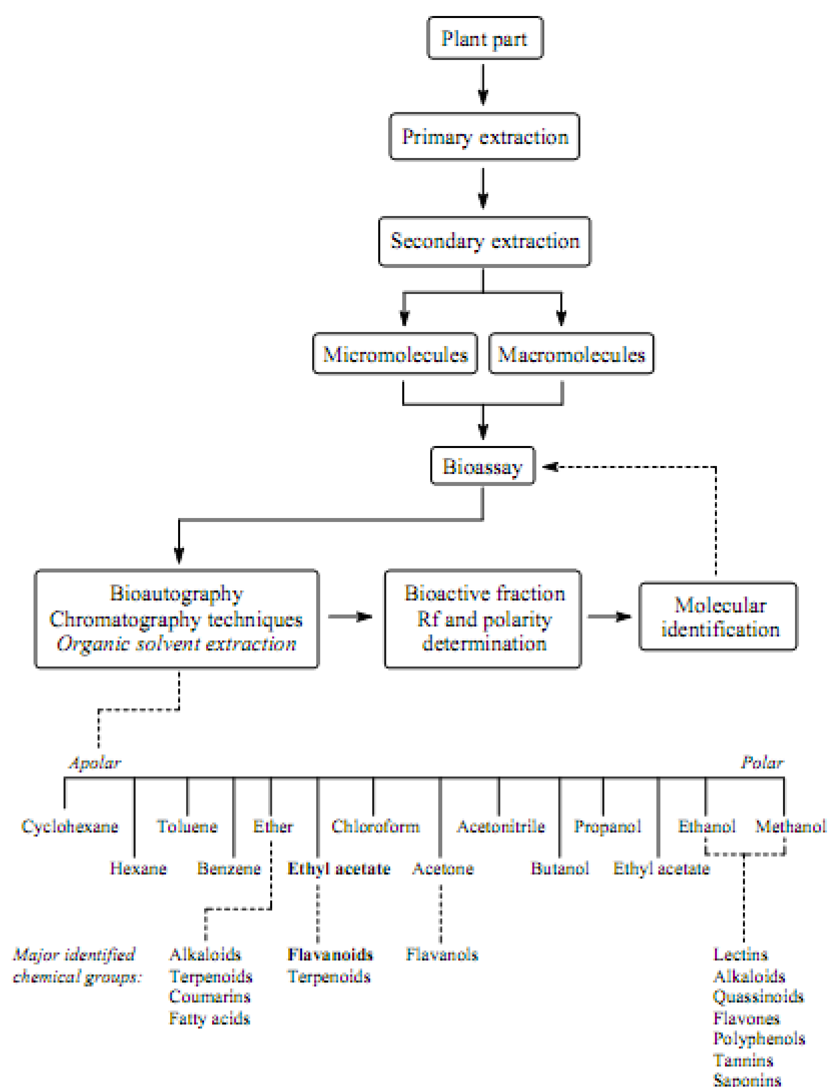

Figure 1: Overview of the procedure from extraction to identification (Modified from Mendonca-Filho, 2006)

1.3 Aims and Objectives

The species of *Gentiana* are not among the commonly studied plants, so their phytochemical constituents and ethnobotanical knowledge remains unexplored and unknown; especially in the context of Nepalese ethnopharmacologic research. This experiment aims to excavate their phytochemical properties and bioactivity along with the estimation of major Gentian phytochemicals with TLC.

The **hypothesis** proposed here is that species of *Gentiana* contains active constituent(s) that are rich in antioxidants, flavanoids, xanthones and other important compounds. These unknown species study will open new frontiers in the export of Non Timber Forest Products from Nepal that can be a potential source of highly potent biochemicals.

The main objective of this study is to establish a novel and basic foundation in the study of various *Gentiana* species found in Nepal and estimate their major compounds. These

species have rarely been studied before from the Nepalese Himalayas. This broad objective is achieved through following activities:

I. To perform qualitative phytochemical screening on those plant extracts.
II. To estimate total phenolics and flavonoids in their methanol and aqueous extracts.
III. To evaluate their in vitro antioxidant properties.
IV. To evaluate their antibacterial activity.
V. To estimate Amarogentin, Swertiamarin and Mangiferin from their methanolic extracts.

1.4 Rationale

As Gentianeaceae species have varied importance in human therapeutic and preventive medicine, its study reveals newer medicinal prospects. So, the species of *Gentiana* that are amply found on the slopes of Himalayas can be a source of many new compounds that may have potential pharmaceutical applications. The present study aims to focus and discover useful and medicinal aspects of *Gentiana* species that have been reported from Nepal but not studied. These unexplored Gentians can be a source of many compounds that can be useful in many diseases. The biological action of most of the chemical components of this important medicinal plant still remains unexplored.

Gentianeaceae species are particularly recognized for many bitter compounds and multiple metabolites, so these Gentians can be a storehouse of many compounds that need to be studied extensively. The present study mainly aims to establish a rough and preliminary framework for their phytochemical analysis. As the compounds like Amarogentin, Amaroswerin and Swertiamarin are reported in other species of *Gentiana*, so the present *Gentiana* samples (*G. urnula, G. depressa, G. ornata and G. capitata*) also should contain these important bitter compounds.

Phytochemicals like Amarogentin, Bellidifolin, Gentianin and Swerchinin have been reported to possess significant bioactive properties. Thus, the screening of *Gentiana* plants for these useful compounds is particularly helpful to open new therapeutic prospects and areas. The present work is novice and is of high importance in studying the Gentian plants at the species level, unveiling their properties.

1.5 Scope of the Study/Justification

Nepal houses 44 different species of *Gentiana* that provides sufficient traditional and pharmacognostic importance in many Nepalese communities and some species are traded from Nepal through various routes to China and India. The various species of *Gentiana* that have been studied thoroughly in China and Europe like *G. lutea, G. kurro*

and *G. macrophylla* in terms of their compound isolation and therapeutic efficiency motivates rigorous research in Gentian species from the Nepalese Himalayas. This basic assessment of different species of *Gentiana* and their primary phytochemical profile will elucidate different species reported from Nepal and their secondary metabolites in methanol and aqueous extracts. It will provide a foundation for further study in these species.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The study of medicinal plants used in traditional medicine requires the effective integration of information on chemical composition of extracts, pharmacological activities of isolated compounds, as well as indigenous knowledge of traditional healers. The acquisition of ethnobotanical information remains an empirical aspect in any such study (Soejarto *et al.*, 2005).

The urgency of the discovery of new agents is a result of impenetrable factors that come into play, including the emergence of new killer diseases, known antimicrobial drug-resistance, the inefficiency of synthetic drug discovery and the high cost of bringing to market a single drug. A shift towards natural product research, which is further driven by remarkable advances in plant extract technology, biotechnology and analytical chemistry, is therefore inevitable (Joy *et al.*, 1998). There is a great need and ethical obligation to accurately document investigative findings on plants used for health purposes. This will additionally aid in the efficient preservation and conservation of traditional knowledge, thereby preventing the further disappearance of indigenous systems of medicine, which may potentially benefit society in general (Paraskeva, 2007).

Plants have been used as the sources of feed as well as medicinal products from time immemorial. Various parts of plants including bark, tuber, root, leaves flowers or the whole plant are consumed by us in the form of plant based medicine since the traditional era. The importance of those parts has been described on various written forms, on many ancient literatures. Ayurveda has been sources of knowledge of drug discovery for many ailments in current pharmaceutical industries and Ayurvedic system is still used to treat many of the diseases. Today this system of medicinal practices is even practiced in remote areas of Nepal, Bhutan and India (Joy *et al.*, 1998). These plants of medicinal importance are cultivated at huge area in many parts of world for extraction of specific compounds to be used as drugs (Joy *et al.*, 1998).

The modern world is suffering from the increasing trend of multidrug resistance and finding the drug with highest efficacy and efficiency has revolutionized the use of medicinal plants. Rapid mutations in the current pathogens and upcoming new pathogenic variants has raised the spectra of untreatable infections and added the urgency to find new strategy against the pathogens (Sieradzki *et al.*, 1999). Plants are and have been the source of various compounds as the cure to infections. They are known to contain various metabolites including phenolics, flavonoids, alkaloids, iridoids, terpenoids, xanthones and their formulated derivatives that has potent role as cure in

various medical implications (Bentley and Trimen, 1980).Medicinal and herbal plants are a potential source of natural antioxidant compound such as flavonoids, anthocyanins, tannins, dietary glutathionine, vitamins and endogenous metabolites which can interfere with the production of free radicals and inactivate them by quenching singlet and tripletoxygen, decompose hydrogen peroxide and inhibit enzymes. These free radicals are implicated in etiology of many diseases including cancer, atherosclerosis and neurodegenerative diseases (Dutta *et al.*, 2012). The chemical compounds derived from the plants are used as such as the pharmacological drug or modified to obtain the compound with best efficacy and efficiency to be used as drug against infections. Besides, they add up the chemical library such that various bioinformatics tool can be used to derive the new drug using combinatorial tool (Newman *et al.*, 2000; Tabassum *et al.*, 2012).

2.2 Medicinal Plants of Nepal

Nepal is a small country that covers a total area of 1,47,181 sq. km with the latitudinal range from 26°22' to 30°27' N and longitudinal ranges from 80°14' to 88°12' E. Although it covers only 0.3% of the total land area of the world it has a huge altitudinal variation that creates biodiversity and divides the nation into six regions including all the climatic condition of the world, tropical, subtropical, warm temperate, cool temperate, subalpine, alpine and arctic regions (Anonymous, 2002).

Nepal Himalayas is a major haven for potent medicinal and aromatic plants since the vedic period (Baral & Kurmi 2006). Nepal comprising only 0.3% the global land area represents 2.2% of the global plants (Anonymous, 2002) of which 245 are endemic (Shrestha and Joshi 1996). Chandra Nighantu, an herbal pharmacopoeia of medicinal value of plants in the 19th century explains the traditional medicinal and ethnobotanical use of various Nepalese medicinal plants (Malla and Shakya, 1999). It is expected that the country is the store house of more than 7000 higher plants, 5% of which are endemic and 10% have medicinal and aromatic values (Joshi and Joshi, 1991). There is an estimation of traditional use of 1000 species of medicinal plants by various communities in Nepal (Chaudhary, 1998). Shrestha *et al.* (2000) enlisted more than 1600 species of medicinal plants of Nepal. Various types of medicinal plants including Non-timber Forest Products (NTFPs) have very high socio-cultural, symbolic and economic values and for its crucial role in increasing revenue, conservation and employment in the local regions (Pyakurel and Baniya, 2011). The trade of medicinal plants within or from Nepal is not known because of illegal trade routes used by vendors. According to Subedi (2006), a total of 161 plants based NTFPs are harvested for commercial purpose in Nepal of which 50% are medicinally important however, Bhattarai and Ghimire (2006) enlisted a total of 143 species as commercial MPs. The trade in Medicinal and Aromatic plants is

the important source of revenue collection for government of Nepal and a sustainable rural income generating source for people of Nepal (Pyakurel and Baniya, 2011; Olsen 2005). Department of Plant Resources says that the proper documentation of trade of *Gentiana* species has not been established due to the illegal trade within and outside of the country by the local traders.

2.3 The family Gentianaceae

The family Gentianaceae consists of flowering plants comprising approximately 84 genera and about 970 species throughout the world. Plants belonging to this family are annual and perennial herbs or shrubs; native to the northern temperate areas of the world (Daniel and Sabnis, 1978; Sharma *et al*., 2011). They are known for the bitter taste and used in traditional remedies against loss of appetite, fever, and are still included in many "tonic" formulations (Negi *et al*., 2011; Jensen and Schripsema, 2002). The plant species of genus *Gentiana* are diverse and large genus populated with more than 400 species (Bajracharya, 1996) and mostly distributed around the Himalayan foothills and higher altitudes like most other member of the gentian family (Negi *et al.,* 2011).

The family of gentianaceae is reputed for comprising versatile bioactive compounds like iridoids, xanthones, mangiferin and glucoflavones. The iridoids (mainly secoiridoid glucosides) appear to be present in all Gentian species, with a predominance of gentiopicroside and gentianine with 90 different compounds have been reported from 127 species in 24 genera. Xanthones are not universally present in Gentianaceae, but about 100 different compounds have been reported from 121 species in 21 genera. Mangiferin (a c-glucoxanthone) has been recorded from 42 species in seven genera. The c-glucoflavones have been recorded from 78 species in nine genera (Jensen and Schripsema, 2002).

The important genus of this family includes *Gentiana, Swertia, Blackstonia, Sabbatia, Halenia, Gentianella, Eustoma, Centaurium, Voyriella* and *Schenkia* among others. (The Plant List, http://www.theplantlist.org/1.1/browse/A/)

2.4 The Genus *Gentiana*

Gentiana is a genus of flowering plants belonging to the gentian family (Gentianaceae), the tribe Gentianeae, and the monophyletic subtribe Gentianinae. With about 400 species, it is considered a large genus. They are notable for their mostly large, trumpet-shaped flowers, which are often of an intense blue (Brickel, 2008). Gentians are thought to be named after Gentius, King of Illyria (present-day Albania), who was the first to use this plant medicinally. The species of genus *Gentiana* are distributed chiefly in mountain regions, especially in the Alps, the Carpathians, the Central Asia mountains and the Andes in South America. Due to their impressive and colorful flowers, gentians

decorate mountain meadows and foothills. Some species are also found in the monsoon zones of India, New Zealand and southern Australia. The yellow gentian root was already mentioned as a "*remedium stomachium*" by Galen and Dioscorides (Skrzypczak *et al.*, 1993). Shaheen *et al.*, (2012) mention that *Gentiana* species are among the more than 2500 plants out of total 10700 Himalayan plants that are reported in Hindukush Himalayas.

Most *Gentiana* plants have been widely used to treat viral, neurological, respiratory, cardiovascular, and other diseases (Zhang *et al.,* 2003). Recently, there are also reports about the use of compounds from *Gentiana* species against human promyelocytic leukemia, human ovarian cancer, and human lung epithelial cancer cell lines in vitro (Wu *et al.,* 2007). Gentiopicroside is a major medicinally effective component in *Gentiana* plants (Skrzypczak *et al.,* 1993) and its content has therefore been used as a main quality indicator for different *Gentiana* species. Apart from *Gentiana lutea*, other medicinal species have also been included in many pharmacopoeias and plant registers of the world. According to most European pharmacopoeias, the official drug may also contain material from *G. pannonica*, *G. punctate* and *G. purpurae*. The active constituents of the drug are bitter tasting seco-iridoid glucosides which are applied in the treatment of gastrointestinal tract diseases (Skrzypczak *et al.*, 1993). Gentian roots are applied as decoction, an extract and tincture. According to the Rote Liste, 1986, "*Radix Gentianae"* is a component of about 100 drugs belonging to the group of drugs namely anara, tonica, cholagoga and laxantia. Once the gentian root was a component of *Pilula estomachicae cum Fallo*; but because of their bitter taste, extracts are used in production of wines and liquors (Skrzypczak *et al.,* 1993).

2.4.1 Distribution and uses of *Gentiana*

Bajracharya *et al.*, (1996) reported 44 species of *Gentiana* found in Nepal out of the 400 species found in the world, among which 3 are endemic. The species of *Gentiana* are mostly distributed around the moist habitats of alpine region in the upper Himalayas. The three endemic species are listed as follows (Joshi *et al.*, 2000):

Gentiana decemfida Buch-Ham.exD.Don. found in Central Nepal and Langtang regions.

Gentiana pentasticta Marq. found in Eastern Nepal around the altitudes of 5000-5700m.

Gentiana radicans Sm. found in Eastern Nepal at the altitude of 5000m.

Among the alpine scrubs, *Gentiana depressa* and *Gentiana urnula* have been observed with associate herbs and climbers like *Lonicera myrtillus*, *Cotoneaster acuminatus*, *Clematis montana* and *Rhodiola himalensis* in Mustang, Dolpo and Phoksundo areas at the altitudinal range of 3000-3600m dominated mostly by xerophile vegetations. The

other associated species are *Astragalus candolleanus*, *Caragana brevifolia*, *Potentilla fructicosa*, *Bistorta affinis, Geranium pretense, Euphorbia stracheyi, Glechoma nivalis* along with *Gentiana robusta a*nd *Gentiana ornata* in the open alpine moist meadows (Shrestha *et al.*, 2005).

A number of endemic species like *Gentiana stipitata*, *Astertapete*, and *Clematis phlebanth* have been reported to be found on the rough rocky or stony slopes of Langtang, Dhaulagiri-Annapurna massif and the heads of inner valley Himalayas. (http://www.biome-explorer.net/Mountains/Himalayan%20Mountain%20Vegetation.html)

The Eastern Himalayan alpine meadows from Bhutan, China, India, Myanmar and Nepal at an altitude from 3000 to 4000m harbors species of *Alchemilla*, *Androsace*, *Diapensia*, *Gentiana*, *Leontopodium*, *Meconopsis*, *Pedicularis*, *Potentilla*, *Primula*, *Rhododendron*, *Sedum* and *Viola*. In the spring and summer, the alpine meadows are covered with brightly colored flowers (Hoekstra *et al.*, 2010).

The Alpine zone consists of moist scrub vegetation of *Rhododendron*, *Juniperus* and *Berberis* along with colorful herbs, grasses and sedges of *Meconopsis*, *Primula*, *Gentiana* and *Saxifraga*. *Gentiana speciosa* has been reported in the moist alpine scrub around 4,000 to 4,600 m of Pipar Pheasant Reserve in Annapurna Conservation Area in the Kaski district (Poudyal *et al.*, 2007).

Chettri and Bhattarai (2013) presented the floristic composition pattern of Manaslu Conservation Area recording a total of 161 species, among which 5 species of Gentianeceae were found. *Gentiana depressa* and *G. ornata* were among the most frequently occurring species in the alpine and sub alpine regions. Other species in Gentianeceae family associated were *Swertia angustifolia*, *S. paniculata* and *Lomatogonium carnithiacum*.

Kunwar *et al.*, (2006) examined the ethnobotany and traditional use of plants extracted from the vulnerable alpine zone in the Dolpa, Humla, Jumla and Mustang districts of Nepal. The genus of *Gentiana* is reported to be used traditionally in respiratory tract infections, gastro-intestinal problems and dermatological problems with varied preparation methods. *Gentiana urnula*, *Gentiana ornata* and *Gentiana pedicellata* were commonly used alpine Gentians used in the form of decoction, paste and raw (unprocessed) plant materials.

Gentiana robusta known as 'Kiyce', Kicchakarba' (Amchi) is used as decoction for stomachache, fever, and edema (swelling of the body) until recovery. And the paste of flowers is applied on cuts and wounds, boils, and edema two times a day until recovery (Bhattarai *et al.*, 2009).

The whole plant of *Gentiana kurro* Royale is used in fever, cough, liver ailments, and headache and as a blood purifier (Sharma *et al.*, 2006).

Gentiana urnula, locally known as gang-ga-chung, is commonly used to allay fever associated with poisoning, blood infection, malfunctioning of bile, tuberculosis and fitful coughing. Its whole plant is also used as hydragogue, deobstruent, attenuant and anti-diarrheal (Wangchuk *et al.*, 2011).

Gentiana depressa is found mostly around Central Nepal to south-western Tibet on open slopes at 3300 to 4300m altitudes. *Gentiana capitata* is also known as *Ericala capitata* or *Gentiana cephalodes* and locally as 'Pangyenmbhu'. Its flower is commonly used as juice; given two to three times a day to treat cold and fever in small children and infants (Swar, 2014).

Gentiana ornata is ranked as one of the most attractive of the compact-growing, autumn-flowering gentians. It forms a neat mat with congested rosettes and, at flowering time the terminal flowers are pale blue, solitary and sessile. White flowering forms have also been noted in nature, most notably in the Gosainkunda regions (http://www.jimjermynalpines.com/plant-of-the-month-octnov/1121).

Gentiana scabra Bunge, commonly known as "Longdan" in Chinese herbal medicine have been traditionally used as a crude drug, *Gentianae scabrae* Radix. They mainly grow in temperate climates such as China, Japan, South Korea, Russia, and North America. The rhizomes and roots of *G. scabra* have been used to treat inflammation, anorexia, indigestion, and gastric infections (Li *et al.*, 2015).

Gentianae radix is used in combinations with many other bitter and/or aromatic herbal substances and herbal preparations, usually for treatment of dyspeptic or choleretic complaints. Typical examples of such preparative combinations are: *Rumicisherba pulvis*, *Sambuciflos pulvis*, *Primulaeflos pulvis*, *Verbenaeherba pulvis*, *Plantaginis folium*, *Curcuma zedoaria* Rosc. *Angelicae radix*, *Fraxinusornus* L. (radix), *Myrrhaexudatum*, *Carlinaacaulis* L. (radix) (EMA, 2009).

Gentiana kurroo Royale flower tops (Gul-e-Ghafis) has been traditionally used for the treatment of inflammation, pain, fever, hepatitis in Unani System of Medicine (Latif *et al.,* 2006). Whole plant of *Gentiana kurroo* Royle (Common name: Tikta) was used a blood purifier, in fever, cough, liver ailments and headache (Gupta *et al.*, 2013).

Gentiana straminea is the famous Tibetan folk medicine thought to treat various diseases. Historically, the Qinghai–Tibetan region has been considered as the store house of medicines, where this species of *Gentiana* has been used to treat fungal and bacterial infections, hepatitis, constipation, rheumatism, pain and hypertension for thousands of years (Yang *et al.*, 2014).

Species such as *Gentiana barbata* (Froel) Ma, *Gentiana algida* Pall, *Gentiana macrophylla* Pall. and *Gentiana decumbens* have been successfully used in Mongolian traditional medicine for the treatment of a variety of diseases (Purev *et al.*, 2002).

2.5 Pharmacological and medicinal aspects of *Gentiana*

Plants belonging to Gentianaceae are very well-known for their pharmacological properties due to the presence of bitter glycosides (Hottestmann-Kalad *et al.,* 1981). The occurrences of taxonomically informative compounds in this family include iridoids, xanthones, mangiferrin and c-glucoflavones. Secoiridoid glucosides appear to be present in all species investigated, with a predominance of Swertiamarin and/or Gentiopicroside. Xanthones however are not universally present in all Gentianaceae. They possess phytochemical like amarogentin, bellidifolin, gentianine and swerchirin have been reported to possess significant anti-inflammatory, analgesic, anti-asthmatic, anticonvulsant, antihistaminic, antimalarial, antiamoebic, cytoprotective, diuretic, hepatoprotective and hypoglycemic properties (Singh, 2008). The genus *Gentiana* comprises over 400 species throughout the world, widely distributed in alpine habitats within the temperate regions of Asia, Europe, and North America (Wang *et al.,* 2014). They have been an integral part of traditional medicine systems that contain bitter tasting secoiridoid glycosides that are commonly used to treat gastrointestinal tract diseases (Skrzypczak *et al.*, 1993).

G. robusta leaves and flowers are used as appetite stimulant, bile disorders, fever, stomach troubles, inflammations, weakness, swelling, food poisoning and pain due to cold and hot weather. Particularly the flowers are commonly used in treating sore throat, stomachache, swelling, fever, rheumatism and allergy (Ghimire and Aumeeruddy-Thomas, 2010).

Citova *et al.*, (2008) have comparatively studied capillary electrophoresis and HPLC method to quantify Gentisin, Isogentisin and Amarogentin in five different samples of G. lutea obtained from Austria and Germany. The investigator reported isogentisin (ranging from 8.97 to 12.30µg/ml) and gentisin (4.27 to 9.72µg/ml) but the values of amarogentin were substantially lower, not exceeding 7.68µg/ml (0.05%).

Georgieva *et al.,* (2005) compared the volatile compounds from fresh flowers and leaves of *Gentiana lutea*, *Gentiana punctate* and *Gentiana asclepiadea* and analyzed and identified 81 compounds using GC/MS.

As published by the European Medicines Agency Evaluation of Medicines for Human Use in its Assessment Report on G*entiana lutea*, the bitter constituents (2-8%) are located mostly in the cortex of the plant roots. The bitterest constituents belong to the class of secoiridoid glycosides, with gentiopicroside (also known as gentiamarine and

gentiopicrine) as main component and a lower amount of amarogentin (0.02 to 0.4%). The occurrence of swertiamarine and sweroside has been reported occasionally. The bitter value of gentiopicroside is 12000; that of amarogentin are 58 million, the bitterest substance known. The quantity of the bitter constituents depends on the season as well as the age of the roots and the altitude. The total content increases with the altitude and reaches its maximum in spring (EMA, 2009).

Zimmermann (1986) has postulated a potential immunological influence of bitter compounds of *Gentiana lutea* by studying immunoglobulin in healthy and control populations and the use of Gentiana extracts caused an elevation of salivary immunoglobulin.

Mahady *et al.*, (2005) assessed the in vitro susceptibility of 15 *Helicobacter pylori* strains to botanical extracts with uses in the treatment of gastrointestinal disorders. Among the methanolic extracts, the least value of MIC (100 µg/ml) was seen in *Gentiana lutea* root extracts.

Kondo *et al.*, (1994) evaluated the hepatoprotective activity of gentiopicroside in immunologically induced acute liver injury mice models. With the use gentiopicroside, the liver injuries were significantly suppressed at doses of 30- 60 mg/kg/day.

Kušar and Baričevič (2006) reported considerable antioxidant properties of methanolic extracts of gentian leaves and roots using DPPH and superoxide radicals compared with butylated hydroxyanisole as standard. This study provides proofs that gentian leaves and roots exhibit considerable antioxidant properties.

In a study done by Ozturk *et al.* (1998) the ethanolic extract from *Gentiana lutea* roots contain 21% gentiopricroside, 5.2% swertiamarin and 2.55% sweroside revealed potential hepatoprotective activity using mice models.

An extensive study by Li *et al.*, (2015) in *G. scabra* revealed the presence of three new secoiridoid glycosides and two novel phenolic compounds. Fifty secondary metabolites were isolated from the roots of *G. scabra*, including sixteen secoiridoid glycosides, four iridoid glycosides, seven phenolic compounds, two lignans and twenty one triterpenoids. He mentioned iridoid and secoiridoid glycosides as the primary components of members of the genus *Gentiana*, especially from a chemotaxonomic viewpoint.

Secoiridoids such as swertiamarin, gentiopicrine and sweroside and xanthone mangiferin are the common compounds that were analyzed in a study by Mihailović *et al.*, (2011) in methanol, chloroform, ethyl acetate and n-butanol fractions of *G. asclepidea* roots by HPLC-DAD analyses. Gentiopicrine was observed as the most abundant secoiridoid compound in the extracts in the range of 14.39 to 442.89 mg/g. The sweroside contents in the extracts were 1.52 to 27.85 mg/g, while the swertiamarin

contents ranged from 5.74 to 16.62 mg/g. The n-butanol fraction possessed the highest amount of secoiridoid compounds and its swertiamarin, sweroside and gentiopicrine contents were found to be 16.62, 27.85 and 442.89 mg/g of extract, respectively. No detectable amounts of mangiferin were found in roots extracts of *G. asclepiadea* (Mihailović *et al.*, 2011).

Chromatographic analysis of extracts of *G. depressa* has been shown to contain depresteroside in addition to isovitexin, sorientin and iridoid depressoside. The authors characterized loganin, isoscoparin, isovitexin 2"-glucoside and isorientin 2"-glucoside, together with the isolation and structural elucidation of 3"-glucosyl depresteroside and depressine, two new iridoid and seco-iridoid glycosides (Chulia *et al.*, 1996).

The phytochemical screening, quantitative estimation (%), TLC and anti-inflammatory screening indicate that the flower tops of *Gentiana Kurroo Royale* possess anti-inflammatory activity against the inflammation induced by carrageenan in acute phase in comparison to diclofenac. The *Gentiana kurroo* significantly inhibited paw edema as anti-inflammatory agent (Latif *et al.,* 2006).

Wani *et al.*, (2011) reported that the plant is rich in tannins, flavonoids, phenolics, cardiac glycosides, terpenes, and alkaloids. Flavonoids, alkaloids and terpenoids in the plant may be responsible for its effects as analgesic, anti-malarial properties and its use in treatment of stomach disorder. The extract showed strong analgesic activity to acetic acid induced writhing response in mice models and Eddy's hot plate method.

In the Nepalese context, the *Gentiana* species like *G. robusta* and *G. depressa* are among the studied plants. A study by Lamichhane *et al.,* (2014) states *G. depressa* as one of the most used medicinal plants around the areas of Langtang National Park. They performed phytochemical screening, antimicrobial tests and cytotoxic assays to prove the traditional uses and medicinal importance. Another study by Bhattarai *et al.*, (2009) checked the biological activities of some Nepalese medicinal plants including *G. robusta.*

2.6 Phytochemical Extraction, Separation and Analysis of Plant derived Compounds

2.6.1 Phytochemical Extraction

The collection, preservation, extraction and storage of the plant material are important steps in a scientific study. For most pharmacological and phytochemical analyses, extraction of dried plant material is preferred to using fresh plant material. The dried plant material is easier to handle and more stable because water content in fresh plant

material changes over time causing a wide variation in plant chemical composition and properties (Makkar, 2000).

The extraction of plant material using different solvents is based on the biological concepts of permeability of the plant cells. Dried plant material extraction can be achieved using different solvent systems. These include the use of a single solvent or a series of solvents of increasing polarity either sequentially or non-sequentially. The dried plant material is extracted using different solvents to obtain the bioactive compound(s) present in it for pharmacological investigation. It is importance to use a similar extraction method employed by the local people for traditional medicinal plant research (Gurib-Fakim, 2006). This will enhance the extraction of the same natural bioactive product used by the people and probably help in validation of a medicinal plant.

It has been estimated that our world is inhabited by 250,000 higher plant species and more than 80,000 plant species are reported to have at least some medicinal value and around 5000 species are known to have specific therapeutic values (Joy *et al.*, 1998; Tabassum *et al*., 2012). According to W.H.O report, 80% of the rural areas and depends on the traditional medicinal systems for their primary health care needs and medicinal plants could be the best sources for obtaining the varieties of drugs (Chhetri *et al.*, 2008, Bajracharya *et al.*, 2008). Several studies on the medicinal plants has revealed that medicinal plants contain compounds like peptides, unsaturated long chain fatty acids, aldehydes, alkaloids, essential oils, phenols, flavonoids which are either water or ethanol soluble. These phytochemicals are significant in therapeutic application against human and animal pathogens including bacteria, fungi and viruses (Bentley and Trimen, 1980).

2.6.2 Extraction Procedures

To obtain organic constituents from dried plant tissues, it is continuously extracted powdered in a soxhlet apparatus with a range of solvent, starting with non-polar solvent (to separate lipids and terpenoids) and then a polar solvent for more polar compounds such as ether, petroleum, chloroform, alcohol and ethyl acetate. The extract obtained is clarified by filtration and is then concentrated in vacuo (in a rotary evaporator) normally at temperatures ranging between 30°C and 40°C (Katz *et al.*, 1966; Salminen 2003; Houtman *et al.*, 2007). The concentrated extract may crystallize on standing. These are normally collected by filtration and their homogeneity tested for by chromatography in several solvents. In the presence of a single substance, the crystals are purified by recrystallisation and further analysis is carried out. With mixture of substances, the crystals are re-dissolved in suitable solvent and chromatographic methods are used to separate those (Katerere *et al.*, 2004).

2.6.3 Separation of Plant Derived Compounds

Chromatography techniques are mainly employed in separation and purification of plant constituents (Wagman and Cooper 1989; Bjornstad *et al.*, 2009). Chromatography involves a sample (or sample extract) being dissolved in a mobile phase (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible stationary phase. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase (Wagman and Cooper 1989; Bjornstad *et al.*, 2009).

There are many types of separation techniques based on the instrumentation and sophistication; the basic and preliminary being the chromatography techniques that includes paper chromatography (PC), thin layer chromatography (TLC), gas liquid chromatography (GC) and high performance liquid chromatography (HPLC). Chromatography can be used to separate and purify a large variety of substances, from chlorophyll and other plant pigments, through amino acids in cell or tissue samples, to dyes commonly found in foods.

High performance liquid chromatography and gas chromatography use narrow tubes called columns packed with stationary phase, through which the mobile phase is forced. The sample is transported through the column by continuous addition of mobile phase. This process is called elution. The average rate at which an analyte moves through the column is determined by the time it spends in the mobile phase (Smith *et al.*, 1965; Wagman and Cooper 1989; Bjornstad *et al.*, 2009).

Amarogentin: It is a seco-irridoid glycoside with one of the bitterest compound knownand the bitter taste persists even at a dilution of 1g in 14000 liter 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 topoisomerase inhibition (Ray *et al.,* 1996), chemopreventive, anti-leishmanial (Medda *et al.,* 1999) and gastroprotective properties (Niiho *et al.,* 2006). It is found in most of the members of Gentianeceae.

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 (Zheng and Lu 1990), anti-atherogenic, immunodilatory (Guha *et al.*, 1996), anti-proliferative and diuretic properties (Sanchez *et al.* 2000). It is also reported to have significant anti-

diabetic activity similar to the clinical drug Glibenclamide (Andreu *et al.,* 2005). Mangiferin significantly increased heart tissue phospholipids in isoproterenol induced cardio-toxic rats suggesting cardioprotective and hypolipidemic effects (Nair and Devi 2006). The content of mangiferin is very less in content in most of the *Gentiana* species (Li *et al.,* 2015; Mihailovic *et al.*, 2013).

Swertiamarin: It is a secoirridoid that has been reported to possess hepatoprotective, anti-inflammatory, anti-bacterial, anti cholinergic, free radical scavenging and antispastic (Vaijanathappa and Badami, 2009). It is one of the most common compounds reports in most of the Gentian genus. 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).

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 and Guiochon, 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-quantification by *GelQuant.NET* software

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 i*ntuitive 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 3: MATERIALS AND METHODS

3.1 Materials

3.1.1. Chemicals and Reagents

All the chemicals and reagents, listed in Appendix A, that were used during this research work were of analytical grade (Merck Co., Mumbai). The total research experiment was conducted at the Central Department of Biotechnology, Tribhuvan University, Kirtipur, and Kathmandu.

3.1.2 Plant Samples

All the plant samples were collected as whole plants from their wild habitat from various districts of Nepal. The whole plants that were collected were identified by a taxonomist from Central Herbarium and Plant Laboratory, Godawari, Lalitpur. Voucher specimens (Herbaria) were deposited at the Central Herbarium and Plant Laboratory.

3.1.3 Bacterial Strains

ATCC cultures of *Escherichia coli* (25922), *Salmonella paratyphimurium* (14028), *Klebsiella pneumonia* (700603), *Staphylococcus aureus* (25923) and *Enterococcus faecalis* (25912) were used.

3.2 Selection of the plant samples

The *Gentiana* plant samples are among the least studied species from Nepal or elsewhere. So, the plants were selected according to the unexplored or new/novel plants from the Nepalese Himalayas and their ethnomedicinal uses as well as ease if accessibility.

3.3 Collection and identification of the plant samples

Whole plants were collected from different places of Central Nepal during their flowering periods of August to October, 2013. The selected samples collected were photographed and altitude of the collection sites was measured using GPS. Identification of the plants material was done by a taxonomist from Central Herbarium and Plant Laboratory. Herbariums were prepared and voucher specimens are submitted for deposition at National Herbarium, Godawari, Lalitpurfor the future references. Following species of *Gentiana* were collected from the different places of Nepal. The *G. urnula* samples were purchased from a local vendor in Kathmandu.

Table 3.1 Collection of different *Gentiana* species from various places of Central Nepal

S.N.	Name of the plant	Place of collection	Altitude	Part of the plant collected
1	*Gentiana ornata*	Way to Cholangpati, Rasuwa	2610 m	Whole plant
2	*Gentiana capitata*	Way to Cholangpati, Rasuwa	3086 m	Whole plant
3	*Gentiana depressa*	Kalinchowk Temple, Dolakha	3407 m	Whole plant

3.4 Preparation of the plant material

The collected plant material were cleaned off mud, fungi and any unwanted materials, then shade dried at 32-35°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 fine powder was collected on sterile and dry polyethylene bag for the extraction of phytochemicals**.**

3.5 Preparation of plant extracts and extracts dilution

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

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

The resulting dry extract was then sealed and stored at 4 $^{\circ}$C until use.

Each 20 mg of plant extract was weighed accurately and dissolved on 1 ml methanol. This 20 mg/ml stock of each plant extract was used for antioxidant activity,

quantification of the total phenol and total flavonoids; and also for the quantification of the major Gentian phytochemicals.

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

3.6 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 *et al*., 1989.

Test for flavonoids:

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

Test for glycosides:

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

Test for steroids:

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

Test for terpenoids:

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

Test for saponins:

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

Test for alkaloids:

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

Test for phenols and tannins:

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

3.7 Quantitative phytochemical analysis

3.7.1 Total polyphenol content

The total polyphenol content of *Gentiana* species was determined using the Folin–Ciocalteu phenol reagent (Chang et al., 2002; Roy *et al*., 2010). 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 ($mgGAEg^{-1}$). The test was triplicated for the reproducibility of results.

3.7.2 Total flavonoid content (Chang *et al*., 2002)

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) 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 four *Gentiana* species and standard (Ascorbic acid) was assessed on the basis of the radical scavenging effect of the stable 1, 1- diphenyl-2 picrylhydrazyl (DPPH) - free radical activity following the protocol of Chang *et al*. (2002).

Different concentration of plant extract (30-270µg/ml) 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.

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

$$\%\ Radical\ scavenging\ activity = [(Control\ abs - sample\ abs)/Control\ abs)]\ x100\%$$

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 $IC50\ = EXP\ (LN\ (conc. > 50\%) - ((pi > 50\% - 50)/\ (pi > 50\% - pi < 50\%) * LN\ (conc. > 50\%/conc. < 50\%)))$using 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 Identification of major phytochemicals by Thin Layer Chromatography

As the family of the *Genatiana* are rich in various compounds, their visualization and their software semi-quantification was done using Thin Layer Chromatography (TLC) to observe the main components of *Gentiana* species: Amarogentin, Mangiferin and Swertiamarin present in their methanol extracts.

Silica TLC plates were prepared for initial detection and analysis. To prepare a 1.0 mm thick Silica TLC 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 area until use. Plastic coated prepared TLC plates were used for subsequent optimization and visualization of extracts.

3.8.1 Preparation of standard

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

3.8.2 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 µl 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 Wagner and Bladt (1996). 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 (Rugutt, 1996; Wagner and Bladt, 1996). 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.

3.9 Semi-quantitative estimate of major compounds using *GelQuant.NET* software

The compounds that were visualized using the TLC were estimated semi quantitatively 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 were used. 3 to 5 µL 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.

3.9.1 Preparation of standard curve

For the preparation of standard calibration curve and to assess linearity different concentration of the marker stock solutions (500 µg/ml, 250µg/ml, 125µg/ml and 62.5µg/ml) were applied in different tracks as bands to furnish in the amounts in the range of 0.5-0.0625 µg per band. The peak areas in terms of pixels were plotted against

the corresponding concentrations and regression analysis was done to obtain the calibration equation.

3.9.2 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.

3.10 Determination of Antibacterial activity

3.10.1 Preparation of Nutrient agar

For the preparation of NA plates and NA slants, about 28 gram of the powder (Hi Media Laboratories Pvt. Ltd, Mumbai, India) was carefully weighed and poured in distilled water. The contents were dissolved on the water completely and the final volume was maintained to 1000 ml followed by boiling for uniform mixing. This media was sterilized on an autoclave at 15lbs pressure at 121°C for 15 minutes. The autoclave tape was used as an indicator for the completeness of sterilization. After cooled to about 45-50°C, the media was poured on sterilized and properly labeled petri dishes. About 25 ml of the media was poured on each petri dish 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 and molten NA media and placing them in an inclined position. These bottles were left for solidification.

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

The Luria Bertani broth, (LB) Miller media was prepared by mixing 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 MHA 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 petri dishes under aseptic conditions.

3.10.3 Preparation of the standard bacterial culture Inoculums

Pure ATCC cultures 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.

3.10.4 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 and 12.5 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.

3.11 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 Student's t test and ANOVA and mean values were compared. Differences at $P<0.05$ were considered to be significant. All the statistical analyses were done using GraphPad Prism7 and Microsoft Excel 2007.

CHAPTER 4: RESULTS

4.1 Percentage yield of plant extracts

All the plant samples collected were subjected to methanol and aqueous extraction. The total amount of methanol and aqueous plant extract isolated and the characteristics of the extracts from 10 grams of the finely powdered whole plant material are shown in Table 4.1.

Table 4.1: Total percentage yields of methanol and aqueous extracts of *Gentiana* species

Plant extracts	Dry weight taken (gm)	Weight of plant extract(gm)	Yield (%)	Part of the plant used	Color of the extract	Consistency of extract
GUR-MET	10	0.87	8.7	Whole plant	Brown	Greasy
GUR-AQ	10	1.04	10.4	Whole plant	Light brown	Greasy
GOR-MET	10	0.81	8.1	Whole plant	Grey	Sticky
GOR-AQ	10	0.78	7.8	Whole plant	Brown grey	Greasy
GDE-MET	10	0.73	7.3	Whole plant	Grey	Greasy
GDE-AQ	10	0.88	8.8	Whole plant	Grey	Greasy
GCA-MET	10	1.19	11.9	Whole plant	Deep green	Oily
GCA-AQ	10	0.84	8.4	Whole plant	Green	Sticky

MET- Methanol **AQ- Aqueous** **GUR- *Gentiana urnula***

GOR- *Gentiana ornata* **GDE- *Gentiana depressa*** **GCA- *Gentiana capitata***

The highest and lowest yield of extract was found in G*. capitata* (11.90%) and *G. depressa* (7.34%) respectively in case of methanol extracts. Similarly the highest and lowest percentage yield was found in *G. urnula* (10.40%) *and G. ornata* (7.8%) respectively in aqueous extracts.

4.2 Qualitative Phytochemical Analysis

Both methanol and aqueous extracts were subjected to various reagents to observe the presence of secondary metabolites present in the samples. The below tables, Table 4.2, lists the findings.

Table 4.2: Qualitative phytohemical analysis of different *Gentiana* species

Plant Extract	Alkaloid	Resins	Phenol	Flavonoid	Glycosides	Diterpenes	Tannin	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
GUR-MET	+	+	+	+	+	+	+	+
GUR-AQ	+	-	+	-	+	-	-	+
GOR-MET	+	+	+	+	+	+	+	+
GOR-AQ	+	-	+	-	+	+	+	-
GDE-MET	+	+	+	+	+	+	-	-
GDE-AQ	+	+	-	+	-	-	-	+
GCA-MET	+	-	+	+	+	+	+	+
GCA-AQ	+	-	-	-	+	-	-	+

+ Positive **-Negative or Not Detectable** **MET- Methanol**

AQ- Aqueous **GUR-** ***Gentiana urnula*** **GOR-** ***Gentiana ornata***

GDE- ***Gentiana depressa*** **GCA-** ***Gentiana capitata***

The table clearly shows the presence of major secondary metabolites in various species of *Gentiana* methanol and aqueous extracts. Comparatively, methanol extracts were found to contain phenols, glycosides, flavonoids and phytosterols; and fewer amounts of resins, diterpenes and tannins than their aqueous extracts.

4.3 Total Polyphenol Content

Standard graph of gallic acid was obtained by plotting the absorbance at 765 nm against concentration ranging from 25µg/ml to 250µg/ml is shown in Figure 4.1.

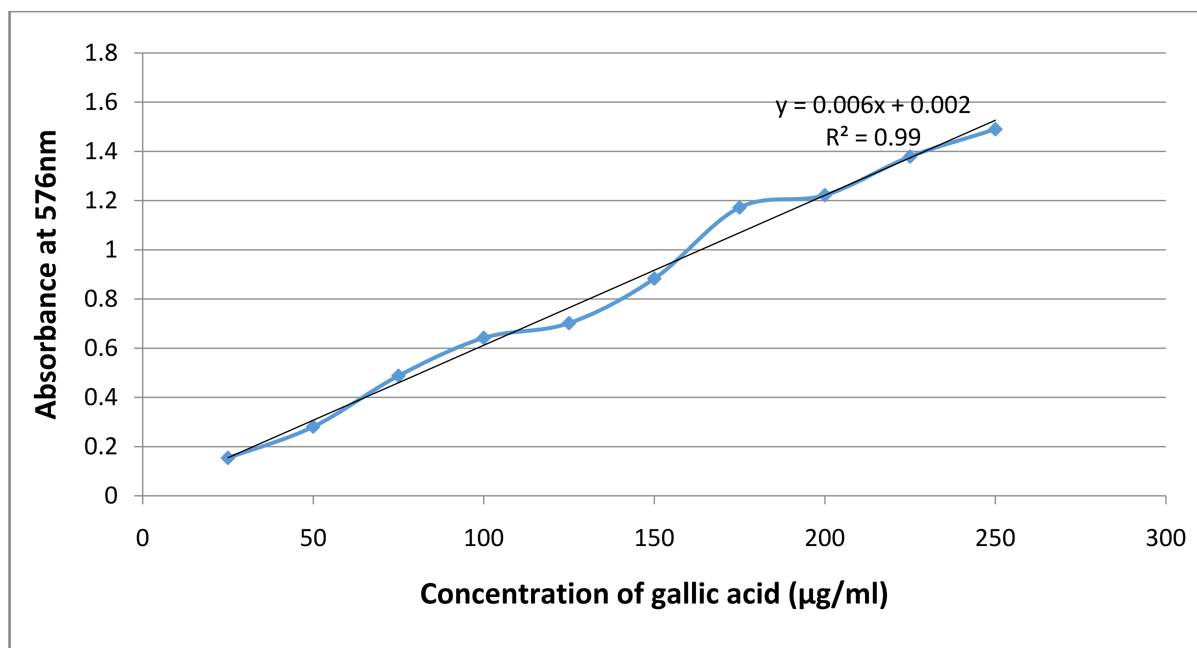

Figure 4.1: Standard graph of Gallic acid

The total polyphenol content present in methanol and aqueous extracts of different species of *Gentiana* are presented in Table 4.3. For the methanol extracts, the highest polyphenol content 79.2±6.39 mgGAE/gm was found in *G. depressa* while the lowest concentration of polyphenol 44.6±3.97 mgGAE/gm was found in *G. capitata.* Similarly in the aqueous extract, *G. depressa* had the highest 59.64±3.64mgGAE/gm polyphenol content whereas the lowest polyphenol content was found in *G. urnula* with 37.11±2.18 mgGAE/gm*.* The polyphenol content of other species remained in between these two extremes. The polyphenol content in the methanol extract of *G. depressa and G. ornata* were found to be significantly higher than other two *Gentiana* species (P<0.05). For the aqueous extracts, the polyphenol content in *G. depressa* was significantly higher than all other species of *Gentiana* (P<0.05), however, no statistically significant difference in the polyphenol content was observed between aqueous extracts of *G. urnula*, *G. ornata* and *G. capitata,* respectively*.* Finally, the polyphenol content was found to be significantly higher (P<0.05) in the methanol extracts of *G. depressa*, *G. ornata* and *G. urnula* compared to their aqueous counterpart.

Table 4.3: Total Polyphenol content of methanol and aqueous extracts of different species of Gentiana

Gentiana sps	100% Methanol	Aqueous
G. capitata	44.6±3.97[c]	39.71±2.16[b]
G. depressa	79.2±6.39[a]	59.64±3.64[a] *
G. ornata	76.84±6.43[a]	40.35±2.06[b] *
G. urnula	60.62±3.27[b]	37.11±2.18[b] *

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.

4.4 Total Flavonoid Content

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

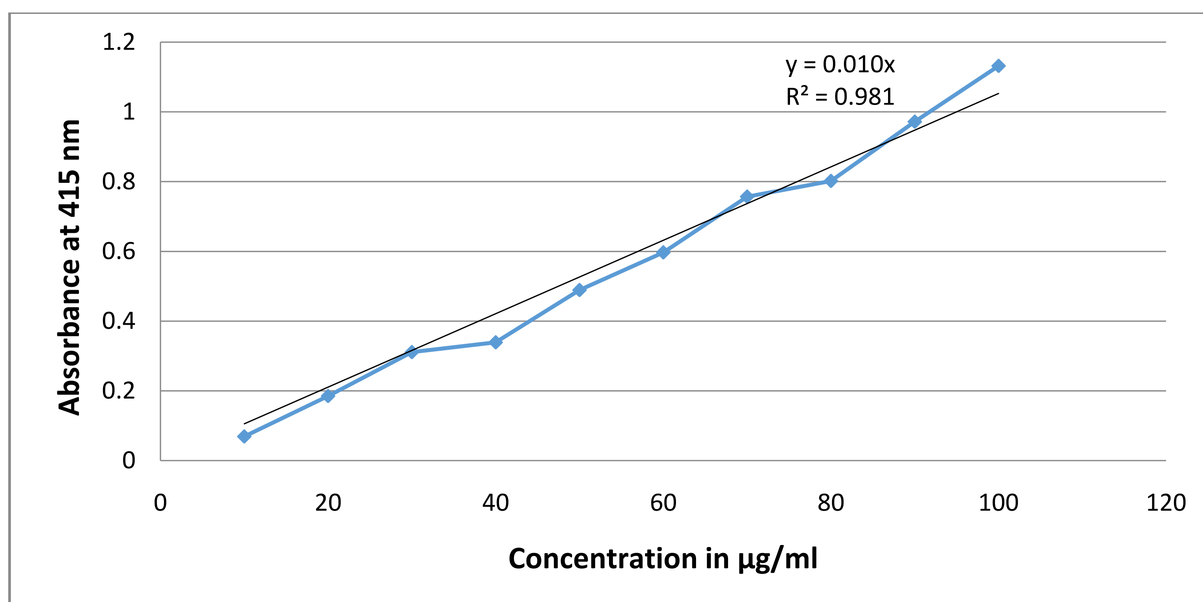

Figure 4.2: Standard graph of Quercetin

Highest flavonoid content of 19.09±0.97 mg QE/g was observed in *G. capitata* while the lowest amount of flavonoid 11.31±0.49 mg QE/g was recorded for *G. ornata* in methanol extracts. The highest and lowest values were 7.85±0.37 for *G. ornata* and 4.22±0.66 for *G. urnula* respectively in aqueous extracts. The flavonoid content in methanol extract of *G. captitata* and *G. depressa* were significantly (P<0.05) greater than that of *G. urnula* and *G. ornata.* However, the flavonoid content in *G. captiata* and *G. depressa* were not significantly different from each other. In the aqueous extract, flavonoid content of *G. ornata* was found to be significantly (P<0.05) higher than the flavonoid content of *G. urnula*, *G. capitata* and *G. depressa*. However, there was no significant difference between the mean flavonoid contents of the three latter species. The flavonoid content in the methnol extracts was found to be significantly (P<0.05) greater than the flavonoid content in aqueous extract for all the Gentian species studied. The flavonoid content of methanol and aqueous extracts were significantly different in all of the *Gentiana* species studied.

Table 4.4: Total Flavonoid content of methanol and aqueous extracts of various *Gentiana* species.

Gentiana sps	100% Methonol	Aqueous
G. capitata	19.09±0.97^{a}	5.07±0.46^{b}*
G. depressa	16.66±1.24^{b}	5.39±0.50^{b}*
G. ornata	11.31±0.49^{c}	7.85±0.37^{a}*
G. urnula	14.93±1.44^{b}	4.22±0.66^{c}*

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.

4.5 Total DPPH Antioxidant activity

The antioxidant activity of methanol and aqueous extracts of *Gentiana* species was determined using DPPH Free Radical Scavenging Assay using ascorbic acid as a standard compound. IC_{50} value was calculated for each sample taking the concentration vs. % radical scavenging activity. The graph of standard ascorbic acid was drawn using various concentrations of ascorbic acid (5µg/ml to 100µg/ml) against the DPPH radical scavenging activity. IC_{50} value was calculated for ascorbic acid and each of the extracts.

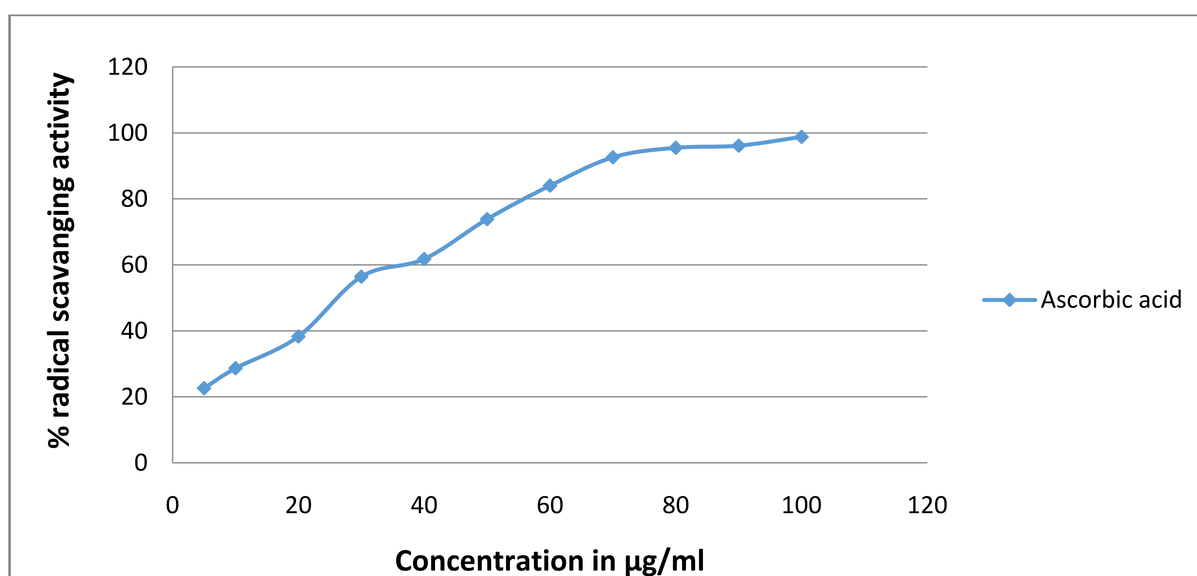

Figure 4.3: Percentage DPPH Radical Scavenging Activity of Ascorbic Acid

For methanol extracts, highest scavenging activity was seen in *G. depressa* accounting 81.68% and lowest observed was 59.76% in *G. capitata* at 100µg/ml (Fig 4.4). Among the aqueous extracts, highest radical scavenging activity was seen in *G. depressa* at 80.03% and lowest was observed 53.63% in *G. capitata* at 500µg/ml (Fig 4.5). Higher concentration of plant extracts showed an increasing percentage of radical scavenging activity, revealing concentration dependent scavenging property.

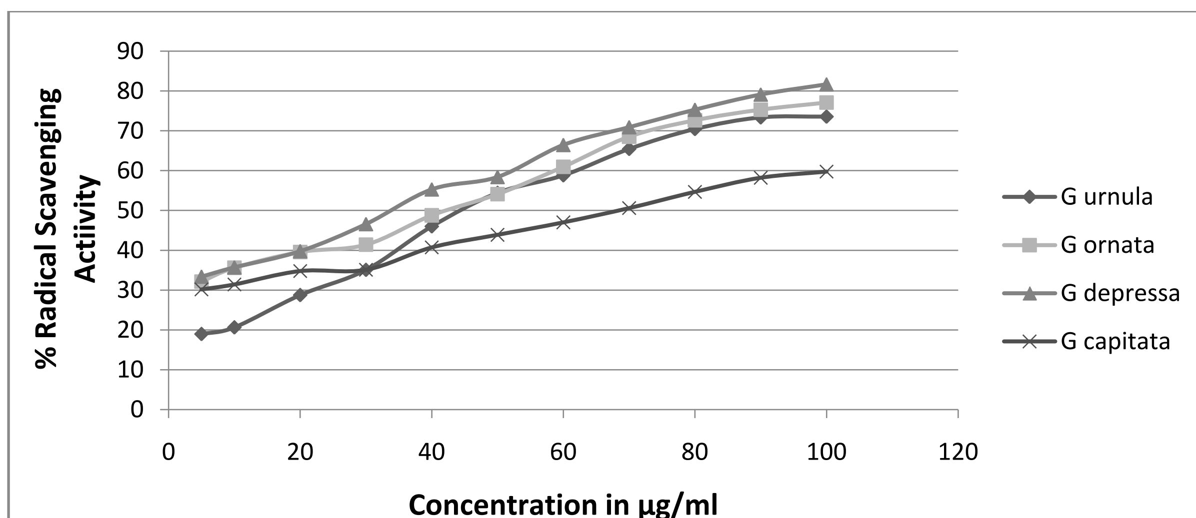

Figure 4.4: Percentage DPPH Radical Scavenging Activity of methanol extracts of *Gentiana* species

The IC_{50} value for ascorbic acid was found to be 29.55±2.13µg/ml. According to this comparison, the lowest and highest IC_{50} values were observed in methanol extracts of *G. depressa* (39.57±0.95 µg/ml) and *G. capitata* (80.12±3.69 µg/ml) respectively (Table 4.4). The species with lower IC_{50} are considered as better antioxidants. Therefore, *G. depressa* has the best antioxidant activity among the studied Gentian species. The IC_{50} value of ascorbic acid was significantly ($P<0.05$) lower than that of all the four Gentian species. Among the species, the IC_{50} value of the methanol extract of *G. depressa* was significantly ($P<0.05$) lower than the three remaining species. Likewise the IC50 value of *G. ornata* and *G. urnula* were also significantly lower than that of *G. capitata*.

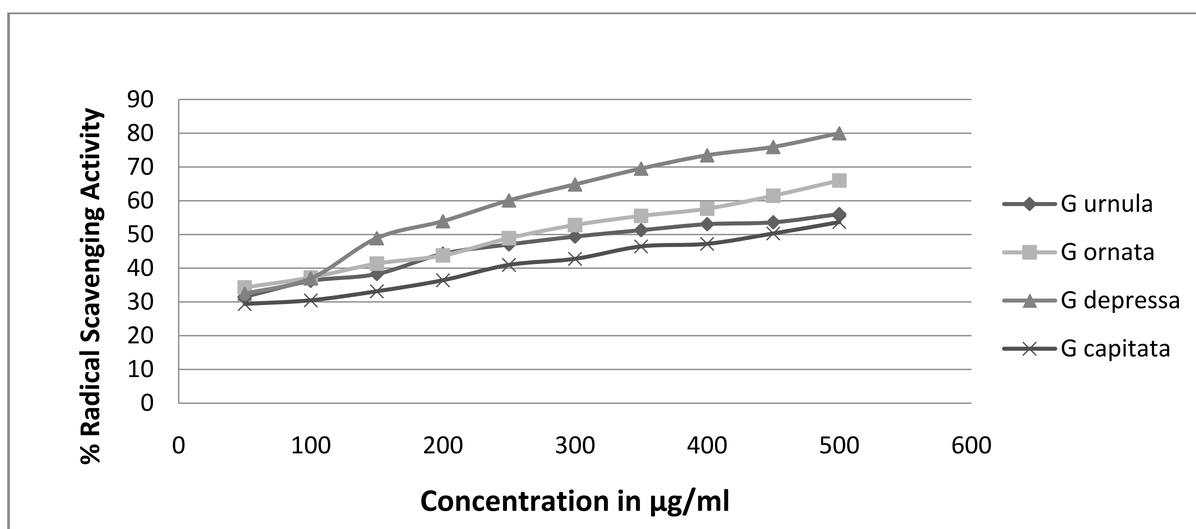

Figure 4.5: Percentage DPPH Radical Scavenging Activity of aqueous extracts of *Gentiana* species

Table 4.5: IC_{50} values of different *Gentiana* species

Gentiana sps	100% Methanol	Aqueous
G capitata	80.12±3.69[a]	433.3±28.87[a] *
G depressa	39.57±0.95[c]	183.19±28.75[c] *
G ornata	49.8±2.22[b]	323.31±54.28[b] *
G urnula	51±2.31[b]	283.24±28.23[b] *

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 contrast to the methanol extracts, aqueous extracts reported significantly reduced anti oxidative capacity. The IC_{50} values of each aqueous extract was found to be greater than 150µg/ml and lagged far behind in comparison to ascorbic acid or their respective methanol extracts. Among these aqueous extracts, lowest IC_{50} value was observed in *G. depressa* with the value of 183.193±28.75 µg/ml (Table 4.4). The plant with highest IC_{50} or least radical scavenging activity was *G. capitata* at 433.30±42.83 µg/ml. The IC_{50} value of the aqueous extract of *G. depressa* was significantly (P<0.05) lower than the three other Gentian species.

4.6 Correlation between TPC and IC_{50} values

Correlation analysis showed a significant negative correlation between IC_{50} values and total phenolic content in the methanol extracts with Pearson's r value of -0.927 at $P<0.05$ (Figure 4.6). The higher the phenolic content lower is the IC_{50} value and vice versa. The correlation between the IC_{50} values and total phenolic content in the aqueous extracts showed an inverse trend but was not significant ($P<0.05$) with Pearson's r value of -0.793 (Figure 4.7).

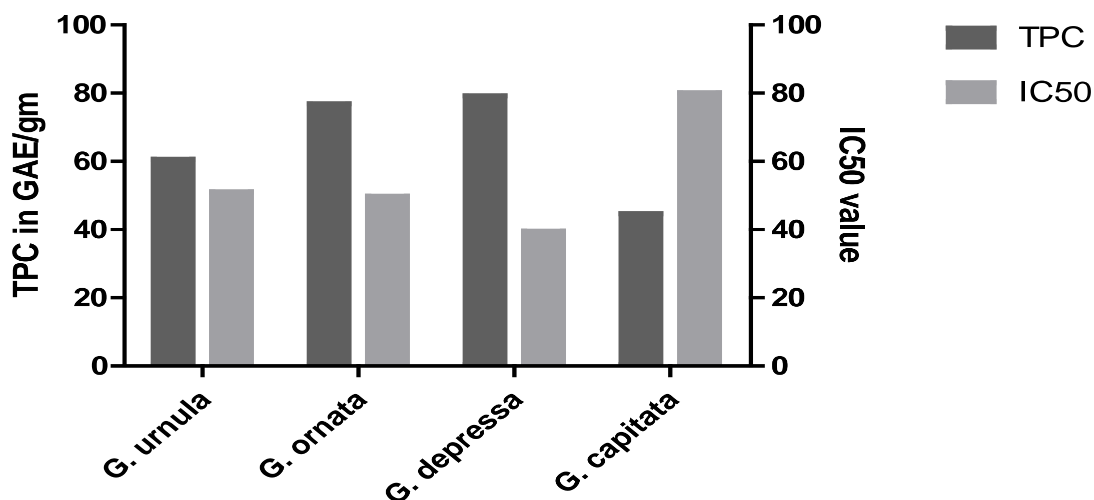

Figure 4.6: Correlation between TPC and IC_{50} value in methanol extracts.

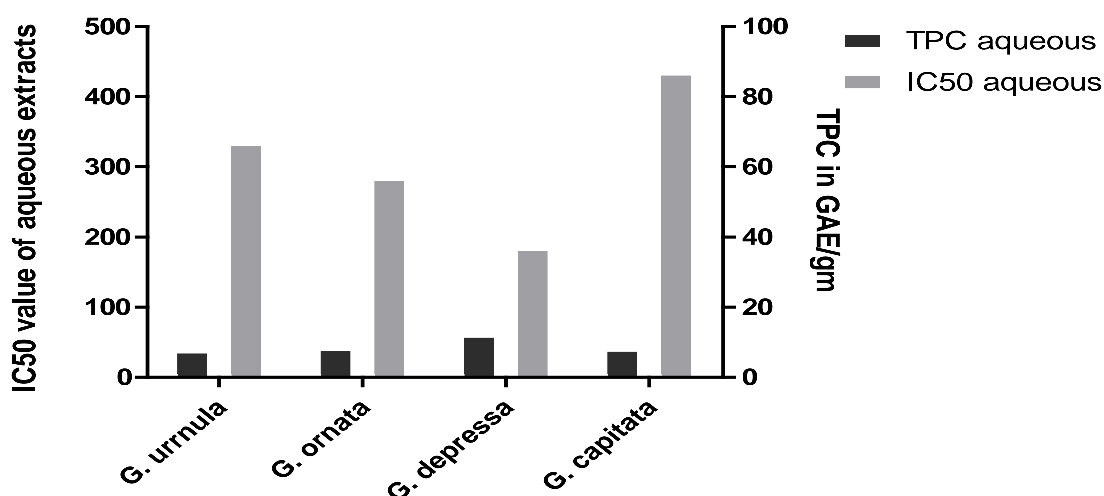

Figure 4.7: Correlation between TPC and IC_{50} value in aqueous extracts.

4.7 Semi-quantitative estimation of compounds by TLC

All the four *Gentiana* species were tested for presence of phytochemicals like Amarogentin, Mangiferin and Swertiamarin in a TLC plate. All the species showed the presence of compounds according to their observed intensities. Only their methanol extracts were used in TLC detection and compound estimation as the aqueous extracts were not completely soluble.

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 *Gentiana* methanol extracts and compared accordingly.

4.7.1 Semi-quantitative estimation of amarogentin

Standard curve of amarogentin was prepared by taking various concentrations of standard swertiamarin compound against respective pixel ratio are shown in Figure 4.8.

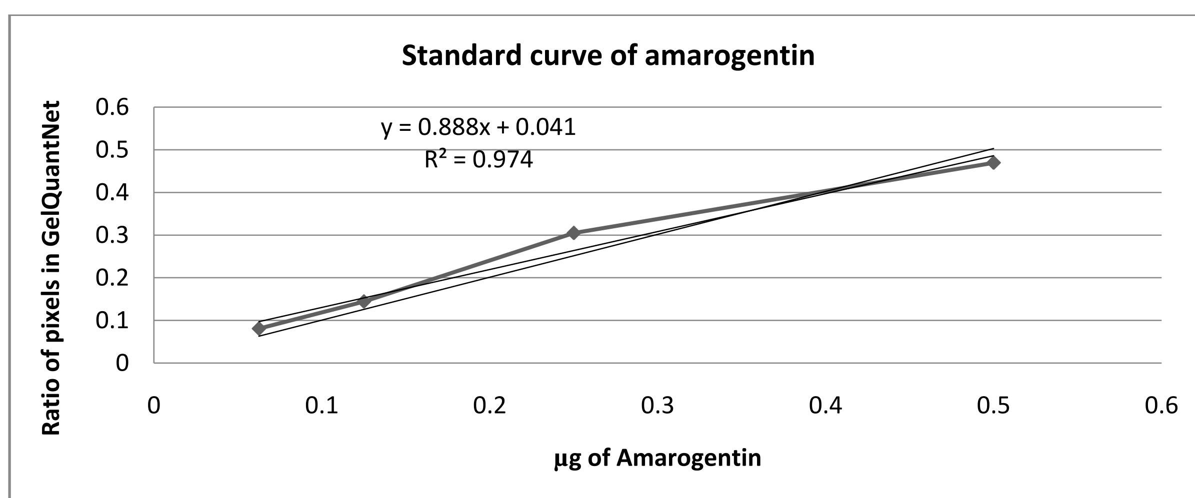

Figure 4.8: Standard curve of amarogentin

The highest content of amarogentin (0.075±0.005 mg/gm DW) was observed in *G. capitata* and lowest (0.026±0.006 mg/gm DW) in *G. depressa* methanol extracts. The content of amarogentin in other species was in between these two extremes and is shown in the Figure 4.9. The amarogentin content of *G. capitata* was significantly ($P<0.05$) greater than the amarogentin content of three remaining species. The amarogentin content among these three species were not significantly different from each other.

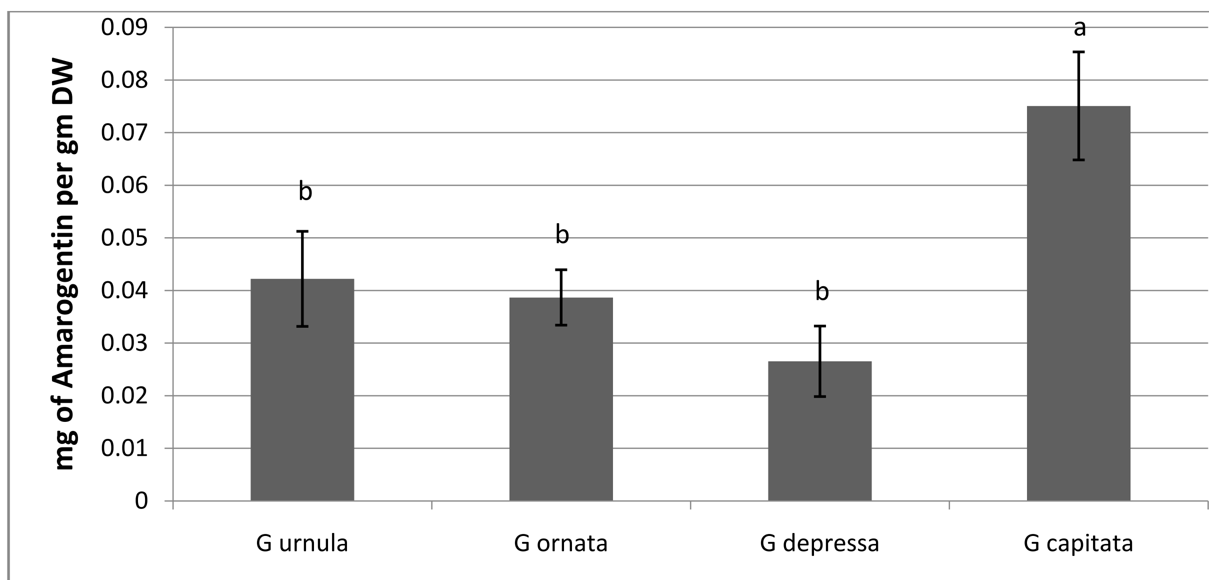

Figure 4.9 Semi-quantitative estimation of amarogentin in different species of *Gentiana.*

Note: Each bar with different letters are significantly different (P<0.05).

4.7.2 Semi-quantitative estimation of Swertiamarin

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

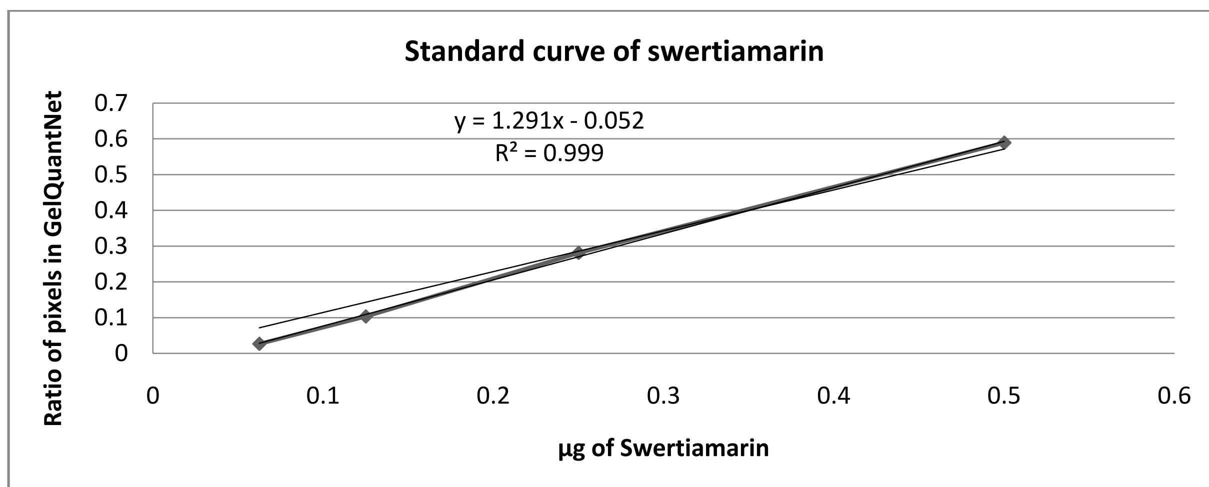

Figure 4.10 Standard curve of Swertiamarin

The highest content (0.109±0.013 mg/gm DW) of swertiamarin was observed in *G. ornata* and lowest (0.038±0.002 mg/gm DW) was observed in *G. depressa*. The content of swertiamarin in other species was observed in between these two extremes and is

shown in the Figure 4.11. The swertiamarin content of *G. ornata* was found to be significantly greater than the content in *G. urnula, G. capitata and G. depressa*. However, the swertiamarin content in *G. urnula* was also found to be significantly (P<0.05) greater than that of *G. capitata* and *G. depressa*.

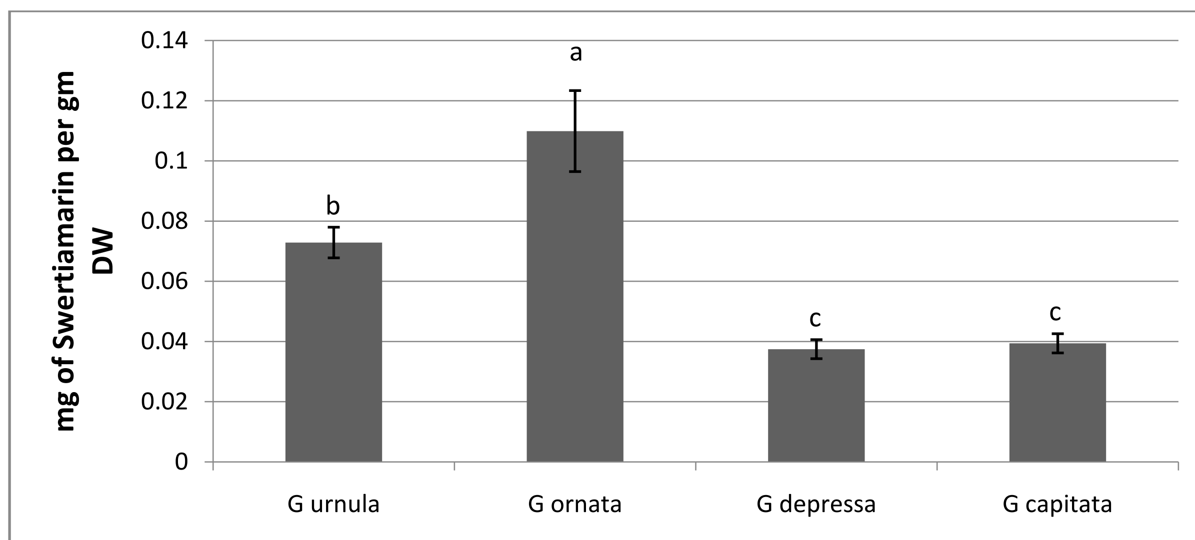

Figure 4.11 Semi-quantitative estimation of swertiamarin in different species of *Gentiana.*

Note: Each bar with different letters are significantly different (P<0.05).

4.7.3 Semi-quantitative estimation of mangiferin

Standard curve of mangiferin prepared by taking various concentrations of standard mangiferin compound against respective pixel ratio is shown in Figure 4.12.

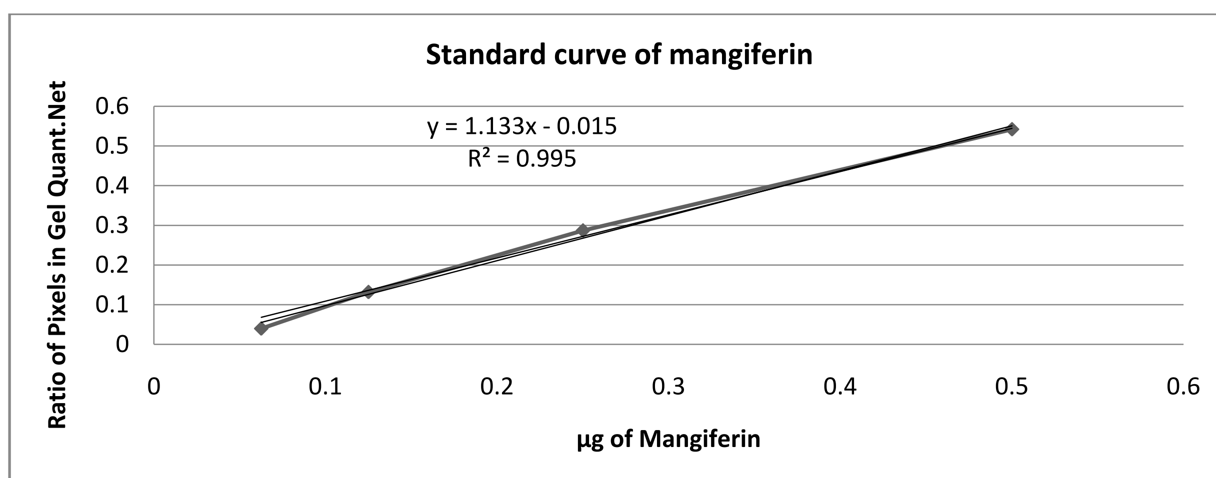

Figure 4.12: Standard curve of mangiferin

The highest content of mangiferin (0.018±0.001 mg/gm DW) was reported in *G. ornata* and lowest (0.012±0.002 mg/gm DW) in for *G. depressa* methanol extracts. The content of mangiferin in other species was in between these two extremes (Figure 4.13). The mangiferin content of the four species of *Gentiana* were not significantly different from each other.

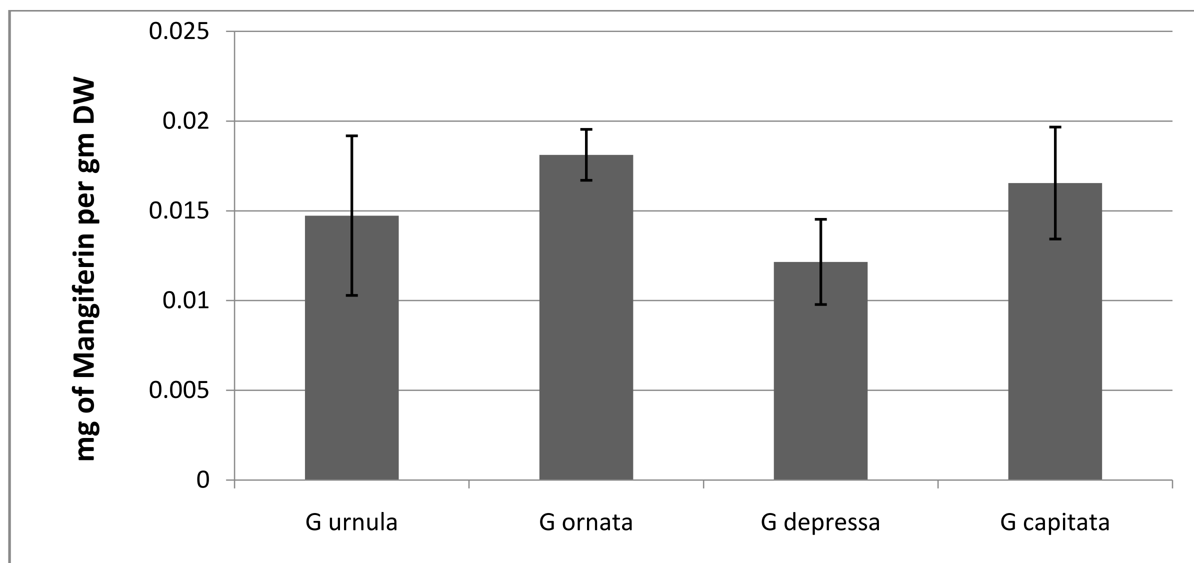

Figure 4.13 Semi-quantitative estimation of mangiferin in different species of *Gentiana.*

4.8 Antibacterial Assay

The antibacterial activity of both methanol extracts of *Gentiana* species were tested against ATCC cultures of *Escherichia coli* (25922), *Salmonella typhii* (14028), *Klebsiella pneumonia* (700603), *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 4.6 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

For the standard drug Streptomycin, all the six bacterial strains gave zone of inhibition that is represented in the above table 4.6. The zone of inhibition for *Klebsiella pneumonia* was comparatively higher than the other strains.

Table 4.7 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	+ve control	-ve control
G. urnula	*E. coli*	10	8	7	-	25	-
	S. typhii	7	6	-	-	30	-
	K. pneumoniae	11	8	6	-	41	-
G. ornata	*E. coli*	14	12	8	6	25	-
	S. typhii	9	7	6	-	30	-
	K. pneumoniae	18	16	16	12	41	-
G. depressa	*E. coli*	12	9	8	8	25	-
	S. typhii	10	8	7	-	30	-
	K. pneumoniae	14	12	10	7	41	-
G. capitata	*E. coli*	7	6	-	-	25	-
	S. typhii	10	7	6	-	30	-
	K. pneumoniae	11	9	7	-	41	-

The above table shows that the methanol extracts of all the Gentian species possessed poor inhibitory activity against *E. coli*, *S. typhii* and *K. pneumonia*. The zone of inhibition increased with the increase in concentration of extract used. Activity against *E. coli* was observed in all Gentian species at concentrations of 50mg/ml and 100mg/ml. The methanol extracts of *G. depressa* and *G. ornata* inhibited *E. coli* at all of the concentrations. The largest zone of inhibition for *E. coli* was recorded at 14mm in methanol extract of *G. ornata* and the smallest zone of inhibition was recorded at 7mm for *G. capitata*. *G. urnula* and *G. depressa* did not show any inhibition of *E. coli* at 12.5mg/ml concentrations. Activity against *Salmonella typhii* was observed at the concentration of 100mg/ml, 50 mg/ml and 25mg/ml for all samples. No zone of inhibition was observed at 12.5mg/ml concentration for any of the samples. Highest zone of inhibition was measured for *G. depressa* and *G. capitata*, 10mm at 100mg/ml concentration and the lowest zone of inhibition was measured at 7mm for *G. urnula*. Activity against *Klebsiella pneumoniae* was observed at all of the concentrations in *G. ornata* and *G. depressa*. No zone of inhibition was observed for *G. urnula* and *G. capitata* at 12.5mg/ml concentration. Highest zone of inhibition was measured for G. ornata i.e., 18mm at 100mg/ml concentration. And lowest zone of inhibition was shown by *G. urnula* and *G. capitata* i.e. 11mm at 100mg/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)**					
		100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	+ve control	-ve control
G. urnula	*S. aureus*	9	18	6	-	29	-
	E. feacalis	12	8	7	6	28	-
G. ornata	*S. aureus*	10	8	6	-	29	-
	E. feacalis	14	12	8	6	28	-
G. depressa	*S. aureus*	12	9	6	-	29	-
	E. feacalis	9	8	6	-	28	-
G. capitata	*S. aureus*	-	-	-	-	29	-
	E. feacalis	7	6	-	-	28	-

For *S. aureus* the highest zone of inhibition was measured at 12mm in 100mg/ml of *G. depressa* extract and the lowest zone of inhibition was found to be 9mm in 100mg/ml of *G. urnula* extract. *G. capitata* did not show any inhibitory effect on *S. aureus* at any of the concentrations. Similarly the highest zone of inhibition against *E. feacalis* was measured at 14mm in 100mg/ml concentration of *G. ornata* extract. *G. capitata* showed lower inhibitory effect on *E. feacalis* compared to other *Gentiana* extracts used. All of species studied reported very less zone of inhibition as compared to the standard drug Streptomycin.

CHAPTER 5: DISCUSSION

The use of medicinal plants for the treatment of various human ailments is evident from their ethno medicinal aspects and is scripted in various old repositories like Ayurveda and various pharmacopoeias. High value medicinal plants have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from medicinal plants. The use of indigenous drugs of plant origin also forms major part of complementary and alternative medicines in the form of herbal drugs. Several approaches have been made for the discovery of drugs from the medicinal plants. But the approach based method on their ethno medicinal uses and bioactivity has been commonly practiced. Nepal is a store house and diverse habitat for different medicinal and aromatic plants. 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.

5.1 Percentage yield of plant extracts

The primary step of phytochemical investigation is the extract preparation from plants. The yield of plant extract depends on the parts of the plant material used, different solvents, content of the plant phytochemicals that are present within the plant species and the employed extraction procedures. A significant variation has been seen on the percentage yield of the plant extracts.

As per the earlier studies, the percentage yield has been reported from 6.11% in methanol and 2.31% in ether in case of *G. depressa* (Lamichhane *et. al.*, 2014). Different types of medicinal plants show varied yield of plant extracts. As the species of *Gentiana* are small stolons like herbs, they are light weight and contribute less to yields of the plant extracts. Aqueous extracts have comparatively more yield than other solvent extracts because of their higher polarity and hence ability to dissolve large number of primary as well as secondary metabolites. Alcohols are the preferred solvents in the extraction of Gentianeceae plants as the secoiriod and xanthones are more soluble in alcohols. Furthermore, higher biological activities have been reported in methanol than other solvents (Kweera *et. al.,* 2011).

5.2 Qualitative Phytochemical Analysis

Phytochemical screening is a primary and important step towards discovery of new drugs as it provides the information regarding the presence of a particular primary or secondary metabolite in the tested plant extracts. The presence of significant bioactive natural product indicates the necessity of their separation and isolation through suitable chromatographic techniques. The preliminary phytochemical screening of different

species of *Gentiana* revealed the presence of major phytochemicals like alkaloids, flavonoids, terpenoids, steroids and glycosides in both type of extracts. The findings of the present study are in accordance with the phyotchemical screening done by Wani *et al.* (2001) for *Gentiana kurro* and Lamichhane et *al.* (2014) for *G. depressa*.

As secoiridoid and xanthones are highly soluble in alcohols (Kweera *et. al.,* 2011), so the methanol extracts showed the presence of more secondary metabolites than their aqueous extracts.

5.3 Total Polyphenol Content

Phenols are the class of secondary metabolites with strong antioxidant features that can donate hydrogen to free radicals and contribute to antioxidant activities. The phenols extracted from natural products have outperformed synthetic antioxidants such as butylated hygroxyanisole and are nontoxic and non carcinogenic. Moreover, polyphenolic compounds are believed to have chemo preventive and suppressive activities against cancer cells by inhibiting metabolic enzymes involved in the activation of potential carcinogens or arresting the cell cycle (Newman *et al.,* 2000).

Wijekoon et *al.* (2011) depicted water to be the least effective solvent for the extraction of phenolic compounds. The phenols dissolve better in alcohols than water, which is also reflected in the present observation of decrease of TPC in aqueous extracts than their corresponding methanolic extracts. Azman *et al.* (2014) reported higher phenolic content in 50% methanol extracts in comparison to the water extract of *G. lutea*. In accordance to the above mentioned studies, higher TPC value was found in the methanol extracts of Gentiana species than their aqueous counterparts in this study as well. However, Nastasijevic *et al*. (2012) determined the total polyphenol content of *G. lutea* in water extract as being slightly higher compared to different concentrations of aqueous ethanol and methanol extracts.

Baba *et al.* (2014) reported total phenolic content in the methanolic root and leaf extract of *G. kurroo* to be 68 ± 2.4 (GAE)/g DW and 34 ± 1.8 (GAE)/g DW respectively. Similarly Nikolova *et al.* (2012) reported the phenolic content in 80% methanol extract of *G. lutea* to range between 20.74 mgGA/g in rhizomes to 37.02 mgGA/g in generative parts. The total phenolic content in methanol extracts of whole plant of *G. depressa,G. ornata* and *G. urnula* were found to be higher or similar to those of *G. kurroo* and *G. lutea*. This variation in TPC may be due to the altitudinal variations, genotypic differences, species diversity, time of sample collection and the procedures of extraction employed.

5.4 Total Flavonoid Content

Flavonoids are a group of more than 4000 polyphenolic phytochemicals that include flavones, isoflavones, flavonols, catechins, anthocyanidins and chalcones. They are commonly found in fruits, vegetables, herbs and most of the medicinal plants. Flavonoids are known to have broad range of biological effects such as anti-inflammatory, antioxidant, antiviral and anticancer effects (Middleton, 2000). 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 (Havsteen, 2002).

The total flavonoid content in methanol extracts was comparatively greater than their aqueous extracts in almost all of the studied species. *G. ornata* and *G. urnul*a showed the lowest TFC in methanol and aqueous extracts respectively whereas *G. capitata* and *G. ornata* contained the highest TFC in methanol and aqueous extracts respectively. Our results are consistent with research carried out previously by Wani *et al.* (2013) which reported TFC of 46 ± 2.05 mg/g of methanol extract of *G. kurroo* roots. Latif et *al.* (2006) also quantified the flavonoid content to be around 0.31% of the chemical constituents of flower tops of *Gentiana kurroo* Royale. In another study, Baba *et al.* (2014) reported total flavonoid content of 41 ± 2.2 rutin equivalent/g DW in root extract and 20 ± 1.5 rutin equivalent/g DW in leaf extract of *G. kurroo* respectively. The variation in TFC can be attributed to the species diversity, geographical locations and extraction methods. In a similar context, the phytochemical screening done in different Swertia species from Nepal by Khanal *et al.* (2015) reported higher TFC content of around 25 mg/g. Similarly, a range of 0.57 to 6.8 mgQE/gm DW of TFC was observed in methanolic extracts of Nepalese *Swertia chirayita* population by Bhattarai (2014), which is similar to the present results.

5.5 DPPH Radical Scavenging Inhibition Assay

Free radicals like super oxide, hydroxyl radicals, nitric oxide and other reactive species produced during aerobic metabolism in the bod are well known reactive molecules mainly derived from univalent reduction of oxygen. They are highly reactive and present challenges to the cellular metabolism leading to cell inactivation and disease conditions. Antioxidants are important compounds which possess the ability to protect the body from damages caused by free radical-induced oxidative stress (Chan *et al.*, 2007). Antioxidants are substances that delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidizing reactions (Schwartz, 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).

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 activity; the reaction between antioxidant molecules and radicals resulting in the scavenging of the radical by hydrogen donation that discolors from purple to yellow (Chan *et al.*, 2007). Antioxidant property is studied on the basis of radical scavenging activity 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. A plant with higher radical scavenging activity has lower IC_{50} value. The plant extract with lowest IC_{50} value is considered having better antioxidant properties.

The methanol extracts reported better scavenging property than their aqueous extracts in all of the *Gentiana* species and a significant variation in their IC_{50} values was observed. This variation can be attributed to the significantly higher presence of total polyphenol and flavonoid content in methanol extract than in the aqueous extract. These findings were in accordance with Azman *et al.* (2014) who reported higher DPPH radical scavenging activity in 50% methanol extracts of *G. lutea* as compared to the respective water extract. Nikolova *et al.* (2012) reported the IC_{50} values of 80% methanol extracts of *G. lutea* to be higher than 200. The IC_{50} values of the Gentiana species investigated by us were lower than that of *G. lutea*. The difference in results may be due to the concentration of the solvent used for extraction, species diversity and geographic location.

5.6 Correlation between TPC and IC_{50} value

Most antioxidant activities from plant sources are correlated with phenolic-type compounds (Chen *et al*., 2011). A significant negative correlation between TPC and IC_{50} value for methanol extracts of different plant parts was found after correlation analysis. A more negative correlation between the IC_{50} value 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.

During aerobic metabolism in the body, free radicals (super oxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypochloric acid and proxynitrite) are produced which can cause oxidative damage of amino acids, lipids, proteins and DNA and have been linked to majority of the systemic diseases including

cancer, cardiovascular diseases, and type 2 diabetes (Phoboo *et al.*, 2012). Phenolic compounds are considered as some of the most important and widely found antioxidants in foods and medicinal plants. *Gentiana* species extracts possess many naturally occurring bioactive compounds that have been associated with a wide range of biological and pharmacological properties including antioxidant, analgesic and hepatoprotective activities (Singh 2008; Mirzaee *et al.*, 2017). The results of the present study indicate that both methanol and aqueous extracts of various *Gentiana* species possess phenolic-linked antioxidant activity. These results also justify the ethno-parmacological uses of *Gentiana* species in the local communities.

5.7 Antimicrobial Assay

With the rise of antibiotic resistance in many pathogenic organics, the need to develop novel therapeutics and antibiotics has been escalated. Bacterial species like *P. aerigunosa* and *S. aureus* are showing resistance against the most antibiotics and are increasing threats to human health. So, medicinal plants serve as the primary tool for the development of alternative drugs for the treatment of emerging and resistant bacteria species. As medicinal plants are the rich source of many bioactive secondary metabolites, they prove to show superior anti-microbial activities (Cowan, 1999). Ethnomedicinally important plants have been used for the treatment of numerous acute and chronic diseases. Different species of *Gentiana* gave different levels of antibacterial activity.

Most of the methanol extracts showed activity against Gram negative organisms like *S. typhiimurium, K. pneumoniae* and *E. coli* at 100 mg/ml concentration. According to Bhattarai *et al.* (2009) antimicrobial activity was reported on *S. aureus, B. subtilis, P. auriginosa and E. coli* using *Gentiana robusta*. Similarly, Lamichhane *et al.* (2014) proved the medicinal importance of *Gentian depressa* by showing the antimicrobial activity of its methanol extract on thirteen bacterial and five fungal strains. *G. depressa* depicted antimicrobial activity against *S. typhii, Cytobacter freundii, P. aeroginosa, E. coli* and *Shigella dysenteriae*. The presence of broad spectrum antibiotic compounds like xanthones, iridoid glycosides and flavones in these plants confer antimicrobial property. These phytoconstituents show antimicrobial activity through varied intra cellular mechanisms such as disruption of cytoplasmic membrane, disrupting the proton motive force and coagulation of cell contents (Kotzekidou *et al.*, 2008). Inhibition by the methanol extract is due to the presence of tannins, flavonoids, alkaloids and glycosides that 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 (Scalbert, 1991).

5.8 Qualitative and Semi-quantitative estimation

The members of Gentianaceae contain many species with interesting phytochemical properties. They have been widely used in traditional medicine, so a considerable amount of work in characterization and biosynthesis has been performed on these members. Most promising groups of compounds like iridoids, xanthones, mangiferin, and c-glucoflavonoids, which are most characteristic for the family and secoiridoid glycosides, triterpenoids, flavonoids and xanthones form an integral part of the members of *Gentiana*. Amarogentin and swertiamarin are the important secoiriod glycosides; and mangiferin is a xanthone that is present in trace quantities in *Gentiana* species (Jensen and Schripsema, 2002; Struwe and Albert, 2002).

Amarogentin, which is the bitterest compound known, tastes bitter even at dilution of 1: 58,000,000. Amarogentin, amaroswerin and amaropanin cause most of the bitter taste of Gentiana, although they are present in the drug only in low concentrations (Quercia *et al.*, 1980). According to a review by Singh (2008), it could be obtained from *Gentiana lutea L.*, *G. macrophylla* Pall, *Swertia chirayita* (Roxb ex. Flem) Karst. and few other members of Gentianaceae. Quercia et al., 1980 reported the amarogentin content to be 0.05 - 0.025% in methanol extracts of Gentian roots by HPLC. Furthermore, in another study by Azman *et al.* (2014) amarogentin was not identified in detectable amounts in 50% methanol extract as well as in aqueous extract. The content of amarogentin found in this study (0.026 – 0.075mg/gm DW) is lower compared to the value reported for G. *lutea*.

Olennikov *et al.* (2015) quantified the amount of swertiamarin in decoctions of *Gentiana algida*, *G. decumbens*, *G. macrophylla* and *G. triflora* by microcolumn (MC)-RP-HPLC-UV procedure. The amount of swertiamarin was reported to be 26.38±0.47μg/ml in *G. algida*, 4.02±0.06μg/ml in *G. macrophylla* and in trace amounts in *G. triflora* and *G. decumbens*.Huang et al. 2013 also determined the presence of swertiamarin in three Taiwan specific Gentiana species (*G. davidii var. formosana, G. arisanensis and G. scabrida var. punctulata) and compared them to G. scabra*. The content in *G. davidii* and *G. scabra* was reported to be 2.66mg/gm DW and 2.52mg/gm DW respectively. Aberham *et al*. (2007) reported the swertiamarin content of *G. lutea* to be consistently between 0.21% and 0.45% in 12 commercial samples of *G. lutea* root. These finding in different Gentiana species are notably higher than the values obtained in this study. This difference in swertiamarin content may be due to species diversity, habitat, harvesting method, extraction method, efficiency of the method used for quantification or other criteria influencing handling of the samples.

Semi-quantitative estimation of mangiferin reported no significant difference between the mangiferin content of different species of *Gentiana* in our study. Mangiferin was

detected in fairly low amounts, with the highest content occurring in *G. ornata* in comparison to methanol extracts of other species used in this study. In a study by Olennikov *et al.* (2015), mangiferin was reported for the first time in *G. triflora* at a concentration of 19.86µg/ml. It was however not detected in *G. macrophylla*, *G. algida* and *G. decumbens*. The author also concluded that this compound occurs sporadically in Gentianacea. Mihailović *et al*. (2013) reported the absence of detectable amounts of mangiferin in chloroform fraction, ethyl acetate fraction and n-butanol fraction of *G. asclepiadea*. However, 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.*

All four species of *Gentiana* used in this study showed presence of swertiamarin, amarogentin and mangiferin albeit in a low concentrations. Detection of these bioactive compounds and the demonstration of certain level of antimicrobial activity to various pathogenic bacteria provide support to their frequent use in traditional medicine in various parts of Nepal.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This present investigation deals with the primary phytochemical assessment of different species of *Gentiana* found in Nepal that are also used among the local communities as traditional medicine. As such kinds of work in these plants have been documented very rarely from Nepal; this research provides a basic platform for further and extensive study of *Gentiana* species from Nepal.

Quantification of phenolics and flavonoids showed the substantial presence of these secondary metabolites that possibly led to antibacterial effects. Among the two extracts, methanol extracts exhibited better antimicrobial inhibition than the aqueous extracts. It is also found that the Gram-negative microorganisms were more sensitive to the plant extracts than the Gram-positive microorganisms. Inhibition of Gram negative organisms by these species may be the reason of its ethnomedicinal and therapeutic use in gastro intestinal and respiratory disorders that are basically caused by common Gram negative organisms such as *Klebseilla, Salmonella and Enterococus*. This in vitro study demonstrated that folk medicine can be linked to modern medicine to combat pathogenic microorganisms. Among the studied species, *G. ornata* and *G. depressa* revealed promising antimicrobial effects due to their higher content of total phenols and flavonoids. All the four species showed a considerate level of anti-oxidant property that serves and justifies their uses in various traditional remedies. Other species also showed considerable results in DPPH assay and antimicrobial property. TLC profiling showed the presence of amarogentin, mangiferin and sweriamarin present in all *Gentiana* species. Swertiamarin was reported in a significant amount whereas mangiferin was seen in traces. The secoiriod glycosides like amarogentin and swertiamarin are the basics components of members of Gentianeceae that were observed in the studied four *Gentiana* species.

The species of *Gentiana* are commonly studied species in Europe and China. This preliminary analysis of the major phyotchemicals, DPPH assay and antimicrobial properties has proven the traditional therapeutic uses and paved a way to indulge in further research in this genus from the Himalayas.

6.2 Recommendations

This study is just an elementary research focused in the basics of phytochemical analysis and bioassays in *Gentiana* species from Nepal. Through this preliminary study, the following recommendations can be made.

I. This study is novice that has covered only a small number of *Gentiana* species from Nepalese Himalayas, so the remaining and endemic species of *Gentiana* should also be studied and explored for their bioactivities.

II. All the documented species of *Gentiana* from Nepal should be studied individually to improve our understanding of their ethnomedicinal uses and laboratory established pharmacologic proofs.

III. Further bioanalytical and functional studies should be the focus of future research. Phytopharmacology, compound isolation and identification, and testing their therapeutic efficiency should be done so as to develop lead molecules for many diseases that can be validated/tested in in-vivo models.

IV. The species of *Gentiana* contain a large number of secoiridoid glycosides that are pharmacologically important; so other highly therapeutic compounds should be looked upon and tested using phytopharmacological studies.

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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 (Merck 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 (Merck 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 MerkPvt. 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 (Merck Specialities 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:

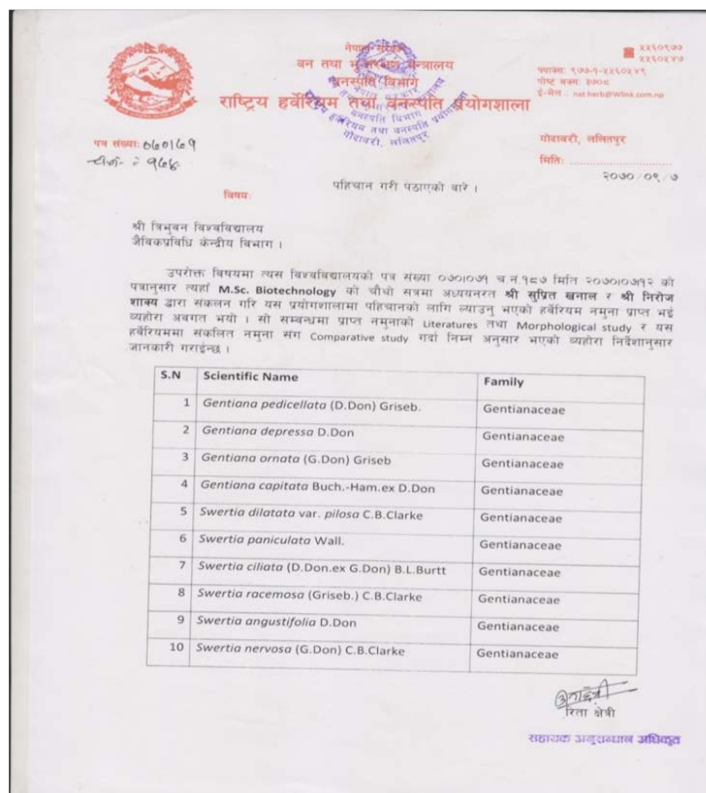
Components	gram/L
Potato	200
Agar	2
Dextrose/Glucose	20

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)



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च.नं. ९६८

मिति: २०७० /०९/ ७

विषय: पहिचान गरी पठाएको बारे ।

श्री त्रिभुवन विश्वविद्यालय
जैविकप्रविधि केन्द्रीय विभाग ।

उपरोक्त विषयमा त्यस विश्वविद्यालयको पत्र संख्या ०७०।०७१ च.नं.१८७ मिति २०७०।०७।१२ को पत्रानुसार त्यहाँ **M.Sc. Biotechnology** को चौथो सत्रमा अध्ययनरत **श्री सुप्रित खनाल र श्री निरोज शाक्य** द्वारा संकलन गरि यस प्रयोगशालामा पहिचानको लागि ल्याउनु भएको हर्बेरियम नमुना प्राप्त भई व्यहोरा अवगत भयो । सो सम्बन्धमा प्राप्त नमुनाको Literatures तथा Morphological study र यस हर्बेरियममा संकलित नमुना संग Comparative study गर्दा निम्न अनुसार भएको व्यहोरा निर्देशानुसार जानकारी गराईन्छ ।

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

रिता क्षेत्री
सहायक अनुसन्धान अधिकृत

Photograph 1 Letter of plant Identification

Photograph 2 *Gentiana ornata* (G. Don) Griseb

Photograph 3 *Gentiana depressa* D. Don

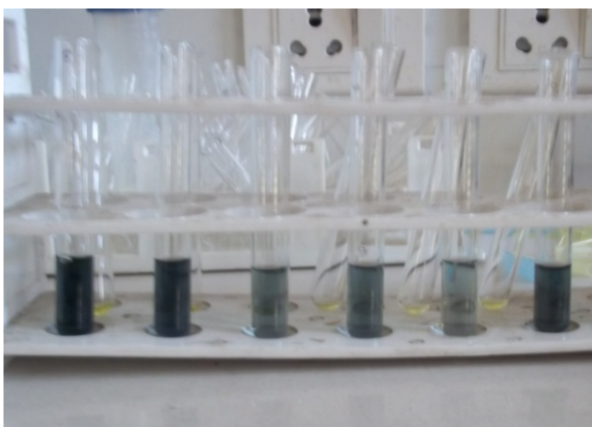
Photograph 4 *Gentiana capitata* Buch Ham ex D. Don

Photograph 5 *Gentiana urnula* H. Smith

(http://biodiversity.bt/observation/show/7365)

Photograph 6 Plant Extract Preparation

Photograph 7 Gallic acid standard solution (25-250 µg/ml)

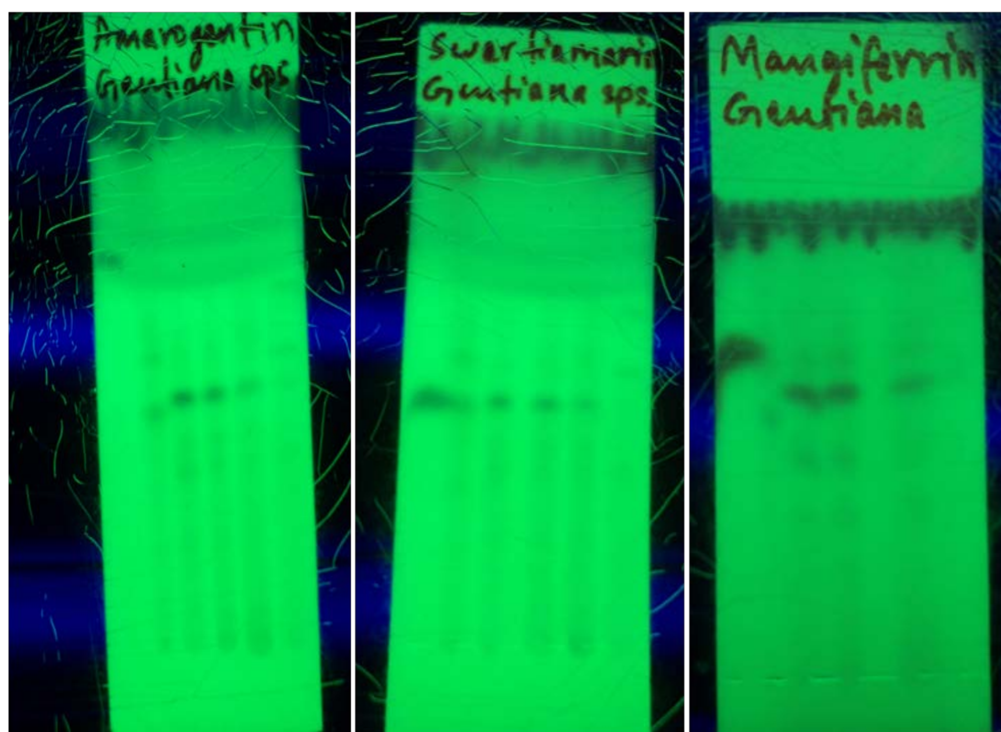


Photograph 8 Quantification of polyphenols

Photograph 9 Quercetin standard (10 - 100 µg/ml)

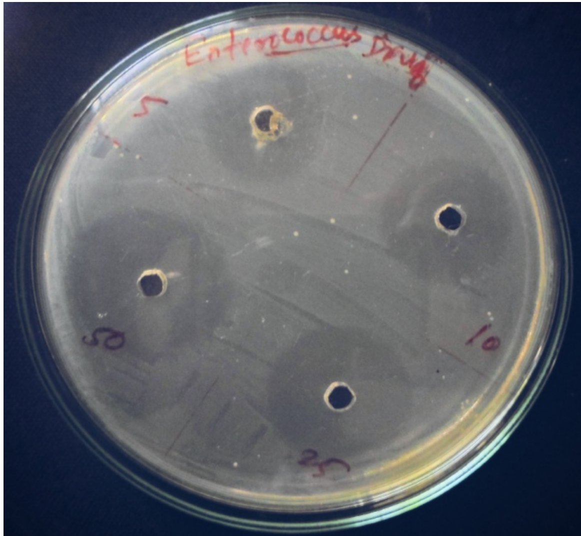
Photograph 10 Detection and semi-quantification of amarogentin, swertiamarin and mangiferin standard

(From left: Amarogentin, Swertiamarin and Mangiferin at 500, 250, 125 and 62.5µg/ml)

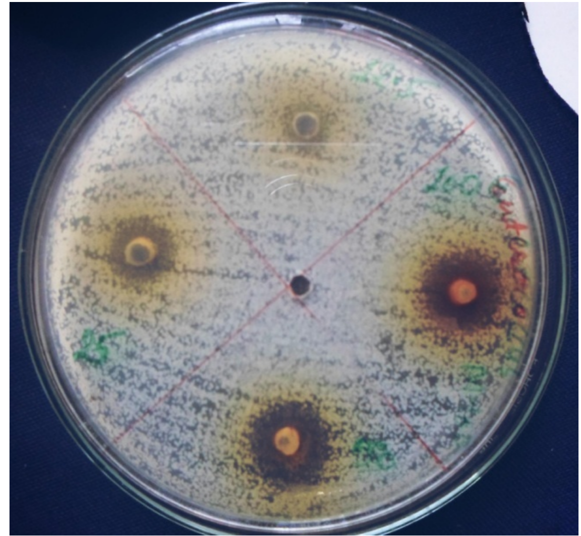


Photograph 11 Detection and semi-quantification of amarogentin, swertiamarin and mangiferrin in different species of *Gentiana*

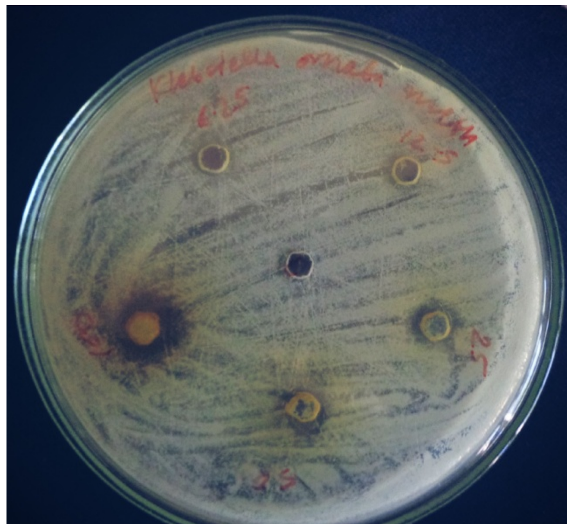
(From left: Standard*, G. depressa, G. ornata, G. urnula* and *G. capitata*)



Photograph 12 Antibacterial activity of Streptomycin on *Enterococcus faecalis*



Photograph 13 Antibacterial activity of *G. ornata* extract on *E. faecalis*



Photograph 14 Antibacterial activity of *G. ornata* extract on *K. pneumoniae*