PHYTOCHEMICAL SCREENING AND BIOLOGICAL ACTIVITIES OF *BERBERIS ASIATICA* FROM ARGHAKHANCHI DISTRICT

A DISSERTATION SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE MASTER OF SCIENCE DEGREE IN CHEMISTRY

Submitted by

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BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation work entitled **"Phytochemical Screening and Biological Activities of *Berberis asiatica* from Arghakhanchi District"** has been carried out by Devendra Dhakal (Exam Roll No. Chem 118/071 and T.U. Reg. No. 5-2-37-668-2010) under the supervision of Asst. Prof. Dr. Khaga Raj Sharma, Central Department of Chemistry, Tribhuvan University, Nepal is hereby submitted for partial fulfillment of Master of Science (M.Sc.) Degree in Chemistry. This dissertation has been accepted for the award of Master degree.

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DECLARATION

I hereby declare that this dissertation entitled **"Phytochemical Screening and Biological Activities of *Berberis asiatica* from Arghakhanchi District"** has been submitted by me to the Central Department of Chemistry, Tribhuvan University, for the partial fulfillment of the requirements for the award of the Master degree program (M.Sc.), is a complete record of original research work carried out by me under the supervision and guidance of Asst. Prof. Dr. Khaga Raj Sharma, Central Department of Chemistry and it has not formed the basis for the award of any other Degree/Diploma/Fellowship or other similar title to any candidate of any University. Any literature, data or work done by others are cited in this research work has been given due acknowledgement and are listed in the reference section.

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LIST OF ABBREVIATIONS

CNPG3	2-Chloro-4-nitrophenyl-α-D-maltotrioside
PPA	Porcine Pancreatic α –Amylase
DM	Diabetes mellitus
DMSO	Dimethyl sulphoxide
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
F-C	Folin ciocalteu
IC_{50}	Inhibitory concentration of drug that gives half-maximal response
mg GAE/g	Milligram gallic acid equivalent per gram
mg QE/g	Milligram quercetin equivalent per gram
mM	Millimolar
µg/mL	Microgram per milliliter
µg	Microgram
µL	Microliter
ppm	Parts per million
w/v	weight by volume
v/v	volume by volume
PPA	Porcine pancreatic α-amylase
ROS	Reactive oxygen species
TFC	Total flavonoid content
TPC	Total phenol content
MHB	Mueller Hinton Broth
MHA	Mueller Hinton Agar
NA	Nutrient Agar

MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
CFU/mL	Colony forming unit per milliliter
ZOI	Zone of Inhibition
GC	Gas Chromatography

LIST OF FIGURES

LIST OF TABLES

Page No.

ABSTRACT

The aims of the present study were to prepare methanolic extract of *Berberis asiatica* and subjected for their phytochemical screening and biological activities. Phytochemical screening of methanolic extract of *Berberis asiatica* showed the presence of different chemical constituents such as alkaloids, flavonoids, polyphenols, terpenoids, saponins, coumarins, reducing sugars, steroids, glycosides but tannin and volatile oils were absent. The total phenolic content of methanolic extract of *Berberis asiatica* was found to be 37.686 ± 2.728 mg GAE/g and the total flavonoid content of was found to be 115.568 ± 8.012 mg QE/g. The radical scavenging activity of the extract was evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical method. The IC_{50} value of methanolic extract of *Berberis asiatica* was found to be 205.7 ± 5.353 µg/mL. The smaller the IC_{50} value, the greater is the antioxidant property. The antibacterial activity methanolic extract of *Berberis asiatica* was studied against *S. aureus, E. coli, K. pneumoniae* and *S. typhi* using agar well diffusion method. The result showed that the plant extract exhibit maximum activity against *S. aureus* and *K. pneumoniae* having ZOI value 14 mm and 19 mm respectively. Minimum inhibitory concentration (MIC) for *S. aureus* was found to be 0.39 mg/mL and for *K. pneumoniae* was 3.125 mg/mL. Minimum bactericidal concentration (MBC) for *S. aureus* and for *K. pneumoniae* was found to be 6.25 mg/mL. Qualitatively, GC of hexane extract of *Berberis asiatica* showed 35 compounds. This can be categorized into ketones, alkanes, cycloalkanes, other are identified as aromatic compounds. The plant under study was potential medicinal plant due to variation and availability of phytochemicals.

Keywords: *Berberis asiatica*, Phytochemicals, DPPH, Antioxidant, Antibacterial, MIC, MBC.

TABLE OF CONTENTS

CHAPTER 4

RESULTS AND DISCUSSION

CHAPTER 5

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The wonder and curiosity man regarding his natural world are evident in his earliest records. People rely on plants and plant products to fulfill their needs viz. fooding, sheltering and clothing. Besides, people are using plants and plant products for other various purposes such as crude aqueous extractions of flowers and certain plants and insects provided a number of pigments e.g., indigo and alizarin used in ancient world for dyeing. Also plant products were extensively used for both killing and healing. Thus, plant and plant products were used for various benefits to the human civilization.[1]

People have been using plants and plant products for healing of various diseases as medicine without knowing the chemical constituents and biological activities. Ethnic people around the world developed their own drug to fight against the diseases by hit and trial methods on their experience and passed the knowledge from generation to generation.[2] This relay of knowledge let the creation of the practice medicine called Ethno-medicine. The long experience of various ethnic groups in early human civilization helped to develop a system of medicine called "The Traditional Medicine", "The Chinese (Tibetan) Medicine", "The Unani Medicine", "Ayurvedic Medicine" and "Traditional faith healing" are grouped as Traditional medical system. Besides these, nowadays Allopathic and Homeopathic medical systems are being practiced. Still, plants and plant products get popularity as an alternative medicine due to their fewer side effects on living beings. Thus, this is an important task to explore the chemical constituents and biological activities of plants and plant products, which influence the human biochemistry in these medicinal plants for establishing relation between traditional use and scientific meaning.[1]

Medicinal plants are used basically in two different forms: i) as complex mixtures obtaining a broad range of constituents (infusions, essential oils, tinctures, extracts, etc.) and ii) as pure chemically defined active principles. Pure compounds are generally employed when the active principles of a medicinal plant exhibit strong, specific activity and/or have small therapeutic index, requiring accurate and reproducible dosage. Therapeutic index is the ratio of amount necessary to kill the patient (i.e., the

maximum tolerated dose) to that required for a maximum curative dose. On the other hand, the use of extracts/tinctures etc. is appropriate for plants exhibiting weaker and/or less specific pharmacological activities and if the active principles of a medicinal plant are as yet unknown.[1]

Today's world is alarmed with various diseases and problems, of which some are life threatening. Efforts are being directed towards the cure of those diseases and problems by the bio-active constituent from the parts of the plants. Thus, this research is focused on the chemical and biological activities of methanolic extract of *Berberis asiatica* of Nepalese origin.

1.2 Nepal

Nepal is a beautiful south Asian country, situated in the laps of Himalayas. This Himalayan country is sandwiched between China in north and India in east, west and south. It is a landlocked country.

Geographically, Nepal lies between latitudes 26º22' north to 30º27' north and longitudes 30º4' east to 88º12' east. The average East-West length is about 885 km and North-South width varies from 160 km to 240 km making a total area of 1,47,181 Sq.km. It is divided into three regions: Mountain region (15%), Hilly region (68%) and Terai region (17%). The elevation ranges from 60 meter (Kechanakalan, Jhapa) to the top of the world 8848 meter (Mount Everest).[3] This mountainous country in the central Himalayas, which occupies about the one third of (800 km) of the entire length of Himalayan mountain range. Nepal alone contains 8 out of the top 10 tallest mountains in the world, including Mount Everest. Nepal occupies about 0.3% of landmass in Asia and about 0.3% of the entire landmass of the world, but contains a disproportionately large diversity of plants and animals.[4]

It is due to such extreme geographical diversity, unique topography and climatic condition, Nepal has wide variety of flora and fauna. The climate varies from tropical climate in southern plainland called Terai to alpine climate in northern belt called Mountain region. Here, occur not only rich elements of vegetation of cosmopolitan type but also several endangered toxa of flora including aromatic and medicinal plants as potential source of different drugs for different disease.[2] Nepal has always been the center of herbal richness with more than ten thousand species of herbs in its alpine

belt. The medicinal databases listing for Nepal shows 1,624 species of medicinal and aromatic species.[5] These herbs have been integral part of traditional medicine practices of indigenous community of Nepal.

Arghakhanchi is one of the districts of Lumbini Province in Nepal. The district headquarter is Sandhikharka. The district lies between 27º45'N and 28º6'N latitude, and 80º45'E to 83º23'E longitude and covers an area of 1,193 km^2 and has a population (2011) of 197,632. Its neighboring districts are Palpa in the east, Gulmi in the north, Kapilvastu in the south and Pyuthan in the west. The altitude of the district varies from 305 to 2515 meter above the sea level and about 40% of the total area is forested. 68% of the district is in the mountainous Mahabharat Range and the rest is in the Siwalik Hills.[6]

It is a land of small holdings and 70 percent of population practice subsistence agriculture, but the most peasant are not self-sufficient. In most localities, no organized medicinal aid is available. Therefore, they depend mainly on the local herbal medicines. The different skin diseases are treated with plant remedies on the basis of empiric knowledge. The district has variation of climate from tropical, subtropical to temperate while most of the area lie under subtropical region.[7]

1.3 Natural Product Chemistry

Historically, natural products have been used in folklore for the treatment of many diseases and illness. Classical natural product chemistry methodology enabled a vast array of bioactive secondary metabolites from terrestrial and marine sources to be discovered. Many of these natural products have gone on to become current drug candidates.[8] The earliest record of natural products was depicted on clay tablets in cuneiform from Mesopotamia (2,600) which documented oil from *Cupressess empervirens* (cypress) and *cammiiphora* species (myrrh) which are still used today to treat coughs, cold and inflammation.[9]

The world health organization estimated that approximately 80% of the world's population relies primarily on traditional medicines as sources for their primary health cares.[10] Over 100 chemical substances that are considered to be important drugs that are either currently in use or have been widely used in one or more countries in the

world have been derived from a little under 100 different plants.[11] Approximately 80% of these substances were discovered as a direct results of chemical studies focused on the isolation of active substances from plants used in traditional medicine.[12]

Mother earth has gifted mankind with lots of plants which has the ability for curing the health disorders of human being. This feature has been identified in the pre-historic times and the world-wide use of the herbal therapies and the health care preparation that are prescribed in the ancient books like Vedas and the Bible given a way for discovery of natural products with medicinal values.[13] By definition the word natural is an adjective referring to something that is present and produced by nature and artificial or man-made. The term natural products today are quite commonly understood to refer to herb's dietary supplements traditional Chinese medicine.[14]

The concept of natural products dates back to the early 19th century. Natural product is the high value chemical entities derived from plant or microbial sources.[15] But in the field of organic chemistry, the purified organic compounds isolated from natural sources that are produced by pathways of primary or secondary metabolism are natural products. Primary metabolites are associated with essential cellular functions such as nutrition assimilation, energy production, growth and development. Thus, they are essential for the survival of the organism that produces them. Secondary metabolites in contrast have an extrinsic function that mainly affects other organisms. Secondary metabolites are not essential for the survival of the organism but do increase the competitiveness of the organism within its environment. Because of their ability to modulate biochemical and signal transduction pathways, some secondary metabolites are pharmacologically or biologically active. Hence, they are the active components not only of most traditional medicines but also of many modern medicines. Natural product chemistry is the branch of chemistry which deals with the study of these primary and secondary metabolites. Natural products may be extracted from the cells, tissues and secretions of microorganisms, plants and animals. A large number of currently prescribed drugs have been either directly derived from or inspired by natural products.[16]

Throughout the ages humans have relied on nature for their basic needs for the production of foodstuffs, shelters, clothing, means of transportation, fertilizers, flavors,

fragrances and medicines.[17] Man cannot survive on this earth for long life without natural products because the natural products and their active constituents played an important role to sustain their lives.[18] Among different natural products, plant products are of vital importance for us since plants are used as food, clothes, fodder, medicine, timber etc. by man since time immemorial.[19]

Natural product chemistry and research deals with chemical compounds found in nature that usually have a pharmacological or biological activity for use in pharmaceutical drug discovery and drug design.[20] Current study on natural molecules and products primarily focuses on plants since they can be sourced more easily and be selected based on their ethno-medicinal uses They can be extracted and used for chronic and infectious diseases.[21] In organic chemistry, definition of natural products are usually restricted to mean purified organic compounds isolated from natural sources that are produced by primary and secondary metabolic pathway. Within the field of medical chemistry, definition is further restricted to secondary metabolites. Major class includes alkaloids, phenols, polyphenols, tannins, saponins and terpenes.[22]

1.4 Phytochemicals

Phytochemicals are chemical compounds produced by plants, generally to help them thrive or thwart competitors, predators or pathogens.[23] They are bioactive chemicals of plant origin regarded as secondary metabolites because the plants that make them may have little need for them. They are naturally manufactured in all parts of the plant body i.e., bark, stem, leaves, root, rhizome, flower, seeds, fruits, etc. Depending on the location of the plant body parts, the quantity and quality of phytochemicals may differ. Knowing as a basic for the traditional herbal medicine, phytochemicals have been practiced in the past and currently in different parts of the world. Ethno-botanical surveys by local healers in a region, researchers usually search the phytochemicals that may be useful to the pharmaceutical industry. Following such suggestions, one can usually screen selected plant parts for phytochemicals that may be present. If the phytochemical of interest is present, further isolation, purification and characterization can be performed. Then it can be formulated further for a new pharmaceutical product. Successful determination of biologically active plants constituents is mainly dependent on the type of solvent used in the extraction process.[24]

1.5 Medicinal plants

Plants possessing secondary metabolites which are potential sources of curative drugs with the very long list of chemicals and its curative nature are called as Medicinal plants.[25] Earth contains a rich wealth of medicinal plants, an important source of medicine which plays a key role in world health. Now-a-days, plant derived substance have become of great importance owing to their multi-purpose uses. Medicinal plants are the richest bio-source of drugs in traditional system of medicine, modern medicine, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs.[26] There are many medicinal plants that are either 'Wild plant species' or Domesticated plant species' whose scientific importance is yet to be investigated. Knowledge gained by ancient people was passed from generation to generation and new knowledge added to it by the next generation. Although many of these plants have not been identified scientifically, a group of people in every generation started specializing in collecting and processing medicinal plants and using them against various diseases.[27]

According to a WHO survey, application of medicinal plant remedies is increasing even in developed countries, especially among the young generation. Recently, WHO (World Health Organization) estimated that 80 percent of people worldwide depend on herbal medicines for some aspect of their primary health care needs. According to WHO, around 21,000 plant species have the potential for being used as medicinal plants. Medicinal plants are considered as rich resources of ingredients which can be used in drug development.[28]

1.6 Diabetes Mellitus (DM)

Diabetes mellitus has emerged as a major health problem in both developed and developing countries and growing at its alarming rate all over the world. Diabetes mellitus is a chronic endocrine metabolic disorder that affects human body in terms of physical, psychological and social health caused by defective insulin secretion, resistance to insulin action or a combination of both. It is defined as a group of disorders characterized by hyperglycemia, in which blood sugar levels are elevated, altered metabolism of carbohydrates, lipids, proteins and electrolytes.[29] It constitutes the sixth

leading cause of global mortality.[30] According to the International Diabetes Federation, currently 246 million people worldwide live with diabetes and it is expected that 380 million will be affected by 2025.[31] It is the fact that diabetes can't be cured completely as it has never been reported that someone had recovered totally from it. Ayurvedic herbs provide better alternatives, owing to lesser side effects and low cost. World Health Organization has also substantiated the utilization of herbal remedies to combat diabetes.[32]

There are three main types of diabetes mellitus according to World Health Organization (WHO) as follows:

- **Type I Diabetes Mellitus**

  It is also called Insulin Dependent Diabetes Mellitus (IDDM). It used to be called juvenile- onset diabetes, because it often begins in childhood and it usually occurs before the age of 40. Type I diabetes result from destruction of pancreatic β-cells due to which pancreas fail to produce insulin. The loss of β-cells may be due to exogenous chemicals from the environment or diet, viral infection, or immunological factors such as an autoimmune disorder in genetically vulnerable individuals. Type I Diabetes can be treated by taking insulin, which needs to be injected through the skin into the fatty tissue below.[33]

- **Type II Diabetes Mellitus**

  It is also called Non-Insulin Dependent Diabetes Mellitus (NIDDM). It is used to be called adult-onset diabetes, because it usually occurs after the age of 40, but with the epidemic of obese and overweight kids, more teenagers are now developing type II diabetes. Type II diabetes is the most known form of diabetes and often a milder form of diabetes than type I, characterized by abnormality in insulin secretion and its resistance. It is simply defined as a metabolism disorder in which either the insulin secretion is not sufficient as per body's need or muscles cells, body fat and liver fail to respond normally to insulin so the glucose can't be grabbed out of bloodstream resulting high glucose level in the bloodstream.[34] Type II diabetes can be controlled with weight management, healthy diet and regular exercise. Treatments includes:

  • Agents that increase the amount of insulin secreted by the pancreas,
  • Agents that increase the sensitivity of target organs to insulin, and

- Agents that decrease the rate at which glucose is absorbed from the gastrointestinal tract.[35]

- **Type III Diabetes Mellitus / Gestational Diabetes (GD)**

  Type III diabetes is temporary disease related to diabetes, known as Gestational diabetes. It is triggered and detected during pregnancy, usually in the second or third trimester. It usually resolves after the birth of baby. It occurs in about 4% of all pregnancies and has 30% to 50% chance of developing diabetes, usually type II diabetes. It refers to the occurrence or initial recognition of high blood glucose during the period of pregnancy. Gestational diabetes must be controlled to protect the baby's growth and development.[36]

Polysaccharides like starches and sugar belong to major constituents of human diet that mainly play a role in the energy supply. The dietary polysaccharides digestion occurs mainly in the gastro-intestinal tract, and it involves the combine action of pancreatic α-amylase and to release the absorbable glucose monosaccharides. α-amylase hydrolyses polysaccharides to form linear and branched glucose oligomers i.e., oligosaccharides and disaccharides as its major products.[37] However, in case of people with type II diabetes, the dietary polysaccharides keep on breaking into glucose with the help of α-amylase but either due to insulin deficiency or resistance, glucose cannot be absorbed into gut so that there is increase in blood glucose concentration. Thus, if the amount of this gastro-intestinal enzyme available for the breakdown can be reduced then complex saccharides can have a better chance of travelling through the gastro-intestinal tract (GIT) without being assimilated and are eventually excreted from the body instead of being convert into storage fat. Therefore, the inhibition of α-amylase enzyme is one of the most effective approaches to significantly reduce the postprandial increases of blood glucose by delaying the process of polysaccharides hydrolysis and absorption.[38]

1.6.1 α-Amylase

The α-amylase, an endoglucanase enzyme (α-1,4-glucan-4-glucanohydrolase) is one of the major secretory products of the pancreas (about 5-6%) and salivary glands, playing a role in digestion of starch and glycogen and can be found in micro-organisms, plants and higher organisms. Thus, α-amylase is a digestive enzyme that catalyzes the initial hydrolysis of starch into shorter oligosaccharides such as maltose and maltotriose which

are the polymers composed of glucose units through the cleavage of α-D-(1→4) glycosidic bonds.[39] The final degradation is carried out by a debranching enzyme α-glycosidase that is present in the mucosal brush border of the small intestine, hydrolyzing the α (1→6) linkages at the branch points converting maltose and maltotriose to D-glucose. These two digestive enzymes together assist glucose absorption into the bloodstream in turn increasing postprandial blood glucose level. Inhibition of α-amylase delays the digestion process by hampering breakdown of starch and hence can be used as an effective strategy for regulating hyperglycemic condition.[40]

It is found to have 496 amino acids residue and 170 water molecules along with 1 calcium ion sphere and 1 chlorine ion sphere as catalytic center which help the enzyme to bind substrate. Salivary amylase folds into a multi-domain structure consisting of three domains, A, B and C. Domain A is the largest and catalytic domain consists of 8 stranded parallel beta barrels surrounded by cylinder of alpha helical segments also it holds chloride ions bounded in V shaped active site. Domain B is located next to the domain A and calcium ions bound to it, necessary for the structural stability. Domain C is composed of the antiparallel beta barrel.[41]

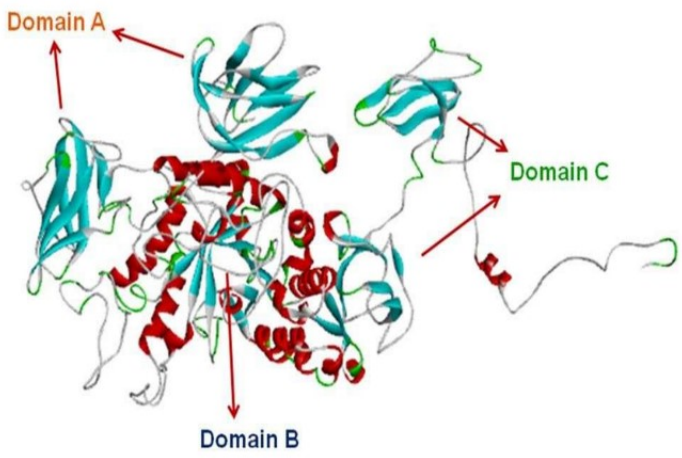


Figure 1: Structure of α-Amylase

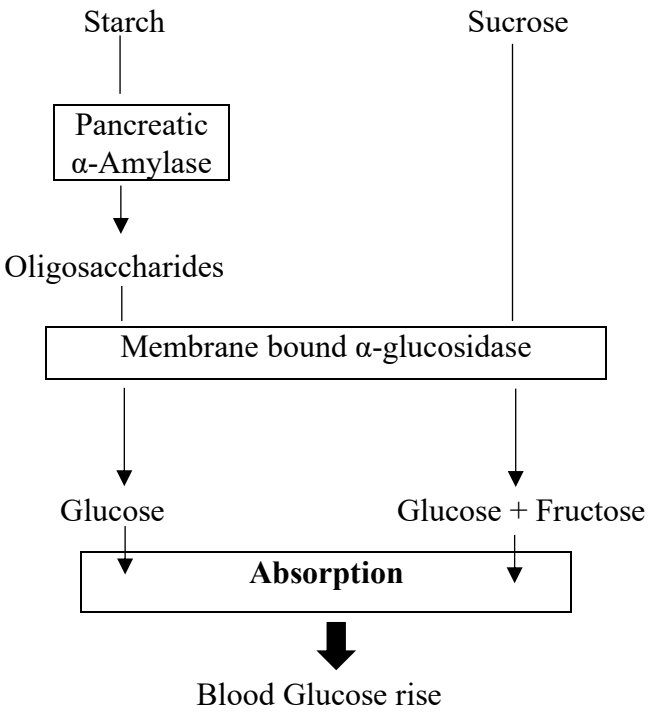


Figure 2: Schematic diagram illustrating the effect of α -amylase and α -glucosidase on digestion of starch.

1.6.2 Mode of action of α-Amylase

α-Amylase acts in a similar way to its corresponding substrate. The normal enzyme-substrate mode of action is shown in figure 3.

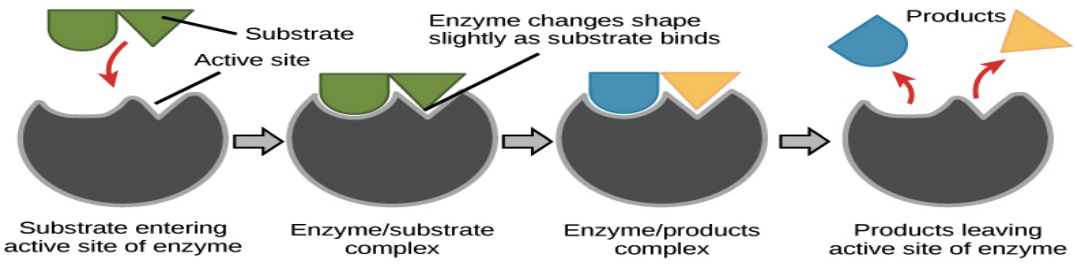


Figure 3: Mechanism of enzyme action on substrate

The above figure shows that the substrate when bind to the active sites of enzyme form enzyme substrate complex which on catalysis forms enzyme-product complex. On hydrolysis of the α-bond of substrate molecule, it forms product. Finally, the products leave the active sites of enzyme. In case of α-amylase mode of action, substrate

molecule is usually starch which is hydrolyzed into oligosaccharides of starch as a product.[41]

1.6.3 α-Amylase Inhibitors

The screenings of chemical constituents as potential α-amylase inhibitor in medicinal plants have received more attention as therapeutic source for the treatment of type Ⅱ diabetes because inhibition would decrease the absorption of glucose and consequently reduce postprandial blood glucose level.[42] Among the phytoconstituents that have been investigated, phenolics and flavonoids have demonstrated the highest inhibitory activities with the potential of inhibition related to number of hydroxyl groups in the molecule of the compound.[41] α-Amylase inhibitors are divided into two classes :

- **Non-proteinaceous inhibitor**

  The class of non-proteinaceous α-amylase inhibitors comprises diverse type of organic compounds such as iso-acarbose, acarbose, acarviosine glucose, hibiscus acid and the cyclodextrins. The inhibitory activity of these compounds against α-amylase is due to their cyclic structures, which resemble α-amylase substrates and bind to α-amylase catalytic sites.

- **Proteinaceous inhibitor**

  Proteinaceous α-amylase inhibitors are found in micro-organisms, plants and animals. Among plants, cereals and legumes are the rich sources of the α-amylase inhibitors. Such inhibitors are classified into six classes according to their tertiary structures and that are lectin-like, knotting-like, cereal-type, kunitz-like, γ-purothionin-like and thaumawtin-like α-amylase inhibitors.[43]

There are two types of assays mostly used to determine the inhibition potential of α-amylase inhibitors on α-amylase. One is based on change in reducing power of the substrate by the dinitro salicylic acid (DNS) reagent, whereas the other is based on the change of the iodine staining properties of blue starch-iodine complexes. The second method i.e., starch-iodine assay is widely used which is based on color development resulting from iodine binding to starch polymers. The more the color intensity of starch-iodine complex, powerful is the inhibition of α-amylase inhibitor.[44]

1.6.4 Mechanism of enzyme inhibition

During α-amylase inhibition, the enzyme inhibitor binds to active site or allosteric of enzyme. Thus, substrate molecule does not fit on the active site of enzyme.[41] There are mainly two possible mechanism of enzyme inhibition and they are:

- **Competitive inhibition**

  The competitive inhibition incorporates the competition of inhibitor and substrate to bind the active sites of enzymes because the inhibitor is structurally and chemically similar to the substrate. The competitive inhibitor blocks the active site and thus prevents substrate binding. However, a competitive inhibition is usually reversible if substrate molecules are available to ultimately displace the inhibitor. Therefore, the amount of enzyme inhibition depends upon the inhibition concentration, substrate concentration and the relative affinities of the inhibitor and substrate for the active site.

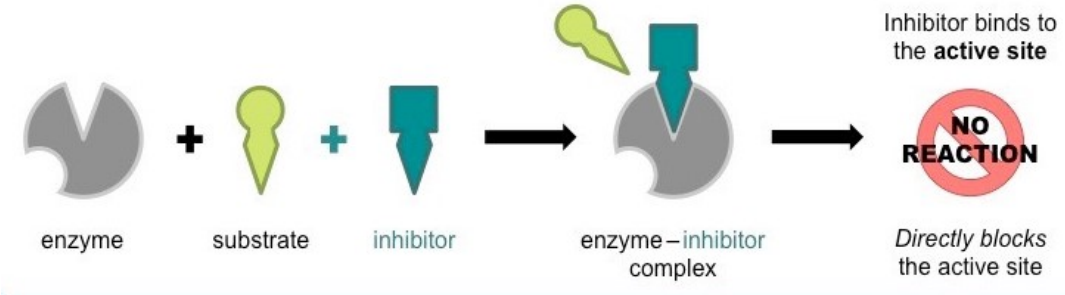


Figure 4: Mechanism for Competitive enzyme inhibition

- **Non-competitive inhibition**

  Non-competitive inhibition incorporates the binding of inhibitor to a site other than the active site called an allosteric site. The binding of the inhibitor to the allosteric site causes a conformational change to the enzyme's active site. As a result of which, the active site and substrate no longer share specificity, i.e., the substrate cannot bind. Since the inhibitor is not in direct competition with the substrate, increasing substrate levels cannot mitigate the inhibitor's effect.[45]

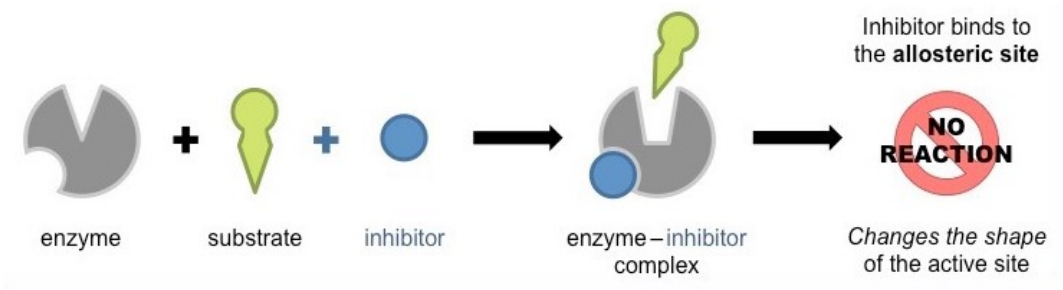


Figure 5: Mechanism for Non-competitive enzyme inhibition

1.7 Oxidative stress and Antioxidants

Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses. An imbalance between antioxidants and reactive oxygen species is the underlying basis of oxidative stress, leading to the cellular damage. Oxidative stress leads to many Patho-physiological conditions in the body. Some of these include neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, gene mutations and cancers, chronic fatigue syndrome, fragile X syndrome, heart and blood vessel disorders, atherosclerosis, heart failure, heart attack and inflammatory diseases. Oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species.[46] Reactive Oxygen Species (ROS) are highly reactive unstable ions formed inside living organisms as a result of oxidative stress, normal and pathological cell metabolic processes and exogenous pollutants such as cigarette smoking, ultraviolet rays, ionizing radiation, toxic chemicals, which include hydrogen peroxide (H_2O_2), free radicals such as hydroxyl radical (•OH), superoxide anion (O_2^-), alkoxy and proxy radicals (RO• and ROO•). ROS are very harmful as they react with various cellular components including DNA, proteins, lipids, fatty acids which primarily results in lipid peroxidation, decreases membrane fluidity, DNA mutation, has major role in progression of various life-threatening conditions like cancer, inflammation, cardio vascular diseases, infection, etc.[47] Oxidative stress is produced during normal metabolic process in the body as well as induced by a variety of environmental factors and chemicals. Thus, oxidative stress is thought to be the cause of many diseases.

Antioxidants are the compound that either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell. However, reactive oxygen species also have useful cellular functions such as redox signaling. Thus, the function of antioxidant systems is not to remove oxidants entirely, but instead

to keep them at an optimum level. In other words, antioxidants protect cells against the damaging effects of reactive oxygen species, prevent the production of such highly reactive free radicals, aids in repairing from oxidative damage and have effective functioning of naturally prevailing antioxidants in our body.[48] Plants are the good source of biologically active compounds known as phytochemicals like phenolic acids, polyphenols and flavonoids, tannins, etc. Presence of polyphenols is reported to be the major secondary metabolite responsible for antioxidant activity. The phytochemicals have been found to act as antioxidants by scavenging free radicals and have therapeutic potential for free radical associated disorders.

The DPPH free radical (DPPH•) scavenging assay has been largely used by various researchers as a quick, easy, reliable and reproducible parameter in search of in-vitro antioxidant activity of pure compounds as well as plant extracts. The DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable radical because of the para magnetism conferred by its odd electron.[49] Its gram molecular weight is 394.32g. Its solution in methanol appears as a deep violet color solution and shows a strong absorption band at 517 nm.[50] This method based on the ability of DPPH radical to reduce and decolorize in the presence of antioxidants. Antioxidants either transfer a hydrogen atom or electron to DPPH free radical to neutralize its free radical character and become a stable diamagnetic molecule. Scavenging of free radicals determines the free radical scavenging capacity or the antioxidant potential of the test sample which shows its effectiveness, prevention, interpretation and repair mechanism against injury in biological system.[51]

1.7.1 Mechanism of DPPH with Antioxidants

The proposed mechanism involves the transfer of a hydrogen atom from an antioxidant to the DPPH molecule to form DPPH-H molecule which is stable with the loss of violet color and loss of its paramagnetic resonance and does not absorb at 517 nm. DPPH solutions show a strong absorbance band at 517 nm due to its odd electron appearing a deep violet color; the absorption vanishes as electron pairs off. The resulting decolorization is stoichiometric with respect to the number of hydrogens taken up.

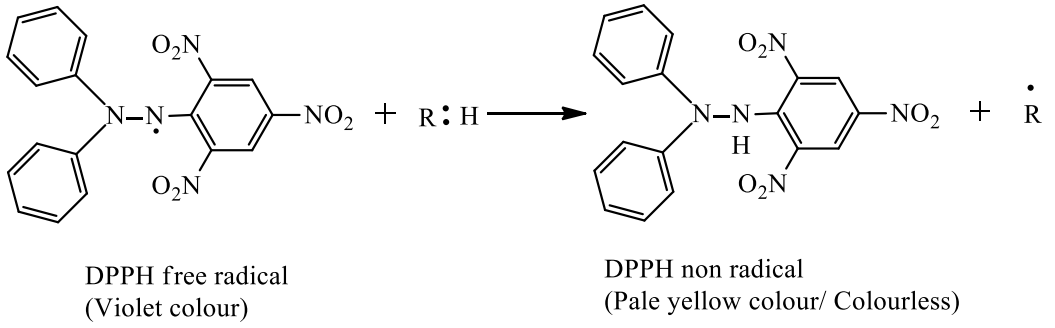


Figure 6: Mechanism of DPPH antioxidant assay

The antioxidant activity of plant extracts or its compounds is evaluated by comparing with standard antioxidant. Some antioxidants are ascorbic acid, cysteine, etc. Ascorbic acid is used as standard in DPPH free radical scavenging method. It is commonly known as Vitamin C (molecular formula $C_6H_8O_6$ and molecular weight 176.12). It is a natural antioxidant.[52]

1.7.2 Mechanism of DPPH and Ascorbic acid

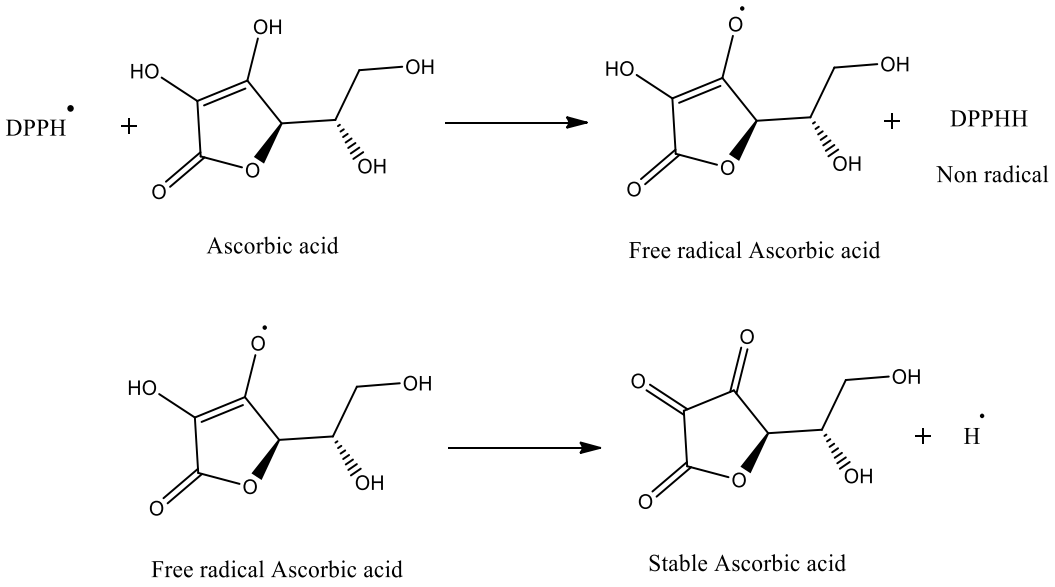


Figure 7: Mechanism of DPPH and Ascorbic acid

Two molecules of DPPH are reduced by one molecule of Ascorbic acid. Similarly, gallic acid and quercetin can donate a pair of hydrogen atoms to DPPH molecule and thus get oxidized acting as an antioxidant.

1.7.3 Alkaloids

Alkaloids are the largest group of secondary metabolites present in the living organism. Alkaloids are a highly diverse group of naturally occurring chemical compounds that contains ring structure and a nitrogen atom. There are also some related compounds with neutral and even weakly acidic properties in this group. Alkaloids are also attributed to some synthetic compounds of similar structure. In addition to carbon, hydrogen and nitrogen, alkaloid molecules may contain sulfur and rarely chlorine, bromine or phosphorus. Alkaloids are formed by a wide range of species, including bacteria, fungi, plants and animals, and are part of a group of natural products. In most cases, the nitrogen atom is located inside the heterocyclic ring structure.[53]

A classification based on biosynthetic pathways is mostly used to categorize different alkaloids. Alkaloids have a wide distribution in the plant kingdom and mainly exist in higher plants, such as those belonging to Ranunculaceae, Leguminoceae, Papaveraceae, Menispermaceae, and Loganiaceae. Moreover, several alkaloids exhibit significant biological activities, such as the relieving action of ephedrine for asthma, the analgesic action of morphine, and the anticancer effects of vinblastine, berberine. In fact, alkaloids are among the most important active components in natural herbs and some of these compounds have already been successfully developed into chemotherapeutic drugs, such as camptothecin (CPT), a famous topoisomerase I inhibitor and vinblastine, which interacts with tubulin.[54] Many of these compounds have powerful effects on pharmacology. The well-known plant alkaloids, for example, include narcotic analgesics, morphine and codeine, apomorphine (a morphine derivative) used in Parkinson's disease muscle relaxant papaverine, and sanguinarine and berberine antimicrobial agents. A number of potent anti-cancer drugs from plant compounds have also been produced.[53]

1.7.4 Phenols and Flavonoids

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation. More than 8,000 polyphenol compounds have been identified in various plant species. They are classified into simple phenols, phenolic acids, hydroxyl cinnamic acid derivatives and flavonoids. Presence of polyphenols is reported to be the major secondary metabolite responsible for antioxidant activity. The position and number of hydroxyl groups on the phenolic compounds are possibly

related to their relative toxicity to micro-organisms, since increased hydroxylation results in increased toxicity. The antioxidant activity of phenolic is mainly because of their redox property which allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators.[55] There are increasing evidences that as antioxidants, polyphenols may protect cell constituents against oxidative damage to lymphocytic DNA and therefore, limit the risk of various degenerative diseases associated with oxidative stress such as cancers, cardiovascular diseases, and diabetes, osteoporosis and neurodegenerative diseases. Thus, it is important task in search of the potential health benefits of dietary plant polyphenols as antioxidant.[56]

Flavonoids are the most common group of polyphenol compounds are found ubiquitously in plants. Flavonoids are potent antioxidants. The capacity of flavonoids to act as antioxidants depends upon their molecular structure, the position of hydroxyl groups and other features in the chemical structure. More than 4,000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of the flowers, fruits and leaves, for prevention of fat oxidation, and protection of vitamins and enzymes. Flavonoids are divided into six subclasses: flavanols, flavones, flavanones, flavanols, anthocyanins and isoflavones. Quercetin, myricetin, catechins are some most common flavonoids. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and also have protective effects against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases.[57]

Different parts of the plants contain substantial amount of bioactive phytoconstituents such as phenolics, flavonoids, tannins, etc. having ability to inhibit free radicals that are excessively produced and hence acts as antioxidants. Other biochemical activities of phenolic compounds isolated from plant species anti-mutagenic, anticarcinogenic and also modify the gene expression. Likewise, flavonoids isolated from medicinal plants have been reported with antioxidant, anti-inflammatory, oestrogenic, antimicrobial, anti-allergic, cardiovascular and cytotoxic antitumor activities.[16]

1.8 Antimicrobial activity

Anti-microbial agents are definitely one of the most important discoveries of the 20th century in the field of medicines.[58] Fungi, bacteria, viruses and nematodes cause serious infections in human beings throughout the world. Now-a-days these pathogenic microbes have become resistant to most of the antibiotics, because of careless use of these antimicrobial agents.[59] Thus, it causes a great problem in treating the infectious diseases. Antibiotics also have a number of side effects. *Staphylococcus aureus*, a gram-positive bacterium is said to be responsible for skin diseases such as pimples boils and infection in wounds.[60] Although *S. aureus* is susceptible to some common antibiotics, there is still the need to find alternative drugs before it develops resistance to the current ones.[61]

To avoid chances of resistance it becomes necessary to search some antimicrobials from natural resources. Traditional plants and their extracts have been used for treatment of various diseases in history for several years. Some plant families showed antibacterial as well as antifungal activities. Plants produce compounds such as secondary metabolites which are active against pathogenic microbes.[62] These compounds having less side effects, low cost and better patient tolerance.[60] These compounds have low molecular weight. In response to microbial infection, plants synthesize phytochemicals, Therefore, it is evident that they may be effective against a large number of microorganisms.[63]

Plant phenolics have many defensive functions such as antibacterial and antifungal potential. Phenolic compounds present in the cytoplasm of the epidermal cells and on the surface of plants, protect from pathogens. Phenolic acids such as pyrogallol and gallic acid, flavonoids such as rutin, myricetin and daidzein are significantly effective as antibacterial agents.[64] The method of antimicrobial activity of secondary metabolites is not clearly known. Phenolic compounds exert antimicrobial activity primarily by their potential to act as nonionic surface-active agent, therefore, disturbing the lipid-protein interface or denaturing the proteins and inactivating of enzymes in the pathogens. Secondly, phenols alter the permeability of the membrane, which results in inhibition of active transport and coupling of oxidative phosphorylation, and also loss of metabolites due to membrane damage.[65]

Flavonoids may show their activity by inhibiting DNA gyrase, the cytoplasmic membrane function and β-hydroxy acyl-acyl carrier protein dehydratase enzyme.[66] Biological activities of phenolic compounds are related to their molecular structure; hydroxyl groups or phenolic ring.[67]

1.9 Rationale of the Study

Medicinal plants are second most valuable bio-resources after water resources in Nepal. From many years more than 80 percent drugs are discovered and designed using natural products from medicinal plants. The plant taken for this research has not been explored from Arghakhanchi district and reported in literature to that extent where it should be based on its local and traditional use to treat various non-fatal diseases. The ethnobotanical aspects of the medicinal plant *Berberis asiatica* suggests that there is presence of important phytoconstituents like alkaloids, flavonoids, phenolic compounds, terpenoids and also may have biological activity such as antioxidant, antidiabetic, antibacterial and this is the major hypothesis of this research.

1.10 Objectives of the study

The research work is inclined to accomplish the following general and specific objectives:

1.10.1 General Objective

The general objective of the study is to carry out phytochemical screening and biological activities of the methanolic extract of *Berberis asiatica.*

1.10.2 Specific Objectives

The specific objectives of the study are:

- To prepare the methanolic extract of *Berberis asiatica* through cold percolation method.
- To analyze different classes of phytochemical present in methanolic extract of *Berberis asiatica*.
- To evaluate the total phenolic and total flavonoid content of methanolic extract of *Berberis asiatica.*
- To determine antioxidant activity of methanolic extract of *Berberis asiatica*

- To determine the α-amylase inhibitory activity of methanolic extract of *Berberis asiatica.*
- To determine the antibacterial activity of methanolic extract of *Berberis asiatica.*
- To determine the minimum inhibitory concentration.
- To determine the minimum bactericidal concentration.
- To identify the bioactive constituent by GC analysis.

CHAPTER 2

LITERATURE REVIEW

Literature review of the work done on the *Berberis asiatica* were composed from information published on different articles from different journals, websites (Sci-hub.io, Google, Google scholar).

2.1 Introduction of Plant

The Berberidaceae is a family of 18 genera of flowering plants commonly called the barberry family. This family is in the order Ranunculales. The family contains about 700 known species, of which the majority are in *Berberis.* The species includes trees, shrubs and perennial herbaceous plants. In Nepal, the Berberidaceae is represented by *Berberis* (including *Mahonia*) and Podophyllum.[68]

Berberis is the largest genus in the Berberidaceae family. In Nepal, 32 species and more than 8 varieties of *Berberis* are found. *Berberis asiatica* and *Berberis aristata* are major two traded species of *Berberis.*[69] The Nepalese species of *Berberis* usually grow in somewhat disturbed habitats such as forest margins, open pastures at higher altitudes, semi-desert vegetation, and the margins of cultivated land. Most of the Nepalese species of *Berberis* have medicinal uses due to the presence of the alkaloid berberine. Extracts from the stem and root of *Berberis* are used in ophthalmic medicine and to treat jaundice, malarial fever, diarrhea and peptic ulcers. Fruits are eaten fresh and are sometimes used locally for making alcoholic drinks. In Nepali, all the species are commonly known as 'chutro'.[70]

2.1.1 Scientific Classification of *Berberis asiatica* Roxb. ex DC.

Kingdom: Plantae

Class: Dicotyledonae

Order: Ranunculales

Family: Berberidaceae

Genus: *Berberis*

Species: *asiatica*

Vernacular name: Chutro

Figure 8: *Berberis asiatica*

Distribution in Nepal: 1200-2500 m, East to West

2.1.2 Habit and Habitat

Berberis asiatica Roxb. ex. DC., is a wild variety species, commonly known as Chutro in Nepali, Daruharidra in Sanskrit and tree turmeric in English, is Shrubberies found in grassy and rocky slopes from 1200-2500 meters elevation in heavy shade, on north-facing slopes and on open hillsides in the drier areas from east to west of Nepal.[71] *Berberis asiatica* is an erect, spiny, deciduous to evergreen shrub that is freely branched from the base, which can grow up to 4 meters tall with yellow bark. It has simple, alternate leaves that are thick, rigid usually have 2-5 spiny teeth, shining dark green colored above and grayish beneath, margins usually with spiny teeth, 1.8-7.5 cm long, leathery. Flowers are pale yellow arranged in flat topped clustered racemes with 1025 branches. Fruit are dark purple berries having sweet and sour taste.[72] The genus Berberis has wide distribution all over the world mainly in India, Pakistan, West and Central Asia, China, Japan, South-east Asia, Europe, East Africa, North America and South America.[73]

2.1.3 Ethnobotany and Pharmacological Properties

The genus *Berberis* is well known for its diversity and pharmacological uses in traditional medicine system since ancient time.[73] *Berberis asiatica* is a very common substitute for *B. aristata* in having a similar percentage of active compounds which is

used in Ayurvedic system of medicine.[74] *Berberis asiatica* is used to cure ophthalmological problem and to cure fever and headache by Tamang community of Rasuwa district, Nepal.[75] *Berberis asiatica* roots are traditionally used for treatment of mouth ulcer, as an anti-inflammatory, as an analgesic, for antipyretic action, as diuretic, as hepatoprotective, as an antimicrobial, as an antioxidant, as a strong wound healer, as an anti-rheumatic, as stimulant, etc.[76] Wood, roots, bark and the plant extract are used as alterative, deobstruent, astringent, antiperiodic and diaphoretic. Extracts are widely used for various enteric infections, bacterial dysentery.[72]

The roots are used in treating ulcers, urethral discharges, ophthalmia, jaundice, fevers, skin disease etc. The roots contain 2.1% berberine, the stems 1.3%.[77] In Nepal, the bark and wood are crushed then boiled in water, strained and the liquid evaporated until a viscous mass is obtained. This is antibacterial, laxative and tonic. It is taken internally to treat fevers and is used externally to treat conjunctivitis and other inflammations of the eyes. Tender leaf buds are chewed and held against affected teeth for 15 minutes to treat dental caries.[78] It is used orally in the treatment of various enteric infections, especially bacterial dysentery.[79]

The plant *Berberis asiatica* has been used for the treatment of various problems and its plant extract has an important role in the drug discovery. Due to the various traditional medicinal uses of *Berberis asiatica*, investigators are interested in performing different biological assay and to find out the contributing chemical compound. Therefore, the study of *Berberis asiatica* in recent period in the scientific field has a growing interest.

According to phytochemical studies, various types of alkaloids were reported. Among all of them, berberine is found to be most powerful with greater yield. The quantity of secondary metabolites in plants, including Berberine, are known to vary across habitats, geographical locations, seasons, and parts of the plant. The quantity of berberine in the various parts of the plant depends on altitude and soil content of the surrounding. Roots and stem bark of the plant contains higher content of berberine, major alkaloid, known for its activity against cholera and severe diarrhea. Roots of *Berberis asiatica* are reported to have anticancer properties. *Berberis* extracts have demonstrated significant antimicrobial activity against a variety of organisms including bacteria, viruses, fungi, protozoans, and helminthes.[80]

It is also experimentally claimed to be hepatoprotective, antidiabetic[81], anti-inflammatory[82] and antimicrobial agent.[83] The use of root bark extract of *Berberis asiatica* to treat conjunctivitis was common and its effectiveness as an antimicrobial, anesthetic, antihypertensive, and pigment inducer has been clinically verified.[84]

Percentage Yield of bark extract in methanol of *Berberis asiatica* Roxb. ex DC. has found to be 6.8% yield of extraction by dry weight.[71]

Methanolic extract of *Berberis asiatica* leaves exhibit total phenolic content about 132.63 mg GAE/g, and total flavonoids content about 31.36 mg QE/g.[85]

The total phenolic content of methanolic extract of *Berberis asiatica* stem bark and root was observed to determine the presence of antioxidant constituent as they are extremely vital plant constituents due to the scavenging ability of their hydroxyl groups.[86] Total phenol in the study was found higher in *Berberis asiatica* root (59.076 ± 0.219mg/100 g) than in the stem bark (52.240 ± 0.119mg/100 g).[87]

It has been reported that the flavonoid constituents of the plant possess antioxidant properties and was found to be useful in the treatment of liver damage and possess an ideal structure for the scavenging of free radicals, which makes them important antioxidant agents.[88] In the present study, root powder of *Berberis asiatica* was found to exhibit less flavonoid content (1.576 ± 0.197) as compared with stem bark (1.735 ± 0.396).[87]

The methanolic extract of stem bark and root of *Berberis asiatica* exhibited very good antioxidant activity in a dose-dependent manner. However, the root of *Berberis asiatica* at different doses exhibited significantly higher (10%) antioxidant activity as compared with stem bark. The IC_{50} value of root and stem bark extract was found to be 102.31 and 120.7 µg/mL respectively, as compared with standard drug ascorbic acid (4.981 µg/mL).[87]

Berberis aristata and *Berberis asiatica* both reduces the blood sugar in sugar loaded rats but the reduction is significant in case of *B. aristata*. No hypoglycemic and antidiabetic action is observed in both drugs.[89]

50% ethanolic extract of roots of *Berberis asiatica* showed significant anti-diabetic activity than the standard drug, Glibenclamide and presence of alkaloids Berberine,

Berbamine, Palmatine and Oxyacanthine. Berberine is its marker compound and it is isoquinoline alkaloid.[76]

The hypoglycemic effect of berberine, a major alkaloid present was similar to that of metformin. Significant decreases in hemoglobin A1c (HbA_{1c}; from 9.5% ± 0.5% to 7.5% ± 0.4%, $P<0.01$), fasting blood glucose (FBG; from 10.6 ± 0.9 mmol/L to 6.9 ± 0.5 mmol/L, $P<0.01$), postprandial blood glucose (PBG; from 19.8 ± 1.7 to 11.1 ± 0.9 mmol/L, $P<0.01$).[81]

Blood glucose and lipid regulatory properties of *Berberis* was due to berberine-induced improvement in insulin sensitivity through regulation of adipokine secretion.[90]

Methanolic bark extract of *Berberis asiatica* Roxb. Ex DC. had highest Zone of inhibition (ZOI) value 24 mm against *S. aureus* that is followed by 23 mm, 22 mm, 10 mm against MRSA, *Bacillus subtilis, Enterococcus faecalis*.[71]

Methanolic extract of stem and leaves of *Berberis asiatica* high inhibitory potential on *S. aureus*, *K. pneumoniae*, *E. coli*, *B. subtilis* and *P. vulgaris* in all concentration.[91]

Crude aqueous extract of *B. asiatica* stem bark exhibited a zone of inhibition by the disk diffusion method. The Gram-positive bacteria *Staphylococcus aureus* and *Enterococcus faecalis* were observed sensitive to the extract at the concentration of 1 mg/disk. Among the Gram-negative bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi-A*, *Shigella dysenteriae-1* and *Pseudomonas aeruginosa* exhibited a zone of inhibition 8 mm in diameter. *P. aeruginosa*, however, had a considerably large zone (16 mm) of inhibition. A total of 5 microorganisms had MIC values 156.25 g/mL, while the rest of the tested organisms had MIC values 2500 g/mL for the pure alkaloid. The alcoholic plant extract showed the broadest spectrum as 12 microorganisms were inhibited when 1250 g/mL was taken as the cutoff point of MIC. With the same cutoff point, the aqueous extract inhibited 8 microorganisms. Among the Gram-positives, *E. faecalis* was inhibited best by the alcoholic extract (MIC 9.76 g/mL). For Gram-negatives, the alkaloid fraction was observed to inhibit the majority of the bacteria at a lower concentration than other extracts.[92]

Methanolic stem bark extract exhibited minimum inhibitory concentration for *S. aureus* was 312.5 µg/mL but best inhibition was found 78.12 µg/mL by berberine iodide.[71]

Minimum bactericidal concentration (MBC) value of bark extract of *B. asiatica* was found 0.1953125 mg/mL against *S. cerevisiae* and that is followed by 0.78125 mg/mL, 3.125 mg/mL, and 12.5 mg/mL against *S. aureus*, Methillicin Resistant *S. aureus,* and *B. subtilis*.[71]

Methanolic root extract of *Berberis* species contains jatrorrhizine exhibited higher antibacterial activity with MIC (0.78 µg/mL) and MBC (1.56 µg/mL) compared with the standard (streptomycin, 10 µg/mL).[93]

Berberine sulphate which is an alkaloid extracted from the roots and bark of various Berberis spp. have been reported to possess antibacterial, antifungal and antiprotozoal activities.[90]

2.1.4 Phytochemistry

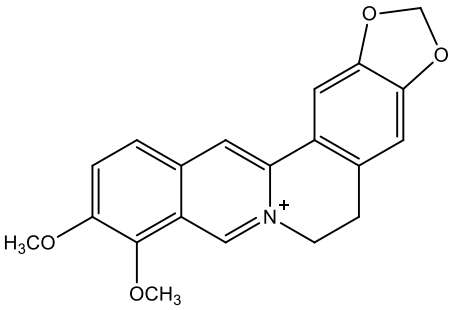
The constituents reported from stem and roots of the plants were found almost same however, variability has been reported in the chemical constituents of leaves.[73] Phytochemical screening of bark extract of *Berberis asiatica* showed the presence of alkaloids, flavonoids, reducing sugar and steroids whereas volatile oils and protein were absent.[71] *Berberis asiatica* is a very common substitute for *Berberis aristata* in having a similar percentage of active compounds, phytochemical screening of *Berberis aristata* stem exhibited the presence of alkaloids, coumarin, flavonoids, glycosides, polyphenol, reducing sugar, saponin, steroids, tannin, tri terpenoids.[94] Phytochemical study root and stem extract of *Berberis aristata* exhibited almost similar characteristic that presence of alkaloids, flavonoids, saponins, terpenoids, glycosides and reducing sugars, being absence of tannins but leaf extract exhibited presence of all those like root and stem except alkaloids, tannins and steroids.[95]

Major alkaloids reported from various *Berberis* species are berberine, berbamine, palmatine, columbamine, jatrorrhizine, oxyacanthine. The berberine and berbamine are the most biologically active compounds among all.[73] *Berberis asiatica* root, stem and bark contain alkaloids such as berberine, berbamine, palmatine, jatrorrhizine, oxyacanthine, oxyberberine, tetrahydropalmatine and columbamine. They are located mainly in the cortical tissues of the roots and stems and have important biological activities.[90, 96]

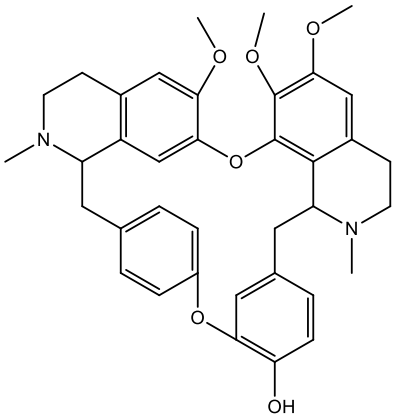
It has been reported that presence of antioxidants xanthophyll, phenol, carotene in the fruits and roots of *Berberis asiatica* Roxb.[97]

The gas chromatography coupled to mass spectrometry (GC-MS) analysis of various parts of *B. vulgaris* revealed that benzaldehyde, benzyl alcohol, 1-hexanol and 2-hexenal were major compounds of the essential oil from fruit, while p-cymene, limonene and ocimene were identified as major compounds of the essential oil from leaves and flowers.[90]

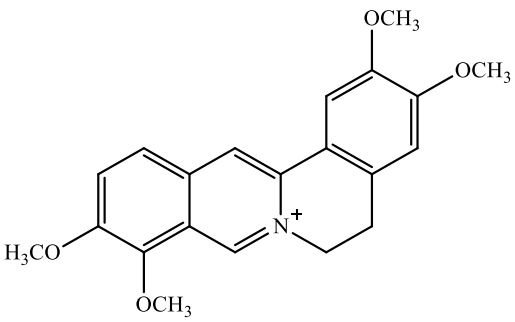
Structures of some major chemical constituents present in *Berberis asiatica* are as below:



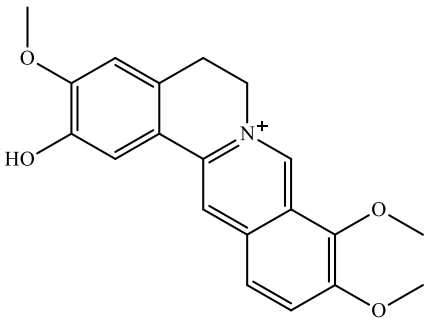
Berberine



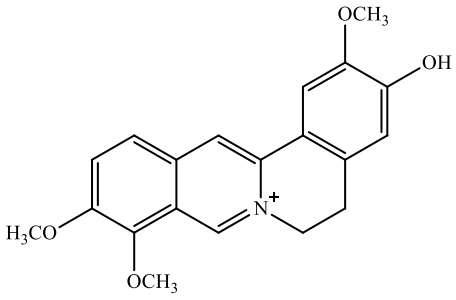
Berbamine



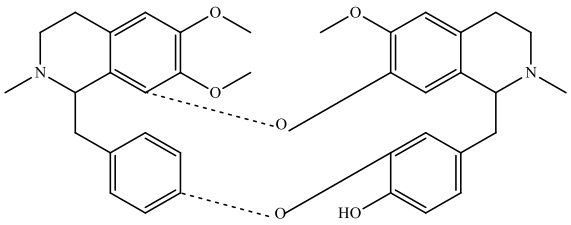
Palmatine



Columbamine



Jattrorrhizine



Oxyacanthine

CHAPTER 3

EXPERIMENTAL METHODS

3.1 Chemicals and Equipments

Most of the chemicals and solvents used were of analytical as well as laboratory grade. Methanol (Fisher Scientific), Hexane (Merck), Dimethyl sulphoxide (DMSO) (Merck), Ethyl acetate. Distilled and doubled distilled water was purchased from local vendor. Enzyme used for α-amylase enzyme inhibition assay was porcine pancreatic α –amylase (PPA) of Sigma Company. Other chemicals and reagents like DPPH, ascorbic acid, gallic acid, quercetin, required for antioxidant, TPC and TFC and were provided by Central Department of Chemistry. Chemicals like aq. Na_2CO_3, $NaNO_2$, NaOH, $AlCl_3$, ethanol., potassium acetate, etc. and other reagents for phytochemical screening were available in laboratory.

Digital weighing balance, electric mixture grinder, mortar and pestle, hot air oven, rotatory evaporator with water bath, multimode 96 well plate reader, micropipette, 96 well plate, vials, vortex shaker, petri disc, pH meter, beaker, conical flasks, test tubes, reagent bottles, etc. used in this work.

3.2 Collection of Plant

The plant sample was collected from Arghakhanchi district of Nepal at the altitude of 1810 m in the month of October based on ethnobotanical aspects. The plant under study was identified from National Herbarium and Plant Laboratories (NHPL), Godawari-5, Lalitpur, Nepal.

3.3 Sample Preparation

The freshly collected plant parts (leaves, bark and roots) were washed with tap water to remove the contaminants. Then, the plant parts were cut into small pieces, dried under shade and grinded into fine powder form. Before grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other foreign matter deposited on the grinder. Grinding improves the efficiency of extraction by increasing surface area. The powdered plant sample was collected in a clean waterproof plastic bag and stored in a cool and dry place until used for further experiment.

3.4 Extraction

The extraction of chemical constituents of selected plant material was carried out using methanol by the cold percolation method.

About 100g shade dried grinded powder of various parts of medicinal plants were kept in clean and dry conical flask and 350mL methanol was added to the flask. Then, it was kept in dark for 2-3 days at room temperature with shaking at intervals (cold percolation). The mixture was decanted and filtered with the help of Whatman filter paper and thus obtained filtrate was concentrated using rotatory evaporator by distillation at temperature below 55°C. This process was repeated for 6-7 times. The concentrated filtrate was air dried for the evaporation of solvent. After evaporation of the solvent, thus obtain solid or semisolid residue is the methanolic extract of plant sample under study. The extract was kept in vials after weighing.

The percentage yield of the extract was calculated using the following formula:

$$\text{Percentage Yield} = \frac{\text{Weight of extract}}{\text{Weight of the powered plant sample taken}} \times 100\ \% \text{ ---------- (1)}$$

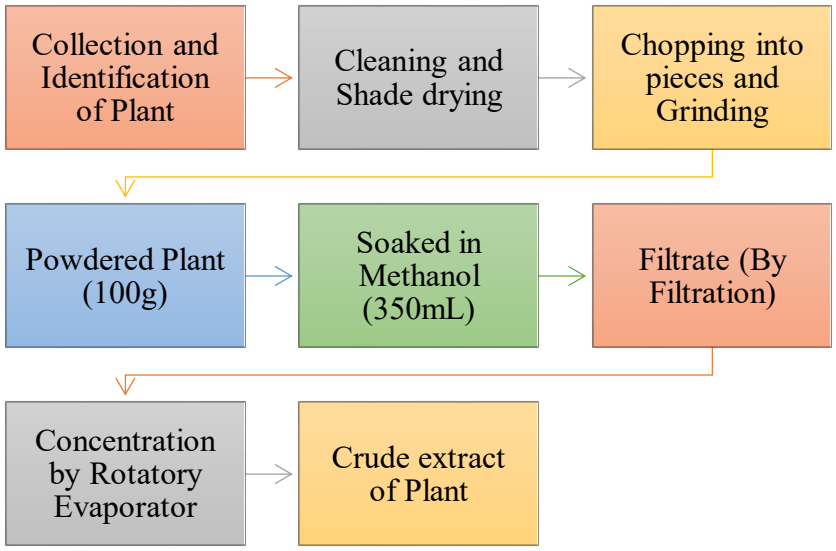


Figure 9: Schematic diagram for extraction of phytoconstituents by cold percolation method

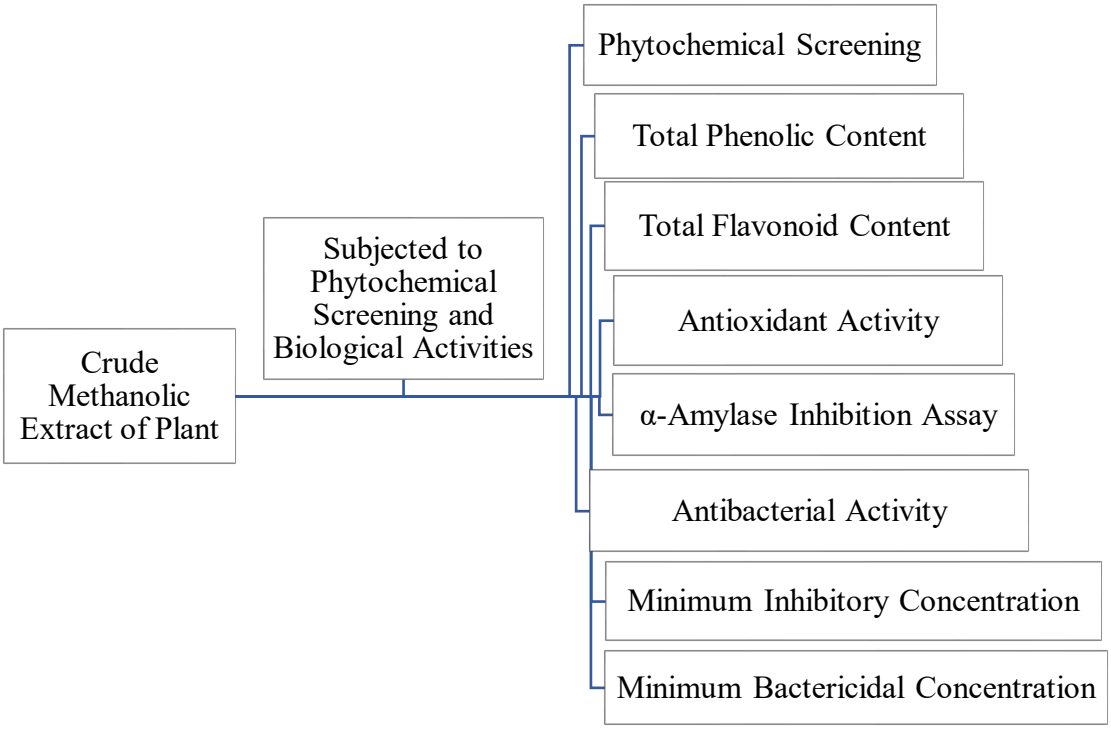


Figure 10: Schematic representation of Biological activities

3.5 Phytochemical Analysis

Freshly prepared extracts were subjected to standard phytochemical analysis to determine the presence of the following phytoconstituents, i.e., alkaloids, phenols, flavonoids, glycosides, tannins, saponins, steroids, terpenoids, sugar, and proteins by following the protocol given by Ciulei I., Sashidharan S., and Tamilselvi N.[98, 99, 100] Tests that were carried out to identify major phytoconstituents in plant extracts are given below:

1. Test for volatile oils

To about 500 mg extract, 0.5 ml, of methanol was added shaken vigorously and filtered. Few drops of filtrate were put on a filter paper by means of a capillary tube. Yellow spot persistent even after evaporation of the solvent indicates the presence of volatile oils.

2. Test for Alkaloids

About 500 mg extract was dissolved in 3 mL of 2 % (v/v) hydrochloric acid solution. The solution was equally divided into two test tubes and following tests were performed:

i. Meyer's Test

Few drops of Meyer's reagent were added to the first part. Formation of pale yellow precipitate indicates the presence of alkaloids.

ii. Dragendorff's Test

Few drops of Dragendorff's reagent were added to the second part, formation of orange red precipitate indicates the presence of alkaloids.

3. Test for Terpenoids

Salkowski test

To about 200 mg extract, 2 mL of chloroform and then 3 mL of concentrated sulphuric acid were added carefully. Formation of reddish-brown coloration at the interface indicates the presence of terpenoids.

4. Test for Coumarins

To about 1 mL of extract, 1 mL of 10 % sodium hydroxide solution was added. Formation of yellow color indicates the presence of coumarins.

5. Test for Flavonoids/ Shinoda's Test

About 200 mg extract was dissolved in 2 mL of methanol. To this solution small piece of magnesium and 4-5 drops of concentrated hydrochloric acid were added. Formation of orange color indicates the presence of flavonoids.

6. Test for Quinones

To about 2 mL of extract, 1 mL of freshly prepared ferrous sulphate solution and few crystals of ammonium thiocyanate were added and the solution was treated with conc. sulphuric acid drop by drop. The appearance of persistent deep red coloration indicates the presence of quinones.

7. Test for Polyphenols/ $FeCl_3$ Test

To about 1 mL of extract, 1 mL of distilled water was added followed by addition of few drops of 10 % (w/v) ferric chloride solution. The appearance of greenish blue coloration indicates the presence of polyphenols.

8. Test for Glycosides

About 500 mg extract was dissolved in 2 mL methanol and divided into two parts and following tests were performed:

i. Molisch's Test

First part was treated with 5 mL of Molisch's reagent and conc. sulphuric acid was added drop by drop from the side of the test tube without disturbing the solution. Appearance of violet ring at the junction of two liquids which on shaking turns the solution into violet color indicates the presence of glycosides.

ii. To the second part, 2 mL of 25 % (v/v) ammonium hydroxide solution was added and shaken vigorously. Appearance of cherry red color indicates the presence of glycosides.

9. Test for Reducing Sugars

To about 1 mL of extract, 1 mL of distilled water was added followed by addition of 1 mL of Fehling's reagent (1:1 mixture of Fehling's solution A and B). Then the mixture was warmed over water bath for 30 minutes. The appearance of brick red precipitate indicates the presence of reducing sugars.

10. Test for Saponins

About 500 mg was treated with hot water followed by shaking for 30 seconds. Formation of thick forth indicates the presence of saponins.

11. Test for Tannins

About 200 mg extract was boiled adding 10 mL of distilled water. The mixture was cooled, filtered and few drops of $FeCl_3$, solution were added to the filtrate. Appearance of blue-black ppt. indicates the presence of tannins.

12. Detection of Steroids

2 mL chloroform was mixed with crude extracts and concentrated sulphuric acid was added sidewise presence of red color in the lower chloroform layer indicates steroids.

(B) Preparation of reagents

1. Meyer's Reagent

Mercuric chloride (0.679 g) was weighed in a 50 mL volumetric flask and dissolved in distilled water. To this solution, 2.5 g potassium iodide was added. The scarlet red precipitate was dissolved by shaking and volume was made up to the mark by adding distilled water.

2. Dragendorff's Reagent

Bismuth nitrate (4.000 g) was dissolved in 5 N nitric acid (10 mL) to make solution A. Next, potassium iodide (13.5 g) was dissolved in distilled water (20 mL) to make solution B. These two solutions were mixed together in a 50 mL volumetric flask.

Picric acid (0.25 g) was dissolved in 50 mL of distilled water to make aqueous picric acid solution. The solution was neutralized with sodium bicarbonate. A strip of Whatman no. 1 filter paper was dipped in the prepared solution. The paper was dried completely and protected from external contamination. Thus, prepared sodium picrate paper was used for cyanogenic glycoside detection.

3. Molisch's Reagent

α-Naphthol (5.000 g) was dissolved in 50 mL of methanol to prepare Molisch's reagent.

4. Neutral Ferric Chloride Solution

Ferric chloride crystals (1.000 g) were dissolved in 100 mL of distilled water. Then, sodium carbonate crystals were added little by little with stirring until the slight turbidity was persistent. Finally, the mixture was filtered and the colorless filtrate was used as neutral ferric chloride solution.

3.6 Determination of Total Phenolic Content

Total phenol content of the methanolic extract of *Berberis asiatica* was measured using Folin-Ciocalteu reagent by 96 well plate methods which was modified from colorimetric method.[101, 102]

Preparation of Reagents

1 M sodium carbonate was prepared by dissolving 5.29 gram of sodium carbonate in 50 mL distilled water and 1:10 v/v Folin-ciocalteu reagent was prepared by diluting 10 mL of commercially available F-C reagent in 100 mL of distilled water.

Preparation Standard Gallic acid solution

500 µg/mL stock solutions were prepared by dissolving 5 mg of gallic acid in 10 mL of ethanol. The dilution of stock solution was done to prepare final concentration of 10, 20, 30, 40, 50, 60, 70, and 80 µg/mL. The solution used for the test should be freshly prepared.

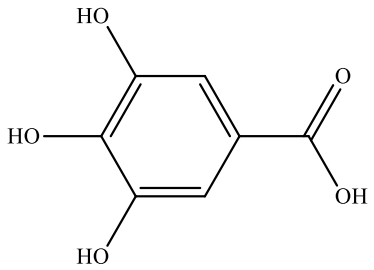


Figure 11: Structure of Gallic acid

Preparation of plant extracts

The plant extracts were prepared 500 µg/mL by diluting the stock solution of 50 mg/mL in 50 % DMSO solution.

At first 20 µL of different concentration of standard 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL gallic acid was loaded on 96 well plate in triplicate by diluting stock solution of 0.5 mg/mL with distilled water. Then 20 µL of plant sample of 500 µg/mL was loaded on 96 well plate in triplicate. After that in each well containing standard and sample 100 µL Folin-ciocalteu followed by 80 µL Na_2CO_3 was added separately. Then it was left in dark for 15 minute and and after 15 minute absorbance was taken at 765 nm using multi-mode plate reader (Synergy/ LX, BioTek, Instruments, Inc., USA). Gallic acid was used for constructing the standard curve (10-80 µg/mL) and the total

polyphenolic compound concentration in the extracts was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of the extract using gallic acid standard curve.

Calculation of Total Phenolic Content:

Total phenolic content in extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g). The total phenolic content was calculated using the following relation:

$$C = \frac{cV}{m} \quad \text{---------- (2)}$$

where, C = Total phenolic content in mg/g, in gallic acid equivalent (GAE)

c = Concentration of gallic acid established from the Calibration curve in mg/mL

V = Volume of extract in mL

Statistical Analysis

Data were recorded as a mean of three absorbance for each concentration, from which linear correlation coefficient (R^2) value was calculated. The regression equation is given as:

$$y = mx + c \quad \text{---------- (3)}$$

where, y = Absorbance of the extract

m = Slope from the Calibration curve

x = Concentration of the extract

c = Intercept

Using this regression equation, concentration of the extract was calculated. Thus with the calculated value of concentration of the extract, the phenolic content was calculated from the above equation (2).

3.7 Determination of Total Flavonoid Content

Total flavonoid content of the methanolic extract of *Berberis asiatica* was determined by 96 well plate method which was modified from colorimetric method.[103]

Preparation of Reagents

10% Aluminum trichloride was prepared by dissolving 1 gram of $AlCl_3$ into 10 mL distilled water and 1 M potassium acetate was prepared by dissolving 0.98 grams of potassium acetate into 10 mL distilled water.

Preparation standard quercetin solution

Stock solution was prepared by dissolving 1 mg of quercetin into 10 mL methanol (0.1 mg/mL). Then final concentrations of the standard solution were prepared 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL by diluting the stock solution of 0.1 mg/mL.

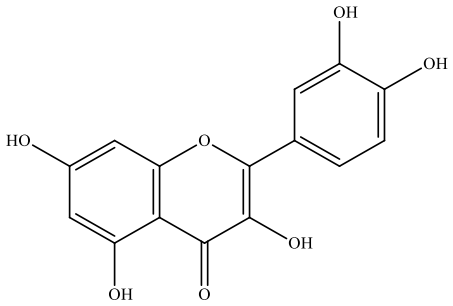


Figure 12: Structure of Quercetin

Preparation of plant extracts

The plant extracts were prepared 500 µg/mL by diluting the stock solution of 50 mg/mL in 50% DMSO solution.

At first 130 µL of different concentration of standard 10, 20, 30, 40, 50, 60, 70 and 80 µg/mL quercetin was loaded on 96 well plate in triplicate by diluting stock solution of 0.1 mg/mL with distilled water. Then 20 µL of plant sample of 500 µg/mL was loaded on 96 well plate in triplicate, and then 110 µL of distilled water was added in each well containing plant sample maintaining the final volume 130 µL. Then in each well containing standard and plant sample 60 µL ethanol, 5 µL $AlCl_3$ and 5 µL potassium acetate was added separately. Then it was left in dark for 30 minutes and after 30 minutes absorbance was taken at 415 nm using multi-mode plate reader (Synergy/ LX, BioTek, Instruments, Inc., USA).

Calculation of Total Flavonoid Content

Total flavonoid content in extract was expressed as milligrams of quercetin equivalent per gram of dry weight. The total flavonoid content was calculated using the following relation:

$$C = \frac{cV}{m} \quad \text{---------- (4)}$$

where, C = Total flavonoid content in mg/g, in quercetin equivalent (QE)

c = Concentration of quercetin established from the Calibration curve in mg/mL

V = Volume of extract in mL

Statistical Analysis

Data were recorded as a mean of three absorbance for each concentration, from which linear correlation coefficient (R^2) value was calculated. The regression equation is given as:

$$y = mx + c \quad \text{---------- (5)}$$

where, y = Absorbance of the extract

m = Slope from the Calibration curve

x = Concentration of the extract

c = Intercept

Using this regression equation, concentration of the extract was calculated. Thus, with the calculated value of concentration of the extract, the flavonoid content was calculated from the above equation (4).

3.8 Determination of Antioxidant Activity

Antioxidant activity of the methanolic extract of *Berberis asiatica* was determined by 96-well plate method which was modified from colorimetric method.[104, 105]

3.8.1 DPPH free radical scavenging assay

The free radical scavenging activity of plant extract was assayed by using a stable free radical, DPPH. This method is simple and sensitive. This assay is based on the theory that a hydrogen donor is an antioxidant. It measures compounds that are radical scavengers. Figure below, shows the mechanism by which DPPH˙ accepts hydrogen from an antioxidant. The antioxidant effect is proportional to the disappearance of DPPH˙. DPPH˙ shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm.

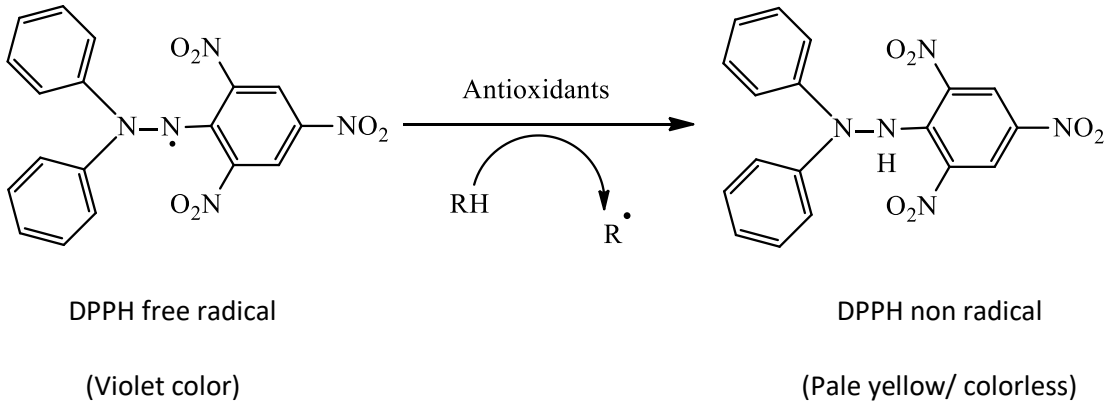


Figure 13: Reaction mechanism of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. RH = antioxidant radical scavenger. R˙ = antioxidant radical.

Preparation of DPPH solution (0.1 mM)

0.1 mM DPPH solution was prepared by dissolving 3.9 mg DPPH in 100 mL methanol in volumetric flask covered with aluminum foil.

Preparation of quercetin solution

Stock solution of quercetin was prepared by dissolving 1 mg of quercetin in 1 mL of methanol. Then, final concentration of 20, 10, 5, 2.5 and 1.25 µg/mL were prepared by diluting the stock solution of 1 mg/mL.

Preparation of plant extracts

Stock solution of 50 mg/mL was prepared by dissolving 50 mg of plant extracts in 1 mL of DMSO using vortex machine. The final concentrations of plant extracts were prepared 1000, 500, 250, 125 and 62.5 µg/mL in 50% DMSO solution.

For DPPH test, quercetin of 20 µg/mL was used as positive control and 50% DMSO was used as negative control. The positive control quercetin, negative control DMSO and plant samples were loaded 100 µL in 96 well plate in triplicate. Then 100 µL of DPPH reagent was added in each well. Then it was incubated for 30 minutes in dark. After 30 minutes, absorbance was taken at 517 nm using multi-mode plate reader (Synergy/ LX, BioTek, Instruments, Inc., USA). The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\% \text{ Inhibition} = (A_0 - A) / A_0 \times 100\ \%$$

Where, A_0 = Absorbance of control

A = Absorbance of sample

The IC_{50} value is the effective concentration of the sample that is required to scavenge 50% of the free radicals present in the solution. The IC_{50} value was calculated using a Graphpad software.

3.9 Determination of Anti-diabetic Activity

Anti-diabetic activity of plant extract was determined by α-amylase enzyme inhibition assay.[106]

3.9.1 α-Amylase enzyme inhibition assay

Preparation of reagents

100 mM of sodium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate were prepared and both buffers were mixed for 50 mL and pH was

maintained 7. Then equal volume of distilled water was added to make buffer 50 mM concentration and 100 mL volume.

Preparation of plant extract

The plant extract was prepared 500 µg/mL in 50% DMSO solution by diluting the stock solution. Plant extract which shows more than 50% inhibition were further diluted as 500, 250, 125, 62.5 and 31.25 µg/mL for IC_{50} calculation.

The α-amylase inhibition activity was evaluated in 50 mM phosphate buffer pH 7.0 with 0.9% NaCl. 80 µL PPA at the final concentration of 1.5 units/mL (prepared in buffer mentioned above) with the various concentrations of 20 µL test compounds (prepared in DMSO) was incubated at 37°C for 15 min. The reaction was started by the addition of 100 µL substrate, CNPG3, at 375 µM final concentrations prepared in the buffer as above. The change in absorbance by released p-nitroaniline was continuously monitored at 405 nm. DMSO tolerance was tested and found no effect up to 5% for the inhibition of α-amylase and therefore 5% DMSO was taken as control. All the experiments were performed in triplicate in a final volume of 200 µL, by using a multi-mode plate reader (Synergy/ LX, BioTek, Instruments, Inc., USA).

% Inhibition = $(A_0 – A) / A_0 \times 100$ %

Where, A_0 is the absorbance of enzyme-substrate reaction with 30 % DMSO and

A is the absorbance of enzyme-substrate with plant extract.

3.10 Determination of Antibacterial Activity

The antibacterial activity of the methanolic extract of *Berberis asiatica* against four bacterial species was performed by agar well diffusion method. Effectiveness of antibacterial substance was evaluated by determination of Zone of inhibition (ZOI) as given by Cavalieri S. J. et al (2005) with little modification.[107]

Preparation of Stock Solution

Stock solution of 50 mg/mL was prepared by dissolving 50 mg of plant extracts in 1 mL of DMSO solvent using vortex machine. Then, the stock solution was sealed and stored in refrigerator at 2-8 ºC until use.

Collection of Test Organism

The test organism includes one gram-positive bacteria (*Staphylococcus aureus*) and three gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*) which were available in Central Department of Chemistry, Tribhuvan University.

Preparation of Standard Culture Inoculum

The bacteria to be tested were aseptically touched with the help of inoculating loop from primary culture plate. Then it was transferred into a test tube containing 10 mL of sterile Muller Hinton broth and incubated overnight at 37 ºC in incubator to adjust the turbidity to 0.5 McFarland standards giving a final inoculum of 1.5×10^8 CFU/mL.

Preparation of Media

The media used in the study were prepared according to the manufacturer's recommendation. The detailed procedure is given below:

Nutrient Agar (NA)

It was prepared by adding the distilled water to 28 g of nutrient agar in appropriate size of conical flask and made final volume 1000 mL (28 g/liter). Then it was boiled with continuous shaking and autoclaved at 121 °C for 30 minutes. The sterilized media was allowed to cool about 50 °C. They were distributed in the sterilized petri-plates of 90 mm diameter in the ratio of 25 mL per plate aseptically and labeled properly. Plates were then left as such for solidification.

Mueller Hinton Broth (MHB) Solution

21 g of powder was weighed and dissolved in 1000 mL distilled water in a conical flask. Then the solution in conical flask was boiled to dissolve the medium completely. Now the solution was sealed with aluminum foil and then allowed to cool down at room temperature. It was poured into tubes or flasks as desired and sterilized by autoclaving at 15 lbs pressure (or 121 °C) for 15 minutes.

Mueller Hinton Agar (MHA) Plates

9.5g of media was suspended in 250 mL distilled water in a conical flask, boiled to dissolve and sterilized by autoclaving at 121 °C for 15 minutes. The medium was then allowed to cool down about 50 °C and poured to sterile petri-plates in 20 mL/plate quantities. The plates were left as such for solidification.

Qualitative Screening and Evaluation of antibacterial activity

Prepared sterile Mueller-Hinton Agar (MHA) plates of approximately 4mm thickness were dried at appropriate temperature to remove excess of moisture from the surface of the media. The agar plates for the assay were prepared by labeling them with the name of the bacteria and the name code of the disc. The fresh inoculums comparable with turbidity standard were prepared. Then a sterile cotton swab was taken out and was dipped into the prepared inoculums. The inoculums of bacteria were transferred into Petri disc. The excess of inoculums was removed by pressing and rotating against the upper inside side wall of the tube above the liquid level and then swabbed carefully all over the plate. The plate was rotated through the angle of 60º after each swabbing. Finally, the swab was passed round the edges of the agar surface. The inoculated plates were left to dry for few minutes at room temperature with the lid closed.

Three wells were made in each inoculated media plate with the help of sterile cork borer no. 6. So, the diameter of a well was 6 mm and labeled properly. Then 50 µL of the working solution of the plant extract, 50 µL of 50% DMSO as negative control (N) and 50 µL of 1 mg/mL Neomycin (antibiotic) as positive control (P) at the same time in the separate well were loaded into the respective wells with the help of micropipette. The plates were then left for half an hour with the lid closed so that the extract diffused into media. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours), the plates were observed for the presence of inhibition of bacterial growth that indicated by a clear zone around the wells. The size of the zone of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of zone of inhibition in millimeters. The absence of zone of inhibition was interpreted as the absence of activity. The ZOI were measured with the help of millimeter ruler and mean was recorded.[108]

3.10.1 Determination of Minimum Inhibitory Concentration (MIC)

The smallest amount of compounds requires to kill or inhibit the growth of micro-organism in-vitro can be determined by the broth microdilution method. This amount is referred as Minimum Inhibitory Concentration (MIC). It is a measure of potency which is expressed in terms of either µg or mg per mL. The methanolic extract of the plant which showed significant antibacterial activity were then subjected to two-fold serial broth microdilution method to determine MIC.[109]

- A sterile 96 well plate was taken under aseptic conditions and labeled.
- A volume of 100 µL Mueller Hinton broth was taken in each well of the microtiter plate with the help of micropipette.
- Then 100 µL of plant extract of 50 mg/mL in 50 % DMSO was pipetted to wells from 1A to 4A.
- Now 100 µL Neomycin (as standard or positive control) of 0.5mg/mL was pipetted to wells from 5A to 7A for triplicate.
- By using multichannel micropipette, the content in the respective wells of first rows were mixed by sucking up and down 5-8 times.
- Now 100 µL solution was withdrawn from row A to row B within respective columns for serial two-fold dilution. Then again mixed by sucking and transfer to next row. This process was repeated to row H.
- 100 µL solution was discarded from last row after dilution.
- Now 5 µL of the prepared bacterial inoculum (of size 10^5 to 10^6 CFU/mL which was equivalent to 0.5 MacFarland turbidity) was poured into the wells from column 2 to column 8. Column 1 contains only MHB media and plant extract. Last column contains only MHB media (Broth media sterility control) while Column 8 contains MHB media and bacteria (Bacteria control).
- The microplate was incubated at 37 ºC or other desired temperature for 12 to 18 hours.
- The Resazurin (0.003 %) solution was prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution. Resazurin is an oxidation–reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays. It is a blue non fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells.[109]

- Now after incubating for 12 to 18 hours, 10 µL of resazurin indicator solution was added to all wells of microplate and again incubated for 2 to 3 hours.
- Any color changes from purple/blue to pink or colorless were recorded as positive. The lowest concentration at which color change did not occurred was taken as the MIC value for the test material and bacterial strain.

3.10.2 Determination of Minimum Bactericidal Concentration (MBC)

MBC was determined by sub culturing the MIC cultures on suitable agar plates. The sterile nutrient agar plate was taken in which solution from micro plate was loaded by streaking with the help of inoculating loop. The solutions of the well showing MIC value and above were loaded in the plate. The plates were incubated at 37 ºC for 18-24 hours. Then plates were examined for the growth of microorganisms. The tubes with minimum concentration of extract in which the growth was completely checked was noted as the MBC of the plant extract. MBC is complimentary to MIC and it is the lowest concentration of antibacterial agent that has reducing capacity of viability of initial bacterial inoculum up to 99.9%.[71]

3.11 Statistical analysis

All the experiments were performed in triplicates and data were presented in mean ± standard error of mean. The TPC, TFC, antioxidant assay and enzyme inhibition were calculated by Microsoft Excel 2007. The IC_{50} value was calculated using Graphpad Prism software.

3.12 Identification and Characterization of compounds

The hexane fraction of the *Berberis asiatica* extract was sent to Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur for GC in order to identify the major compounds present in it on the basis of tailing and multi-spots on TLC plate.

3.12.1 GC-MS analysis of hexane fraction

Based on TLC report hexane fraction was subjected for GC.

3.12.2 Analytical condition for GC

The instrument GCMSD was used for analysis under following analytical conditions;

Instrument	:	Agilent 7890 GC
		Agilent 5975C inert MSD with Triple-Axis Detector
Column	:	Agilent 190915-433
		30 m × 250 m × 0.25 m
Gas carrier	:	Helium, 1mL/min
Pressure	:	6.6018 psi
Average velocity	:	36.112 cm/sec
Sample Injection volume:		2 µL
Inlet heater	:	230 ºC
Split ratio	:	75:1
Oven temperature	:	32 ºC
Maximum oven temperature:		320 ºC
Programmed	:	32 ºC for 5 min + up to 230 ºC with 5 ºC/min and then hold time 15 min = Total time 59.6 min

The obtained result was given in chapter-4.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Percentage Yield of Extract

The percentage yield of the methanolic extract of *Berberis asiatica* Roxb.ex DC. was found to be 10.66% (w/w) which was shown in table as follows:

Plant	Colour	Dry weight of Plant (in g)	Weight of extract (in g)	Percentage Yield
B. asiatica	Yellow	75	8	10.66

Table 1: Physical characteristics and percentage yield of plant extract

It can be noticed from the results that percentage yield of methanolic extract of *Berberis asiatica* was comparatively higher than the yield (6.8%) reported in the literature.[71]

4.2 Phytochemical Screening

The result of the phytochemical screening of the methanolic extract of *Berberis asiatica* is tabulated as below:

S.N.	Phytochemicals	*Berberis asiatica*
1	Alkaloids	+
2	Flavonoids	+
3	Tannins	−
4	Saponins	+
5	Terpenoids	+
6	Steroids	+
7	Glycosides	+
8	Reducing sugars	+
9	Coumarins	+
10	Polyphenols	+
11	Volatile oils	−

Table 2: Phytochemical screening of plant extracts

Where, (+) = Present and (−) = Absent

Presence of different bioactive compounds present in plant extract were analyzed by different preliminary phytochemical screening which are summarized in the Table no. 2. The positive test results were indicated by (+) sign and negative test results are indicated by (−) sign. Compounds such as alkaloids, flavonoids, saponins, terpenoids, steroids, glycosides reducing sugars, coumarins, polyphenols were present in extracts of plants after phytochemical screening. But tannins and volatile oils were found to be absent. The result of phytochemical screening is in accordance with the literature.[71, 95]

4.3 Total Phenolic Content

The total phenolic content present in the methanolic extract of *Berberis asiatica* was estimated by Folin-Ciocalteu reagent according to the standard protocol using gallic acid as standard. Polyphenols in the plant extracts react with specific redox reagent (F-C Reagent) to form a blue complex exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The observation for absorbance for different concentration of standard gallic acid was illustrated through graphical representation, X-axis being plotted for concentration and Y-axis for absorbance. The numerical data is shown in Appendix I. The absorbance curve for standard gallic acid is shown in figure 14.

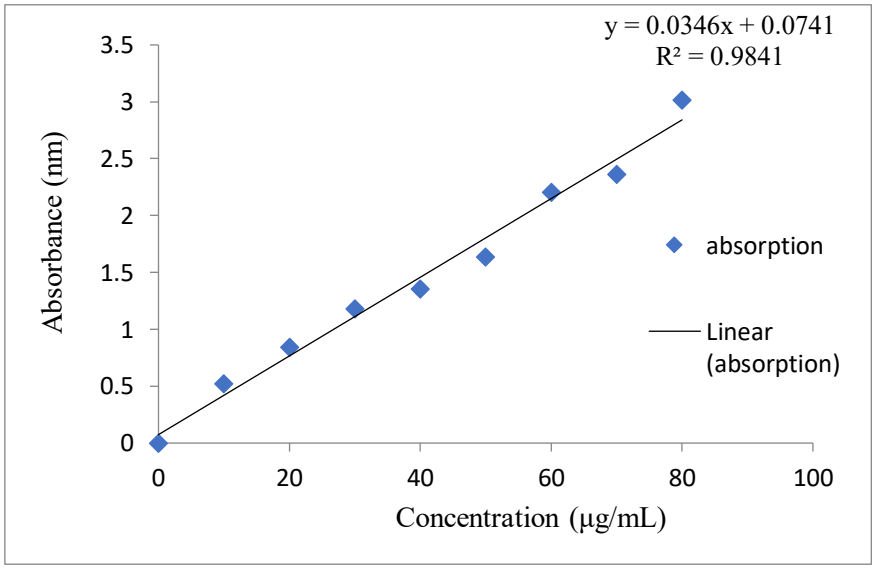


Figure 14: Calibration curve for standard gallic acid

The Total Phenolic Content in the plant extract was calculated by using regression equation y = 0.0346x + 0.0741, R^2 = 0.9841, of the curve obtained from above graph followed by the formula cV/m and expressed as mg GAE/g of dry weight of the extract.

The TPC (mean ± standard error) of methanolic extract of *Berberis asiatica* was shown in table below:

S.N.	Plant	TPC (mg GAE/g)
1	*Berberis asiatica*	37.686 ± 2.728

Table 3: Total Phenolic Content of methanolic extract of *Berberis asiatica*

Phenolic compounds react with F-C reagent only in basic condition adjusted by Na_2CO_3 solution. Under basic condition phenolic compound undergoes dissociation to form phenolate anion which reduces F-C reagent to blue color solution. The total phenolic content of methanolic extract of *Berberis asiatica* was found to be 37.686 ± 2.728 mg GAE/g. The total phenolic content of *Berberis asiatica* showed slightly different result than that reported in the literature. This variation in the phenolic content may be due to types of solvent used for extraction, environmental factors, different part of the plant used. From literature review, methanolic extract of *Berberis asiatica* leaves exhibit total phenolic content about 132.63 mg GAE/g.[85] Total phenol content in *B. asiatica* was found higher in roots (59.076 ± 0.219mg/100 g) and stem bark (52.240 ± 0.119mg/100 g).[87]

Phenolic compounds have been known to possess high antioxidant properties due to their free radical scavenging properties. It has been reported that extract containing large amount of polyphenol content possesses a greater antioxidant activity. Although quantitative determination of phenolic compounds in plant extracts are hampered by their structural complexity, diversity, nature of analytical assay method, selection of standard and presence of interfering substances.

4.4 Total Flavonoid Content

The total flavonoid content present in the methanolic extract of *Berberis asiatica* was estimated by Aluminum chloride 96 well plate method which was modified from colorimetric method using Quercetin as standard. The flavonoids of the plant extracts in the presence of aluminum chloride forms an acid liable complexes, has an intense yellow fluorescence which was observed with a maximum at 415 nm. The intensity of

light absorption at that wavelength is proportional to the concentration of flavonoids. The numerical data of absorbance of different concentration of standard quercetin is shown in Appendix II. The absorbance curve for standard quercetin is shown in figure 15.

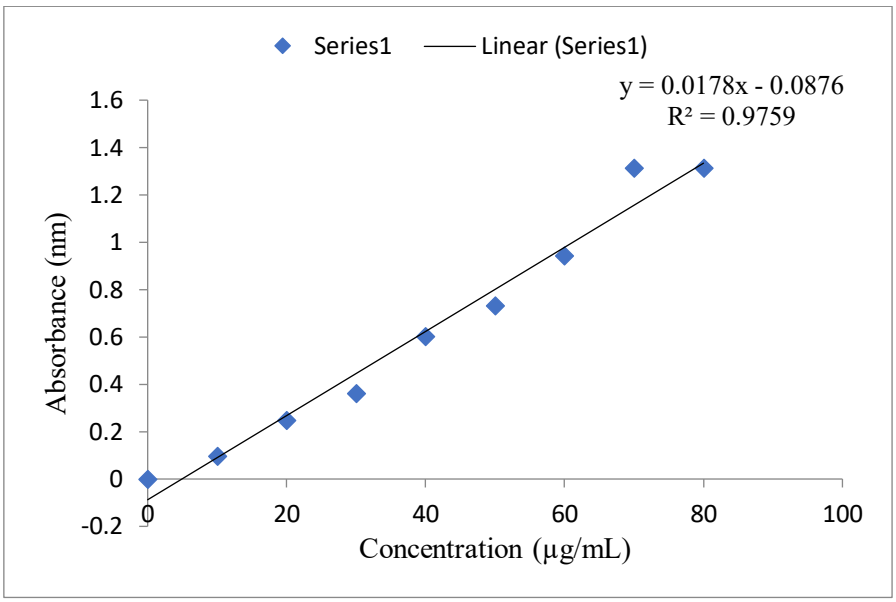


Figure 15: Calibration curve for standard quercetin

The total flavonoid content in the plant extract was calculated by using regression equation y = 0.0178x - 0.0876, R^2 = 0.9759 of the curve obtained from above graph followed by the formula cV/m and expressed as mg QE/g of dry weight of the extract. The TFC (mean ± standard error) of methanolic extract of *Berberis asiatica* was shown in Table 4.

S.N.	Plant	TFC (mg QE/g)
1	*Berberis asiatica*	115.568 ± 8.012

Table 4: Total Flavonoid Content of methanolic extract of *Berberis asiatica*

The total flavonoid content of methanolic extract of *Berberis asiatica* was found to be 115.568 ± 8.012 mg QE/g. From literature review, root and stem bark powder of *B. asiatica* was found to exhibit significantly less flavonoid content 1.576 ± 0.197 and 1.735 ± 0.396 respectively.[87] Also, methanolic extract of *Berberis asiatica* leaves

exhibit total flavonoids content about 31.36 mg QE/g.[85] The result showed significantly higher value of total flavonoid content in *Berberis asiatica* plant than that reported in the literature. This variation in the flavonoid content may be due to types of solvent used for extraction, environmental factors, different part of the plant used.

It has been reported that the flavonoid constituents of the plant possess antioxidant properties and was found to be useful in the treatment of liver damage and possess an ideal structure for the scavenging of free radicals, which makes them important antioxidant agents.[88]

4.5 Antioxidant Activity

1, 1-diphenyl-2-picrylhydrazyl (DPPH) was used to determine antioxidant activity of methanolic extract of *Berberis asiatica* and the result was expressed as IC_{50} (half inhibitory concentration).

4.5.1 DPPH Free Radical Scavenging Activity

The DPPH radical assay was performed for plant extract using quercetin as standard following standard protocol and absorbance was recorded at 517nm.

The percentage radical scavenging of plant extract at different concentration was calculated and listed in the Table 5.

S.N.	Concentration (µg/mL)	% radical scavenging
1	62.5	28.547
2	125	34.760
3	250	51.301
4	500	70.025
5	1000	89.588

Table 5: Percentage radical scavenging with different concentration of *Berberis asiatica* extract

Percentage radical scavenging effect on the DPPH radical was concomitantly increased with the increase in concentration of the methanolic extract from 62.5-1000 µg/mL. The *Berberis asiatica* plant extract showed highest percentage radical scavenging or inhibition of 89.588 % at the concentration of 1000 µg/mL.

The IC_{50} value of methanolic extract of *Berberis asiatica* and quercetin were shown in Table 6.

Plant	IC_{50} value (µg/mL)
Berberis asiatica	205.7 ± 5.353
Quercetin (standard)	6.29 ± 1.02

Table 6: IC_{50} value of methanolic extract of *Berberis asiatica* and Quercetin

The result was compared with standard quercetin with IC_{50} value 6.29 ± 1.02 µg/mL. *Berberis asiatica* showed significant radical scavenging activity with IC_{50} value 205.7 ± 5.353 µg/mL. The IC_{50} values of plant extract with standard are represented in bar diagram in Figure 16.

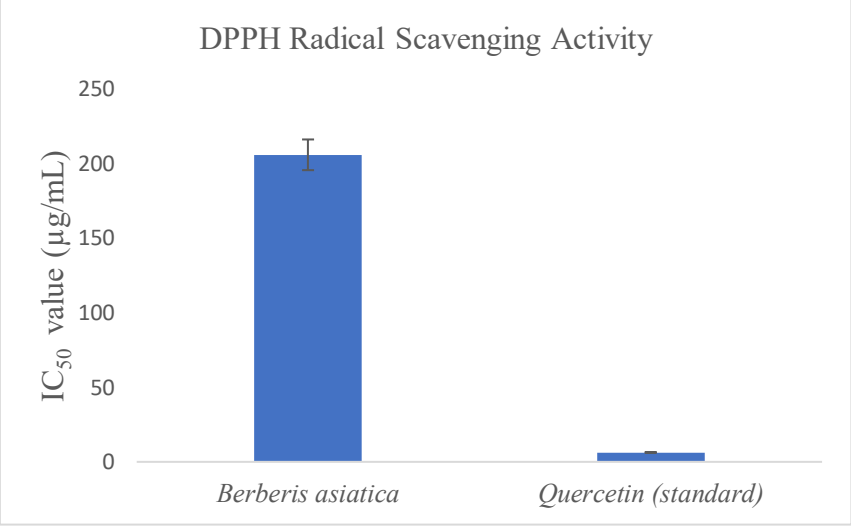


Figure 16: Bar diagram showing IC_{50} value of DPPH of *Berberis asiatica* and quercetin.

The above result of IC_{50} value of methanolic extract of *Berberis asiatica* and its bar diagram suggests the significant antioxidant activity. Thus, the plant are sources of potential antioxidants. The methanolic extract of stem bark and root of *Berberis asiatica* exhibited very good antioxidant activity in a dose-dependent manner. However, the root of *Berberis asiatica* at different doses exhibited significantly higher (10%) antioxidant activity as compared with stem bark. The IC_{50} value of root and stem bark extract was found to be 102.31 and 120.7 µg/mL respectively, as compared with standard drug ascorbic acid (4.981 µg/mL).[87]

Phenolic compounds in plants function as scavengers of free radicals. Thus, they impart antioxidant activity. Different classes of phenols have free radical scavenging mechanism which takes place through metal ion chelation or hydrogen ion donation. The plant extract having phenolic and flavonoids as major portion showed higher free radical scavenging property.[110]

4.6 Antidiabetic Activity

4.6.1 α-Amylase enzyme inhibition assay

α-Amylase enzyme inhibition assay was carried out using enzyme substrate method to determine antidiabetic potential of plant extract. Percentage inhibition of α-amylase by methanolic extract of *Berberis asiatica* of 500 µg/mL in 50% DMSO solution was calculated and the result was tabulated in Table 7.

Concentration (µg/mL)	% inhibition
500	37.732 ± 1.189

Table 7: Percentage inhibition of α-amylase by methanolic extract of *Berberis asiatica*

The above result showed the percentage inhibition less than 50%. Thus, plant extract which show more than 50% inhibition were further diluted as 500, 250, 125, 62.5 and 31.25 µg/mL for IC_{50} calculation. Literature also supports the above result as crude extract shows lesser inhibition of enzyme. Therefore, for best inhibition and antidiabetic activity, more concentrated extract should be used.

Besides above result the plant was better known to have antidiabetic property according to some literature. The hypoglycemic effect of berberine, a major alkaloid present in *Berberis asiatica* was similar to that of metformin.[81] Blood glucose and lipid regulatory properties of *Berberis* was due to berberine-induced improvement in insulin sensitivity through regulation of adipokine secretion.[90] The result may vary due to the time, geography of place from where the plant was collected, extraction process, solvent used, laboratory conditions, etc.

4.7 Antibacterial Activity

Antibacterial activity of methanolic extract of *Berberis asiatica* was determined in-vitro against one gram-positive and three gram-negative bacteria by agar well diffusion method and the zone of inhibition (ZOI) was measured and was tabulated in Table 8.

S.N.	Name of Bacteria	Zone of Inhibition (mm)	
		Plant Extract (50 mg/mL)	PC
1	*S. aureus*	14	13
2	*E. coli*	-	16
3	*K. pneumoniae*	19	18
4	*S. typhi*	-	15

PC = Positive control (Neomycin) (1 mg/mL)

Table 8: Zone of Inhibition of methanolic extract of *Berberis asiatica* against different bacteria.

The result showed that the methanolic extract of *Berberis asiatica* has significant inhibition in the growth of one gram-positive bacteria (*S. aureus*) and one gram-negative bacteria (*K. pneumoniae*) having ZOI values 14 mm and 19 mm respectively. Here, gram-negative bacteria (*K. pneumoniae*) is highly inhibited by the plant extract than gram-positive (*S. aureus*) bacteria. This signifies that the plant extract exhibit higher antibacterial property due to presence of some antibacterial agents. But methanolic extract of *Berberis asiatica* showed no inhibition for *E. coli* and *S. typhi*. This may be due to the presence of outer lipid membrane in gram negative bacteria which provides an additional mechanism of protection.

According to literature, the ZOI values for *S. aureus* and *K. pneumoniae* is similar with the result obtained in this study. But for *E. coli* and *S. typhi* the result obtained is different from literature. Crude aqueous extract of *Berberis asiatica* stem bark exhibited a zone of inhibition by the disk diffusion method. The Gram-positive bacteria *Staphylococcus aureus* was observed sensitive to the extract at the concentration of 1 mg/disk. Among the Gram-negative bacteria, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi-A* exhibited a zone of inhibition of 8 mm in diameter.[92] The variation in result from literature may be because of the part of plant used during study, solvent used for extraction, environmental conditions and lab condition, etc. Literature also suggests that the antibacterial activity of methanolic extract of *Berberis asiatica* is

due to presence of major alkaloid berberine, jatrorrhizine and phenolic compounds.[71,93,110]

4.8 Minimum Inhibitory Concentration

The methanolic extract of the plant which showed significant antibacterial activity were then subjected to two-fold serial broth microdilution method to determine MIC. It is a measure of potency to inhibit the growth of micro-organism in-vitro which is expressed in terms of mg/mL. In this study, minimum inhibitory concentration (MIC) value of methanolic extract of *Berberis asiatica* was determined against two bacteria i.e.one gram-positive bacteria (*S. aureus*) and one gram-negative bacteria (*K. pneumoniae*) taking Neomycin as standard. The MIC values of plant extract and Neomycin against test bacteria was shown below in Table 9.

S.N.	Bacteria	*Berberis asiatica*	Positive control
		MIC (mg/mL)	MIC (mg/mL)
1	*S. aureus*	0.39	0.0039
2	*K. pneumoniae*	3.125	0.0019

Table 9: Minimum inhibitory concentration of methanolic extract of *Berberis asiatica* against test bacteria

Methanolic extract of *B. asiatica* plant exhibited minimum inhibitory concentration for *S. aureus* was 0.39 mg/mL and for *K. pneumoniae* was 3.125 mg/mL. The result showed that the plant extract exhibited best inhibition for *S. aureus.*

According to literature, methanolic stem bark extract exhibited minimum inhibitory concentration for *S. aureus* was 312.5 µg/mL.[71] This reported data in literature is similar to the MIC value obtained. Methanolic root extract of *Berberis* species contains jatrorrhizine exhibited higher antibacterial activity with MIC (0.78 µg/mL) and MBC (1.56 µg/mL) compared with the standard (streptomycin, 10 µg/mL).[93] But for *K. pneumoniae*, aqueous stem bark extract of *Berberis asiatica* exhibited MIC value of 2500 µg/mL.[92] The obtained MIC values vary due to plant parts used, solvent used for extraction, amount of phytoconstituents having antibacterial property because of geographical variation, etc.

4.9 Minimum Bactericidal Concentration

MBC was determined by sub culturing the MIC cultures on suitable agar plates. MBC is the lowest concentration of antibacterial agent that has reducing capacity of viability of initial bacterial inoculum up to 99.9%. MBC is complimentary to MIC. The MBC values of plant extract against test bacteria was shown below in Table 10.

S.N.	Bacteria	*Berberis asiatica*	Positive control
		MBC (mg/mL)	MBC (mg/mL)
1	*S. aureus*	6.25	0.015
2	*K. pneumoniae*	6.25	0.0078

Table 10: Minimum bactericidal concentration of methanolic extract of *Berberis asiatica* against test bacteria

Methanolic extract of *Berberis asiatica* plant exhibited minimum bactericidal concentration for *S. aureus* was 6.25 mg/mL and for *K. pneumoniae* was 6.25 mg/mL. The result showed that the plant extract exhibited same MBC value for *S. aureus* and *K. pneumoniae.*

According to literature, methanolic stem bark extract exhibited minimum bactericidal concentration for *S. aureus* was 0.78125 mg/mL.[71] MBC value obtained is different from the literature. This is due to the plant parts used for extraction, solvent used, etc.

4.10 Chemical Analysis of Constituents of hexane fraction of *Berberis asiatica* by GC

The GC analysis of the hexane fraction of *Berberis asiatica* revealed the presence of 35 compounds. Some compounds are ketones, alkanes, cycloalkanes, other are identified as aromatic compounds. The major constituents present in the hexane extract of *Berberis asiatica* are given in Table 11. The GC chromatogram is given in Appendix III.

S.N.	Name of Compound	Retention Time ($R_{t)}$	Area %	Mol. Wt.
1	3,3-dimethylpentane	2.354	0.78	100.2
2	Cyclohexane	2.479	86.25	84.16
3	2-methylhexane	2.518	4.14	100.2
4	3-methylhexane	2.632	3.45	100.2
5	1,3-dimethylcyclopentane	2.758	0.40	98.19
6	1,2-dimethylcyclopentane	2.850	0.53	98.19
7	Heptane	3.036	0.55	100.2
8	Methylcyclohexane	3.494	0.93	98.186
9	2,2-dimethyl-3-pentanol	10.542	0.28	116.2
10	2-methyl-2-pentanol	10.662	0.84	102.17
11	2,5-dimethyl-4-hydroxy-3-hexanone	11.252	0.58	144.21
12	3-hexanol	12.135	0.16	102.17

Table 11: Major chemical constituents detected by GC analysis.

CHAPTER 5

CONCLUSIONS

Thus, from the above experiment, these conclusions were made:

The Percentage yield of the methanolic extract of *Berberis asiatica* was found to be 10.66%. The phytochemical analysis of the methanolic extracts of *Berberis asiatica* shows the presence of alkaloids, terpenoids, flavonoids, quinones, glycosides, polyphenols, saponins, coumarins, reducing sugars but tannin and volatile oil were absent.

The total phenolic content of the methanolic extract of *Berberis asiatica* was found to be 37.686 ± 2.728 (mg GAE/g). The total flavonoid content the methanolic extract of *Berberis asiatica* was found to be 115.568 ± 8.012 (mg QE/g). In the DPPH free radical scavenging assay for the determination of antioxidant activity, the IC_{50} value of the methanolic extract of *Berberis asiatica* was found 205.7 ± 5.353 µg/mL.

In α-amylase inhibition assay for the determination of antidiabetic activity, the percentage inhibition is less than 50 % for the methanolic extract of *Berberis asiatica*.

For determination of antibacterial activity, the methanolic extract of *Berberis asiatica* showed significant inhibition in the growth of one gram-positive bacteria (*S. aureus*) and one gram-negative bacteria (*K. pneumoniae*) having ZOI values 14 mm and 19 mm respectively. Methanolic extract of *Berberis asiatica* exhibited minimum inhibitory concentration (MIC) for *S. aureus* was 0.39 mg/mL and for *K. pneumoniae* was 3.125 mg/mL. While minimum bactericidal concentration (MBC) for *S. aureus* was 6.25 mg/mL and for *K. pneumoniae* was 6.25 mg/mL.

From GC analysis of hexane extract of *Berberis asiatica* showed presence of 35 compounds. Some compounds are ketones, alkanes, cycloalkanes, other are identified as aromatic compounds.

RECOMMENDATION

The plant species chosen is said to have a good medicinal value. Phytochemical analysis of the plants showed the presence of many biologically active compounds, such as alkaloids, glycosides, tannins, terpenoids, flavonoids and reducing sugars, which can be attributed to the potential antibacterial and antioxidant properties in the tested samples. Total phenolic and flavonoid content validated the idea behind use of traditional medicinal plants to treat different diseases and could be used as a source of active compounds in future study. Since, extract of *Berberis asiatica* contains good amount of bioactive chemical constituents. So, it could be used for isolating the active compound and it could be used for drug discovery process in future. Further, the *in-vitro* and *in-vivo* bioactivity of these extracts needs to be assessed that can shape to the potential drug discovery.

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APPENDIX

Appendix I: Absorbance values of standard Gallic acid at different concentration

Concentration (µg/mL)	Absorbance (nm)
10	0.522333
20	0.842333
30	1.181667
40	1.356333
50	1.636333
60	2.203667
70	2.360667
80	3.015

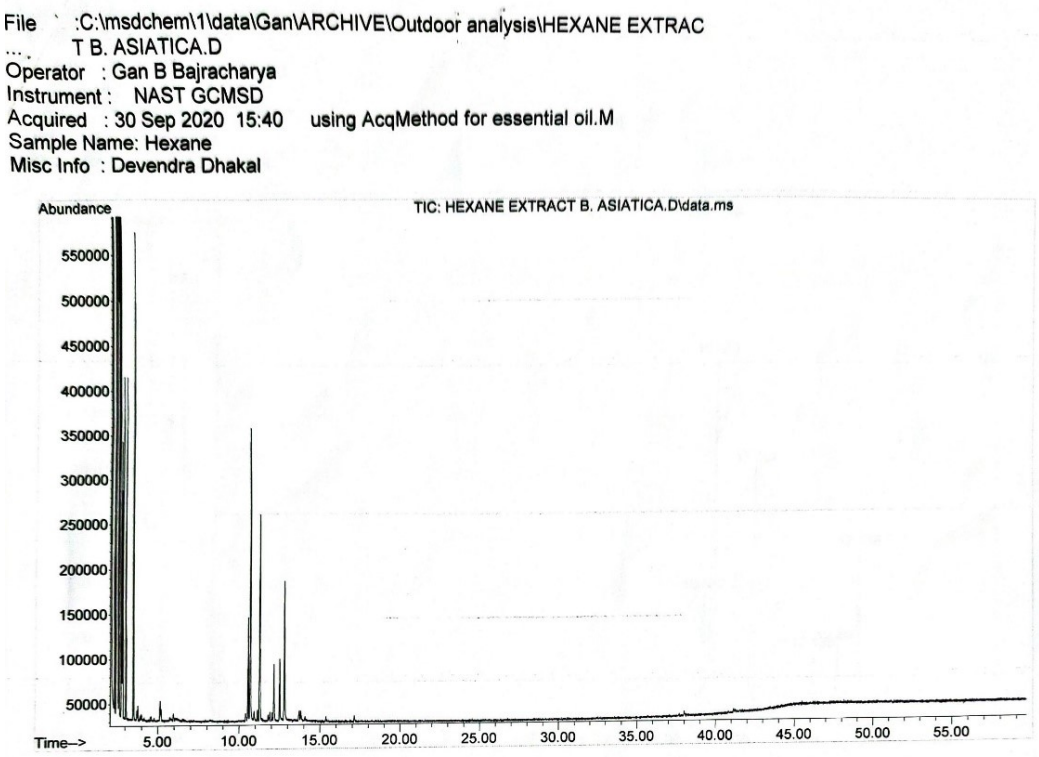
(Each value is a mean of triplicate data)

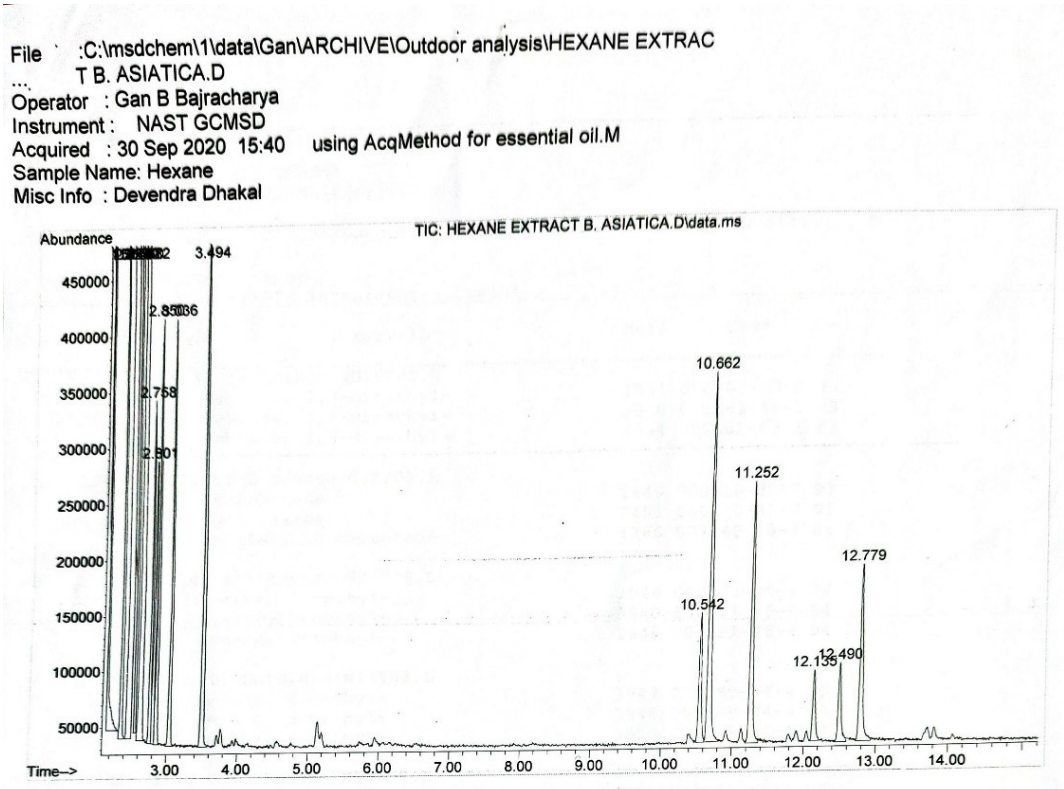
Appendix II: Absorbance values of standard Quercetin at different concentration

Concentration (µg/mL)	Absorbance (nm)
10	0.095
20	0.249
30	0.361
40	0.602
50	0.731
60	0.942
70	1.313
80	1.3126

(Each value is a mean of triplicate data)

Appendix III: GC chromatogram of hexane extract of *Berberis asiatica*





GC chromatogram

Appendix IV: Photographs showing determination of ZOI, MIC, MBC by methanolic extract of *Berberis asiatica*

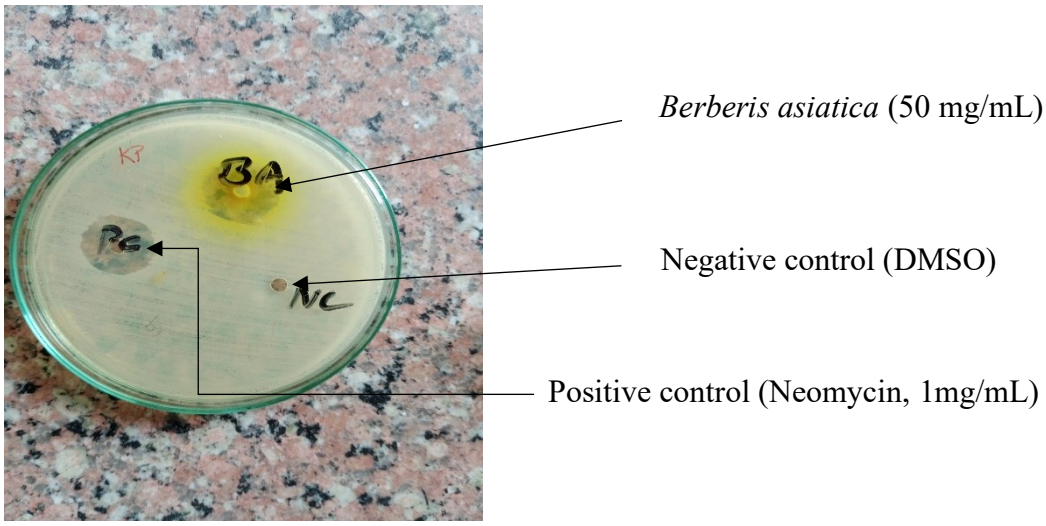


Photo: ZOI showed by plant extracts against *K. pneumoniae*

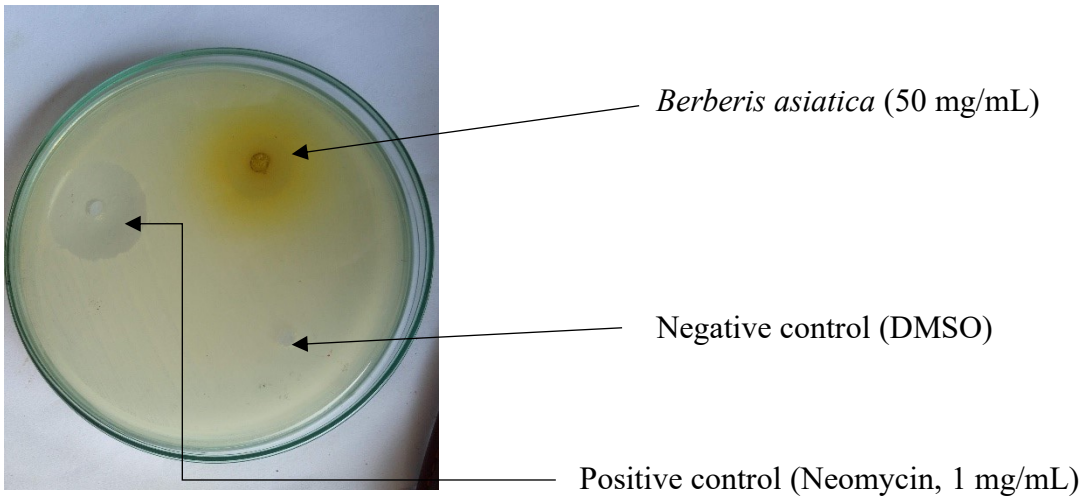


Photo: ZOI showed by plant extracts against *S. aureus*

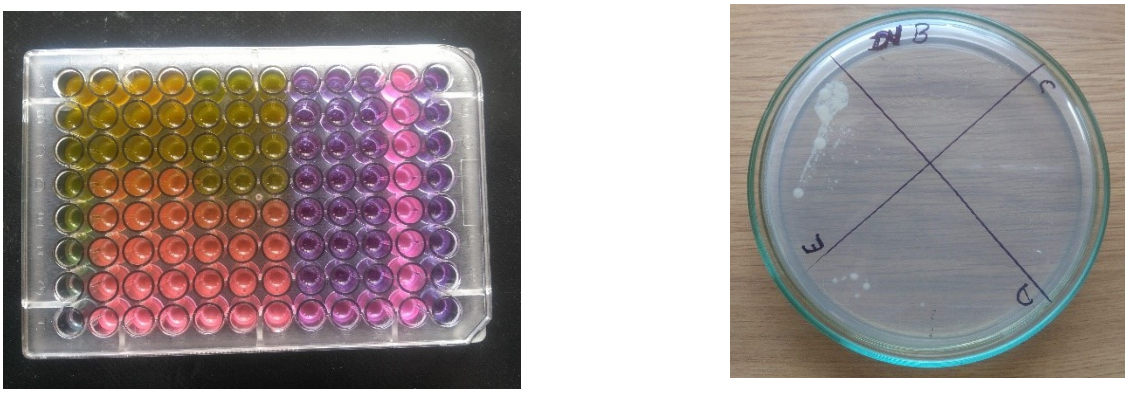
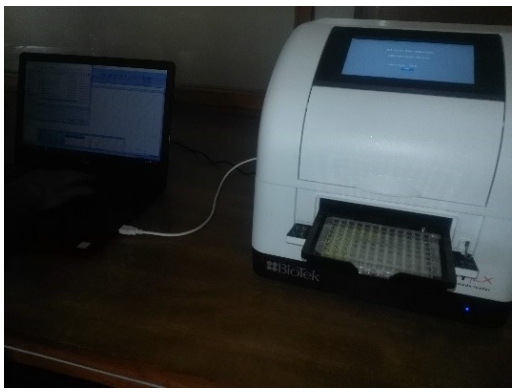


Photo: MIC and MBC determination of plant extract against *K. pneumoniae*

Appendix V: Some photographs of instrument used and doing experiment work.



Multi-mode plate reader

Photo taken during MBC determination