PHYTOCHEMICAL SCREENING AND BIOLOGICAL ACTIVITIES OF METHANOLIC EXTRACTS OF SELECTED SPECIES OF *SWERTIA* L.

A Dissertation Submitted for Partial Fulfillment of the Requirement for the Master's Degree in Science

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RECOMMENDATION

This is to certify that the dissertation work entitled **"PHYTOCHEMICAL SCREENING AND BIOLOGICAL ACTIVITIES OF METHANOLIC EXTRACTS OF SELECTED SPECIES OF *SWERTIA* L."** submitted by **Ms. Laxmi Thapa** was accomplished under our supervision. It is original research carried out by the candidate and to the best of my knowledge the work has not been submitted elsewhere for any academic purpose. We hereby recommend for the acceptance of this dissertation as a partial fulfillment of the requirement of Master's Degree in Botany at Tribhuvan University.

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LETTER OF APPROVAL

The M. Sc. Dissertation entitled **"PHYTOCHEMICAL SCREENING AND BIOLOGICAL ACTIVITIES OF METHANOLIC EXTRACTS OF SELECTED SPECIES OF *SWERTIA* L."** submitted at the Central Department of Botany, Tribhuvan University by Ms. **Laxmi Thapa** for the partial fulfilment of her Master's degree in Botany, has been accepted.

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DECLARATION

I, **Laxmi Thapa**, M. Sc student of botany certify that this dissertation entitled "**PHYTOCHEMICAL SCREENING AND BIOLOGICAL ACTIVITIES OF METHANOLIC EXTRACTS OF SELECTED SPECIES OF *SWERTIA* L**." submitted to the Institute of Science and Technology, Tribhuvan University, for completion of Master's Degree in Botany is a record of genuine work carried out by me under the supervision of **Dr. Deepak Raj Pant,** Associate Professor, Central Department of Botany. I further declare that the work reported in this research has not been previously submitted either in part or in full for the award of any degree, in this or any other institute or University.

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March 2, 2022

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ABSTRACT

Swertia is a genus of traditionally used plants pertaining to the family Gentianaceae. There are about 250 species of *Swertia* L. worldwide out of which 28 species (including three endemic species) with five varieties are reported from Nepal. In the present study, five species comprising eight samples were tested for their phytochemical constituents and biological activities. Methanol was used as solvent and sonication was used for extraction process. In quantitative assay, the total phenolic content and total flavonoid content was measured using Folin-Ciocalteu reagent and Aluminium chloride colorimetric method. In biological assay, antioxidant activity was determined by DPPH Radical Scavenging Assay method, α-glucosidase activity by using PNPG solution and antibacterial activity by agar well diffusion method. Column chromatography method was used for separation of samples into fractions using series of solvents. The highest yield percentage was obtained from *S. multicaulis* roots (35.67±3.52%) while lowest from *S. chirayita* (12.79±2.23%). Qualitative phytochemical analysis of selected species of *Swertia* showed the presence of alkaloids, saponins, phenols, flavonoids, steroids, tannins and terpenoids. The total flavonoid content was found to be highest in *S. multicaulis* leaves (54.13±0.002mg QE/g) and lowest in *S. multicaulis* root (7.67±0.001 mg QE/g). Highest amount of total phenol content was found in *S. barunensis* leaves (107.38±2.35 mg GAE/g) and lowest in *S. cuneata* (37.44±2.28mg GAE/g). In DPPH scavenging assay lowest IC50 value was observed in *S. barunensis* leaves with value i.e, 17.74 µg/mL while highest IC50 value was observed in *S. nervosa* i.e, 399.84 µg/mL. In α-glucosidase assay, highest inhibitory activity was observed in *S.barunensis* leaves (36.65±0.74%) while lowest in *S. cuneata* (-26.15±0.3%). The fractionated sample of *S. barunensis* and *S. multicaulis* leaves when tested for their α-glucosidase inhibition activity fraction E exhibited maximum inhibition activity (97.2±1.86%) in *S. barunensis* leaves while fraction G (94.4±0.66%) in *S. multicaulis* leaves. The antibacterial activity was carried out against five bacterial strains. None of the plant sample showed inhibition against all the strains. *S. barunensis* leaves showed best antibacterial activity against *Bacillus subtilis* that can be comparable with the standard drugs Gentamycin. The fractionated sample of *S. barunensis* and *S. multicaulis* leaves when tested for antibacterial activity didn't show much significant zone of inhibition. Based on the above mentioned results, it can be concluded that *Swertia* species are rich in medicinal properties and are needed to further research and explore

on more species of *Swertia* which can be useful in development of drugs and pharmaceutical areas.

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ABBREVIATIONS AND ACRONYMS

µg	microgram
µL	microliter
asl	above sea level
DM	Diabetes mellitus
DPPH	1, 1-Diphenyl-2Picrylhydrazyl
GAE	Gallic Acid Equivalent
IC_{50}	Inhibition concentration 50
KATH	National Herbarium and Plant Laboratories, Godawari
mg	milligram
MHA	Mueller Hinton Agar
mL	milliliter
mM	millimolar
NB	Nutrient Broth
nm	nanometer
QE	Quercetin Equivalent
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RSA	Radical Scavenging Activity
SD	Standard deviation
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TUCH	Tribhuvan University Central Herbarium
WHO	World Health Organization

CHAPTER ONE: INTRODUCTION

1.1 Background

Nowadays, most of us abode in mechanized urban settings, leading a sedentary life and having a highly processed synthetic food that results into overweight or obesity (O'Kefee and Cordain, 2004). A lifestyle with devoid of physical activity is one of the major causes of preventable mortality worldwide (Senapati *et al*., 2015). Such physiologically-stressed lifestyle results in increased levels of risk factors including hypertension, dyslipidemia, diabetes, respiratory diseases, and obesity (Jamison *et al*., 2006). Hence, these non-communicable diseases (NCDs) are the major health problems globally (WHO, 2018) and are estimated to account for 71% of deaths globally (Balwan and Kour, 2021).

In this modern age, with the advancement of medical science, there is also an upsurge of diseases. The new medicines that are developed for treatment of complicated ailments themselves result into number minor to severe side effects (Nisar *et al*., 2018). In this era, there is also another global problem that is irrational use of antibiotics which results into antimicrobial resistance that is growing rapidly worldwide and is causing significant morbidity and mortality (Levy, 2005; Livemore, 2003).

Despite the advancement in modern medicine people in low income countries, especially in rural area are still relying on medicinal plants to cater their healthcare needs (Shihabudeen *et al*., 2010). These plants have been in use throughout the world to treat and preclude various diseases and ailments for centuries (Aleem and Kabir, 2018). In modern world, medicinal plants have gained attention due to their cost effectiveness, easy availability and with little or no side effects. Herbal products are often perceived as safe because they are "natural". Hence there is tremendous increase of demand and interest of new drug discovery made from plants and new chemical compounds to heal many diseases.

There are large numbers of medicinal plants which are used as therapeutic application (Kweera *et al*., 2011). Among the estimated 250,000-500,000 plant species, only a small portion has been examined phytochemically and even smaller is the fraction subjected to biological or pharmacological screening (Bader, 2014). Gentianaceae is a

family of flowering plants consisting of about 70-80 genera and about 900-1200 species (Chandra *et al.*, 2016). Plants pertaining to various genera of this family are medicinally important and have been exploited over years in various parts of the world to treat large number of ailments, hence this family holds distinct place in traditional medicinal plants (Bader, 2014). In the Pyrenees, Himalayas and alpine mountains throughout Eurasia, many Gentians species are found extensively. This family comprises of the genera such as *Centaurium* Hill., *Gentiana* L., *Swertia* L., *Menyanthes* L., etc. (Budniak *et al.,* 2021). Plants categorized under these genera are a source of pharmacologically significant active phytochemicals that possess antimicrobial, antipyretic, anti-inflammatory, cyto- and hepato-protective, stomachic, analgesic, and gastro-protective properties (Singh, 2008; Tomiczak *et al.,* 2016). Among the plants often used in traditional medicine, *Swertia* species are quite important and have been used as crude drugs in Asian pharmacopeia.

Swertia L. belonging to the family Gentianaceae is a morphologically diverse and taxonomically distinct genus. There are about 250 species of *Swertia* L. worldwide out of which 28 species with five varieties occur in Nepal with two species being endemic (Rajbhandari *et al*., 2017). The *Swertia* genus of family Gentianaceae has been used globally for centuries for their traditional medicinal properties (Kshirsagar *et al.,* 2019). Countries including Bhutan, China, India, Japan, and Nepal, some *Swertia* species are well-known in traditional medicines to treat array of diseases and ailments. Every underground and aerial parts of *Swertia* species are used in the preparations of mixtures and decoctions to treat the different ailments (Joshi and Dhawan, 2005). The botanical, phytochemical, ethnobotanical and pharmacological aspects of *Swertia* spp. have been delineated in different books and modern text (Khare, 2007; Kumar and Van Staden, 2016).

1.2 Plants and their antioxidant potential

In the human body, about 5%–7% or more of the inhaled oxygen (O_2) is transformed into reactive oxygen species (ROS) such as O_2^-, H_2O_2 and •HO (Gupta and Sharma 2006). The imbalance between production of ROS and the capacity of the normal detoxification systems in favor of the oxidants results into oxidative stress, which itself contributes to cellular damage caused by the interaction of ROS with cellular constituents leading to many acute as well as chronic diseases including cancer, cell

aging, cardiovascular problem and neurodegenerative diseases. Antioxidants have the capacity to remove these free radical intercede by suppressing oxidation reactions.

Antioxidants are those compounds that either can cause dilatory or inhibit the oxidation of lipids, protein and nucleotides by suppressing the initiation of oxidative chain reactions, thus preventing damage done to the body cells by ROS (Tachakittirungrod *et al*., 2006). Numerous methods are there for determination of antioxidant potential such as peroxyl radical scavenging (Oxygen Radical Absorbance capacity, ORAC), Total Radical-trapping Antioxidant Power (TRAP), metal reducing power (like FRAP-Ferric Reducing Antioxidant Power, CUPRAC- Cupric Reducing Antioxidant Power etc), hydroxyl radical scavenging (deoxyribose assay), organic radical scavenging (ABTS- 2,2-2'-Azino-bis-3-ethylbenzothiazoline-6-Sulfonic Acid, DPPH- 2,2-Diphenyl-1- picrylhydrazyl), quantification of the products formed during the lipid peroxidation (Thiobarbituric Acid Reactive Substances, TRAPS), Low-density Lipoproteins (LDLs) oxidation, etc. (Pérez-Jiménez and Saura-Calixto, 2008).

Different methods work on the basis of different principles. The DPPH method is a simple, rapid and convenient method independent of sample polarity for screening of many samples for radical scavenging activity (Marxen *et al*., 2007). The method DPPH is widely used for measurement of free radical scavenging ability of antioxidants (Perez-Jimenez and Saura-Calixto, 2008) DPPH (2,2-Diphenyl-1- picrylhydrazyl) is a free radical which absorbs free electrons from antioxidant to form a stable compound $DPPH_2$ (2,2-Diphenyl-1- picrylhydrazine). DPPH shows maximum absorption at 517nm. Initially DPPH is dark purple in color which changes to yellow color upon addition of antioxidant as it absorbs hydrogen ion from the antioxidant. The antioxidant activity is inversely proportional to the absorbance of the sample. Higher the absorption, lower is the antioxidant activity.

The medicinal plants act as the treasure house of various kind of naturally occurring antioxidants (NOAs) like polyphenolics, phenolic acids, flavonoids, tannins, steroids, carotenoids, and so forth (Mazzoni *et al*., 2016; Niki, 2016). These NOAs are well known for their biological potential viz. anti-hypertensive, anti-inflammatory, anticancer, antidiabetic, anti-proliferative, and so forth (Alarcon *et al*., 2015; Azevedo *et al*., 2015; Tulipani *et al*., 2014). Thus it is pertinent to discover plants rich in antioxidants in order to alleviate diseases/ disorders related to oxidative stress. It is reported that species of *Swertia* show good to moderate antioxidant activity against free

radicals; hence more study is needed in order to identify active components in *Swertia* species (Kshirsagar *et al*., 2019).

1.3 Plants and their anti-diabetic activity

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia, hyperlipidemia, hyper amino acidemia and hypo insulinaemia which may be either due to impairment of insulin secretion or resistance to peripheral insulin action (Goyal and Jialal, 2021). Diabetic hyperglycemia occurs either due to absolute lack of insulin secretion (Type -1) or due to insulin action (Type-2). Type-2 diabetes is connected to oxidative stress due to hyperglycemia and hyperlipidemia which induce inflammatory-immune responses and oxidative stress reactions, leading to an increased production of reactive oxygen species (ROS), including the superoxide radical, hydrogen peroxide and hydroxyl radicals or reduction of antioxidant defense system in the body. Therefore, the generation of free radicals accounts for complexion of type-2 diabetes and cardiovascular complications (Baynes and Thorpe, 1999; Pickup, 2004).

Diabetes mellitus once considered a disease of minor significance to world health is now taking its place as one of the main threats of human health in the 21st century (Zimmet *et al*., 2001). Diabetes mellitus occurs as a result of abnormal carbohydrate metabolism and mainly linked to abnormal insulin level or insensitivity of target organs to insulin (Hahm *et al*., 2011). Enzyme glucosidase catalyzes the final step of carbohydrate metabolism in biological systems (Liu *et al*., 2006). Inhibition of this enzyme is one of the ways to decrease postprandial blood sugar and manage type-2 diabetes. Even a number of hypoglycemic synthetic drugs have been synthesized to treat Diabetes mellitus; many synthetic drugs have shown many adverse side effects (May *et al*., 2002). Management of hyperglycemia with less or no side effects in clinical experience and with low costs is still a challenge to the medical system (Sun *et al*., 2008). Plant phenolics are known for their α-glucosidase inhibitory potential and when combined with their antioxidant activity could be useful in early stage management of type-2 diabetes (Phoboo *et al*., 2010). *Swertia* plants have been used widely as traditional medicines in the treatment of diabetes. The major class of chemical compounds of this genus has been reported to show significant hypoglycemic activities (Ghosal *et al*., 1980).

1.4 Medicinal Plants and their antimicrobial capacity

Antibiotics are one of the most significant tools to fight against bacterial infections and have played a major role in improving the quality of human life. However, nowadays antibiotics are proving to be less effective due to the emergence of antibiotic resistance in different pathogens. The evolution of new strains of diseases and accelerated rate of antibiotic resistance is a global public health challenge (Yadav *et al*., 2016) which has created intense clinical hazard in the treatment of infectious diseases (Davies, 1994). Additionally, antibiotics are sometimes associated with adverse effects on host including severe infections, complications, longer hospital stays and increased mortality (Livermore, 2012). WHO warns that the antimicrobial resistance will not only eliminate the advances in healthcare made over 100 years but also will lead to unfeasible to manage the simple infection causing it to be fatal (WHO, 2012). Therefore there is an urgent need to discover substitute antimicrobial drugs or the antibiotics with minimum or no bacterial resistance at all and minimum or no side effects as well. The use of medicinal plants and the extracts containing phytochemical components can be one of the alternatives in therapeutics and also be helpful in dealing with the threats of microbial pathogens resistant to various antibiotics (Kubo *et al*., 1981). In the present scenario screening of local medicinal plants may help in the discovery of novel compound which may be useful to solve the antibiotic resistance problem with minimal or no side effects.

1.5 Botanical description of genus *Swertia*

Swertia has been given name in honor of Emanuel Sweert (rarely spelled Swert, 1552-1612), a Dutch gardener. Plants of this genus are usually annual, biennial or perennial herbs ranging from 2.5cm – 1.5m in height. Roots of *Swertia* species are either fibrous or woody. Stems may be absent, scapiform, or well developed, ascending or erect, terete, striate or angled or winged, may be simple or sometimes branched. Leaves are mostly opposite, rarely alternate or whorled or rosulate and are sessile or petiolate. The margin of leaves is entire in all species of genus *Swertia* L. The inflorescences that can be observed in these plants are cymose type, usually grouped into simple or paniculate thyrses, rarely strictly dichotomous, sometimes raceme like or solitary. Flowers pedicellate, 4-or 5-merous, that mostly subtended by a pair of sessile, opposite and leaf like bracts. Calyx and corolla arrange in rotate, lobed to base, tubes less than 3 mm.

Nectaries are present that is 1 or 2 per corolla lobe, naked or covered by a scale or flaps, glabrous, fringed or fimbriate. Number of stamens is equal to the number of corolla lobes, attached at base of corolla lobe sinuses, sometimes surrounded by long hairs. Ovary 1-celled; Style size ranges from short to elongate. Stigma is bilobed and fruit is a capsule that is enveloped by persistent calyx and corolla, ovoid or flattened that dehisce into 2 valves. Seeds of *Swertia* L. are usually small and are few to many in numbers (Rijal, 2009).

1.5.1 Distribution and ethnobotanical use of selected *Swertia* species.

Swertia barunensis Chassot- It is an endemic species of Nepal found at an altitude of about 4200 m towards east Nepal. No ethnobotanical uses and phytochemical experiment has been reported on this plant till date.

Figure 1. 1: Photographs of *S. barunensis* Chassot (A) Whole plant in natural habitat after flowering, (B) Inflorescence.

Swertia chirayita (Roxb.) H. Karst.- It is the most important species of genus *Swertia* in terms of medicinal application and commercial value. It is found at an altitude of 1200-2500 m and is used as tonic, stomachic, febrifuge and laxative. It is also used in the treatment of skin diseases like eczema and pimples (Manandhar, 2002). Infusion of this plant has been used as stomachic, anthelminthic and bitter tonic (Bajpai *et al*., 1991).

Figure 1. 2: Photographs of *S. chirayita* (Roxb.) H. Karst. (A) Plant with habitat (B) Enlarged part.

Swertia cuneata Wall.-It is found at an altitude of 3900-5000 m east, west and central Nepal (Joshi, 2008). *S. cuneata* has been used as blood purifiers, antimalarial, anti-inflammatory, febrifuge and as an important ingredient of the herbal medicine for diabetic patients (Khetwal and Pande, 1997).

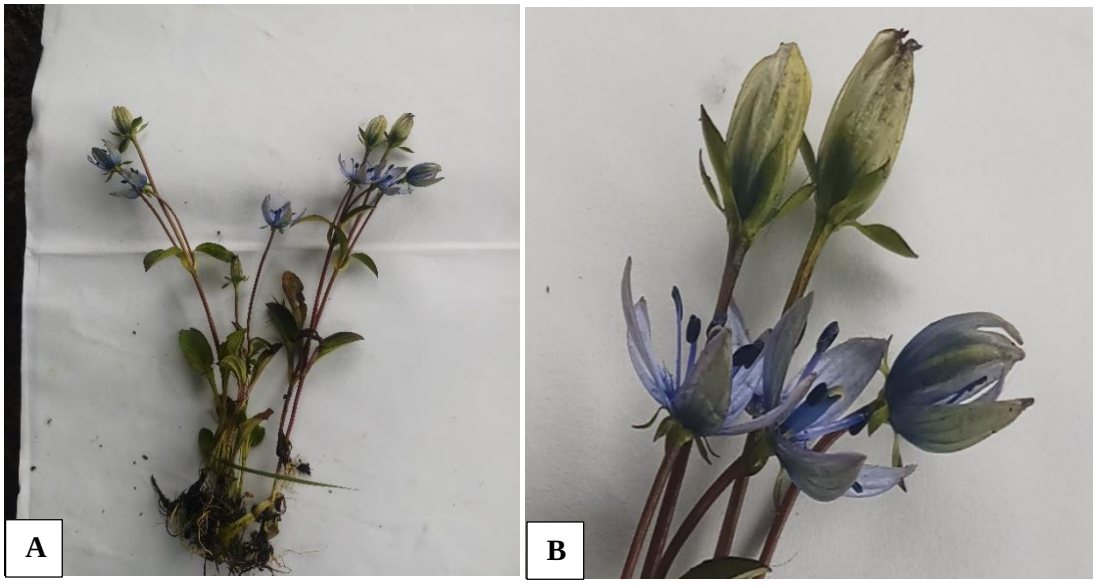

Figure 1. 3: Photograph of *S. cuneata* Wall. (A) Whole plant (B) Flower.

Swertia multicaulis D. Don-It is distributed at an altitude of 4000-4900 m central and east Nepal. The rhizome juice of this plant is reported to be used to cure cough, cold, and fever. The decoction of the plant is also used as an anthelmintic (Manandhar, 2002).

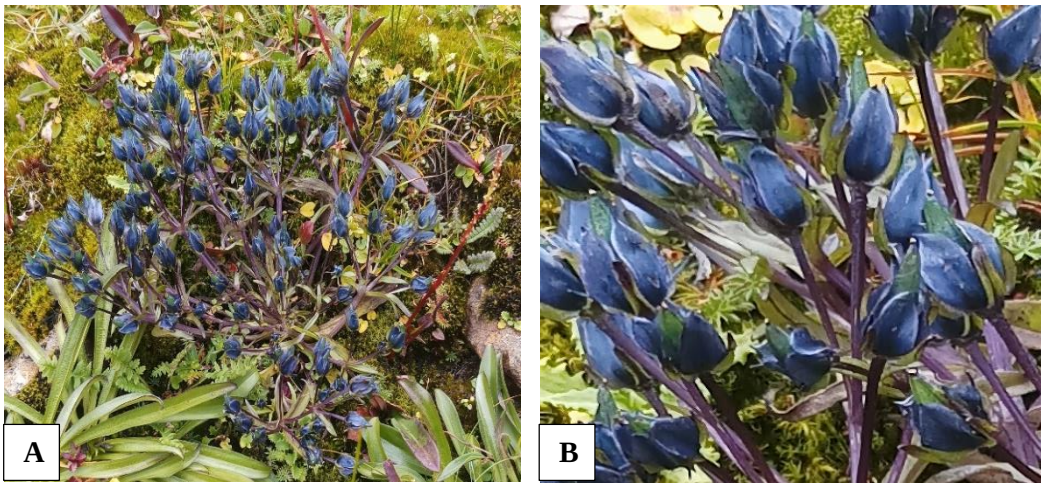

Figure 1. 4: Photographs of *S. multicaulis* D. Don (A) Whole plant in natural habitat (B) Inflorescence.

Swertia nervosa Wall.-It is found at an altitude of 700-3000 m east, west and central Nepal. The extract of the plant is used in the treatment of malarial fever (Manandhar, 2002). Extract of this plant is also given in the morning to treat gasball (Gano) as well as in stomach problem (Joshi, 2008).

Figure 1. 5: Photographs of *S. nervosa* Wall. (A) Whole plant (B) Inflorescence.

1.5.2 Medicinal utility of genus *Swertia* L.

Plants belonging to different species of *Swertia* L. are among the most commonly used plants of family Gentianaceae for treatment of various maladies in various systems of traditional medicine such as the Ayurveda, Unani, Siddha, Chinese, Tibetan, etc. (Mukherji, 1953; Kirtikar and Basu, 1984; Joshi and Dhawan, 2005; Lv *et al*., 2010).

In Chinese traditional medicine, about 20 species of this genus have been used for the treatment of hepatic, choleric and inflammatory diseases (Brahmachari *et al*., 2004) where *S. mileensis, S. bimaculata, S. kouitchensis, S. yunnanensis, S. mussoti*i, and *S. franchetiana* are frequently used for treatment as febrifuge, stomachic tonic, gall and liver tonic and to cure jaundice (Liu *et al*., 2011; Li *et al*., 2017).

Biologically active phytochemicals, like xanthones, flavanoids, iridoid, secoiridoid glycosides and terpenoids are reported to be found large amounts in different species of *Swertia* L. (Pant *et al.,* 2000; Phoboo *et al*., 2010).

The traditionally used *Swertia* species are found to be rich source of xanthanoids. It is recorded that more than 50 xanthanoids and about 40 terpenoids have been isolated from different species of *Swertia* L. (Kshirsagar *et al*., 2019). *Swertia* species are also described to be rich in oleanolic and ursolic acid (Yang *et al*., 2004). Phytochemical investigation of genus *Swertia* demonstrated that these species are also well-stocked in iridoid glycosides such as swertiamerin, amarogentin, amaroswerin and gentiopicroside which serve as chemotaxonomic marker and characteristics compound within species of *Swertia* (Pant *et al*., 2000).

The crude extract of some *Swertia* species are demonstrated to possess wide range of biological and pharmacological activities including anticancer, antibacterial, anti-diabetic, antioxidant, anthelminthic, anti-hepatitis, anti-leishmanial, neuroprotective etc. (Kshrisagar *et al*., 2019).

1.6 Research question

Research question: the present work is trying to work on is; whether the different species of *Swertia* L. differ from one another in terms of different phytochemical constituents and biological activities or not .

1.7 Objectives of the study

General Objective:

- Phytochemical screening of selected species of *Swertia* L.

Specific objective:

- To test antioxidant activity of selected species of *Swertia* L.
- To test anti-diabetic potential in selected species of *Swertia* L.

- To test anti-bacterial activity of selected species of *Swertia* L.
- To identify the major phytochemicals through two chromatographic technique- Column Chromatography and LCMS and examine their anti-diabetic activity.

1.8 Rationale of the study

The world is in traumas due to the surge of novel diseases which is deteriorating the healthy life of living organisms. Due to less side effects of natural compounds made from plants, there is an increasing interest of researchers towards the search of various compound from medicinal plants to treat different ailments. Traditionally, number of plants have been used for curing different kind of diseases. These medicinal plants heal different diseases due to the bioactive compounds present in them. These compounds if identified and isolated can scientifically validate the traditional use as well as can increase their use in pharmaceutical areas.

Swertia L. is considered as panacea for its bitter compound to treat different diseases. *S. chirayita* is highly exploited and studied species of this genus while other species have not been given much scientific attention. The other species of *Swertia* if explored may contain similar constituents as *S. chirayita* or new compound of pharmaceutical importance. The study may give species tantamount to *S. chirayita* in constituents which may ultimately help in minimizing pressure on *S. chirayita*. The marker compound variation may be used to delimit taxon. Evaluation of anti-oxidant, anti-microbial and anti-diabetic compound of unstudied species will help in assessing the potential of different species in carrying out the above mentioned activities. The study of these unexplored species may give out new marker compound in the field of pharmaceuticals. So phytochemical screening of its other species is also needed for the identification of new chemical compounds.

CHAPTER TWO: LITERATURE REVIEW

2.1 Swertia L. diversity and distribution

Swertia L. is a native Himalayan genus (named in honor of Emanuel Sweert 1552-1612, a Dutch gardener) belonging to the family Gentianaceae. It comprises about 170 spp. worldwide (Brahmachari *et al*., 2004), where Nepal harbors 28 species & five varieties with *S. acualis, S. barunensis* and *S. nepalensis* as being endemic species (Khatri, 2019). This genus is widely distributed in the mountainous terrains of Eastern, Central and Western regions at an altitude ranging from 600 m asl (*S. angustifolia*) to 5600 m asl (*S. petiolata*) (Rijal, 2009). Out of 75 districts of the country, *Swertia* species have been documented from 54 districts so far (Joshi, 2012).

Relatively few works have been done on the taxonomy of *Swertia* L. Hara *et al*. (1982) listed 27 species of *Swertia* L. in book entitled "An Enumeration of Flowering Plants of Nepal". Polunin and Stainton (1984) described 8 species of *Swertia* along with their photographs in "Flowers of the Himalayas". Press *et al*. (2000) enlisted 32 taxa (28 species and 4 varieties) of *Swertia* L in Annotated Checklist of Flowering Plants of Nepal". Rijal and Joshi (2015) published the taxonomic study of species of *Swertia* from Nepal. Recently, Rajbhandari *et al*. (2015, 2017) listed 26 species of *Swertia* from Nepal. The revisionary work done by Khatri (2019) enlisted 28 species of *Swertia* from Nepal with five varieties and 3 endemic species.

2.2 Ethnobotany of Swertia and its medicinal value

Swertia is a traditionally important plant widely used in treatment of various ailments for generations. The species of *Swertia* have been used in traditional Indian, Chinese, and in Tibetan medicinal system to treat wide array of diseases (Joshi and Dhawan, 2005; Lv *et al*., 2010).

Different countries including Bhutan, China, India, Japan and Nepal has recognized different species of *Swertia* as medicinal to treat various ailments (Kshirsagar *et al*., 2019). Almost every part of *Swertia* species are used medicinally. They are used in the form of decoction, infusion, paste and juice to treat many diseases (Joshi, 2008; Joshi and Dhawan, 2005). Among the species used, *S. chirayita* is one of the highly esteemed one (Pradhan and Badola, 2015).

A fair number of *Swertia* species has been used in indigenous medicinal system for various illnesses. For instance, *S. chirayita* has been exploited in traditional medicine as a panacea for chronic fever, malaria, anemia, bronchial asthma, liver disorders, hepatitis, gastritis, constipation, dyspepsia, skin diseases, worms, epilepsy, ulcer, scanty urine, hypertension, melancholia and certain type of mental disorder, secretion of bile, blood purification and diabetes 1 (Ghosh *et al*., 2012). Likewise, *S. multicaulis* has been used for treatment of wounds, cough, cold, fever, stomach pain and constipation as well as expelling roundworms from the alimentary canal in Nepalese traditional medicinal practice (Upreti *et al*., 2010; Timsina *et al*., 2018). Similarly, as a blood purifiers, antimalarial, anti-inflammatory, febrifuge and as an important ingredient of the herbal medicine for diabetic patients, *S. cuneata* has been exploited for centuries (Khetwal and Pande, 1997). *S. nervosa* is used traditionally to treat malarial fever, skin problem, gasball and stomach trouble (Joshi, 2008). Other medicinally important species of this genus are *S. minor* used as a substitute for *S. chirayita* in the treatment malarial as well as other kind of fever (Ambasta, 1986), *S. alata* used as palate tonic and febrifuge (Chopra *et al*., 1956). *S. cordata* and *S. petiolata* both have been used for treatment of typhoid, pneumonia fever, throat problems and bronchitis (Khan and Khatoon, 2008).

Additional species, including *S. angustifolia, S. corymbosa, S. decussata, S. hookeri, S. macrosperma, S. petiolata, S. lawii, S. paniculata, S. punctata, S. calycina, S. purpurascens, S. bimaculata, S. ciliata, S. densifolia, S. japonica*, and *S. frachetiana* have also been used as substitutes for *S. chirata* in India, China, Pakistan, Japan, and other Asian countries to treat liver disorders, fevers, dysentery, diarrhea, stomach problems, and various other illnesses (Brahmachari *et al*., 2004).

Different members of the genus are known to possess large amount of structurally diverse different bioactive compounds including xanthanoids, terpenoids, iridoids/ secoiridoids, flavonoids, and alkaloids. The different pharmacological and biological activities which includes anticancer, anti-diabetic, antioxidant, anti-hepatitis, anthelmintic, anti-inflammatory, antimicrobial, hepatoprotective, insecticidal, anti-leishmanial, neuroprotective etc. (Brahmachari *et al*., 2004; Kshirsagar *et al*., 2019) reported in different species may be attributed to the presence of these bioactive compounds.

2.3 Phytochemistry of genus *Swertia* L.

The broad and extensive investigation on phytochemicals of genus *Swertia* has led to the discovery of numerous structurally diverse bioactive compounds (Kshirsagar *et al*., 2019). This phytochemical investigation has led to an identification of about 200 compounds from genus *Swertia* which have various structural patterns (Brahmachari *et al*., 2004). A fair number of these bioactive compounds isolated from different *Swertia* species include xanthonoids, iridoids/ secoiridoids, flavonoid, alkaloids, steroids and terpenoids (Brahmachari *et al*., 2004; Jamwal, 2012).For example, there are reports of the presence of alkaloid, terpenoids, tannins, flavonoids (Begum *et al*., 2020), coumarins, and sterols (Shrestha *et al*,. 2015), triterpenes, carbohydrate, glycosides (Mehajbeen *et al*., 2017, Subedi & Karki, 2018) in various extract of *S. chirayita* using various solvents. Similarly, Timsina *et al*., (2018) carried out xanthones quantification using HPLC, LC-MS, and LC-NMR technology and reported about 13g of xanthones from 1kg of dry matter and identified four major xanthones. It was reported that *S. multicaulis* xanthone content was much higher than other species of *Swertia* used for comparision. Similarly, a new glycosides and tetraoxygenated xanthones were isolated from *S. cuneata* by Khetwal and Pandey (1997).

A total of 176 xanthones, 108 iridoids, 5 alkaloids (Li *et al*., 2017) and about 40 terpenoids (Brahmachari *et al*., 2004; Kshirsagar *et al*., 2019) with basic steroidal frameworks are known to be isolated so far from different *Swertia* species.

This genus is reported to be rich source of iridoids or iridoid glycosides like as swertiamarin, amaroswerin and gentiopicroside that serve as chemotaxonomic markers and characteristic compounds within species of *Swertia* (Pant *et al*., 2000). Amarogentin one of the bitter most compounds found till date is present in different species of *Swertia* (Korte, 1955).

2.4 Biological Activity

2.4.1 Anti-oxidant activity

Antioxidants are those compounds that play significant role in minimizing oxidative stress that could cause damage to biological molecules (Tepe *et al*., 2005). The main factor involved in production of different kind of diseases is free radical damages. The most common free radicals produced in the cells are Reactive Oxygen Species (ROS).

These ROS are responsible for the production, spread and maintenance of both acute and chronic inflammatory processes as well as pain causing tissue damages leading to various kinds of ailments (Dar *et al*., 2005). The use of suitable antioxidants has been reported to reduce the oxidative damage caused by ROS either by preventing the formation of oxygen free radicals or by scavenging them before they can react with sites such as unsaturated lipids in the cell membrane (Khalil *et al*., 1999; Cuzzocrea *et al*., 2001). Thus the natural antioxidant present in fruits, vegetables, spices and herbal medicines reduce the risk of chronic diseases and help to improve health of humans (Niki, 2010). Therefore, there is an increasing avid of researchers in the antioxidant and free radical scavenging properties of medicinal plants and their isolated compounds (Zhu *et al*., 2004, Lee *et al*., 1998).

Various extracts from different species of *Swertia* have been investigated for their antioxidant properties. For example, a comparative study of anti-oxidant activity of *S. chirayita, S. nervosa* and *Andrographis paniculata* in aqueous and ethanolic extract revealed higher antioxidant activity in *S. chirayita* followed by *S. nervosa* and *Andrographis paniculata* (Phoboo *et al.*, 2010). Furthermore, they also reported that the antioxidant activity of aqueous as well as ethanolic extracts was similar in *S. chirayita* while it was higher in ethanolic extract than in aqueous extracts for the remaining two species. Phoboo *et al*. (2012) compared anti-oxidant activity in aqueous and ethanolic extracts of different parts of *S. chirayita* and reported comparatively higher in leaf and inflorescence mixture followed by stem and lowest in root.

Roy *et al.* (2015) studied antioxidant activity of *S. chirayita* and *S. cordata* using aqueous and methanolic solvents and reported better antioxidant activity in methanolic extracts. Furthermore, they also reported superiority of *S. chirayita* over *S. cordata* in terms of anti-oxidant activity. Khan *et al*. (2017) examined antioxidant property of extracts of *S. chirayita* roots *in vitro* using several methods such as DPPH radical scavenging activity, H_2O_2 scavenging activity, β-carotene bleaching assay, total antioxidant activity by phosphomolybdenum method, hydroxyl radical method and ABTS radical scavenging activity using different concentrations(3.0, 1.5, 0.75 and 0.37 mg/mL) of extracts. The maximum anti-oxidant activity was recorded at dose of 3mg/ml in all methods which was 88.1±0.1% in DPPH scavenging method, 92±0.2% in total antioxidant capacity, 84.33±0.2% in hydroxyl radical scavenging activity, 80.33±0.2% in H_2O_2 scavenging activity, 80.88±0.1% in ABTS radical scavenging

activity and 87.03±0.3% in β-carotene bleaching assay. Subedi and Karki (2018) studied an anti-oxidant activity in *S. chirayita* following DPPH radical scavenging activity method and demonstrated antioxidant activity to be 62.54% at 500 µg/mL and 15.32% at 100 µg/mL.

Recent study focuses on finding IC_{50} and there is reported that some species exhibit value lower than those of some natural or synthetic antioxidants. The xanthone derivatives bellidifolin, swertianin and desmethylbellidifolin from *S. japonica* were found to exhibit higher antioxidant capacity than those of commercial synthetic antioxidant butylated hydroxytoluene (BHT) and α-tocopherol (Ashida *et al*., 1994). Das *et al*. (2013) reported the IC_{50} value of *S. bimculata* leaves to be 4.80 g/ml which is even less than the IC_{50} found in standard drug ascorbic acid. Ahirwal *et al*. (2014) reported IC_{50} value in *S. chirayita* following the DPPH method, hydroxyl radical and nitric oxide radical scavenging activity to be 222.74±0.19, 307.93±0.10 and 870.55±0.20 µg/ml respectively. Khanal *et al*. (2015) studied seven species of *Swertia* for their antioxidant activity and calculated IC_{50} value was found that it was lowest for SCH as 23.35 ± 0.6 µg/ml and highest for SAN as 45.81 ± 1.54 µg/ml. The IC_{50} value studied by Jamwal (2014) on five species of *Swertia* L. following DPPH radical scavenging assay revealed that it was minimum in *S. chirayita* (16.46 µg/ml) and maximum in *S. lurida* (32.7 µg/ml). The IC50 value found by Ahirwal *et al*. (2014) in *S. chirayita* was found to be more than the IC_{50} calculated by Khanal *et al*. (2015) and Jamwal (2014) when following DPPH scavenging method.

The documented species of *Swertia* revealed that different species of *Swertia* exhibit moderate to good antioxidant potential against different reactive oxygen species viz. free radicals (Kshirsagar *et al*., 2019), hence many new antioxidant compounds may be discovered if investigated other species that has not been screened and studied.

2.4.2 Anti-diabetic activity

Diabetes mellitus (DM) is a chronic metabolic disorder which results in hyperglycemia that occur either due to defects in the insulin secretion, insulin action or combination of both. DM reduces the individual potential to regulate normal glucose level in body that leads to several complications in the body of an individual (ADA, 2005). In a normal human body, regulation of glucose level takes place by the secretion of two hormones which are insulin and glucagon. When the glucose level in blood increases,

the β-cells of islets of Langerhans are stimulated in pancreas to releases insulin. This insulin signals the liver to convert extra glucose to glycogen for storage and also triggers other cells in the body including adipose tissue, skeletal muscle cells etc., to take up more glucose which results in lowering the glucose level down to normal level in blood stream. On the other hand when glucose level in the blood becomes lower than normal, then triggering of α-cells of pancreas takes place that leads to secretion of hormone known as glucagon from these cells. This glucagon hormone signals the liver to convert stored glycogen to glucose which is released into the blood stream and normal homeostasis is maintained (Kaul *et al*., 2013).

The development of diabetes follows several pathogenic processes which ranges from autoimmune destruction of β-cells of pancreas to abnormalities in insulin action. The main symptoms that can be observed in patient with increased glucose level include polyuria, polydipsia, weight loss, blurred vision. Long term hyperglycemia is often associated with retinopathy, nephropathy, peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Hypertension and abnormal lipoprotein metabolism is also most often seen in patients suffering from DM (ADA, 2005).

Different species of *Swertia* has been used largely in traditional medicinal system to treat disease associated with diabetes (Chettri *et al*., 2005). Both *in vitro* and *in vivo* methods are used to study anti-diabetic activity using various models (Kshirsagar *et al*., 2019). A number of xanthones such as bellidifolin, mangiferin and methylswertianin, Swerchirin isolated from various species of *Swertia* has been reported to be responsible for hypoglycemic activity (*Bajpai et al*., 1991; Basnet *et al*., 1994; Muruganandan *et al*., 2005; Zheng *et al*., 2014,). Various species of *Swertia* have been examined for anti-diabetic potential and are reported to possess hypoglycemic activity. These include *S. chirayita* (Chandrasekar *et al*., 1990; Bajpai *et al*., 1991; Saxena *et al*., 1993; Phoboo *et al*., 2010 and Verma *et al*., 2013), *S. japonica* (Basnet *et al*., 1994), *S. punicea* (Tian *et al*., 2010); *S. mussotii* (Zheng *et al*., 2014); *S. bimaculata* (Liu *et al*., 2013); *S. corymbosa* (Mahendran *et al*., 2014), *S. kouitchensis* (Wan *et al*., 2013), *S. longifolia* (Saeidnia *et al*., 2016) . The antidiabetic acitivities in these species are achieved through lowering of the blood glucose level (due to inhibition of enzymes of carbohydrate metabolism like α-glucosidase, α-amylase and

glucose-6-phosphatase) protecting pancreatic β-cells, stimulating insulin secretion, increasing the glucose consumption, etc. (Li *et al*., 2017).

Different extracts and parts of various species of *Swertia* are reported to possess different degree of inhibition of enzymes of carbohydrate metabolism. For instance, Basnet *et al*. (1994) reported *in vitro* anti-diabetic activity in the extracts of *S. japonica*, a traditional medicinal plant in Japan that has never been used to cure diabetes mellitus in its history. The study on α-glucosidase inhibitory potential of ethanolic extract of *S. chirayita* and its substitute *S. nervosa* and *Andrographis paniculata* revealed that *S. nervosa* had the highest α-glucosidase inhibitory potential (22.6 % inhibition) followed by *Andrographis paniculata* (20.6 % inhibition) and *S. chirayita* (18.7 % inhibition) (Phoboo *et al*., 2010). Similarly, the study carried out by Phoboo *et al*. (2012) revealed that leaf and inflorescence showed maximum α-glucosidase inhibition activity than stem and root extract in *S. chirayita*.

Similarly, Roy *et al.* (2015) reported significant inhibition of a-amylase activity *in vitro* by methanolic and aqueous leaf extracts of leaf, stem and roots of *S. chirayita* and *S. cordata.* They reported highest inhibitory activity in leaf extracts followed by stem and root extracts. Root extracts also showed minor anti-diabetic activity. Therefore, *Swertia* plants may be a rich source for the discovery of new drugs.

2.4.3 Anti-bacterial activity of *Swertia* L.

The failure of chemotherapeutics and antibiotic resistance is one of the main problems of today's generation and this has resulted into screening of many medicinal plants for their antimicrobial properties (Colombo and Bosisio, 1996). Medicinal plants are attributed with rich source of antimicrobial agents (Ahirwal *et al*., 2011), hence are screened to evaluate antimicrobial potential. For the evaluation of antibacterial activity inhibition zone diameter (IZD) and minimum inhibitory concentration (MIC) approaches are used (Kshirsagar *et al*., 2019).

A number of *Swertia* species has been screened for their antibacterial property such as *S. chirayita*, (Ghosh *et al*., 2012; Ahirwal *et al*., 2011; Kweera *et al*., 2011; Sammadar *et al*., 2013; Yadav *et al*., 2016; Subedi and Karki, 2018), *S. cordata* (Joshi *et al*., 2013), and *S. dilatata* (Sammadar *et al*., 2013), against various bacterial strains and reported various degree of antibacterial activity.

Swertia species are known to be rich in flavonoids, tannins, alkaloids, xanthones and glycosides (Kweera *et al*., 2011) which are demonstrated to exhibit antimicrobial properties against various bacterial and fungal strains (Haslam, 1996; Scalbert, 1991, Brahmachari *et al*., 2004). Similarly Swertiamarin, amarogentin isolated from different *Swertia* species are also reported to exhibit antibacterial activity against various bacterial strains (Samaddar *et al*., 2013; Brahmachari *et al*., 2004). Different species of *Swertia* L. has been studied for their antibacterial activity which is proclaimed to exhibit varying degree of inhibition against various strains of bacteria. The species of *Swertia* L.which were demonstrated to show moderate to good antibacterial activity against various bacterial strains includes *S. japonica* against *Staphylococcus aureus* (Brahmachari *et al*., 2004), *S. corymbosa* against *S. aureus* and *Salmonella typhii* (Ramesh *et al*., 2002), *S. chirayita* against *Bacillus subtilis*, *Escherichia coli*, *Pseudonomonas aeruginosa* (Sammaddar *et al*., 2013), *Salmonella typhii, Providencia alcalifaciens,* and *Vibrio cholera* (Ahirwal *et al*., 2011), *Clostridium perfringens, Clostridium perfringens* and *Staphylococcus aureus* (Roy *et al*., 2015; Yadav *et al*., 2016) *Klebsiella pneumonia, Citrobacter freundii , E. coli, S. typhii, S. aureus, and Shigella dysenteriae* (Subedi and Karki, 2018) and *S. dilatata* against *B. subtilis*, *P. aeruginosa* and *E. coli* (Sammadar *et al*., 2013). Similarly, the xanthone, 1, 2-dihydroxy-6-methoxyxanthone-8-O-β-D-xylopyranosyl isolated from *S.corymbosa* (Grieb.) was reported to exhibit strong antibacterial activity against *S. pneumoniae* and *E. coli* (Mahendran *et al*., 2015). Hence the study indicates that if the unscreened and unstudied species of *Swertia* species are explored may present a problem solving compound against these bacterial resistance and diseases.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Preliminary Study

For the collection of different species of *Swertia* L., distribution pattern of different species was observed through herbarium specimen deposited at National herbarium and Plant Laboratories (KATH) as well as in Tribhuvan University Central Herbarium (TUCH).

3.2 Research Design

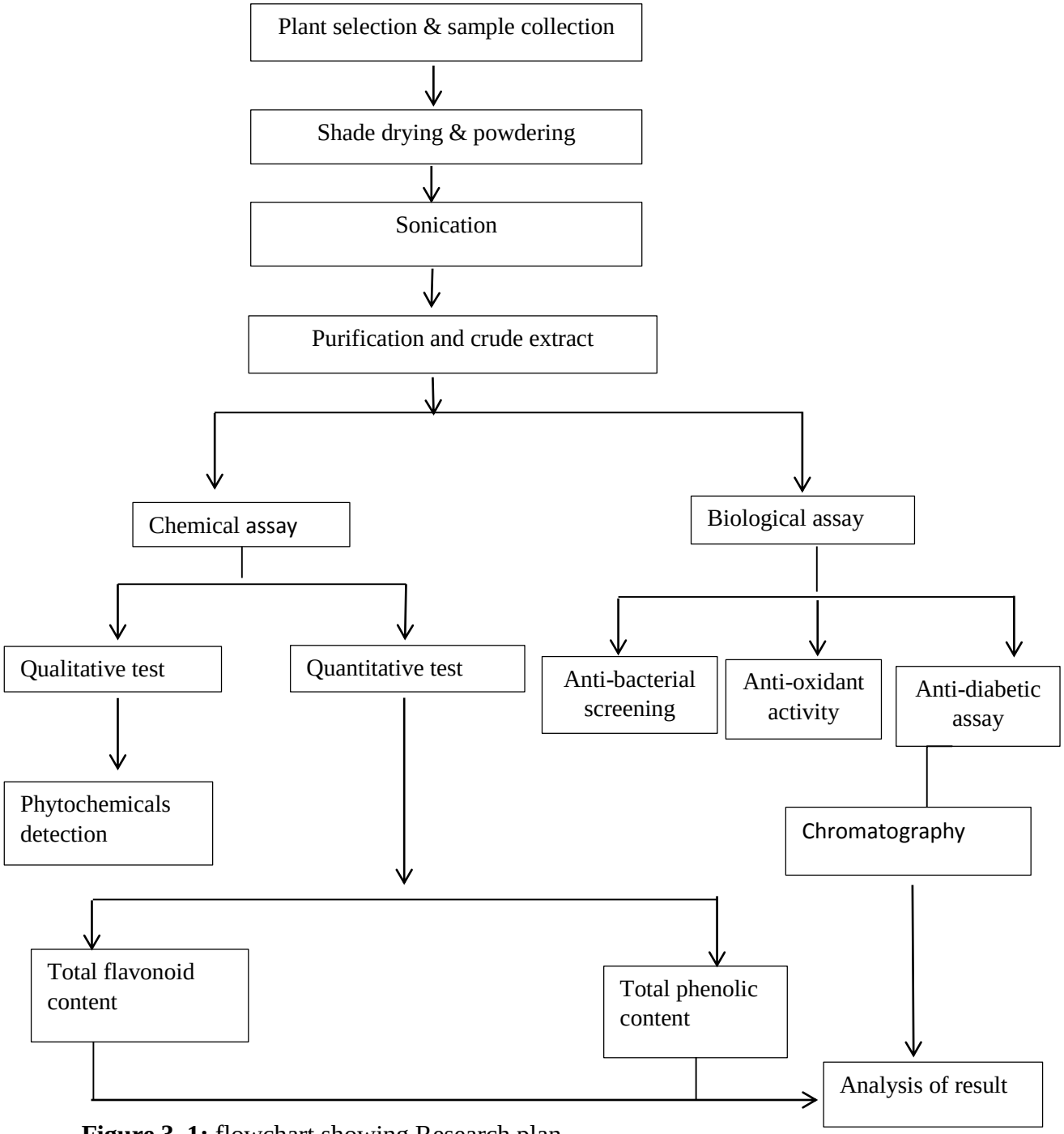

Figure 3. 1: flowchart showing Research plan

3.3 Selection of plant samples

Species of *Swertia* L. are among the most familiar medicinal herbs belonging to the family Gentianaceae (Joshi and Dhawan, 2005). For the phytochemical screening, the criteria used for selection of different species of *Swertia* were ethno-medicinal value and availability of sufficient plant samples that have not been screened till date in the collection site. The plant species used for screening were:

1. *S. barunensis* Chassot
2. *S. chirayita* (Roxb.) H. Karst.
3. *S. cuneata* Wall.
4. *S. multicaulis* D. Don
5. *S. nervosa* Wall.

3.4 Collection and identification of the plant sample

Different species of *Swertia* L. were collected from different parts of Nepal at the end of flowering season in late August to October. The collected species were identified by the experts and cross checked with the herbarium specimen deposited at National herbarium and Plant Laboratories (KATH) as well as in Tribhuvan University Central Herbarium (TUCH). Herbarium specimens were prepared and voucher specimens were submitted at TUCH for future references.

Table 3. 1: List of plant under study, their collection area, elevation and parts used.

S.N	Plant name(Latin Name)	Place of collection	Elevation (m)	Parts used
1	*S. barunensis* Chassot	Jaljale	4500	Root and leaf
2	*S. chirayita* (Roxb.) H. Karst.	Gatlang, Rasuwa	2200	Entire plant
3	*S. cuneata* Wall.	Jaljale	4300	Entire plant
4	*S. multicaulis* D. Don	Jaljale	4500	Root and leaf
5	*S. nervosa* Wall.	Chandragiri	2400	Entire plant

3.5 Preparation of plant materials

The collected plant materials were cleaned by removing mud and other unwanted materials. The cleaned materials were shade dried until constant weight. Then the dried plant material was powdered with the help of grinder and the fine powder was kept in air tight plastic bottles.

3.6 Preparation of plant extracts and dilution

Three gram of fine powder of each plant sample was weighed separately and dissolved in 30 ml of methanol (Fisher Scientific Ltd., Mumbai) in falcon tube. Then mixture was subjected to sonication for 1 hours at 40 KHz at a temperature of 40^0-50^0C for the first time. The mixture was filtered through filter paper. Then the residue was again mixed with 30 ml methanol and again sonicated for next 1 hour and filtered again. The filtrates were then concentrated under reduced pressure in a rotatory evaporator by evaporating methanol till very little extract was left. The extracts were then transferred to a clean and pre-weighed petriplate with the help of pipette and allowed to dry at room temperature under a laminar air hood till constant weight. The dried extract thus obtained was weighed carefully. The yield of extract was expressed as percentage of dry sample powder used for extraction. The dried mass was then scrapped off with blade and then collected in 2ml propylene vials. Then each of 100mg/ml stock was prepared by dissolving 100mg of plant extract in 100ml of methanol. This 100mg/ml stock was used for chemical and biological assay. The remaining solid mass in vials were labeled properly and stored at -20^0C for further use. The percentage yield of plant extract was calculated by using the formula:

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

3.7 Chemical Assay

3.7.1 Qualitative phytochemical Assay

The methanolic extract of plant samples were subjected to preliminary phytochemical screening to detect the presence of major phytochemicals constituents following Harborne and Baxter (1995) and Todkar *et al*. (2010).

3.7.1.1 Test for Alkaloids

The crude extract was dissolved in methanol and then mixed with few drops of Dragendroff reagent. Appearance of orange red color indicated the presence of alkaloids.

3.7.1.2 Test for flavonoids

Small amount of crude extract was mixed with 2ml of 2% NaOH which resulted in formation of intense yellow color that becomes colorless on addition of few drops of dilute acid. Hence it indicated the presence of flavonoids.

3.7.1.3 Test for Phenols and tannins

The crude extract was mixed with about 2ml of ferric chloride ($FeCl_3$) solution. An appearance of dark blue-green or black color indicated the presence of phenols and tannins.

3.7.1.4 Test for Saponins

The crude extract was mixed with about 5ml of distilled water and it was shaken vigorously. Formation of stable foam indicated the presence of saponins.

3.7.1.6 Test for Steroids

2ml of chloroform ($CHCl_3$) was added to the crude extract followed by careful addition of 2ml conc. H_2SO_4 and it was shaken gently. An appearance of reddish brown color ring indicated the presence of steroids.

3.7.1.7 Test for Terpenoids

The crude extract was treated with few drops of copper acetate (prepared by dissolving copper acetate monohydrate in warm water until a solution forms blue color) solution. The formation of emerald green color indicated the presence of terpenoids.

3.7.2 Quantitative phytochemical analysis

3.7.2.1 Total Flavonoid Content

Total flavonoid content in methanolic extract of selected species of *Swertia* was determined using Aluminium Chloride ($AlCl_3$) colorimetric method (Chang *et al*., 2002; Roy *et al*., 2011). Firstly 250µLof the plant extract (10 mg/mL) was taken in a test tube. Then 750µL of $AlCl_3$ solution (10% w/v) was added to the test tubes followed

by 50µl (1M) potassium acetate. Then the solutions were diluted by adding 1.4 ml distilled water and incubated at room temperature for 30 minutes. The blank was prepared by replacing the plant extract with same volume of absolute methanol. The absorbance was measured at 415 nm using UV-visible spectrophotometer (CT 8600, E-chrome Tech, Taiwan). The calibration curve was obtained using different concentrations (25-250 µg/mL) of Quercetin as standard. The total flavonoid content was expressed in terms of milligram of Quercetin equivalent per gram of dry mass (mg QE/g).

3.7.2.2 Total Phenolic Content

Total phenolic content of selected species of *Swertia* were determined using Folin-Ciocalteau phenol reagent following Ainsworth and Gillespie (2007) with slight modification. For this about 100µL of the plant extract (2.5 mg/mL) was taken in a 2 mL polypropylene tube. To this 1 mL of Folin-Ciocalteau reagent (1:10 dilution with distill water) was added followed by addition of 800µL (1M) Na_2CO_3. Then the solution was incubated for 15 minute at room temperature. Then absorbance was measured at 765 nm. The blank was prepared by replacing the extract with same volume of absolute methanol. The calibration curve was obtained using Gallic acid of different concentration (25-250µg/ml). Total phenolic content was expressed in terms of milligram of Gallic acid equivalent per gram of plant extract (mg GAE/g).

3.8 Assessment of Biological Activity

3.8.1 Antioxidant Activity assay

The anti-oxidant activity of plant extract was determined on the basis of free radical scavenging activity of plant extract and standard ascorbic acid solution in methanol to react with stable DPPH (1,1 –diphenyl-2 picryhydrazyl) free radical. The method was carried out following Singh *et al*. (2002) with slight modification. For this DPPH solution of 0.2mM was prepared by dissolving 7.88g of DPPH powder (molecular weight 394.32 gm/mol) in 100 ml of methanol. Three replicates of plant sample 1 mL of each of different concentration of plant extract and ascorbic acid (25-250µg/ml) were prepared in methanol in a 2ml propylene vial. To this 1 mL of freshly prepared DPPH solution (0.2 mM) was added and it was incubated for 30 minute in dark. The control was prepared as above but without the plant extract or ascorbic acid. The absorbance of the solution of each tube was measured at 517nm using visible spectrophotometer

and methanol as blank. The free radical scavenging activity of the plant samples were calculated in percentage using the formula:

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

Standard graph was plotted taking concentration on X-axis and percentage radical scavenging activity on Y-axis based on the standard curve. The IC_{50} value was also calculated by using linear equation of the curve obtained:

$$Y=a*X+b$$

$$IC_{50}= (50-b)/a$$

Where X=concentration, Y=%RSA, a and b are the coefficient and constant of the linear equation respectively.

3.8.2 In-vitro Anti-diabetic Assay

Anti-diabetic activity of selected species of *Swertia* L. was carried out by inhibitory potential of plant extract to the enzyme α-glucosidase.

3.8.1.1 α-glucosidase Inhibition Assay

α-glucosidase inhibition assay was carried out following the protocol of Si *et al*. (2010) with slight modification. The reaction medium was prepared by dissolving P-nitrophenyl β-D, glucopyranoside (Sigma-Aldrich, Germany) in 0.1M potassium phosphate buffer (pH 6.4) to make a final concentration of 10mM. Two sets of reactions were prepared in 96 well plates were used for α-glucosidase inhibition assay.10µl of plant extracts of respective species were added two vertical columns followed by addition of 150µL of PNPG solution. Then 40 µL of 1M Na_2CO_3 was added in samples kept in one of the vertical columns for each species (marked as -ve). Then the plate was incubated at 30^0C for 5 minute. After that 10µl α- glucosidase solution prepared in phosphate buffer was added in all the cells and the plate was and was incubated for next 20 minutes. After incubation, 40µL of 1M Na_2CO_3 was added in all the remaining cells (columns marked as +ve). The control was prepared as above but by replacing the plant extract with pure methanol. Then the absorbance was taken at 405 nm in Elisa Plate Reader (Thermo Fisher) Percentage inhibition of enzyme activity was determined by using following formula:

% inhibition $= \frac{control\ absorbance - sample\ absorbance}{control\ absorbance} \times 100$

3.9 Anti-bacterial Assay

3.9.1 Bacterial Strains

Different strains of ATCC bacteria were purchased from National Public Health Laboratory (NHPL), Teku, Kathmandu Nepal.

Gram positive Bacterial Strains: *Bacillus subtilis, Enterococcus faecalis*

Gram negative Bacterial Strains: *Escherichia coli, Pseudomonas aeruginosa, and Salmonella typhii*

3.9.2. Preparation of Culture media

3.9.2.1 Nutrient Broth (NB)

Nutrient Broth is a general purpose medium used for cultivating a broad variety of fastidious and non-fastidious microorganisms with non-exacting nutritional requirement. For the preparation of NB media, 6.5 gram of NB powder (HI-media laboratories Pvt. Ltd, Mumbai) was mixed with distill water to make a final volume of 500 mL. The media was then sterilized by autoclaving at 15lbs pressure and 121 ^{0}C for 15 minutes which was then cooled under laminar airflow hood. The prepared media was used for suspension type of bacterial culture and kept in aseptic condition for further use.

3.9.2.2 Muller Hinton Agar media (MHA)

Muller Hinton Agar (MHA) media was prepared by dissolving 38 gram of MHA powder (HI-media laboratories Pvt. Ltd, Mumbai) with distill water to make final volume of 1 litre. The media was then sterilized by autoclaving at 15 lbs pressure and 121 ^{0}C for 15 minutes. Then it was allowed to cool for about 1 hour in laminar airflow hood. The media was then transferred to the sterilized petri plates by pouring about 20 mL of media on each plates of 9 cm diameter. Then the media was allowed to cool and placed in aseptic condition for further use.

3.9.2.3 Standard culture inoculum preparation

For inoculum preparation, different strains of pure bacterial strain were revived by transferring aseptically to Nutrient broth for suspension culture and was kept overnight

in a shaking incubator at 37ºC of temperature for its growth. This culture was then just touched in a test tube containing Nutrient broth with the help of cotton ear bud and the turbidity of bacterial suspension was adjusted at the 0.5 McFarland standards for the antibacterial test.

3.9.2.4 Antibacterial screening via Agar well diffusion method

For antibacterial screening Agar well diffusion method was used with slight modification (Perez *et al*., 1990). Paper discs were made by punching the filter paper with a punching machine. All the materials required for bacterial culture i.e, cotton swabs, toothpick, forceps, beaker, cork borer and paper disc was sterilized using autoclave. Five different concentrations (100mg/mL, 50mg/mL, 25mg/mL, 12.5mg/mL, 6. 25mg/mL) of the plant extract was prepared for antibacterial test.

Seven wells were prepared on petri dishes containing solid MHA media with a sterile cork borer of 5mm diameter and labeled with a permanent marker. The sterilized paper disc of 5mm diameter was placed in the six well in petri dish while Gentamycin at concentration 10µg was placed in a well- marked as positive control. 10µl of methanol was poured in a well- marked as negative control while remaining five wells were poured with 10µl of different concentration of prepared plant extract with the help of micropipette. Then the petri dish was swabbed evenly with the particular bacteria with the help of cotton bud and the petri dish was incubated at 37ºC for 24 hour. All the samples were tested in triplicates. The zone of inhibition was noted in mm for each individual plant extract and individual bacteria.

3.10 Column Chromatography

Column chromatographic technique was used for the isolation and purification of secondary metabolites following Bajpai *et al*. (2016) with slight modification. For the separation, silica gel was prepared by adding dichloromethane to a silica powder until a slurry was formed. In a cylinder shaped glass column, a very small amount of cotton was inserted to the mouth of column and then silica gel (stationary phase) is encountered slowly and kept undisturbed for some time to settle the silica. Once the column is ready, the sample is loaded (plant extract dissolved in methanol) inside top of column. The mobile solvent (in Table 3.2) is then allowed to flow down through the column. The compounds in mixture have different interactions ability with stationary phase (silica gel) and mobile phase, thereby will flow along the mobile phase at

different time intervals or degrees (Bajpai *et al.*, 2016). The fractions were separated based on visual observation of color of the extract and extracts with similar color were mixed together. The fractions were then evaporated under reduced pressure in a rotary evaporator (D-Lab, China). The dried fractions were later used for their antibacterial and anti-diabetic activity *in vitro*.

Table 3. 2: Gradient solvent system used in column chromatography for separation of compound from mixture.

Solvent system	**Ratio**	**Volume (mL)**	**Fraction**
Hexane	100%	50	1
Hexane: ethyl acetate	9:1	50	2
Hexane: ethyl acetate	4:1	50	3
Hexane: ethyl acetate	1:1	50	4
Hexane: ethyl acetate	1:4	50	5
Hexane: ethyl acetate	1:9	50	6
Ethyl acetate	100%	50	7
Ethyl acetate: methanol	9:1	50	8
Ethyl acetate: methanol	4:1	50	9
Ethyl acetate: methanol	1:1	50	10
Ethyl acetate: methanol	1:4	50	11
Ethyl acetate: methanol	1:9	50	12
Methanol	100%	50	13

3.11 Liquid Chromatography-Mass Spectrometry Analysis

Crude extracts and selected fractions of *S. barunensis* and *S. multicaulis* were subjected to LC-MS analysis to find out the potential compound responsible for α-amylase inhibition. LC-MS was carried out in following the protocol of Timsina *et al.* (2018) with modification. The different parameters used for LC-MS analysis are as follows.

MS system: Shimadzu LCMS 2020

LC System: Shimadzu prominence

Software for acquisition and data analysis: LabSolutions

Column:Shim-pack XR-ODS 3*50 mm, id 2.2 um

Sample prep: 150 µL sample solution + 600 µL water-> vortex-> inject after filteration

Mobile phase A: 0.1% formic acid in water

Mobile phase B: 0.1% formic acid in acetonitrile

Flow rate: 0.3mL/min

Injection volume: 5 µL

Column oven temp: 30 ° C

Run time: 35 min

uv: 315 nm

Time (min)	% B
0.01	5
20	95
30	95
30.01	5

MS parameter

Ionization source: ESI in positive mode

Acquisition mode: Scan

start m/z: 35

end m/z: 750

DL temp :250 °C

Nebulizing gas flow: 1.5 L/min

Heat block temp: 200 °C, Drying gas flow: 15 L/min.

CHAPTER FOUR- RESULTS

4.1 Percentage yield of plant extracts

The percentage yield of extracts of selected species of *Swertia* L. is listed in Figure 4.1. The yield percentage was highest in *S. multicaulis* root (35.67±3.52%) and lowest in *S. chirayita* (12.79±2.23%) whole plant while other revealed the yield percentage between these two extremes. Percentage yield was observed higher in root than leaf of the same plant species in *S. barunensis* and *S. multicaulis*.

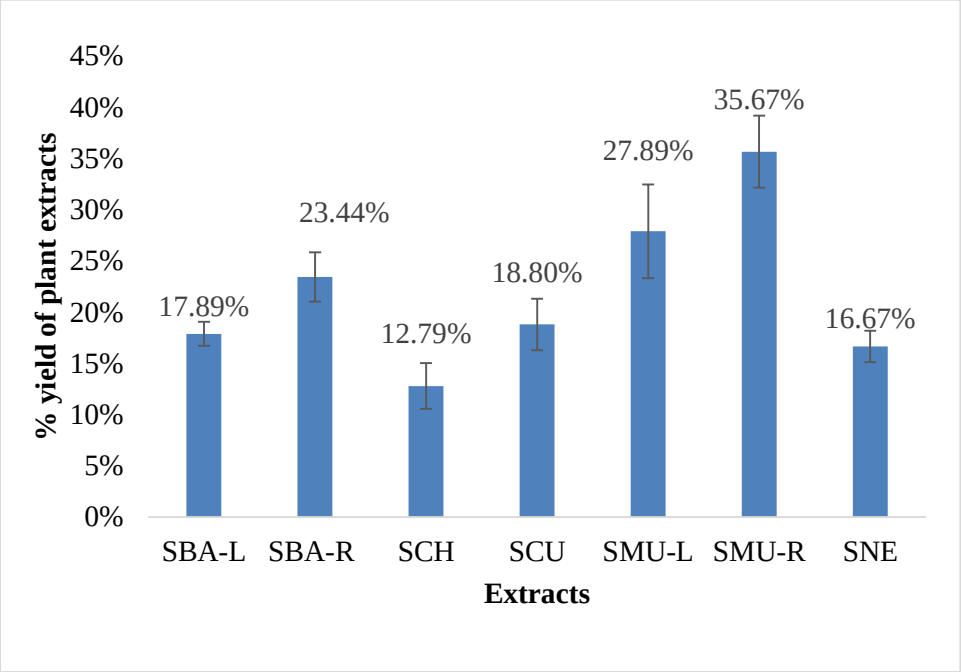

Figure 4. 1: percentage yield of extracts in selected species of *Swertia* L. Legend: (SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata*, SMU-L= *S. multicaulis* leaf SMU-R= *S. multicaulis* root, SNE= *S. nervosa*).

4.2 Qualitative Phytochemical Assay

Qualitative phytochemical screening of methanolic extract of selected *Swertia* species is tabulated in Table 4.1. The study revealed that alkaloids, flavonoids and phenols as well as tannins, steroids and terpenoids were present in the extracts of all the tested species of *Swertia* L. On the basis of color change it seems higher amount of tannin and phenol may be present in roots than leaves. Saponins were present in all the species of *Swertia* except root extracts of *S. barunensis* and *S. multicaulis.*

Table 4. 1 Qualitative Phytochemical screening of selected species of *Swertia* L**.**

Extract	Alkaloids	Flavonoids	Phenols and tannins	Saponins	Steroids	Terpenoids
SBA-L	+	+	+	+	+	+
SBA-R	+	+	+	-	+	+
SCH	+	+	+	+	+	+
SCU	+	+	+	+	+	+
SMU-L	+	+	+	+	+	+
SMU-R	+	+	+	-	+	+
SNE	+	+	+	+	+	+

Legend: + indicate presence and – indicates absence**.** (SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata*, SMU-L= *S. multicaulis* leaf, SMU-R= *S. multicaulis* root, SNE= *S. nervosa*).

4.3 Quantitative Assay

4.3.1 Total Flavonoid Content

The total flavonoid content was expressed in terms of milligram of Quercetin equivalent per gram of dry mass (mg QE/g). The standard curve of Quercetin is depicted in Figure 4.2. The highest amount of flavonoid was observed in *S. multicaulis* leaf (54.13±0.004) followed by *S. chirayita* (40.17±0.005) and *S. barunensis* leaf (37.25±0.003). The lowest amount of flavonoid content was found in *S. multicaulis* root (7.67±0.002). The total flavonoid content of other selected species lied between these two values.

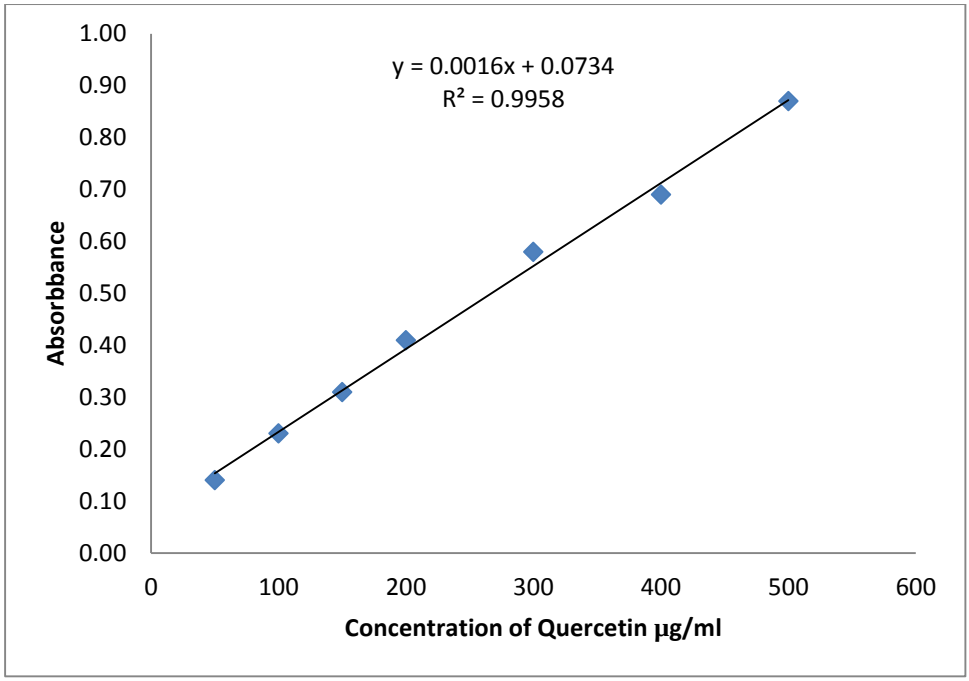

Figure 4. 2: Calibration curve of Quercetin

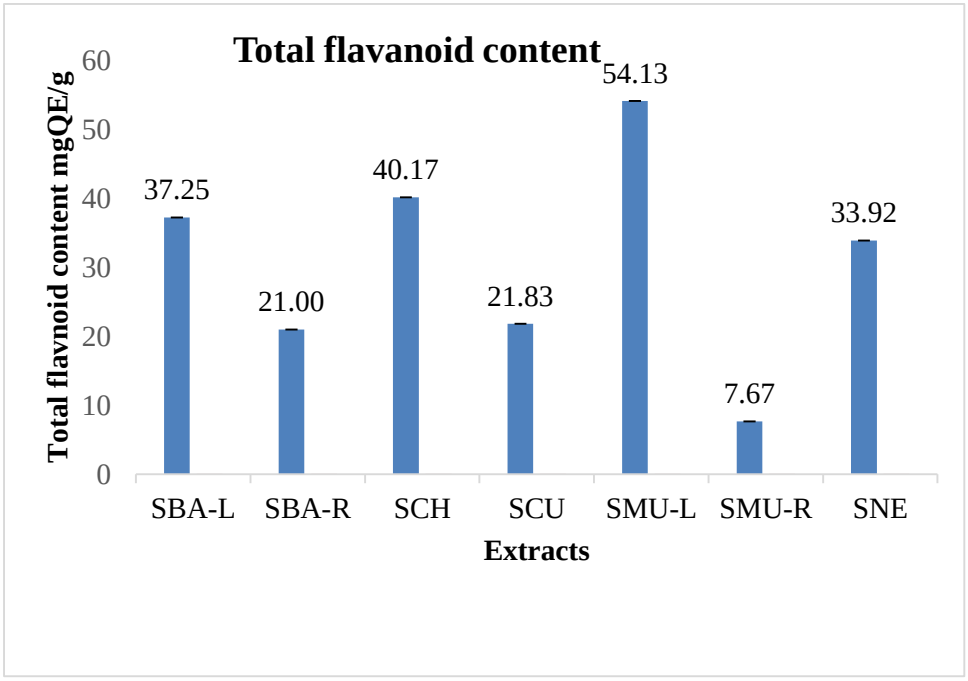

Figure 4. 3: Total flavonoid content in methanolic extract of selected species of Swertia L. (Legend: SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata* SMU-L= *S. multicaulis* leaf, SMU-R= *S. multicaulis* root and SNE= *S. nervosa*).

4.3.3 Total Phenolic Content.

The total phenolic content was expressed in terms of milligram of Gallic acid equivalent per gram of plant extract (mg GAE/g). The calibration curve of Gallic acid is presented in Figure 4.4 and total phenolic content of selected species of *Swertia* L. is depicted in Figure 4.5. Highest amount of polyphenol was found in *S. barunensis* leaves (107.38±2.34) followed by *S. chirayita* (100.10±0.74) and lowest amount was found in *S. cuneata* (37.44±2.28). The total phenolic content of other selected species lied between those two mentioned extremes.

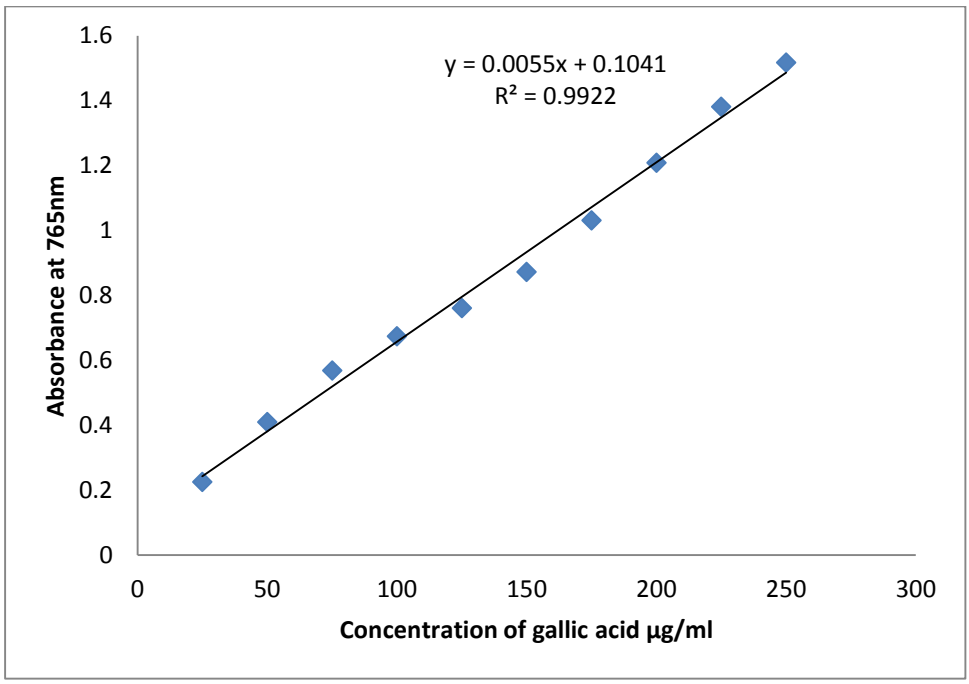

Figure 4. 4: Calibration curve of Gallic acid

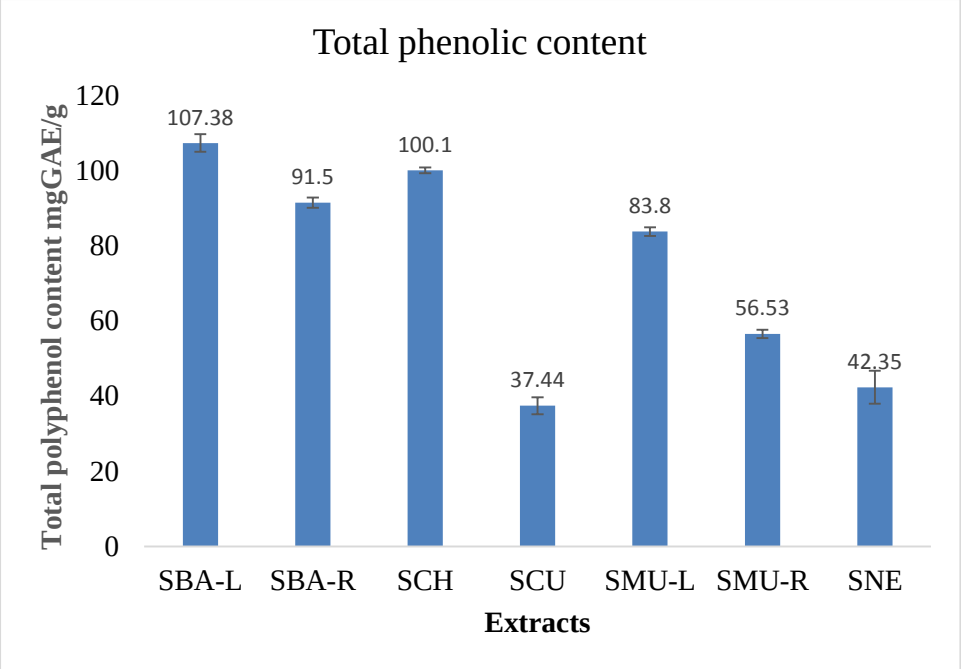

Figure 4. 5: Total phenolic content in selected species of *Swertia* L. (**Legend:** SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata* SMU-L= *S. multicaulis* leaf SMU-R= *S. multicaulis* root, SNE= *S. nervosa*).

4.4 Biological Assay

4.3.1 DPPH Radical Scavenging activity

Antioxidant activity of methanolic extracts of selected *Swertia* species is expressed in terms of their DPPH radical scavenging activity and compared with the standard curve of ascorbic acid as shown in Figure 4.6 and 4.7.

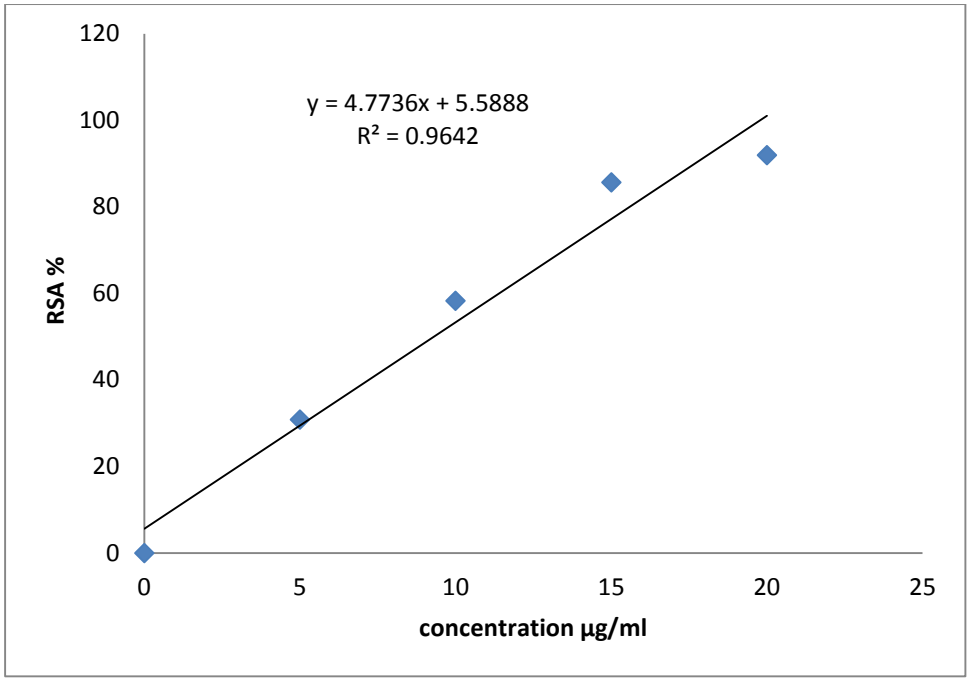

Figure 4. 6: Percentage DPPH Radical Scavenging Activity of standard curve of Ascorbic acid

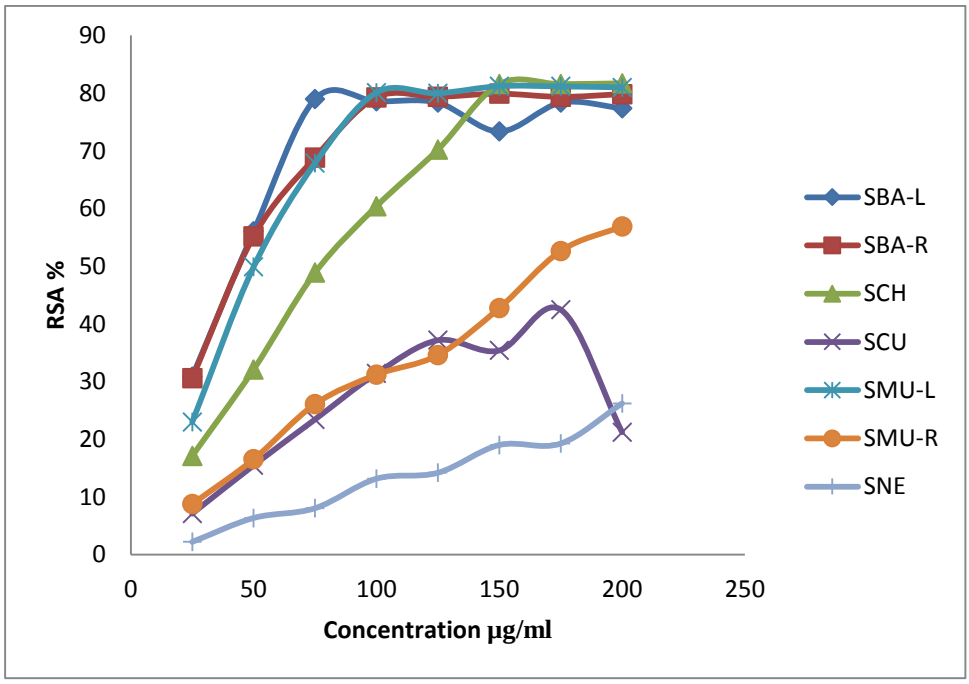

Figure 4. 7: Percentage DPPH Radical scavenging activity of methanolic extract of selected species of *Swertia* L. (**Legend:** SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata* SMU-L= *S. multicaulis* leaf SMU-R= *S. multicaulis* root, SNE= *S. nervosa*).

The various species of *Swertia* revealed concentration dependent scavenging activity. Among the selected species of *Swertia*, highest radical scavenging activity was observed in leaf extracts of *barunensis* (IC_{50} 17.74 µg/mL) while the lowest radical scavenging activity was observed in *S. nervosa* (IC_{50} 399.84 µg/mL). The values of radical scavenging activity in other species were between these two extremes.

The **IC_{50} value** is that concentration of a drug that reduces the activity (or binding) of another drug to an enzyme by 50%. The IC_{50} value of *S. nervosa* (399.84µg/ml) was highest while this value was lowest in case of *S. barunensis* leaves (17.74µg/ml). The IC_{50} of other selected species lies between these two extremes.

Anti-oxidant activity and IC_{50} are inversely related to each other. Higher IC_{50}value indicates that it has lower anti-oxidant activity. IC_{50} of *S. barunensis* roots and leaves are lower that means it has comparatively good anti-oxidant activity which is comparable with the IC_{50} value of ascorbic acid that was found to be 9.30 µg/ml.

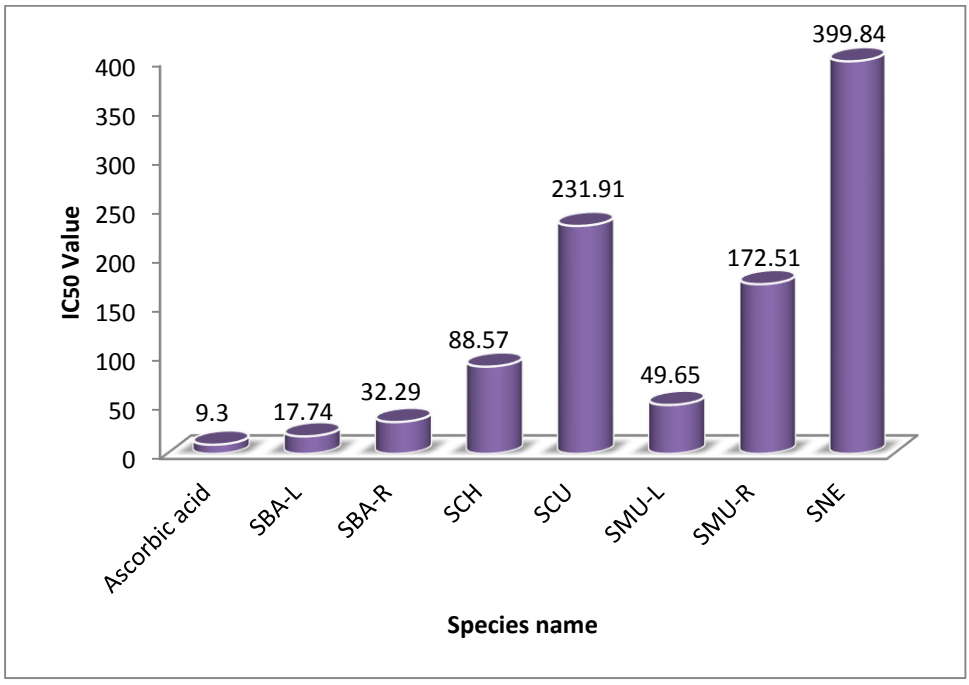

Figure 4. 8: IC_{50} value of methanolic extract of selected species of *Swertia* L. and Ascorbic acid. (**Legend:** SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata* SMU-L= *S. multicaulis* leaf SMU-R= *S. multicaulis* root, SNE= *S. nervosa*).

4.4.1 α-glucosidase inhibition assay

The percentage of α-glucosidase inhibiton activity of methanolic extract of selected species of *Swertia* is depicted in Figure 4.9. The α-glucosidase inhibiton activity of crude sample was tested using 0.1mM PNPG and 1u/ml glucosidase. Highest inhibition activity was found in *S. barunensis* leaves (36.65±0.74%) followed by *S. multicaulis* leaves (33.7±0.15%) while lowest was found in *S. cuneata* (-26.15±0.3%). The leaves of *S. barunensis* and *S. multicaulis* showed α-glucosidase inhibition potenial while the roots of same mentioned species found to be showing α- glucosidase induction activity.

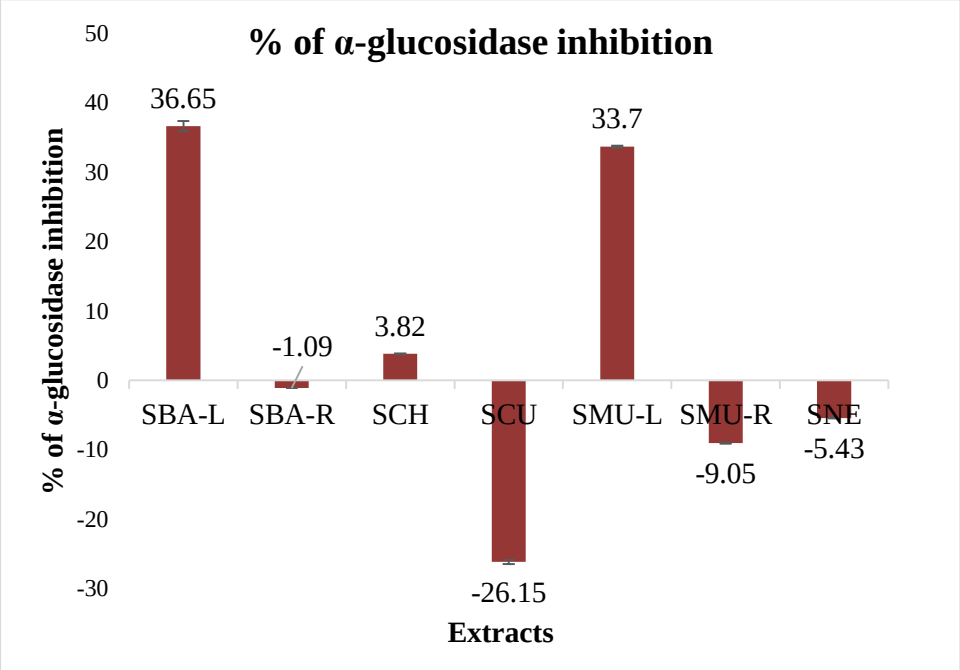

Figure 4. 9: α-glucosidase inhibition activity *in-vitro* in methanolic extract of selected species of *Swertia* L. (Legend: SBA-L=*S. barunensis* leaf, SBA-R= *S. barunensis* root, SCH= *S. chirayita*, SCU= *S. cuneata* SMU-L= *S. multicaulis* leaf, SMU-R= *S. multicaulis* root, SNE= *S. nervosa*).

The two plant sample *S. barunensis* and *S. multicaulis* leaves that showed glucosidase inhibition activity was fractionated using column chromatography and its fraction sample was again tested for α-glucosidase inhibition activity. The result revealed that in *S. barunensis* leaves (as depicted in Figure 4.10), fraction E exhibited maximum inhibition activity (97.2±1.86%) and fraction A has maximum induction activity against α-glucosidase enzyme. In case of *S. multicaulis* (as depicted in Figure 4.11), fraction G was found to be exhibiting maximum inhibition activity (94.4±0.66%) and fraction I exhibiting maximum induction activity against α-glucosidase enzyme.

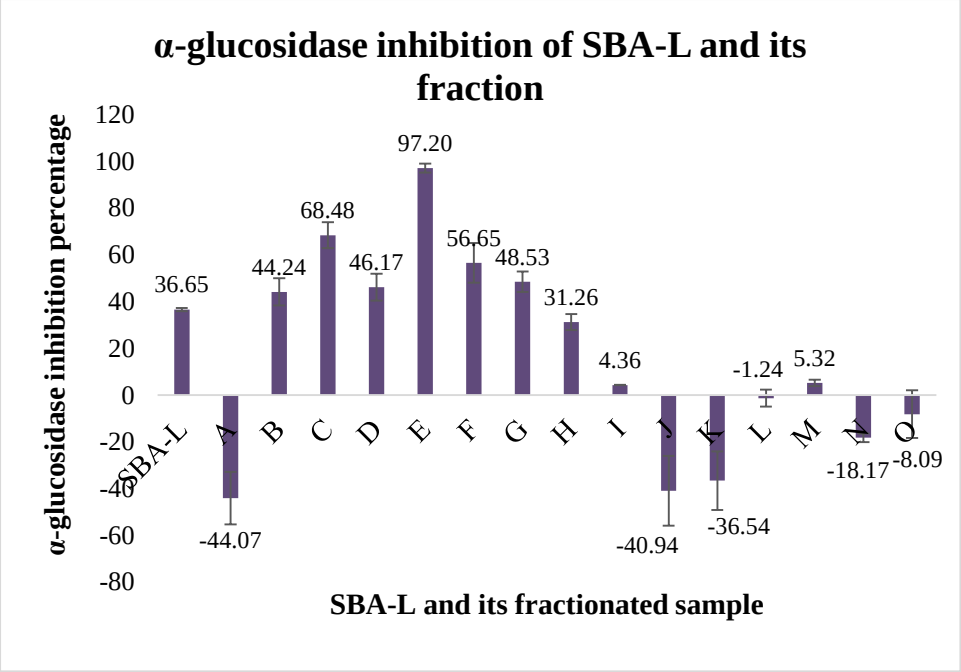

Figure 4. 10: α-glucosidase inhibition activity *in-vitro* in *S.barunensis* leaf and its fraction; (**Legend:** SBA-L= *S. barunensis* leaf).

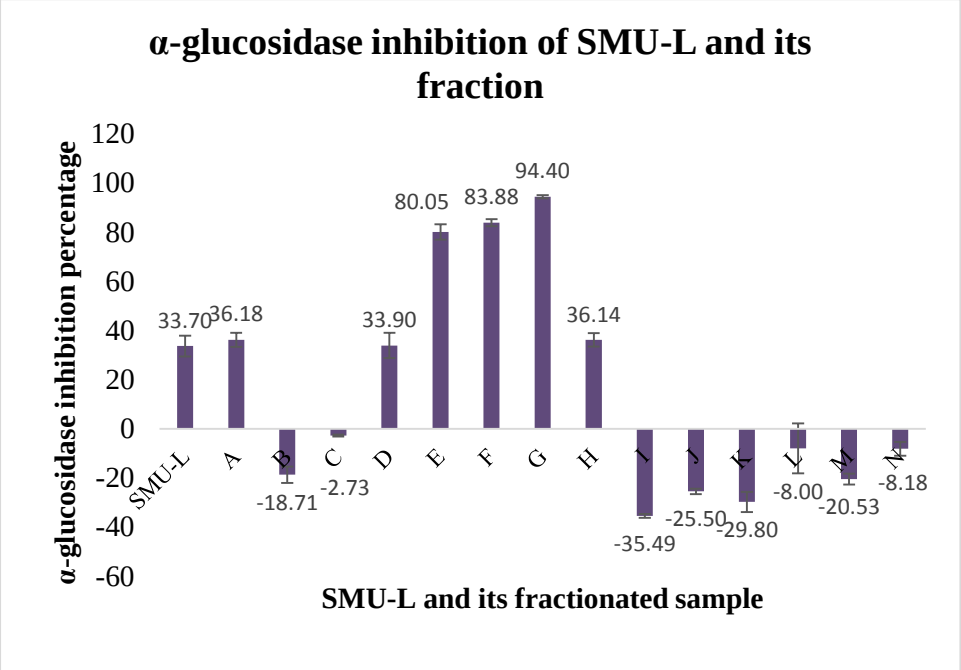

Figure 4. 11: α-glucosidase inhibition activity *in-vitro* in *S. multicaulis* leaf and its fraction; (**Legend:** SMU-L= *S. multicaulis* leaf).

Table 4. 2: LC-MS Analysis of selected fractions of SBA-L.

Retention Time (Minutes)	**m/z ratio**	**Presence of major peaks in different fractions of SBA-L**			
		Crude extract	**Fraction E**	**Fraction F**	**Fraction J**
5.997	227	√			
6.859	471	√			
8.258	453	√	√	√	
9.224	340	√	√	√	√
15.07	412	√	√	√	√
21.117	466	√	√	√	√
33.891	152	√	√	√	√
Glucosidase inhibition (%)		**36.65**	**97.2**	**56.65**	**-25.5**

Data on LCMS analysis of crude methanolic extract of SBA-L and its selected fractions is presented in Table 4.2. The crude extract gave 7 major peaks while fraction E and fraction F gave only 5 major peaks. The fraction J on the other hand gave only 4 major peaks. Looking at the data the presence of a peak with Rt value of 8.258 min and mass to charge ratio of 453 could be linked to high percentage inhibition of glucosiddase by SBA-L extracts.

Table 4. 3: LC-MS Analysis of selected fractions of SMU-L.

Retention Time (Minutes)	**m/z ratio**	**Area of major peaks in different fractions of SMU-L**			
		Crude extract	**Fraction F**	**Fraction G**	**Fraction I**
5.557	363	√			
6.772	375	√			√
7.317	359	√			√
9.224	340	√	√	√	√
10.026	393	√			
15.07	412	√	√	√	√
21.117	466	√	√	√	√
33.891	152	√	√	√	√
Glucosidase inhibition (%)		33.7	83.9	94.4	-35.5

Data on LCMS analysis of crude methanolic extract of SMU-L and its selected fractions is presented in Table 4.3. The crude extract gave 8 major peaks while fraction F and fraction G gave only 5 major peaks. The fraction I on the other hand gave 6 major peaks. Looking at the data the presence or absence of none of the peaks could be linked to high percentage inhibition of glucosiddase by SMU-L extracts.

4.5 Anti-bacterial Assay

The effect of methanolic extracts of selected species of *Swertia* L. against different bacterial strains is summarized in Table 4.4. Anti-bacterial activity of methanolic extract of selected species *Swertia* were tested against five cultured bacterial strains (two gram-positive; *Bacillus subtilis, Enterococcus faecalis* and three-gram negative bacteria; *Salmonella typhii, Escherichia coli, Pseudomonas aeruginosa*). For positive control Gentamycin 10mc was used while methanol was used as negative control. Five different concentration (6.25, 12.5, 25, 50, & 100mg/ml) of plant extract was used to examine their activity against the selected bacterial strains and comparison was made with standard drug Gentamycin. Among the selected sample, none of the extract was found to show zone of inhibition against all tested bacterial strains. *S. barunensis* leaf showed concentration dependent antibacterial activity against *B. subtilis , E. coli* and *P. aeruginosa*. It showed inhibition in all concentration against *B. subtilis* while the same plant showed effect from a concentration of 50mg/ml in *E. coli* and *P. aeruginosa.* The extract of *S. barunensis* root was found to be effective against *E. coli* and *P. aeruginosa* at a concentration of 100mg/ml. The extract of *S. chirayita* showed antibacterial effect against *B. subtilis* (from 12mg/ml), *E. coli* (25mg/ml) and *P. aeruginosa* (at 100mg/ml). *S. cuneata* extract was found to be effective against *B. subtilis* (from 6.25mg/ml) and *P. aeruginosa* (100mg/ml).The extract of *S. multicaulis* leaf was found to be effective against *B. subtilis* (from 6.25mg/ml) and *P. aeruginosa* (50mg/ml). *S. multicaulis* root extract was found to be effective against *B. subtilis* only while the extract of *S. nervosa* showed no effect against the tested bacterial strains.

Table 4. 4: Antibacterial activity of methanolic extracts of selected *Swertia* species.

Extract	Test organisms	Zone of inhibition in mm (Excluding zone diameter of well)					
		6.25mg/ml	12.5mg/ml	25mg/ml	50mg/ml	100mg/ml	Gentamycin
SBA-L	*Bsu*	0.6	9.3	13	16	16.7	21.7
	Efa	0	0	0	0	0	21
	Eco	0	0	0	3.33	5.67	17
	Pae	0	0	0	6	8	21
	Sty	0	0	0	0	0	20
SBA-R	*Bsu*	0	0	0	0	0	21.2
	Efa	0	0	0	0	0	21
	Eco	0	0	0	0	3.66	16.33
	Pae	0	0	0	0	7	21
	Sty	0	0	0	0	0	20
SCH	*Bsu*	0	1.67	6	10	11.3	20
	Efa	0	0	0	0	0	21
	Eco	0	0	0.33	3.67	5.67	19.67
	Pae	0	0	0	0	7	21
	Sty	0	0	0	0	0	25
SCU	*Bsu*	5	5	5	5	6	21
	Efa	0	0	0	0	0	26
	Eco	0	0	0	0	1	19.33
	Pae	0	0	0	0	0	21
	Sty	0	0	0	0	0	24
SMU-L	*Bsu*	0.6	4	10	13.3	15.33	19.33

	Efa	0	0	0	0	0	21
	Eco	0	0	0	2.67	4.33	20
	Pae	0	0	0	0	0	21
	Sty	0	0	0	0	0	20
SMU-R	*Bsu*	5	5.7	7	8.66	11.33	19.67
	Efa	0	0	0	0	0	22
	Eco	0	0	0	0	0	21
	Pae	0	0	0	0	0	21
	Sty	0	0	0	0	0	20
SNE	*Bsu*	0	0	0	0	0	20
	Efa	0	0	0	0	0	21
	Eco	0	0	0	0	0	20.67
	Pae	0	0	0	0	0	21
	Sty	0	0	0	0	0	20

CHAPTER FIVE: DISCUSSION

5.1 Percentage yield of plant extracts

Extraction is a process where the desired constituents of a plant are extracted using a solvent. The primary way of extraction of organic molecules of interest involves breaking upon the cells which can be achieved through various techniques like sonication, maceration, percolation etc. The extraction yield is directly affected by type of extraction methods/solvents, plant parts (root, stem, leaves, fruits or flower), and their extraction efficiency (Azmir *et al*., 2013, Handa, 2008).

For the extraction of studied species methanol was used as solvent and sonication was used for extraction process. The percentage yield of extracts observed under the studied species of *Swertia* was between 12.79±2.23% (*S. chirayita*) to 35.67±3.52% (*S. multicaulis* root). High extract yield for methanol indicates that there are high amount of alkaloids in the plant (Shrestha *et al*., 2015). The variation in yield percent using same solvent might be due to species specific factor including differences in plant part used (as percentage yield was higher in root than leaf of the same plant species in *S. barunensis* and *S. multicaulis* in studied species). It might also be due to difference in maturity of plants parts used, and environmental factor like locality on which they were grown (Kumal *et al*., 2020).

5.2 Qualitative Phytochemical Assay

Therapeutic properties of the crude drugs are mainly because of physiologically active chemical constituents present in the drugs and lower percentage of chemical constituents may result in lesser therapeutic values of the drugs and hence are considered as low standard drugs. A little deviation from the normal in terms of quality and quantity of the chemicals may alter the effect of drug (Latif and Rehman, 2014).

Qualitative phytochemical screening in methanol extracts of selected species of *Swertia* in the present study revealed the presence of different secondary metabolites like alkaloids, flavonoids, phenols, tannins, saponins, steroids and terpenoids. This might be due to the reason that the plants may possess natural compounds in large amount.

The findings are in accordance with the previous studies on other species of *Swertia* that reported the presence of alkaloids, flavonoids, phenols, saponins, tannins, steroids

and terpenoids (Ramesh *et al*., 2002; Ahirwal *et al*., 2011; Mahmood *et al*., 2014; Badar, 2014; Shrestha *et al*., 2015; Khanal *et al*., 2015).

Kweera *et al*. (2011) reported the absence of steroids and terpenoids in methanol extract of *S. chirayita* but it was present in the present study. Mehajbeen *et al*. (2017) and Subedi and Karki (2018) reported the absence of saponin in *S. chirayita* whereas Khan *et al*. (2017) and during the present investigation saponin was found to be present in *S. chirayita* methanol extract.

5.3 Total Flavonoid Content

Flavonoids are polyphenolic compounds that are ubiquitous among vascular plants and occur as a glycones, glucosides, and methylated derivatives (Rajkumar *et al*., 2018).There are different classes of flavonoid present in plants which are known to possess different pharmacological activity (Middleton *et al*., 2000).

Similar studies have been carried out in various species and variation in TFC was reported in several studies. Chen *et al*. (2011), Ghimire *et al*. (2011), and Roy et al. (2015) reported TFC in *Swertia chirayita*. Kshirsagar *et al*. (2014) and Khanal *et al*. (2015) had also carried out total flavonoid content in different species of *Swertia* and reported varying degree of TFC. This variation in TFC might be due to the differences in extraction techniques, solvents used and nature of chemical compounds present in these plants (Kalt *et al*., 2001).

5.4 Total Phenolic Content

The total phenolic content in *S. chirayita* had been reported by various researchers including Phoboo *et al*. (2012), Ghimire *et al*. (2011), Chen *et al*. (2011), Singh *et al*. (2012), and Roy *et al*. (2015) using different solvents and different extraction techniques. Similarly Kshirsagar *et al*. (2014) and Khanal *et al*. (2015) studied TPC in various *Swertia* species and reported their presence in varying degree among different species which is comparable to the present study as in present study varying degree of phenolic content was found. All reviews of TPC revealed variation depending upon the extraction and solvent system used. The variation in TPC reported by the various workers and in present study might be due to the differences in methods of extraction, solvent variation and complex nature of compounds present in plants (Kalt *et al*.

2001).Several studies have reported that the yield of phenolic and flavonoids had strong correlation with solvent polarity used for extraction.

5.5 DPPH Radical Scavenging Activity

The anti-oxidant activity was carried out using one of the methods called DPPH Radical Scavenging activity. In this method, an antioxidant scavenges the free radical. This test is used to measure the efficiency of extracts to scavenge the stable radical DPPH formed in solution by donation of hydrogen atom or an electron (Rather *et al*., 2016). The main antioxidant constituents of plants are known to be phenolic compounds which are composed of phenolic acids and flavonoids (Kahkonen *et al*., 1999). The antioxidant activity of phenolic is mainly because of their redox properties that allow them to act as reducing agents, hydrogen ion donor, and oxygen singlet oxygen acceptors. Reducing power assay involved $FeCl_3$/ $K_3Fe(CN)_6$ complex as a source of ferric ions, which may reduce to ferrous ions in the presence of certain phytochemicals such as phenolic and flavonoids. These phytochemicals donate their hydrogen to Fe^{+3} ions and convert them to reduced form Fe^{+2} resulting in the production of intense green color and greater absorbance. Scavenging of extracts may be attributed to the presence of high phenolic contents, that donate their electrons to H_2O_2, hence neutralizing to water. Although H_2O_2 itself is not very reactive but it may cause toxicity by giving rise the hydroxyl radicals level in the cell (Yen and Duh, 1993, Mahmood *et al*., 2014). Therefore, phenolic compounds are important plant constituents because of their potential to scavenge free radicals facilitated by their hydroxyl groups and the total phenolic concentration could be used as a basis for rapid screening of antioxidant activity. Phenolic compounds are also involved in conferring plants with oxidative stress tolerance (Sato *et al*., 2011). Flavonoids on the other hand are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals implicated in several diseases (Bravo, 1998). It has been identified that flavonoids contain hydroxyl functional groups, which are responsible for antioxidant activity (Pourmorad *et al*., 2006). Flavonoids suppress reactive oxygen formation, chelate trace elements involved in free-radical production, scavenge reactive species, and up-regulate the antioxidant defenses (Rather *et al*., 2016).

Studies carried on other related *Swertia* species have shown similar results. Phoboo *et al*. (2010) reported similar DPPH radical inhibition in ethanol root extracts of *S.*

chirayita. The ethanol extract of *S. chirayita* revealed both *in vitro* and *in vivo* antioxidant activity (Chen *et al*., 2011). Antioxidant activities of methanol extracts studied by Hajimehdipoor *et al*. (2013) revealed that *S. longifolia* aerial parts and roots possess considerable radical scavenging activity. Similar results have also been observed in case of *S. bimaculata* as well (Das *et al*., 2013).

The present study also revealed the significant antioxidant activity. The different species possess various degree of antioxidant activity. The varying degree of antioxidant activity could be might be due to the varying degree of flavonoids and phenolic compound present in different species.

5.6 α-Glucosidase Inhibition Assay

The digestion of carbohydrate will be delayed if the enzymes like α-glucosidase are inhibited retarding the entry of glucose into systematic circulation; hence allowing β-cell enough time to secrete insulin against the increased plasma glucose level (Gaede *et al*., 2003). About 411 compounds belonging to different structural frameworks, i.e., terpenes, alkaloids, quinines, flavonoids, phenols, phenylpropanoids, steroids, and compounds with other structural and functional motifs are reported to be isolated from medicinal plants possessing potent α-glucosidase inhibitory activity (Sato *et al*., 2014).

Traditionally *Swertia* spp. have been used in the treatment of diabetes (Chettri *et al*., 2005).Researchers have reported that natural products like terpenoids, alkaloids, flavonoids, phenolic and some other categories have good anti-diabetic potential but the presence of flavonoid and phenolic only under the study species doesn't reveal good anti-diabetic potential. Possible cause might be due to that there may be other compounds as well playing significant role in hypoglycemic activity (Gaikwad *et al*., 2014). A number of xanthones isolated from this genus, including bellidifolin, mangiferin and methylswertianin, have been confirmed as anti-diabetic agents (Basnet *et al*., 1994; Muruganandan *et al*., 2005; Tian *et al*., 2010; Zheng *et al*., 2014). Methyl swertianin and bellidifolin in *S. punicea* (Tian *et al*., 2010) are reported to be responsible for anti-diabetic activity. Bellidifolin has been demonstrated to be present in different *Swertia* species and act as potential anti-diabetic drug (Tian *et al*., 2010). Saxena *et al.* (1993) and Sekar *et al.* (1987) reported that phytochemical swerchirin (1, 8- dihyroxy-3, 5 dimethoxyxanthone) is responsible for anti-diabetic potential in *S. chirayita*. Hence the plant showing anti-diabetic activity could be due to presence of

these phytochemical like bellidifolin, mangiferin, swerchirin and methylswertianin in large quantity while diabetic proliferation in other plant may be due to lack of these phytochemical.

Previously different species of *Swertia* including *S. chirayita* (Roxb.) H. Karst. (Chandrasekar *et al*., 1990; Bajpai *et al*., 1991; Saxena *et al*., 1993 and Verma *et al*., 2013), *S. japonica* (Schult.) Makino (Basnet *et al*., 1994), *S. punicea* Hemsl. (Tian *et al*., 2010); *S. mussotii* Franch. (Zheng *et al*., 2014); *S. bimaculata* (Sie. & Zucc.) H.f. Thom. Ex C. B. Clarke (Liu *et al*., 2013); *S. corymbosa* Knobl. (Mahendran *et al*., 2014), *S. kouitchensis* Franch. (Wan *et al*., 2013), *S. longifolia* Boiss. (Saeidnia *et al*., 2016) were reported to possess anti-diabetic activity.

The fractionated sample of SBA-L and SMU-L revealed that fraction E in SBA-L and fraction G in SMU-L possess greater α-glucosidase inhibition potential. This might be due to the reason that they contain similar chemical compound that inhibit α-glucosidase enzyme while those fraction that showed opposite action may lack that chemical compound.

5.7 Anti-bacterial Assay

In recent years, infection has increased tremendously to a greater extent and antibiotic resistance has become an everlasting problem. Natural products present in higher plants may possess a new source of antimicrobial agents (Ahmad and Aquil, 2007). Pathogens are destroyed by antimicrobial agents in several ways. The major mode of action includes interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, and inhibition of a metabolic pathway (Neu, 1992). The antibacterial agents that interfere with the bacterial cell wall are the most selective ones like Penicillin, cephalosporin as these drug bear high therapeutic index.

Plant extracts were screened for antibacterial test against five cultured bacterial strains (two gram-positive; *Bacillus subtilis, Enterococcus faecalis* and three-gram negative bacteria; *Salmonella typhii, Escherichia coli, Pseudomonas aeruginosa*). These bacterial strains are responsible for variety of diseases like *Enterococcus faecalis* is attributed to diseases like endocarditis, urinary tract infections, intra-abdominal infection, and cellulitis and wound infection. Infection attributed to *Bacillus subtilis* includes bacteremia, endocarditis, pneumonia and septicemia. *Salmonella typhii,* infect the intestinal tract and blood and is responsible for the disease called typhoid.

Escherichia coli live in the intestines of both healthy people and animal which is in most cases remains harmless and helps to digest food. But certain strains of it can cause symptoms including diarrhea, stomach pain and cramps affects boils, cuts and wounds. *Pseudomonas aeruginosa* causes diseases like urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, and bone and joint infections.

The present investigated species of *Swertia* revealed variation in antibacterial activity against different bacterial strains. This activity might indicate that *Swertia* species either possess broad spectrum antibiotic compounds or simply general metabolic toxins (Srinivasan *et al*., 2001).Phytochemical analysis of *Swertia* species revealed the presence phenols, flavonoids, tannins, steroids etc. Tannins and flavonoids were reported to possess antimicrobial potential. Antibacterial activity against these bacterial strains might be due to the presence of these phytochemicals.

Similar results were revealed in previous study carried out in different species of *Swertia*. Several studies reported the antibacterial activity possess by different *Swertia* species including *S. chirayita* (Sultana *et al*., 2007; Ahirwal *et al*., 2011; Kweera *et al*., 2011; Ghosh *et al*., 2012; Roy *et al*., 2015; Subedi and Karki, 2018), *S. cordata*(Joshi *et al*., 2012; Roy *et al*.,2015); *S. ciliata* (Saeed *et al*., 1998), *S. corymbosa* (Ramesh *et al*., 2002; Mahendran *et al*., 2015), and *S. petiolata* (Bader,2014) against different microbial strains.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Swertia species have very wide range of distribution. Aboriginal people have acclaimed and used various species of *Swertia* L. as a medicine for centuries to cure different ailment, hence it is a significant herbs in ethno botanical as well as in pharmaceuticals arena.

In the present investigation, evaluation of antioxidant activity, anti-diabetic activity and anti-bacterial activity has been carried out along with the determination of phytochemicals constituents, total polyphenol content and total flavonoid content using methanol as solvent i.e., methanolic extracts. The investigation was carried out of seven *Swertia* species.

The highest yield percentage was obtained from *S. multicaulis* (35.67±3.52%) and lowest from *S. chirayita* (12.79±2.23%) while the remaining species revealed the percentage between these two extremes. The qualitative phytochemical screening of these species revealed that *Swertia* species are rich in alkaloids, phenols, flavonoids, steroids, saponins, tannins and terpenoids. In quantitative assay total flavonoid content and total phenol content was estimated. The total flavonoid content was found to be highest in *S. multicaulis* leaves (54.13±0.002 mg QE/g) and lowest in *S. multicaulis* roots (7.67±0.001 mg QE/g) while the total phenol content was found to be highest in *S. barunensis* leaves (107.38±2.34 mg GAE/gm and lowest in *S. cuneata* (37.44±2.28 mg GAE/gm) while other species possessing TPC and TFC between those extremes.

In biological assay, antioxidant activity, anti-diabetic activity and anti-bacterial activity has been determined. The DPPH assay revealed that highest anti-oxidant activity was found in *S. barunensis* with lowest IC_{50} value i.e, 17.74 µg/mL while lowest scavenging activity was observed in *S. nervosa* with highest IC_{50} value i.e, 399.84 µg/mL. The values of radical scavenging activity in other species were between these two extremes. Lower the IC_{50} value, higher is the Radical Scavenging Activity.

In α-glucosidase inhibiton assay, highest inhibitory activity was observed in *S.barunensis* leaves (36.65±0.74%) while lowest was found in *S. cuneata* (-26.15±0.3%) among the studied species of present investigation. The two species *S.*

barunensis leaves and *S. multicaulis* leaves were fractionated using column chromatography and the fraction was tested for their for α-glucosidase inhibition potential. *S. barunensis* leaves were fractionated into 15 samples (A-O) while *S. multicaulis* into 14 fractionated sample (A-N). It was observed that in *S. barunensis* leaves, fraction E exhibited maximum inhibition activity (97.2±1.86%) and fraction A exhibited maximum induction activity against α-glucosidase enzyme while in the case of *S. multicaulis* fraction G was found to be exhibiting maximum inhibition activity (94.4±0.66%) and fraction I exhibiting maximum induction activity against α-glucosidase enzyme.

The antibacterial activity was tested against five bacterial strains. Among five species studied, four species showed antibacterial activity against tested bacteria but none of the species showed zone of inhibition against all the species. *S. barunensis* leaves showed best antibacterial activity against *Bacillus subtilis*. The fractionated sample of *S. barunensis* and *S. multicaulis* using leaves column chromatography when tested for their anti-bacterial activity didn't reveal much significant activity.

6.2 Recommendations

This study is only a preliminary one which compares different species of *Swertia* in terms of their biological activities and phytochemical constituents. Following recommendations are based recommended on the basis of present investigation results.

- ✓ In present study, only one solvent i.e., methanol has been used for extraction. So using various solvents like ethanol, hexane, chloroform, petroleum ether, ethyl acetate, one should test the various bioactive compounds as well as different biological activities.
- ✓ Only five species has been selected in present study, similar studies need to be done in other unexplored species of *Swertia*.
- ✓ Further study should focus on anticancer, anti-fungal, anti-inflammatory, cytotoxicity and insecticidal activities.
- ✓ Further research should include isolation and identification of bioactive compounds responsible for various biological activities.

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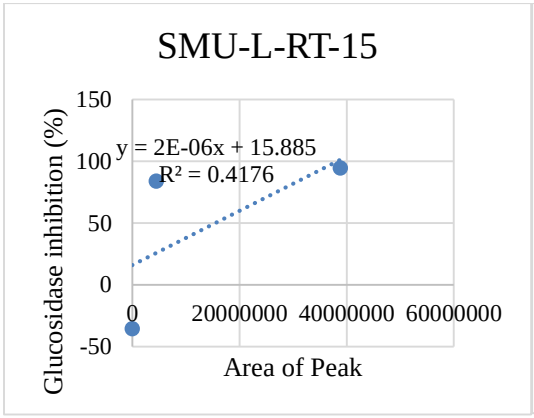
APPENDICES

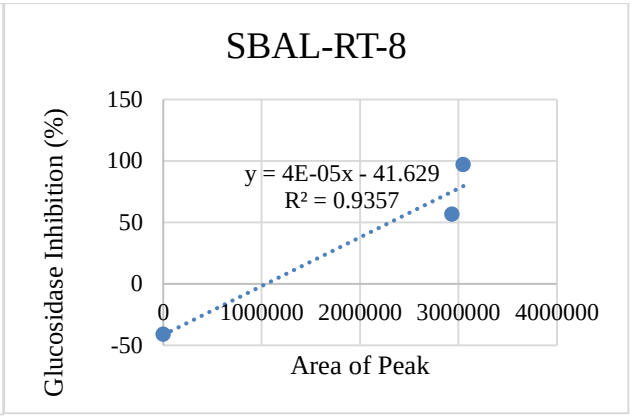
Appendix 1: List of Materials used for the study

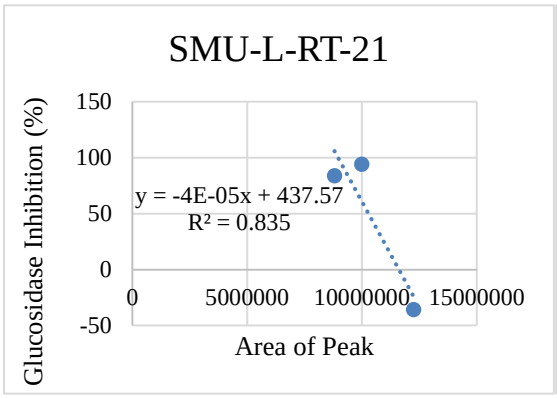
Apparatus and Equipment:

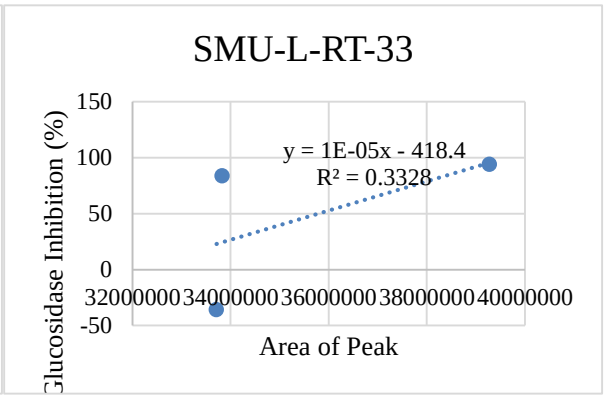
Aluminium foil	Eppendorf tubes	Parafilm
Autoclave	Falcon tube	Petriplates
Beaker	Filter paper	Refrigerator
Camera	Forceps	Rotatory evaporator
Conical flask	Glass rod	Spectrophotometer
Cottons rolls	Gloves	Sonicator
Cotton swabs	Incubator	Spirit lamp
Calculator	Laminar air flow	Test tubes
Earbuds	Measuring cylinder	Toothpick
Electric balance	Micropipette	Vortex
Electric grinder	Micropipette tubes	
Elisa plate Reader	96-well microplate	

Appendix 2: Linear Regression curve of SBA-L & SMU-L in LCMS









Appendix 3: Photo plates

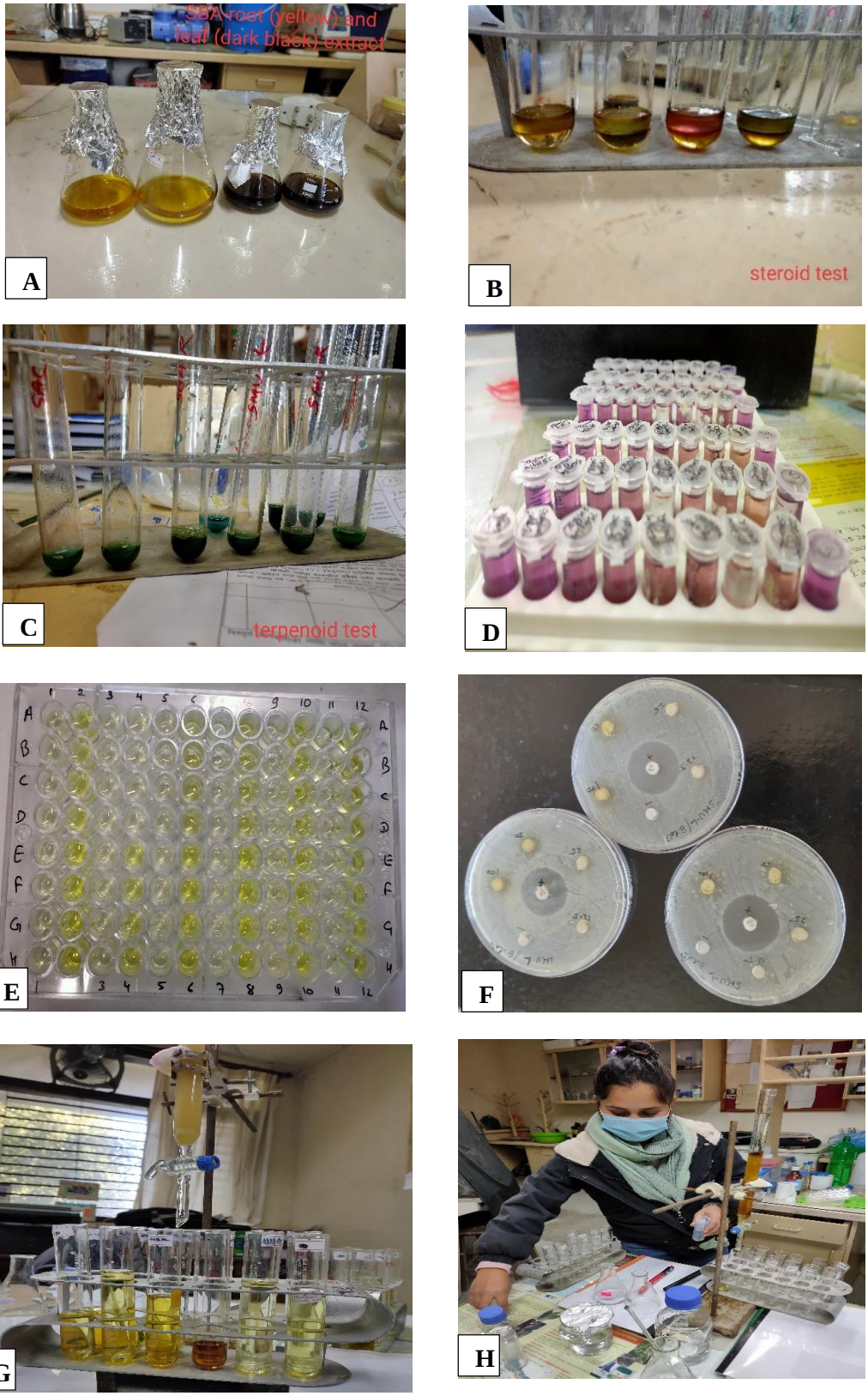


Plate 1. A. Extract preparation, B. steroid test, C. Terpenoid test, D. DPPH assay for antioxidant activity, E. α-glucosidase test using 96-well microplate, F. Antibacterial test using agar well diffusion method, G. & H. Column chromatography for separation of sample into fractions.