CHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS AND GREEN SYNTHESIZED SILVER NANOPARTICLES OF MEDICINAL PLANTS FROM MUSTANG AND KASKI DISTRICTS OF NEPAL

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF CHEMISTRY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN CHEMISTRY

BY

LEKHA NATH KHANAL

FEBRUARY 2023

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DECLARATION

Thesis entitled "**Chemical Analysis and Biological Activities of Crude Extracts and Green Synthesized Silver Nanoparticles of Medicinal Plants from Mustang and Kaski Districts of Nepal**" which is being submitted to the Central Department of Chemistry, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Associate Prof. Dr. Surya Kant Kalauni of Central Department of Chemistry, Tribhuvan University and co-supervised by Associate Prof. Dr. Khaga Raj Sharma of Central Department of Chemistry, Tribhuvan University, and Associate Prof. Dr. Yuba Raj Pokharel of South Asian University, New Delhi, India.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

Lekha Nath Khanal

February 2023

February 2023

RECOMMENDATION

This is to recommend that **Lekha Nath Khanal** has carried out research entitled "**Chemical Analysis and Biological Activities of Crude Extracts and Green Synthesized Silver Nanoparticles of Medicinal Plants from Mustang and Kaski Districts of Nepal**" for the award of Doctor of Philosophy (Ph.D.) in **Chemistry** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

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LETTER OF APPROVAL

Date: /01/2023

On the recommendation of **Associate Prof. Dr. Surya Kant Kalauni, Dr. Khaga Raj Sharma, and Dr. Yuba Raj Pokharel**, this Ph.D. thesis submitted by **Lekha Nath Khanal** entitled "**Chemical Analysis and Biological Activities of Crude Extracts and Green Synthesized Silver Nanoparticles of Medicinal Plants from Mustang and Kaski Districts of Nepal**" is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, T.U.

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ACKNOWLEDGEMENTS

First of all, I would like to extend my heartfelt gratitude to my supervisor Associate Prof. Dr. Surya Kant Kalauni for his continuous support, guidance, and motivation during my Ph.D. study. His valuable supervision has helped me throughout my research. I am also grateful to my co-supervisors Dr. Khaga Raj Sharma from the Central Department of Chemistry and Dr. Yuba Raj Pokharel from South Asian University, India for their valuable support and guidance.

The present study focuses on the evaluation of biological activities, chemical profiling of some active compounds, and fabrication of silver nanoparticles from medicinal plants in Nepal. In this context, I would like to offer my sincere gratitude to Prof. Dr. Jagadeesh Bhattarai, HOD of the Central Department of Chemistry, Kirtipur. I am highly obliged to former HOD of the CDC Prof. Dr. Kedar Nath Ghimire, Prof. Dr. Megh Raj Pokhrel, and Prof. Dr. Ram Chandra Basnyat. I am gratefult to Prof. Dr. Akkal Dev Mishra, Prof. Dr. Paras Nath Yadav, Prof. Dr. Amar Prasad Yadav, Prof. Dr. Niranjan Parajuli, Dr. Santosh Khanal, Dr. Bhanu Bhakta Neupane, Dr. Achyut Adhikari, Dr. Hari Paudyal, Dr. Bipeen Dahal, Mr. Purna Prasad Dhakal, and Dr. Kshama Parajuli as well as the non-teaching staff members of the Central Department of Chemistry for their priceless inspiration and support.

I would like to express my sincere thanks to Prithvi Narayan Campus, Pokhara for providing Ph.D. study leave. I am highly indebted to the respected madam Dr. Aleyamma John, who always guided me in my academic career. I would like to offer my sincere thanks to Dr. Liladhar Paudel, a senior scientist of Lubrizol Corporation, USA, Dr. Binod Babu Pageni from Agriculture and Agri-Food, Canada, Dr. Ramesh Raj Pant, Dr. Subash Adhikari from Policy and Planning Commission, Gandaki Province, Pokhara, Nepal, Namita Paudel Adhikari from Institute of Tibetan Plateau Research, Chinese Academy of Sciences, Beijing, China, Dr. Gopal Prasad Ghimire, Mr. Shiv Tripathi, Mr. Homnath Pathak, Dr. Hari Sharan Adhikari, Dr. Ishwar Mani Adhikari, Mr. Jeevan Regmi, Prof. Dr. Narayan Prasad Adhikari of the Central Department of Physics, TU, for their valued encouragements. I am equally thankful to all the faculties of Prithvi Narayan Campus, lab mates Mr. Bijaya Bahadur Thapa, Bhoj Raj Paudel, Ram Lochan Aryal, Sanjay Singh, Upendra Chaudhary, Basanta Sapkota,

Girija Mani Aryal, Mani Kandel, Prakash Gautam, Janak Adhikari, Janaki Baral, Ramina Shrestha, and Upendra Chaudhary. I would like to acknowledge Mr. Hut Raj Sharma, Prof. Dr. Lekha Nath Bhattarai, the Ex-campus chiefs of the Prithvi Narayan Campus, Mr. George John, Mr. Gehendreshor Koirala, and Prof. Dr. Chandra Bahadur Thapa for their valuable motivation. In this regard, I would like to offer my sincere appreciation to Prof. Ragnar from Norway and Dr. Chitra Bahadur Baniya, for their prolific classes on philosophy.

I am grateful to Mr. Ganga Datta Bhatta of National Herbarium and Plant Laboratories, Godawari for the identification of plant specimens. Similarly, I would like to remember the National Academy of Science and Technology (NAST), Nepal, and the National Food and Feed Reference Laboratory, Nepal for the GC-MS analysis. In this context, I express my sincere thankfulness, to the Jeonbuk National University, Republic of Korea for the XRD, FESEM, EDX, and TEM analysis of the synthesized silver nanoparticles. Similarly, I would like to remember the villagers of Jomsom, who supported me during the collection of plant samples. In this concern, I like to remember my brother Mr. Titrtha Raj Khanal, for providing information on medicinal plants from the Kaski district of Nepal.

Finally, I would like to offer my cordial gratitude to my mother Mrs. Radhika Devi Khanal who always encouraged, strengthened, and taught me lessons of endurance and hard work in life. I am much obliged to my spouse Mrs. Usha Devi Khanal, and lovely kids Sangam and Sandhya for their cooperation and assistance through my difficulties. I am grateful to my extended family members, neighbors, and relatives for their continuous support and inspiration. At last, I wish to thank all the individuals who directly or indirectly helped to complete my research.

Lekha Nath Khanal

(February 2023)

ABSTRACT

Medicinal plants contain numerous secondary metabolites with significant biological activities. Due to diverse geographical and climatic conditions, several indigenous plants that comprise unique phytochemicals having a wide spectrum of biological assets are found in Nepal. This study aims to synthesize, characterize, and evaluate the biological activities of silver nanoparticles (AgNPs) by using some of the active medicinal plants of the study area. Methanol extracts of the selected plants were evaluated for antioxidant, antibacterial and α-amylase inhibition activities by using the DPPH radical scavenging, agar well diffusion, and CNPG3 methods respectively. The chemical profiling of essential oil isolated from the aerial parts of *Ephedra pachyclada* and *Ayenia grandifolia* was performed by the GC-MS analysis. This study exposed the phytochemical and biological activities of methanol extract, chemical profiling of essential oil, and green synthesis of AgNPs by using an aqueous extract of *A. grandifolia* for the first time. Among the 22 plants evaluated, *Rubus ellipticus, E. pachyclada, Pyrus pashia, Drynaria coronans, Mimosa rubicaulis*, and *Ziziphus mauritiana* extracts exhibited significant antioxidant properties with the highest activity of *A. grandifolia* (IC_{50} = 12.87 ± 0.14 μg/mL). The GC-MS analysis of the essential oil (EO) of stem barks of *A. grandifolia* contained di-n- octyl phthalate (28.39%), 2,6,11 trimethyl dodecane (15.77%), 4,6 dimethyl dodecane (12.79%), and o-guaiacol (7.07%). The methanol extracts of *R. ellipticus* and *P. pashia* exhibited the highest antibacterial activity. The resazurin microtiter assay method revealed the MIC and MBC of the methanol root extract of *R. ellipticus* as 3.12 and 12.5 mg/mL respectively against *Staphylococcus aureus* ATCC 25923. The methanol stem bark extract of *P. pashia* exhibited the highest α-amylase inhibition activity with an IC_{50} value of 24.22 ± 0.10 μg/mL.

From the preliminary investigation, *A. grandifolia, R. ellipticus, P. pashia*, and *Z. mauritiana* which exhibited the highest biological activities were used for the fabrication of AgNPs. Each of the plant extracts and $AgNO_3$ (1 mM) in the ratio of 1:9 by volume were mixed with constant stirring at lab temperature (25 ± 2ºC), and neutral pH with constant stirring over a magnetic stirrer. The change of color into light brown within an hour was considered a visual indication of the growth of AgNPs which was further confirmed by the appearance of sharp SPR peaks in the UV-visible spectra. The

UV-visible spectra at different reaction conditions of temperature, pH, and concentration were used to optimize the fabrication of AgNPs. FTIR spectra of the extract and the AgNPs were examined to detect the functional groups responsible for the reduction, capping, and stabilizing of AgNPs. The face-centered crystalline nature of the silver nanoparticles was established by the X-ray diffraction patterns by matching the diffractogram with the Joint Committee on Powder Diffraction Standards **(**JCPDS file no: 03-0921). It was further confirmed by the selected area electron diffraction (SAED) pattern having four discrete rings corresponding to the crystal planes at 110, 200, 220, and 311. The energy dispersive X-ray (EDX) analysis showed the presence of silver in the highest proportions and trace quantities of oxygen, chlorine, calcium, and carbon in the AgNPs. The surface morphology and nearly spherical shapes were determined by field emission scanning electron microscopy (FESEM) images. Transmission electron microscopy (TEM) was used to confirm the topographical, compositional, and morphological status of the AgNPs. Further, TEM images were used to find the sizes of the synthesized AgNPs which ranged from 28.05 ± 11.8 nm of *A. grandifolia* extract-mediated AgNPs to 16.73 ± 4.94 nm of *Z. mauritiana* extract-mediated AgNPs. The antioxidant activities of AgNPs synthesized by using *Z. mauritiana* (with an IC_{50} value of 37.02 ± 1.0 μg/mL) and *A. grandifolia* (with an IC_{50} value of 142.77 ± 10.75 μg/mL) were found to be nearly twice as potent as their respective crude extracts. The AgNPs synthesized using *A. grandifolia* demonstrated notable antibacterial activity, whereas its crude extract showed no such activity.The process of transforming plant material into AgNPs not only enhanced their antioxidant and antibacterial properties but also indicated the potential biomedical applications of these plant-based nanoparticles. Further investigation involving the synthesis of controlled-sized AgNPs from other plants, toxicity testing, and exploring potential applications would greatly benefit humanity.

Keywords: *Antibacterial activity, Antioxidant activity, Green synthesis, Kaski district, Mustang district, Silver nanoparticles.*

LIST OF ACRONYMS AND ABBREVIATIONS

ADP : Adenosine Diphosphate

AFM : Atomic Force Microscopy

AgNPs : Silver Nanoparticles

Ag-AgNPs : *Ayenia grandifolia*-Mediated Silver Nanoparticles

ATCC : American Type Culture Collection

ATP : Adenosine Triphosphate

$CNPG_3$: 2-Chloro-p-nitrophenyl-α-D-maltotrioside

DLS : Dynamic Light Scattering

DMSO : Dimethyl Sulphoxide

DNA : Deoxyribonucleic Acid

DPPH : 2, 2-Diphenyl-1-picrylhydrazyl-hydrate

EDX : Energy Dispersive X-ray Analysis

FCR : Folin-Ciocalteu Reagent

FID : Flame Ionization Detector

FTIR : Fourier Transform Infrared Spectroscopy

GCMS : Gas Chromatography-Mass Spectrometry

IC_{50} : Half-Maximal Concentration

MBC : Minimum Bactericidal Centration

MIC : Minimum Inhibitory Concentration

MTCC : Microbial Type Culture Collection and Gene Bank

NADH : Nicotinamide Adenine Dinucleotide + Hydrogen

NADPH : Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

Pp-AgNPs : *Pyrus pashia*-Mediated Silver Nanoparticles

PTCC : Persian Type Culture Collection

RPM : Rotation Per Minute

RARE : *Rubus ellipticus* Root Extract

Re-AgNPs : *Rubus ellipticus*-Mediated Silver Nanoparticles

RNS : Reactive Nitrogen Species

ROS : Reactive Oxygen Species

SD : Standard Deviation

SAED : Selected Area Electron Diffraction

SEM : Scanning Electron Microscopy

SPR : Surface Plasmon Resonance

TEM : Transmission Electron Microscopy

TFC : Total Flavonoid Content

TPC : Total Phenolic Content

XRD : X-Ray Diffraction

Zm-AgNPs : *Ziziphus mauritiana*-Mediated Silver Nanoparticles

ZME : *Ziziphus mauritiana* Extract

ZOI : Zone of Inhibition

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CHAPTER 1

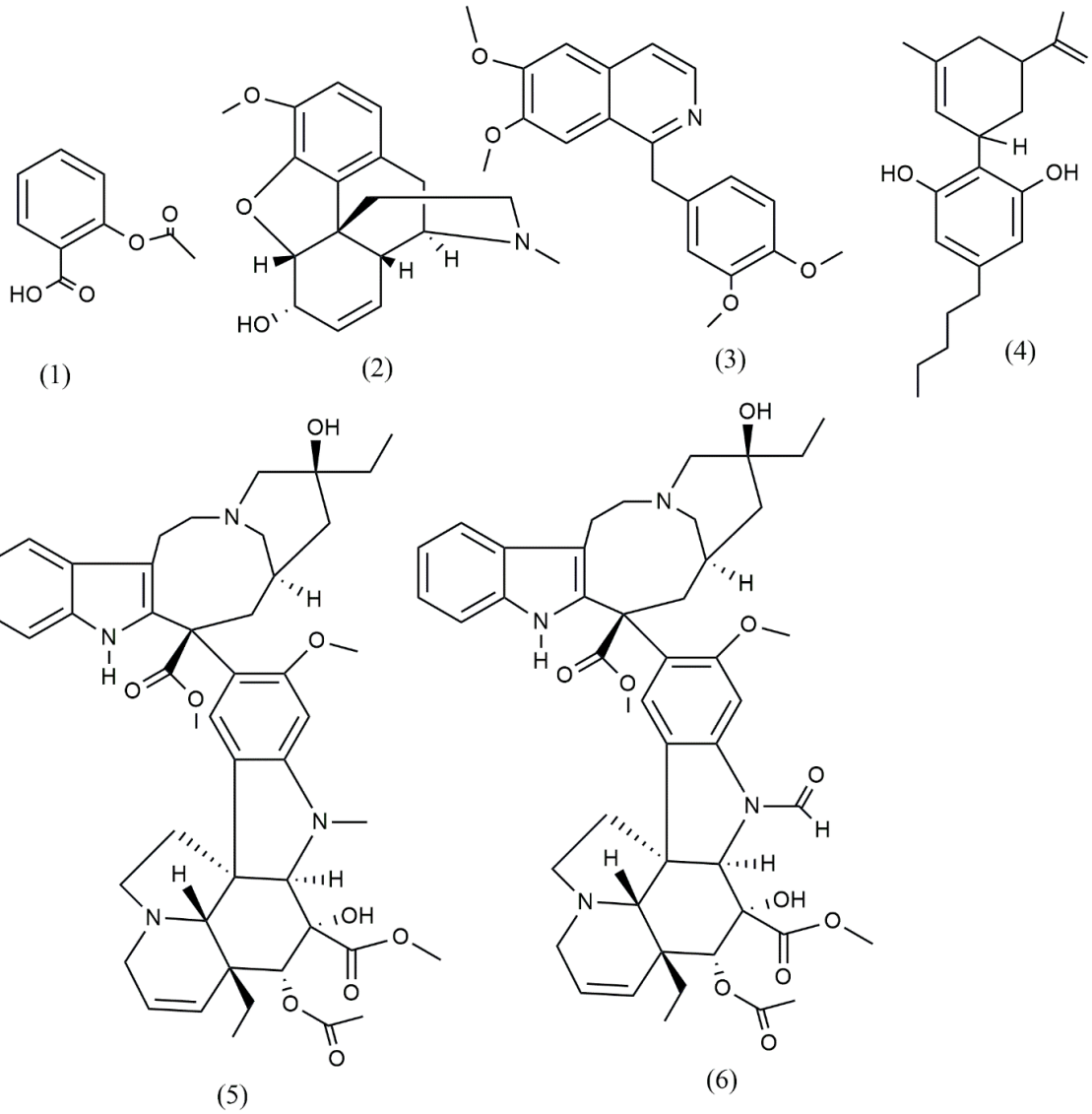
INTRODUCTION

1.1 Historical background of medicinal plants

Terrestrial plants have long been used as medicines and a significant number of modern drugs have their origin from the secondary metabolites of plants (Manju *et al*., 2012). The medicinal plants that have been considered the foremost practice of management of various ailments in developing countries are becoming popular in the developed world. The performance of herbs to work with body defense systems leads to providing healthy lives on the verge of chronic stress and pollution-related illness (Chevallier, 2004). Most of the conventional medicinal systems like Ayurveda, Arabian medicinal systems, Chinese and Tibetan medicinal systems, Japanese Kempo medicinal systems, etc. are merely based on plants (Watanabe *et al*., 2005). The systematic documentation of herbal medicine is found in different ancient pieces of literature. The Hindu holy book 'Ayurveda' describes the use of more than 1000 herbs for medication from around 1000 BC. The sophisticated medicinal system of Mesopotamia of 2600 BC explained 1000 plant-derived medicines in written form. Egyptian medicine, which dates back to about 1550 BC, illustrates more than 700 drugs of plant origin. Similarly, Traditional Chinese Medicine (TCM) has extensively been documented over thousands of years. The Greek philosopher and scientist, Theophrastus, wrote the book *History of Plants* in 300 BC in which he described the cultivation skill and medicinal qualities of different herbs (Cragg & Newman, 2013). Several small molecules which are produced by plants are not required for their normal growth but have significant roles in reproduction, and defense mechanisms against pathogens, vertebrates, and predators. These secondary metabolites exhibit substantial potential against various diseases and can act as drugs or drug leads (Anand *et al*., 2019). From early times, medicinal plants were used without logical knowledge of the pharmacological activities or the active compounds. The systematic application of medicinal plants with the clinical investigation was started only in the 18th century when Anton von Storck explored the poisonous aconite and colchicum. William Withering concluded foxglove for the management of edema based on a comprehensive investigation. Drug discovery from medicinal plants by rational investigation and isolation of active compounds can be considered open from

the 19th century. A German apothecary, Friedrich Serturner isolated morphine from the opium plant and established it as an analgesic and sleep-inducing agent (Atanasov *et al*., 2015).

Higher plants have been used by humans as food and spices for a long time so they are more advantageous in drug discovery than the synthetic route. Natural products having a large diversity isolated from plants are safer than synthetic drugs. Natural products cover a wider area of chemical space than synthetic small molecule libraries (Ahmad & Ahamad, 2020; Atanasov *et al*., 2021). Traditional ethnobotanical knowledge is explored by modern researchers to discover new biologically and chemically active natural compounds for the development of drugs against different diseases (Garnatje *et al*., 2017).



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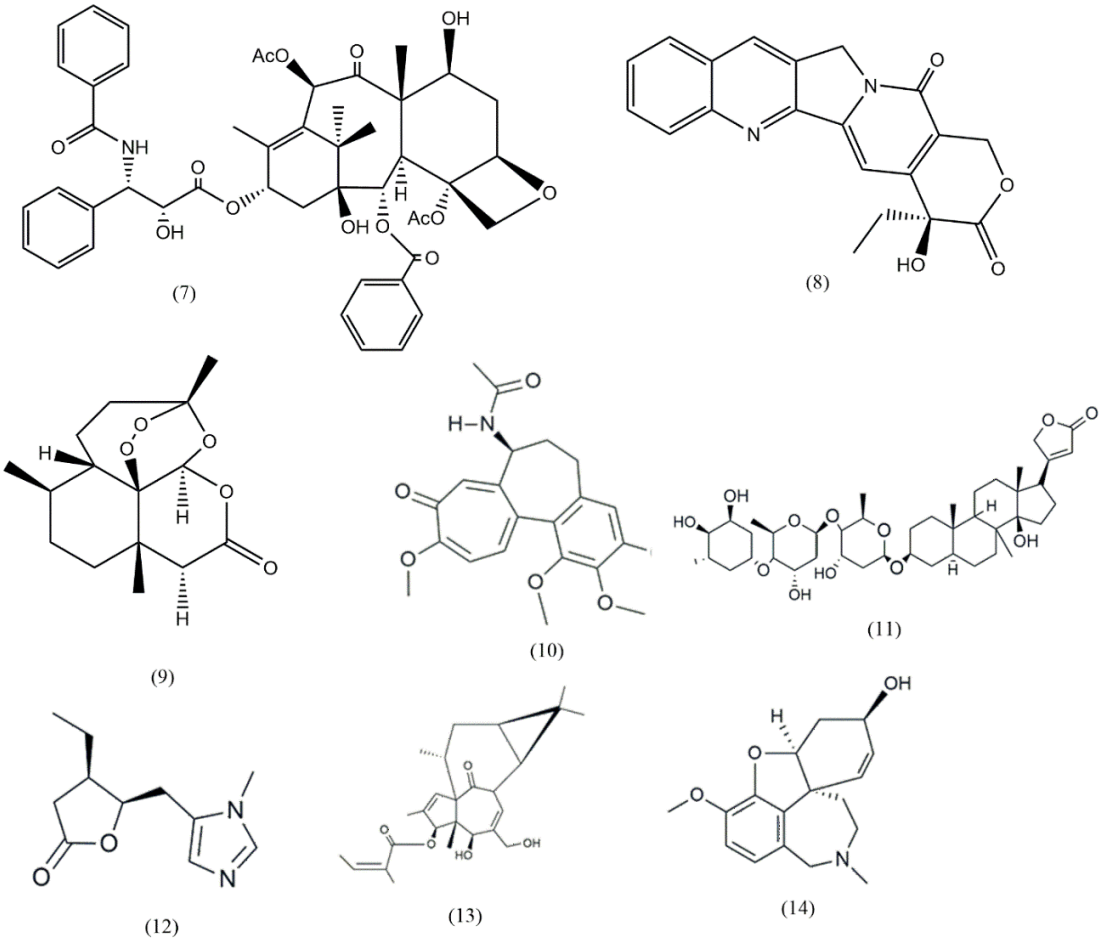


For instance, flowers of *Spiraea ulmaria* were used as an analgesic and anti-inflammatory agent in the middle ages. Later, pharmacologically active salicylic acid identified in the plant was developed into a significant analgesic drug commonly known as aspirin (Mahdi, 2010). The bark of *Cinchona succirubra* was traditionally used in the treatment of malaria, cancer, throat diseases, fever, and indigestion. In 2004, the Food and Drug Administration (FDA) of the United States approved quinine isolated from the plant as an antimalarial drug (Dias *et al*., 2012). Many substances obtained from plants have been successfully employed as medicines, some of which are included in Table 1. The modern lifestyle is plagued by poor dietary composition, decreased physical activity, environmental pollution, etc., which has led to several detrimental metabolic disorders and age-related illnesses (D'angelo *et al*., 2019). Modern people suffer from abnormally high levels of insulin, fat, and cholesterol due to abnormalities in socioeconomic status. This has led to the development of metabolic

Table 1: List of some plant-derived drugs and their therapeutic actions

S.No	Plant species	Drugs	Therapeutics	Ref.
1	*Fillapendula ulmaria* (L.) Maxim	Aspirin (1)	Analgesic	(Anand *et al*., 2019; Dias *et al*., 2012)
2	*Papaver somniferum* L.	Codeine (2) Papaverine (3)	Analgesic, spasms	(Anand *et al*., 2019; Dias *et al*., 2012)
3	*Cannabis sativa* L	Cannabidiol (4)	Epilepsy, dystonia, Parkinson's disease	(Anand *et al*., 2019; Dias *et al*., 2012)
4	*Catharanthus roses* (L.) G. Don	Vinblastine (5) Vincristine (6)	Anti-cancer	(Anand *et al*., 2019; Dias *et al*., 2012)
5	*Taxus brevifolia* Nutt. & *Taxus chinensis* Pilg.) Rehder	Paclitaxel (7)	Anti-cancer	(Dias *et al*., 2012)
6	*Camptotheca acuminate* Decne.	Camptothecin (8)	Anti-cancer	(Pu *et al*., 2019)
7	*Artemisia annua* L.	Artemisinin (9)	Antimalarial	(Numonov *et al*., 2019)
8	*Colchicum autumna* L.	Colchicine (10)	Pain killer, gout, Pericarditis	(Akram, 2012)
9	*Digitalis purpurea* L.	Digoxin Digitoxin (11)	Heart	(Whayne, 2018)
10	*Pilocarpus jaborandi*	Pilocarpine (12)	Glaucoma	(Cho *et al*., 2013)
11	*Euphorbia peplus*	Ingenol-3-O-angelate (13)	Skin cancer	(Dias *et al*., 2012)
12	*Galanthus nivalis*	Galantamine (14)	Alzheimer's disease	(Dias *et al*., 2012)

syndrome, a complicated bodily ailment that causes a wide range of health issues, including stress, obesity, coronary heart disease, cardiovascular problems, and type 2 diabetes (Kassi *et al*., 2011). Diverse biochemical reactions in our cells produce reactive oxygen species (ROS) during physiological and pathological circumstances. Most of

the ROS are free radicals, molecular oxygen in the excited state, carbonyl compounds, dioxetanes as well as ozone which are of biological interest (Sies, 1986). Different reactive chemical species like superoxide (O_2^-), hydroxyl (OH^-), peroxyl ($ROO^·$), hydroperoxyl ($HO^·_2$), hydrogen peroxide (H_2O_2), hypochlorous acid, and ozone that can produce radicals belong to the class of ROS. Important reactions catalyzed by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), and xanthine oxidoreductase (XOR) are the major sources of ROS in living cells (Bayir, 2005). The ROS generated by the enzymatic reactions is involved in respiratory chain reactions, synthesis of prostaglandin, phagocytosis, and the cytochrome 450 system (Pizzino *et al*., 2017). Analogous to ROS, reactive nitrogen species (RNS) like nitric oxide (NO), peroxynitrite ($ONOO^-$), etc. are found in the cardiovascular, nervous, and immune systems that cause various pathological conditions like neurodegenerative disorder, rheumatoid arthritis, acute respiratory distress syndrome, and the inflammatory bowel diseases (Dedon & Tannenbaum, 2004). The free radicals produced in the cells are destroyed by the natural antioxidant system. If the body's system is not enough to manage the concentration of these species, a state of oxidative stress is set up that plays a crucial role in the pathogenesis of chronic complaints like cardiovascular, neurodegenerative, cancer, and diabetes. It brings about various chain reactions leading to the destruction of the cell membrane, inhibition of enzymes and various cellular reactions, hindrances to cell division, DNA damage, and blocking energy production (Sharifi-Rad *et al*., 2020). Plants contain a myriad of secondary metabolites like polyphenols, flavonoids, steroids, saponins, organosulphur compounds, and vitamins. When the plants are in stressful conditions due to extreme weather, ultraviolet radiation, predators, pollinations, etc. there is an imbalance between ROS and their scavenging capacity by the body system, and an oxidative burst is triggered. Oxidative burst activates enzymatic and non-enzymatic functions to synthesize important phytochemicals (Forni *et al*., 2019). The naturally occurring phytocompounds like flavonoids, phenolic acids, tocopherols, alkaloids, chlorophyll derivatives, amines, amino acids, carotenoids, and ascorbic acid inhibit or delay chain reactions of oxidation. So, they have been serving as alternatives in complementary medicines for decades. The use of chemically synthesized antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has long been criticized due to their carcinogenicity, and searching for benevolent substitutes is preferred (Al-Abd *et al*., 2015).

The emerging trend of antibacterial resistance has created havoc on the effectiveness of existing drugs. Despite many antibiotics, malpractice, overdose as well as microbial mutation have led to the development of new drug-resistant strains. Many bacteria develop inherent resistance to a mutation in the chromosomal genes and acquire the capacity to survive with particular antibiotics (Blair *et al*., 2015). The infections of anti-biotic resistant pathogens have brought about considerable morbidity and mortality with a large economic burden on the healthcare system. The infection and subsequent contamination of the soil, water, and air have spread multidrug resistance strains in the environment. Moreover, the waste generated from hospitals, clinics, livestock farms, and other causes comprises enormous stocks of antimicrobials that stimulate the spreading of multi-drug resistant (MDR) bacteria (Khare *et al*., 2021). Antibacterial resistance has now become a global issue of public health. Several MDR strains including *Eneterbacter*, *Enterococcus*, *Klebsiella* spps. *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii,* etc., are creating serious clinical cautions for humans and animals. The exact situation of antimicrobial resistance is not clear in Nepal due to inadequate research, government policy, and lack of trustworthy publications (Dahal & Chaudhary, 2018). World health organization (WHO) suggested a series of coordinated activities for the prevention of transmission as well as research to innovate new strategies against infection (Barbieri *et al*., 2017). A diverse range of bioactive compounds from plants has been extensively used for the pharmacological and health effects against a wide range of pathogens. Only a negligible figure from an estimated number of about 500,000 species of the plant kingdom have been studied for their antimicrobial activities (Mickymaray, 2019).

Diabetes mellitus is a chronic metabolic disorder triggered by the complete lack of secretion, dysfunction of insulin, or both. Type 1 diabetes mellitus (T1DM) is caused due to autoimmune destruction of pancreatic β-cells. Type 2 diabetes mellitus (T2DM) has become an epidemic that increases the failure of β-cells and insulin resistance leading to the elevation of hepatic glucose (Hasanpour *et al*., 2020). Diabetes mellitus especially T2DM which is prevailing in more than 90% of cases and results in more than one million deaths per year has become a serious health burden in the world. The changed lifestyle, diet behaviors, and lack of physical exercise are responsible for the growth of diabetes in the contemporary population (Willcox *et al*., 2021). Chronic hyperglycemia under diabetes conditions increases the concentration of ROS in the

cells. Overproduction of ROS by hyperglycemia induces oxidative damage by accelerating the stimulation of protein kinase C (PKC) isoforms, hexosamine and polyol pathway flux, and advanced glycation end products (AGE) in the body (Moussa, 2008). A high level of ROS has negative impacts on insulin signaling cascades, dysfunction of β-cells, reduced glucose tolerance, and mitochondrial activity. The constant exposure of pancreatic β-cells to ROS reduces the secretion of insulin due to the low level of antioxidant enzymes (Oyedemi *et al*., 2017). Long-lasting diabetes results in many complications like disturbances in the vascular system, eyes, nerves, and kidneys causing neuropathy, nephropathy, retinopathy, morbidity, and mortality (Dodda & Ciddi, 2014). Several new types of research, technology, and treatments have been improving the management of diabetes but no biochemical or chemical agent is available for the complete treatment of diabetes patients yet. Education, awareness, and intensive support are very imperative in controlling the risk of long-term complications (Tripathy *et al*., 2021). Oral antidiabetic agents like insulin, metformin, biguanides, thiazolidinediones, and sulfonylureas have increased the possibility of effective treatment but suffer from serious side effects like hyperglycemia, diarrhea, weight gain, edema, flatulence, and mild anemia (Grover *et al*., 2002). In countries of low socioeconomic status, traditional medicine plays a significant role in primary health care. Vegetables, spices, foods, and medicinal plants have become targets in the management and treatment of chronic diseases including diabetes. Despite modern drugs, traditional herbal medicine reserves a special place in Asiatic countries due to climatic conditions, indigenous people's beliefs, and the abundance of plant species (Al-Aboudi & Afifi, 2011; Shabab *et al*., 2021). Several plants such as *Abroma augusta* L., *Allium sativum* L., *Aspalathus linearis, Bauhinia variegate, Camellia sinensis, Curcuma longa, Ficus religosa, Houttunia cordata* thumb, *Oryza sativa, Picrorhiza kurroa, Psidium guajava* L., *Rhodiola rosea, Syzgium aromaticum* L., *Syzgium cumini, Terminalia arjuna, Vitis vinifera, Zingiber officinalis,* etc. have been reported to exhibit significant antidiabetic activity (Shabab *et al*., 2021).

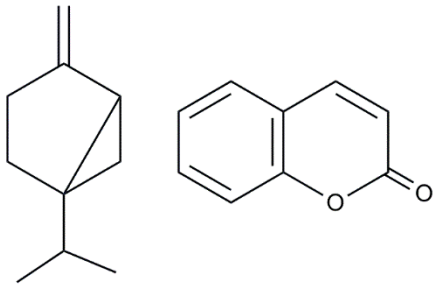
Plant-based phenolic and flavonoids like quercetin, anthocyanins, catechin, ferulic acid, and resveratrol regulate glycemia by insulin secretion, lipid peroxidation, and enzyme actions. These chief phytochemicals exhibit enormous health benefits because of their antioxidant, anticancer, antimicrobial, antidiabetic, and hepatoprotective activities (Aryal *et al*., 2021). Folk medicinal practices are abundant in traditional societies and

ethnic groups that transfer to new generations orally. Integration of the benefits of different traditional medicinal systems with their rational, affordable, and evidence-based application can be used for the dynamic healthcare service of modern people. Because of rigorous investigations and evidence on the theoretical and clinical aspects of plant-based medicines, the traditional systems have been developed into contemporary alternative medicine (Gewali, 2008). Plants are the foremost basis of the cultural system of traditional medicine which is existing for thousands of years and continue to provide new therapeutics (Karunamoorthi *et al*., 2013). The researchers and the practitioners should be educated to use herbal compounds in modern and traditional curative systems to integrate the plants into the contemporary medicinal system (Jamshidi-kia *et al*., 2018). There are several plants in the world and about a half million; species are yet to be investigated for their medicinal value. Their activities could be significant in the treatment of many ailments. The rationalized interest in the research on medicinal plants coupled with the development in information technology has facilitated the isolation, characterization, and purification of the active ingredients of the plants. Drug industries and academic institutions are adopting advanced technology to screen plants for their medicinal importance and the isolation of lead compounds to manufacture drugs.

Essential oils (EO) are a complex mixture of hydrophobic natural products isolated from odoriferous plants. They are obtained from different parts of the plants like the stem, leaf, flower, bark, etc. (O'Shea *et al*., 2012). They are low molecular weight volatile aromatic compounds consisting of terpenoids, aromatic compounds with alcohol, ketones, alkanes, and phenylpropanoids. They have an eccentric odor that protects plants from microbes, insects, and herbivores. The intensity of odor depends on the plant species, chemical composition, and quantity of the EO (Tongnuanchan & Benjakul, 2014). Since ancient times, human beings have been using EOs for medicines, perfumes, incense, food preservatives, and culinary practices (Wangchuk *et al*., 2013). In plants, EOs are accumulated in the trichomes, oil, and resin ducts. Most of the EOs contain about 20 – 100 compounds of different chemical classes (Sowndhararajan *et al*., 2017). EOs exhibit wonderful biological properties like antioxidant, antibacterial, anti-inflammatory, etc. The methods of extraction including distillation, solvent extraction, hydrodistillation, and microwave-assisted extraction

result in a substantial modification in the chemical profiling of the EO (Fokou *et al*., 2020).

The chemical composition of EO can be identified by using the gas chromatography-mass spectrometry (GC-MS) analysis. The EO isolated from the rhizomes of *Alpinia officinarum* collected from China was reported to contain a total of 53 compounds upon GC-MS analysis. The dominant compounds were 1,8-cineole, α-farnesene, γ-cadinene, α-bergamotene, globulol, and α-terpineol (L. Zhang *et al*., 2020). The essential oil isolated from leaves of *Juniperus indica* from the Langtang region of Nepal contained sabinene (15), p-thujone, trans-sabinyl acetate, and terpinen-4-ol as the major compounds (Adams & Chaudhary, 1996). Essential oil isolated from *Artemisia dubia* contained coumarin (16), chrysanthenone, and camphor (17) as the major components. Similarly, EOs of *A. indica* contained ascaridole, isoascaridole, trans-*p*-mentha-2,8-dien-1-ol as the major compounds (R. Joshi *et al*., 2016). Essential oil isolated from the fruit extract of *Zanthoxylum armatum* collected from different altitudes of Nepal contained linalool (18), cinnamate (E) methyl, myrcene, limonene, terpinen-4-ol, and sabinene as the major compounds by GC-Ms analysis (N. Phuyal *et al*., 2020a).



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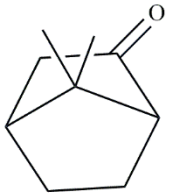

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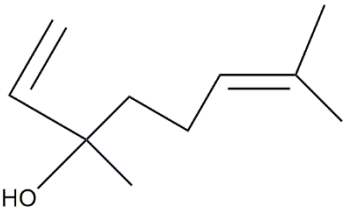


(18)

EOs of aerial parts of four samples of *Ocimum basilicum* L., two from Nepal and 1/1 from Tajikistan and Yemen were reported to contain 179 compounds with a majority of linalool, eugenol, estragole, 1,8-cineole, geraneol, and methyl eugenol. The EO exhibited moderate toxicity on the brine shrimp lethality test (Sharopov *et al*., 2016). In a separate study, EO obtained by the hydrodistillation of *Lantana camara* collected from Cuba, Yemen, and Nepal was evaluated by GC-MS analysis. The sample from Nepal was a davanone, Yemen β-caryophyllene and that of Cuba belonged to (E)-nerolidol chemotypes. Among the three chemotypes, β-caryophyllene, (E)-nerolidol exhibited showed cytotoxic and antimicrobial properties (Satyal *et al*., 2016). EO isolated from *Murraya paniculata* (L.) was reported to contain a total of 76 volatile

compounds with major components as methyl palmitate, isospathulenol, benzyl benzoate, and (E, E)-geranyl linalool present in more than 5%. The EO showed a moderate toxicity against *A. salina* (LC_{50} = 41 μg/mL) and nematicidal activity against *Caenorhabditis elegans* (LC_{50} = 37 μg/mL) (Dosoky *et al*., 2016).

1.2 Medicinal plants in Nepal

Due to distinctive disparities in altitude (500 - 8848 m) and climate, unique types of plants having different potential medicinal values are found in Nepal. The ethnobotanical knowledge of different communities of Nepal is limited to the older group that transforms verbally into the new generation. The lack of proper documentation is posing a potential threat to the extinction of indigenous knowledge (Manandhar, 2001). There are nearly 7000 higher plants in Nepal and an average of 56% are ethnobotanically important, and 54% have been used as ethnomedicine. The practice of herbal medicine is superior in higher altitude areas due to the lack of modern facilities, poverty, cultures, and traditional practices (Kunwar & Bussmann, 2008). Kaski district lies in the Gandaki province in central Nepal. It is one of the densely populated districts with a total area of 2017 km^2. Local people practice herbal and Ayurveda medicine against diabetes using different plants such as *Asparagus racemosus, Mormordica charantia, Berberis aristata, Syzygium cumini, Azadiracta indica, Aegle marmelous,* and *Gymnema sylvestre* (Shrestha & Jamarkattel-Pandit, 2018). Many medicinal plants available in Nepal have been reported to exhibit notable antibacterial activity against many strains. Subba and Basnet (2014) reported significant antibacterial susceptibility of *Pogostemon cablin, Colebrookea oppositifolia, Mussaenda macrophylla*, and *Mallotus philippensis* by agar well diffusion method. Methanol extracts of 25 medicinal plants collected from different parts of Nepal were screened for the anti-biofilm formation activity against five strains of *Escherichia coli* by resazurin assay method. The extracts of *Calotropis gigantea, Moringa oleifera, Eupatorium adenophorum, Ocimum tenuifolium, Oxalis lantifolia, Eclipta prostrata, Prunus persica,* and *Urtica paviflora* showed significant activity against the tested strains (Bhandari *et al*., 2021). Crude methanol extracts of *Myrica esculenta, Mahonia nepaulensis, Schima wallichii*, and *Madhuca longifolia* collected from Kabhrepalanchowk district of Nepal inhibited the growth of human pathogenic *Salmonella typhi, Staphylococcus*, and *Escherichia coli* (Gyawali *et al*., 2015). The

antimicrobial activity of 18 medicinal plants collected from different parts of Nepal was evaluated. The extracts of *Ampelocissus tomentosa,* and *Aleuritopteris anceps* showed significant antibacterial activity against *S. aureus* (MIC = 35 and 649 μg/mL), and *Pseudomonas aeruginosa* (MIC = 15 and 38 μg/mL) respectively. *Rhododendron arboretum* and *Adhatoda vasicca* extracts were active against *Salmonella enterica*. Similarly, *Kalanchoe pinnata, Paris polyphylla, Ampelocissus tomentosa*, and *Terminilia chebula* were active against different viral strains (Joshi *et al*., 2020). Different parts of medicinal plants from Nepal exhibited significant α-amylase and α-glucosidase inhibitory activities. Seeds of *Acacia catechu,* the rhizome of *Acorus calamus,* leaf gel of *Aloe vera,* roots of *Asparagus racemosus,* leaves of *Azadirachta indica,* barks of *Berberis aristata,* rhizome of *Bergenia ciliata*, the leaf extract of *Ocimum sanctum,* fruits of *Phyllanthus emblica,* leaves of *Psidium guajava,* aerial parts of *Scoparia dulcis,* fruits of *Terminalia chebula* and leaves of *Urtica dioica* are reported to exhibit notable activities indicating the possible candidates to conquer T2DM (Shrestha *et al*., 2021).

In comparison to herbal drugs, synthetic medicines are expensive, cause many side effects, and should be handled with strict protocols which are not easy in remote areas lacking contact with medical physicians. The global data shows that more than 80% of the world's population mainly depends on ethnobotanical remedies and plant drugs for various health concerns. Some of the well-known plant-derived drugs such as taxol, vinblastine, camptothecin, etc. against cancer, artemisinin, quinine for malaria, quinidine for heart problems, caffeine and nicotine for the brain, codeine, and morphine for analgesics, allicin for diabetics are used (Luca *et al*., 2012). Due to the huge chemical diversity of the plants and extensive medicinal values of the plant-derived compounds, scientists are focusing on the poorly understood areas of herbs regarding their genetic backgrounds, agricultural traits, and medicinal quality (Chakraborty, 2018). Five medicinal plants out of 41 selected from the Manang and Mustang district exhibited strong cytotoxic activity by dye-uptake method on Vero cells with CC_{50} values ranging from 13.5-25 μg/mL (Rajbhandari *et al*., 2009).

The significant antineoplastic property of alcoholic extracts of *Berberis aristata* (Chutro) was observed in Ehrlich Ascites Carcinoma (EAC) bearing mice by taking cis-platin as a positive control (Pai *et al*., 2012). The methanol extracts of *Glycyrrhiza glabra* (Jethimadhu) of Nepalese origin showed a substantial antioxidant activity on

DPPH free radical scavenging assay with IC_{50} values 40 ± 0.17 μg/mL on taking ascorbic acid as reference (Thapa *et al*., 2017). *In-vitro* antioxidant potential of methanolic extracts of *Stephania elegans* (Gujargano) from India was assessed by 2-2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) and ferric reducing antioxidant power (FRAP) assays. The IC_{50} value was found as 41.66 ± 0.015 μg/mL indicating a significant antioxidant property (Sharma *et al*., 2017). The antibacterial activity of the leaf extracts of *Astilbe rivularis* (Thulo okhati) was evaluated against Gram-positive and Gram-negative bacteria by the disc diffusion method. The minimum inhibitory concentration (MIC) values were found to be ranged from 4-32 and 8-64 μg/mL against Gram-positive and Gram-negative bacteria with kanamycin as the standard indicating that the plant possessed potential antimicrobial properties (Ghosh *et al*., 2018).

Many plant secondary metabolites have been used as lead compounds and converted into important drug molecules by derivatization of functional groups. Nanoencapsulation and nanoprecipitation of plant extracts ameliorate the potency of phytochemicals in several biomedical, agricultural, cosmetic, etc. applications by increasing biocompatibility and solubility while reducing the risk of toxicity.

1.3 Synthesis of nanoparticles

1.3.1 Nanotechnology

Nanotechnology has become a growing paradigm of material science that deals with the study of very small particles ranging in size from 1 – 100 nm at least in one dimension. It is a rapidly emerging field of interdisciplinary area of research since the early 90s and has become the key technology of the 21st century (Pulit-Prociak & Banach, 2016). Although nanotechnology is emerging as a potential field of research in modern times, it has been used since ancient times for making weapons, utensils, decorative articles, and cave paintings. Ayurved Bhasmas, Wootz steel, Damascus blades of the sword, etc. also used nanotechnology. The designs of old paintings in Ajanta and Ellora caves, and the color combination of the sword of the Tipu sultan used nanotechnology (Baboo, 2015). Similarly, the famous Lycurgus cup (Figure 1) of late Romans in the 4th century AD which is in the British Museum is an excellent example of the foremost use of nanotechnology. The cup seems green by reflected light and becomes red when lit from the inside. The fascinating appearance of the cup with

light was later clarified in the 1990s, by Barber and Freestone based on TEM and EDX explanations. The magnificent transformation of color was due to the colloidal dispersion of gold and silver nanoparticles with traces of copper. Similarly, "rose-windows" of medieval cathedrals like the gothic Notre-Dame cathedral in Paris also used noble NPs of different sizes on the glass (Rodrigues *et al*., 2021).

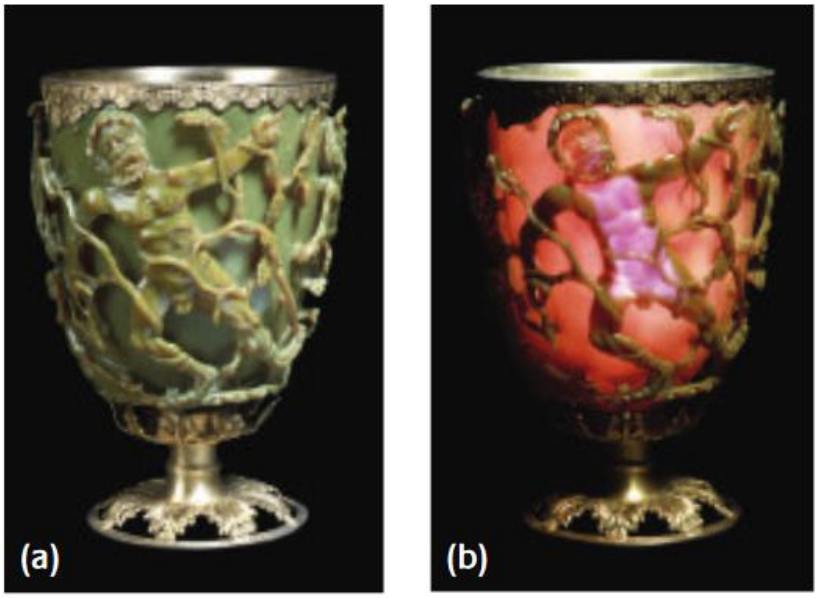


Figure 1: The Lycurgus cup (a) with reflected light (b) with transmitted light (Rodrigues *et al*., 2021)

The legendary lecture of Richard Feynman in 1959 entitled "there is plenty of room at the bottom" highlighted the excessive potential of research works on the nanoscale. The famous query "why cannot we write the entire Encyclopedia Britannica on the head of a pin?" disclosed a new paradigm of research in nanotechnology (Feynman, 1960).

In current times, it is developing exponentially to design, manage, and engagement of atoms or molecules at the nano range. Nanoparticles have a larger surface area to volume ratio which leads them to exhibit novel properties, and widespread applications in biomedical and human health sectors. Unlike bulk materials, the physical, chemical, and biological properties of nanoparticles are dependent on their size and other parameters. At the nanoscale, the fundamental physical, chemical, and biological properties are significantly different from the corresponding bulk counterpart due to the quantum effect (Bhattacharya & Mukherjee, 2008). Nanoparticles are zero-dimensional nanomaterial that have all of their length scales along x, y, and z-axes within the nano range. Metallic nanoclusters, heterogeneous particle arrays, core-shell quantum dots, nanospheres, nanoclusters, etc., are examples of nanoparticles (Tiwari *et al*., 2012). The

noble metal nanoparticles are non-toxic for gene and drug transfer applications. They are regarded as extremely valuable for biomedical purposes with astonishing sensitivity to investigative assays, radiotherapy, thermal ablation, and gene and drug delivery (Yaqoob *et al*., 2020).

1.3.2. Types of nanoparticles

Nanoparticles (NPs) are broadly classified as organic, inorganic, and ceramic nanoparticles (Ijaz *et al*., 2020). The organic nanoparticles are biodegradable, non-toxic and are commonly used in biomedical fields like targeted drug delivery. Dendrimers, micelles, ferritin, and liposomes are common examples of organic NPs. The nanoparticles which do not contain carbon are known as inorganic NPs. Metals like gold, silver, cobalt, copper, lead, zinc, etc. are commonly used to synthesize NPs. They have a high surface area to volume ratio, surface charge, pore size, spherical shape, color, etc., which are affected by environmental factors like sun, air, moisture, and heat. Certain oxides of metals like ZnO, Fe_3O_4, Al_2O_3, etc. are used to synthesize NPs. Ceramic nanoparticles are the oxides, carbides, carbonates, and phosphates of calcium, silicon, titanium, etc. They are chemically inert and stable to heat so used in the biomedical field for drug delivery in glaucoma, bacterial infections, and cancer (Thomas *et al*., 2015). There are many nanomaterials containing carbon in different forms. They are also very small materials that have any one dimension less than a micrometer. Fullerenes, carbon nanotubes, graphene, and carbon nanofibers are common examples of nanomaterial that are extensively used in different products (Ealias & Saravanakumar, 2017).

1.3.3 Silver nanoparticles

Silver is one of the precious metals that is used for the preparation of large quantities of nanomaterial having different physicochemical properties like thermal, electrical, catalytic, optical, and thermal in comparison to bulk material. They have significant antibacterial, antiviral, antioxidant, and antifungal activities so they are used in different biomedical products (Zahoor *et al*., 2021). They have been used in a large variety of commercial products including food packaging, medical devices, cosmetics, catalyzing agents, bioengineering, and electrochemistry. Due to the exponential growth of AgNPs into everyday consumer products, prognostic models have been imposed for the fate,

transport, and antagonistic effects of AgNPs in the organisms and environment in Europe and the USA (Calderón-Jiménez *et al*., 2017). Silver nanoparticles have remarkably greater marketing and publicity than other NPs. The global manufacture of nanotechnology goods and the market of AgNPs is soaring significantly and it is expected to touch about 800 tons by 2025 (Pulit-Prociak & Banach, 2016).

The high toxicity of silver ions and compounds against microbes was known for a long time and has been used for dental, catheters, burns, and wounds (Khalil *et al*., 2014). Silver-containing substances release silver ions which interact with the thiol group (-SH) in the proteins and enzymes that inhibit respiration and kill the cells. When the silver ions meet halide, sulfide, phosphate, or organic acids, in the environment they combine and form insoluble products and reduce bioavailability. So the free silver ions which are either adsorbed or dissolved in the AgNPs are responsible for the antibacterial activity (Nakamura *et al*., 2019; Xiu *et al*., 2012). Silver nanomaterials have exclusive electrical, optical, and catalytic properties. They have noticeable applications in the fields of diagnosis, detection, imaging, and drug delivery. Moreover, researchers are much more focused on the fabrication of AgNPs due to their exceptional antibacterial activity against several infectious microbes including MDR (Bruna *et al*., 2021). Silver nanoparticles have a wide spectrum of applications including agriculture, biomedical, healthcare, food industries, water treatments, textiles, antiinsectal, electronics, renewable energy, etc. (Abdelghany *et al*., 2018) (Figure 2).

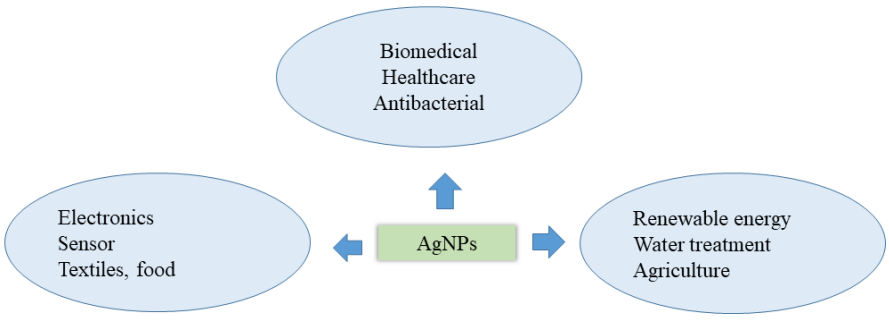


Figure 2: Applications of AgNPs in diverse fields

1.3.4 Synthesis of AgNPs

Nanoparticles can be manufactured by various physical and chemical methods. The most common “top-down” and “bottom-up” approaches are employed in the synthesis of nanoparticles. In the “top-down” method bulk material is broken down into

crystalline materials with dimensions of nanometers and in the "bottom-up" method nanostructure is fabricated by consolidating atoms-by-atoms or molecules-by-molecules (Meyers *et al*., 2006). Certain chemical, physical and optical parameters are the fundamental issues that should be considered for the synthesis of AgNPs. The size, morphology, surface properties, rate of dissolution in a particular solvent, and capping as well as stabilizing agents are the key factors that should be considered during the synthesis of AgNPs. Fabrication of the desired shape and size of controlled AgNPs for a specific purpose can be achieved by an appropriate selection of sources, chemicals, the concentration of reactants, and reducing and capping agents (Lee & Jun, 2019). The chemical, and physical properties, as well as production of AgNPs, are controlled by the use of proper reducing agents, reaction conditions, and capping and stabilizing agents. The proper choice of capping agent is crucial to regulate the stability and functionality of AgNPs (Sidhu *et al*., 2022).

Synthesis of AgNPs can be categorized as physical, chemical, and biological methods as shown in Figure 3. Common physical methods include ball milling, electric arc discharge, and laser ablation. In the ball milling method, metal material with a specific ratio is milled at high speed in an inert atmosphere. The speed of rotation, milling time, and medium determine the size and morphology of the AgNPs (Xu *et al*., 2020). Evaporation-condensation and laser ablation are the general physical methods of AgNPs synthesis. Silver electrode is itched with high electric discharge in a dielectric medium in the evaporation-condensation method.

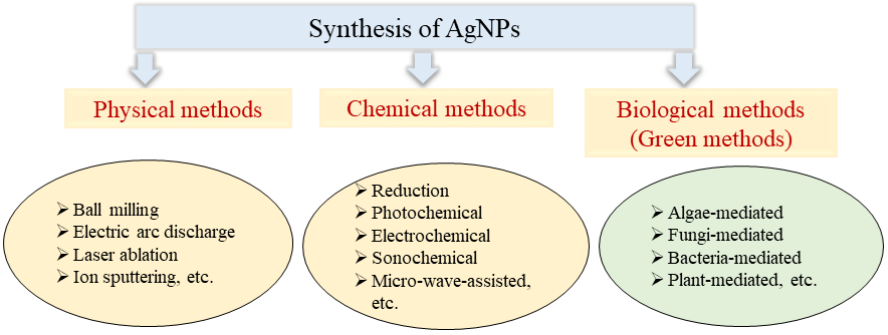


Figure 3: Different methods of synthesis of AgNPs

Metallic silver is vaporized and condensed into AgNPs in the solvent. Energy consumption and temperature factors are the challenges of the evaporation-condensation method. In laser ablation, AgNPs are synthesized from metallic bulk

material by using a laser beam on a metallic target. As no chemicals and solvents are used, the method is efficient from the points of greenness and production of pure and crystalline nanoparticles (Vishwanath & Negi, 2021).

In chemical methods, aqueous silver nitrate is reduced by using citrate, ascorbate, borohydride, and hydrogen gas. The prepared AgNPs are stabilized using surfactants, ligands or polyvinyl pyrrolidone, and hydrogen gas. This method is extensively used for large-scale production of AgNPs at a low cost with desired size, structure, and dimension (Beyene *et al*., 2017). Solvents and reducing agents like ethylene glycol, oleyl amine, liquid paraffin, sodium borohydride, sodium citrate, polyvinyl alcohol, etc. used in the chemical synthesis of AgNPs cause lasting toxicity to humans and animals. They are also harmful to the environment and violate the basic guidelines of "Green Chemistry" (Vishwanath & Negi, 2021). Toxicity is a prominent challenge in the production of nanoparticles by using hazardous reducing agents, organic solvents, and stabilizers that could exert the potential risk of carcinogenicity and environmental pollution. It limits the application of the products for biomedical and clinical purposes (Zhang *et al*., 2020).

The green synthesis of silver nanoparticles by using natural-based materials including bacteria, fungi, yeast, and plants is simple, low-cost, and environmentally benign. It may be the best alternative to chemical and physical methods (Kakakhel, Sajjad, *et al*., 2021). Different species of fungi secrete a large number of metabolites that can be used for the extracellular and intracellular synthesis of AgNPs. The regulating of the quantity of fungal biomass, culture conditions like time, pH, and the metabolism of fungi can be manipulated to get the nanoparticles of the preferred size, shape, and morphology (Guilger-Casagrande & Lima, 2019). Various compounds from fungi like nitrate-dependent reductase, xylanases, naphthoquinones, anthraquinones, and quinine derivatives are involved in the reduction of silver precursors (Xu *et al*., 2020). Numerous bacteria including *Novosphingobium* spp., *Microvirga rosea*, *Paenibacillus anseongensis*, *Pseudomonas* sp., etc. have been used for the synthesis of AgNPs. Bacteria-mediated synthesis is favored due to its easiness, high growth rate, susceptibility to MDR, and large-scale production (Huq & Akter, 2021). Algae are autotrophic organisms growing in different habitats such as water, sea, or damp places. They have been used to synthesize silver nanoparticles due to their high potential to accumulate metal, and are easy to grow and handle. Both the dead and living biomass,

as well as algae, can be used to synthesize AgNPs in a short time. AgNPs can be synthesized by intracellular pathways within the algal cells and extracellular pathways by certain bioactive compounds secreted by the algae (Chugh *et al*., 2021).

The plant-mediated synthesis has grown attention to nanotechnology due to its low toxicity, cost-effectiveness, easy availability, and non-pathogenicity (Sidhu *et al*., 2022). Although the green synthesis of AgNPs using algae, bacteria, fungi, and yeast has many advantages over the traditional physical or chemical methods, the pre-synthesis requirements, such as culturing of biomass, safety precautions, low reaction kinetics, etc., forced the researchers to look after other bio-resources (Moradi *et al*., 2021). The use of plant extracts has lower expenses in comparison to microbe-mediated synthesis as it does not need isolation, or culture maintenance in a sterile environment (Rahuman *et al*., 2022). In plant-mediated nanotechnology, different phytocompounds having significant antioxidant and reducing properties e.g., polyphenols, ascorbic acid, proteins, aldehydes, ketones, carboxylic acids, terpenoids, flavonoids have been used for the synthesis of stable nanoparticles by the reduction of silver nitrate solution (Garibo *et al*., 2020). The optical, magnetic, electronic, and catalytic properties of nanoparticles are influenced by their shape, size, surrounding media, stabilizers as well as a preparation method. The size-controlled synthesis of appropriate nanoparticles for a specific application has become a challenge for researchers (Khodashenas & Ghorbani, 2019).

Several researchers have reported that silver nanoparticles exhibit enhanced electrical, thermal, chemical as well as antimicrobial, and catalytic properties (Almasoud *et al*., 2021). Silver nanoparticles exhibit remarkable antimicrobial action against several bacteria, fungi, and viruses. So, they have been widely used in different products like plasters, bandages, clothes, toothbrushes, catheters, scalpels, cosmetics, refrigerators, and cellphones (Raza *et al*., 2016). Bacterial infections and the development of MDR bacteria have created serious pressure on the existing healthcare system throughout the world. The practice of overdose antibiotics and adverse side effects like strain, allergies, immune suppression, etc. have been connected to the reasons for the emergence of many drug-resistant bacteria. The application of AgNPs for the treatment of bacterial infection has been reported to show good effects against drug-resistant bacteria (Akintelu *et al*., 2020). The safe use of AgNPs in biological and clinical applications is

still to be authenticated in terms of potential risks to animals, humans, and the environment regarding their toxic effects (Rafique *et al*., 2017).

1.4 Rationale

The Himalayan region of Nepal is rich in different types of endemic medicinal and poisonous plants. The plants are being used by the local people for their primary healthcare. These plants might contain significant bioactivity towards anticancer, antioxidant, antimicrobial, etc. due to the presence of secondary metabolites. Only a limited study has been done about these plants by different governmental and non-governmental institutions. International Union for Conservation of Nature (IUCN) has suggested that thousands of important plants are facing extinction threats due to extensive exploitation, loss of habitat, deforestation, and lack of knowledge of their value and conservation (Ramakrishnan *et al*., 2017). In Nepal, many precious plants are being postured to a threat of extinction due to natural habitat loss, non-scientific management and application practices, indiscriminate collection, and lack of knowledge about their importance. The scientific documentation of the plants for their pharmacological values and medicinal use is imperative for the development of herbal drugs. The development of antimicrobial resistance (AMR) worldwide has threatened the positive impacts of antibiotics globally. The limited access to essential antibiotics in many low-income and middle-income countries increased morbidity and mortality whereas improper dosing and poor pharmaceutical quality are contributing to the growth and expansion of AMR (Browne *et al*., 2021). The overconsumption of antibiotics during the 21st century has led to the exponential increase of antibiotic-resistant bacteria that consequently developed the crisis in global public health. The development of effective antimicrobial nanotherapeutic drugs is imperative to fight against the wide-spreading MDR bacteria (Anand *et al*., 2022). So, this research aimed to find the biological activities of the plants as well as synthesized AgNPs, evaluation of phytochemicals, isolation, and characterization of the active compounds present in some of the commonly used medicinal plants in the study area.

1.5 Research objectives

General objective

The general objective of this study is to synthesize, characterize, and analyze the biological activities of silver nanoparticles of medicinal plants from the Mustang and Kaski districts of Nepal. The research aims to establish a green pathway by using the active plants of the study area to synthesize biogenic silver nanoparticles and compare their antioxidant and antibacterial activities with the crude extracts.

Specific objectives

- Phytochemical screening, estimation of total phenolic and total flavonoid contents of the medicinal plant extracts
- Isolation of essential oil and perform chemical profiling by GC-MS of *A. grandifolia* and *E. pachyclada*
- Evaluation of the biological activities of the plant extracts on anti-microbial, anti-diabetic, and anti-oxidant activities
- Synthesis, characterization, and evaluation of biological activities of the green synthesized silver nanoparticles from the active plant extracts

CHAPTER 2

2. LITERATURE REVIEW

A large variety of endemic, medicinal, and poisonous plants are found in the Himalayan region of Nepal which has been used by local people for different types of health problems. Nepalese people have been using several plants for rituals, ceremonial, spirituals, dietary, pharmaceutical, and nutraceutical purposes. The application of herbal medicine is deep-rooted in the cultural and religious backgrounds of different ethnic groups mostly residing in villages (Thorsen & Pouliot, 2016). These plants contain significant phytochemicals posing substantial biological properties like antioxidant, anticancer, antimicrobial, etc. activities. A literature survey reveals that only a limited amount of research has been done on these plants. Some research works conducted by University scholars and organizations are found in scientific publications. This chapter deals with the previous works done by different research scholars about the plants which are collected for the study. The plants were selected based on the recommendations of ethnobotanical users, local healers, and the elderly citizens of the study area.

2.1 Plants used in the study

2.1.1 *Angiopteris helferiana*

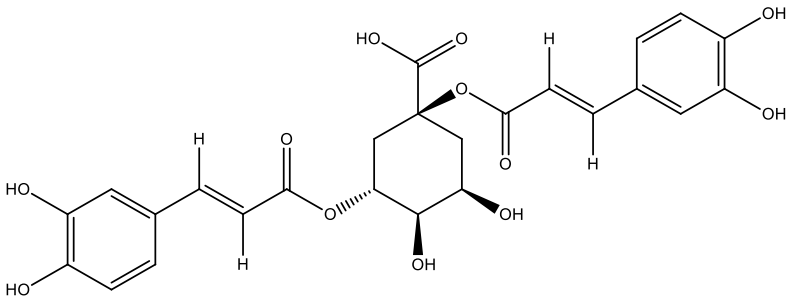
Angiopteris helferiana presl (Figure 4a) is a large pteridophytic fern and belongs to the family Marattiaceae. It is distributed in moist forest areas of Nepal, China, India, Sri Lanka, and South-East Asia. The plant grows on a large fleshy rhizome with many outgrowths like that of a cow's hoof so the local people called it 'Gaikhure' in the Kaski district (Figure 4a). The matured rhizomes of the plant were collected from Rupa rural municipality for the study.

Figure 4: (a) *A. helferiana* (b) *A. lappa* (c) *A. roxburghiana* (d) *A. stricta*

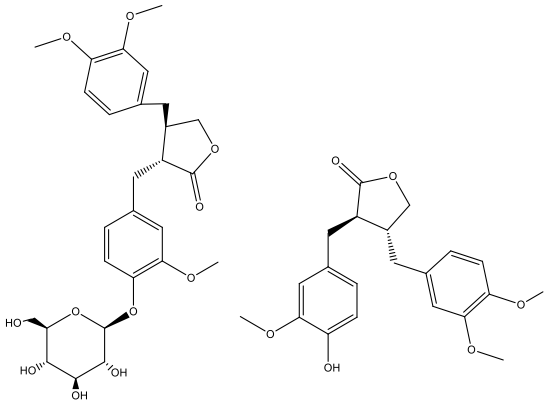
It is also used for the treatment of snake bites, cough, rheumatism, and pain. The rhizome of the plant is used for scabies, and diarrhea as well as a tonic, especially for backache. In India, the rhizome is used for the treatment of scabies (Shaikh *et al*., 2014). The ethyl acetate, water, and butanol fractions of the rhizome collected from the Tanahun district of Nepal exhibited significant antioxidant and α-glycosidase inhibition activities. The anti-obesity and antidiabetic activity of the butanol fraction of the plant were evaluated in the *in vivo* model. A dose of 300 mg/kg/day in male C57BL/6j mice models showed substantial antiobesity and antidiabetic activity (Lamichhane *et al*., 2019). The antibacterial activity of the extracts of the fronds of the plant was tested on multidrug-resistant bacteria by the disc diffusion method. The highest zone of inhibition (9 mm) was observed for the ethyl acetate extract against *Staphylococcus aureus* (ATCC 25293). The bacteria were observed at the concentration of 600 mg/mL concentration also indicating non-significant MIC and MBC (Nath *et al*., 2017).

2.1.2 *Arctium lappa*

Arctium lappa of the family Asteraceae is commonly known as 'Chisung' and is found in the agricultural fields at the altitudes of 2000-4000 m in Mustang. It is a small herb growing about 1 m tall with heart-shaped, simple, and big leaves. Spherical flowers with pink heads grow from June-September (Figure 4b). Local people use the plant for fever, blisters, pimples, and gallbladder. Local 'Aamchis' in Jomsom use the plant in their medicinal mixtures against certain treatments. The leaf extracts of *A. lappa* were found to contain a potent gastroprotective agent, 1, 3-O-dicaffeoylquinic acid. The compound exhibited a potent activity with ED_{50} at 57μg/kg against an ethanol-induced gastric ulcer in rats (Carlotto *et al*., 2015).



1, 3-O- Dicaffeoylquinic acid



Arctiin Arctigenin

A new lignan (+)-7, 8- Didehydroarctigenin together with two known lignans (±) arctigenin and (±)-matairesinol were isolated from the aqueous ethanolic fruit extracts of *A. lappa*. (±) arctigenin was found to exhibit the most potent antiproliferative activity against MH60 cells with IC_{50} of 1.0 μM and the activity was suggested due to apoptosis (Matsumoto *et al*., 2006). Similarly, arctiin, and arctigenin were isolated from 80% methanol extracts of leaves of *A. lappa* by polyamide chromatography in combination with HPLC-PAD and HPLC-ESI/MS (Liu *et al*., 2005). The nutrient-deprived PaNC-1 cancer cells exposed to arctigenin at a concentration of 0.01 μg/mL and 1 μg/mL caused 100% cell death within 24 hours, and 12 hours of starvation respectively (Awale *et al*., 2006).

2.1.3 *Artemisia roxburghiana*

The plant belongs to the family Asteraceae and is locally known as Kalopati/Damana in the Mustang district (Figure 4c). It is an herbaceous plant growing up to 2- 4 m in height with an aromatic smell. It has a tough stem which is green with black stripes. The leaves are much-branched into many leaflets and light-yellow flowers grow from July to August. Local people used this plant for the treatment of colds, coughs, channel

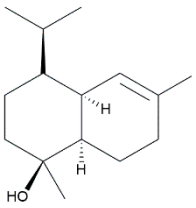
disorders, etc. Important sesquiterpenoid artemisinin was isolated from the plant and compared its percentage yield to that of other Artemisia species. They isolated 0.23 ± 0.01% of the dry weight and the plant was found to contain a higher percentage of artemisinin (9) in its leaves (Mannan *et al*., 2010).

2.1.4 *Artemisia stricta*

Artemisia stricta (family: Asteraceae) grows up to 2 - 4 m in height and has a silvery color on the stem and leaves. It has a specific aromatic smell and the leaves are much segmented but are larger than that of *A. roxburghiana*. Yellowish flowers grow from July to August (Figure 4d). Locally the plant is called 'Setopati' and is used for the treatment of cold cough and channel disorder. Manika *et al*. (2016) evaluated the antimicrobial properties of the essential oil of the plant by the disc diffusion method. The oil was found effective against *Staphylococcus epidermis* (MIC = 0.625 mg/mL) followed by *S. aureus* (MIC =1.25 mg/mL). Similarly, the oil was found to be effective against *Asperagillus flavus*, *A. niger,* and *Sporothrix schenckii* (MIC = 0.625 mg/mL).

2.1.5 *Aster indamellus*

Aster indamellus Grieson is locally called 'Metok' and belongs to the family Asteraceae. This is abundant in Afghanistan, China, India, Pakistan, and Nepal (Nesom, 2020). It is an herbaceous plant that grows in small bunches in dry lands with lilac flowers that have beautiful light yellow color (Figure 6a). The aerial parts of the plant were collected from the Thini village of Jomsom where it is used on wounds, fever, and headaches. The antifungal activity of essential oil of *Aster indamellus* was investigated by the disc diffusion method against *Fusarium oxysporum, Helminthosporium myadis, Alternaria solani, Rhizoctonia solani,* and *Sclerotonia sclerotiorum. H. myadis* and *R. solani* were found most inhibited with IC_{50} values of 186.1μg/mL and 267.5 μg/mL respectively (Mathela & Kumar, 2018). The essential oil of *A. indamellus* was found to contain α-muurolol as the major component (18.15%) and exhibited significant insecticidal activity against *Lipaphis erysimi* (mustard aphid) with LC_{50} of 3.53 mg/mL (Kumar & Mathela, 2017).



α-muurolol

2.1.6 *Ayenia grandifolia*

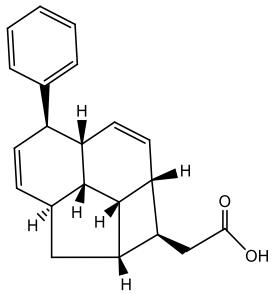
Ayenia grandifolia (DC.) Christenh & Byng (Malvaceae) is abundant in Nepal, India, China, and other Asian countries (Somkumar, 2020). The plant is a medium-sized liana growing in wet places. The mature plant grows up to 30-40 m in length with a maximum stem diameter of nearly 7-8 cm. The adventitious buds grow from the nodes when it is in contact with the ground. The stem grows by binding on stronger support, flowers are in small clusters and spherical thorny fruits with seeds in the capsule grow from December-January (Figure 5). The plant was collected from Rupa-1, Kaski district for the study. Local people know the plant with 'Kholebrale'. The whole plant is crushed in water to get a slippery slurry which is fed to the cattle or humans to relieve heat on strong summer days.

Figure 5: *A. grandifolia* showing buds and fruiting body

2.1.7 *Beilschimedia roxburghiana*

Beilschimedia roxburghiana (Lauraceae) is a pantropical genus that contains about 250 species distributed in Asia and Africa. The plants of this genus have been widely studied in the past decades because of their extensive application in traditional medicine. These investigations resulted in the isolation of important secondary metabolites like

endiandric acid derivatives, kingianins, epoxyfuranoid lignans, and alkaloids having significant antibacterial, anti-inflammatory, enzyme inhibitory, and anticancer activities. (Lenta *et al*., 2015). *B. roxburghiana* Nees is an evergreen tree of medium size that grows in tropical forests of China, India, and Myanmar. Leaves are alternate, elongated, petiolate, and pinnately veined. Small, bisexual flowers with six tepals result in the growth of ellipsoid, pyriform, or spherical greenish fruits (Nishida, 1999).



Endiandric acid A

In the Southeast Asian region, the plant is found in the subtropical forest at altitudes of 200- 400 m and is used for treating bone-related difficulties like arthritis, renal problems, and rheumatism, and as timber in Bhutan (Salleh *et al*., 2015). The stem barks were collected from Rupa-1, of the Kaski district where it was known as 'Hadchur' and local people used its stem bark in bone wounds and fractures. To the best of our knowledge, it is the first scientific investigation of the biological and phytochemical properties of the plant.

Figure 6*:* (a) *A. indamellus* (b) *C. graveolens* (c) *D. coronans* (d) *E. umbellata*

2.1.8 *Bigonia megaptera*

Begonia megaptera (Begoniaceae) is an herbaceous plant that grows in shady and wet places near small water bodies. Leaves are relatively larger than other species of begonia and ovate. The sepal cup is prominently 3-winged and the wings are quite large.

The plant is found in the Kaski district of Nepal. Locally, the plant is called 'Makarkanjy'. The plant is used to prepare local Mehndi to color hands in Shravan Sakranti, eaten raw or pickled, and believed to be beneficial to kill intestinal germs (Rajbhandary, 2013). The extracts of the plant in different solvents contained flavonoids, tannins, phenols, and glycosides. The extract showed significant phenolic and flavonoid contents and DPPH radical scavenging activity (Bhattarai & Rana, 2020).

2.1.9 *Clematis graveolens*

Clematis graveolens Lindl. belongs to the family Ranunculaceae and grows as a woody vine of 2 - 5 m in length. The stem is tough and strong with climber-like structures at the nodes. The yellow flowers leave a hair-like structure when they mature (Figure 6b). The plant is locally called 'Baghjunghe' in Mustang and the stem, leaves, and flowers of the plant are used by the local people for cough, cold, tumors, and joint pain. The triterpenoid saponins Tomentoside, huzangoside, clematoside, and clematogravelenoside were isolated from the root and rhizomes of the plant. Their anti-insecticidal activities were tested by Potter's spray tower method. Tomentoside was found to be the most effective against aphids (*Aphis craccivora*) with LC_{50} values of 1.2 mg/mL and 0.5 mg/mL after treatment for 72 hours and 96 hours respectively (Rattan *et al*., 2015).

2.1.10 *Crataeva unilocularis*

It is a medium-sized tree belonging to the family Capparidaceae which is abundant in Nepal and has been used for wound healing, laxative, anthelmintic, and urinary antiseptic (Pathak, 2010). The leaves are simple and alternate, white flowers bloom with long stamens. The tender leaves and twigs are harvested and used as medicine to increase appetite and reduce stomach pain. The sample was collected from the Rupa rural municipality of the Kaski district. The methanol leaf extract of the plant contained higher phenolic (168.55 ± 0.1 mg GAE/g) and flavonoid contents (292 ± 0.1 mg QE/g). The extract showed significant DPPH radical scavenging activity (IC_{50} = 46.45 μg/mL) (Giri *et al*., 2019).

2.1.11 *Dischidia bengalensis*

The plant belongs to the family Apocynaceae and is characterized as an epiphytic herb growing on larger trees. The green vine (stem) and leaves are fleshy with white latex.

Adventitious roots grow from each node of the plant. Leaves grow with small petioles, opposite, fleshy, and oval to nearly elliptical shapes. From April to May, beautiful creamy flowers grow from the nodes. It was collected from Rupa-1, of the Kaski district for the study. Locally, the plant is called 'Thirjo' and is used for the treatment of body aches and as a tonic. The plant is the newly recorded flora in central Nepal (Bhandari & Shrestha, 2016). The crushed leaves are useful to reduce pain and twigs paste is applied for healing fractured bones by the *Shompen* tribe of the Great Nicobar Islands (Elanchezhian *et al*., 2007).

2.1.12 *Drynaria coronans*

Drynaria coronans Wall. Ex. Mett (Polypodiaceae) is named Kamaru in different parts of Nepal. It grows from a rhizome as an epiphytic plant with a soft mass covered by red scales (Figure 6c). The locals used the rhizome for diarrhea, and bone fractures, and as a tonic for body pain (Khanal *et al*., 2020). The rhizomes were collected for the study from Rupa village in the Kaski district. Chang *et al*. (2014) studied the antidiarrheal effect of the ethanol extract of the rhizome on heat-liable enterotoxin (LT) induced diarrhea. The inhibition effect was evaluated on the enterotoxigenic *Escherichia coli* (ETEC) LT subunit B (LT B) and monosialotetrahexosylganglioside (GMI) interaction by GM1 enzyme-linked immunosorbent assay and patent mouse gut assay. The extract of the plant was concluded to inhibit LT-induced diarrhea.

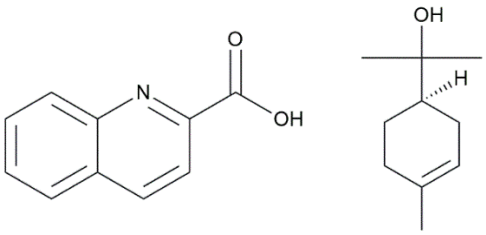
2.1.13 *Elaeagnus umbellata*

Elaeagnus umbellata Thunb belongs to the family Elaeagnaceae and is found in the Himalayan regions of Pakistan, India, and Nepal. The ripen berry-like fruits are a good source of vitamins A, C, and E, minerals, phenolic, and flavonoid compounds (Nazir *et al*., 2018). It is a shrub growing up to 2 - 5 m tall with alternate leaves having a silvery back. The berries and the leaves (Figure 6d) were collected from the Jomsom area of the Mustang district for the study. Local people eat the fruits and use to prepare pickles and practice to cure indigestion. The antimicrobial properties of the different extracts of the plant were assessed by the disc-diffusion method. The ethanol extract of the leaves showed antimicrobial activity against *Escherichia coli* (17.46 ± 0.16 mm), *P. aeruginosa* (18.83 ± 0.06 mm), *P. syringae* (15.66 ± 0.33 mm), *S. aureus,* (12.90 ± 0.35 mm), *B. subtilis* (15.83 ± 0.16 mm), *B. bronchisiptica* (14.09 ± 0.2 mm), *S. typhae* (15.83 ± 0.17 mm), *E. faecium,* (13.90 ± 0.1 mm), *S. cerevisiae*, (12.66 ± 0.06 mm) and

A. flavus, (10.23 ± 0.1 mm). Similarly, the ethanolic extract of the roots of *E. umbellata* exhibited moderate activity against *S. cerevisiae* and *A. flavus.* The mean diameter of zones of inhibition of the extract against *B. subtilis* (11.83 ± 0.17 mm), *S. aureus* (10.67 ± 0.33 mm), *E. faecium* (13.00 ± 0.00 mm), *E. coli* (15.83 ± 0.16 mm), *P. aeruginosa* (12.76 ± 0.23 mm), *B. bronchisiptica* (10.66 ± 0.33 mm), *P. syringae* (11.66 ± 0.33 mm), *S. typhae* (9.06 ± 0.06 mm), *S. cerevisiae* (14.11 ± 0.1 mm) and *A. flavus* (11.75 ± 0.15 mm), respectively (Minhas *et al*., 2013). The fruit extract of the plant in different extracts exhibited significant DPPH radical scavenging and anti-hyperglycemic activity against streptozotocin-induced diabetic rats (Nazir *et al*., 2018).

2.1.14 *Ephedra pachyclada*

Ephedra pachyclada Boiss is a xerophytic plant growing in the mountains and alpine arid zones of the Himalayan region from Nepal to Iran. It grows with light blue tufts and cylindrical shoots in small bushes with beautiful red flowers at the nodes (Figure 7a). The aerial parts of the plant with floral parts were collected from the Thini village of Mustang district. Local people know the plant as Somlata and the 'Aamchis' use the plants against different health problems like asthma, gastric disorder, and blood pressure (Khanal, *et al*., 2022). Pirbalouti *et al*. (2013) evaluated the anti-ulcer effect of the hydroalcoholic extracts of the plant on Wistar rats. The histological analysis revealed that the extract at the concentration of 1000 mg/Kg was effective in experimentally healing rat ulcers. Lee *et al*. (2014) isolated quinoline-2-carboxylic acid from the chloroform extracts of *E. pachyclada* of Korean origin. The antidiabetic effect was evaluated by α-amylase and α-glucoside inhibition assay using acarbose as a positive control. Based on the values of IC_{50} (15.5 ± 1.9 μg/mL for α-amylase inhibition and 9.1 ± 2.3 μg/mL for α-glucoside inhibition) assays, the compound was found to exhibit a strong antidiabetic activity. The antibacterial activity of the methanol extracts of Iranian *E. pachyclada* was evaluated against five Gram-negative bacteria: *Klebsiella pneumoniae* (PTCC-1053), *Escherichia coli* (PTCC-0157), *Escherichia coli* (ATCC-25922), *Serratia marcescens* (PTCC-1111), *Shigella dysenteriae* (PTCC-1188) and *Pseudomonas aeruginosa* (ATCC-27853) by agar dilution method. The minimum inhibitory concentration (MIC) value of *P. aeruginosa* was 0.5 mg/mL and that of other bacteria was 1mg/mL. The result indicated the extract to be the most sensitive toward *P. aeruginosa* (Dosari *et al*., 2016).



Quinoline-2-carboxylic acid α-terpineol

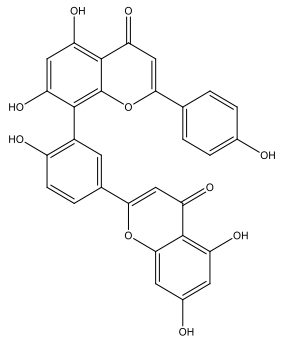
The essential oil isolated by the hydrodistillation of *E. sinica* collected from China contained α-terpineol, p-vinylanisole, 3-methyl-2-buten-1-ol, terpine-4-ol, α-linalool, phytol, eudesm-7(11)-en-4-ol, and tetramethylpyrazine as the major compounds (Wang *et al*., 2006). The chemical composition of essential oil obtained from different species of ephedra contained diverse compositions. They had different compounds in different proportions. The EO of, *E. foliata* and *E. intermedia* contained camphene, p-cymene, limonene, 1,8-cineol, camphor, α-terpineol, and myrtenol in significant proportions but were absent in *E. major*, *E. sarcocarpa*, and *E. distachya* (Ehtesham-Gharaee *et al*., 2017).

Figure 7*: (a) E. pachyclada (b) J. indica (c) M. rubicaulis (d) P. pashia*

2.1.15 *Juniperus indica* Bertol

Juniperus indica Bertol. belongs to the family Cupressaceae and grows in the Himalayan region including Nepal, India, and China at altitudes of 3300 - 4500 m (Chapagain *et al*., 2020). It is a medium-sized- shrub growing 0.5 to 2 m in height in small bushes (Figure 7b). The aerial parts of the plant were collected from the Thini village of Mustang where people use stems, fruits, and barks of the plant against fever, cough, cold, sores, and paralysis. The petroleum ether extracts of the plant showed significant antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli,* and *Klebsiella pneumoniae* by cup plate method (Maharjan *et al*., 2012). The protective effect of amentoflavone, the compound isolated from *Juniperus indica* was studied

against H_2O_2-induced oxidative damage in human erythrocytes and leucocytes. The extract significantly decreased the level of lipid peroxidase (LPO) (10.54 ± 2.90 nmol/mg G1 protein) in a dose-dependent manner against the control showing a significant effect (Bais & Prashar, 2015).



Amentoflavone

2.1.16 *Mimosa rubicaulis*

Mimosa rubicaulis (Figure 7c) is a medium-sized plant growing at altitudes of 300 - 1900 m from Bhutan to Afghanistan. It belongs to the Fabaceae family which is a deciduous shrub having a ribbed stem with thorns and hairy prickles at the nodes and internodes (Tamboli & Wadkar, 2019). The mature root barks were collected from the forest areas of Pokhara city of Kaski district where the local people use it for diarrhea, rheumatism, and some urinogenital disorders. The stem bark extract of the plant of this region contained significant phenolic (281.83 ± 1.98 mg GAE/g), and flavonoid content (381.06 ± 5.23 mg QE/g) (Gurung, 2020). In a separate study, the plant collected from the same locality exhibited considerable antibacterial and antioxidant activities. Leaf, stem, and root extracts were analyzed for antioxidant activity by DPPH and NO scavenging methods. Based on IC_{50} values, the antioxidant property of different parts of the plant followed the order of leaf > root > stem (Gurung *et al*., 2020). Quantitative analysis of different phytochemicals in *M. rubicaulis* of Margalla hills of Pakistan revealed the presence of alkaloids (0.74 ± 0.02%), phenols (0.26 ± 0.00%), tannins (15.75 ± 0.04%), flavonoids (0.87 ± 0.01%) and saponin (3.06 ± 0.03%) (Khan *et al*., 2011). The ethanol root extract of *M. rubicaulis* from India was found to contain a flavonoid glycoside. The structure elucidation by spectroscopic methods revealed the compound as 5,7,4’-trihydroxy-6, 3’,5’-trimethoxy flavone-7-O-α- L-arbinopyranosyl-(1→6)-O-β-D-glucopyranoside (Yadava & Agrawal, 1998). Ganji *et al*. (2010) studied

the antibacterial activity of the root extracts of *M. rubicaulis* by cup-plate method on Gram-positive and Gram-negative bacteria. The methanolic extracts were found to possess a significant antibacterial effect on *Bacillus subtilis* (ZOI = 10 mm), *Bacillus pumilis* (ZOI = 19 mm), and *Escherichia coli* (ZOI = 22 mm) at concentrations of 1000 μgm/ml. The methanol extracts of the stem of *M. rubicaulis* exhibited a significant antioxidant activity with RC_{50} (concentration reducing 50% free radical concentration) of $1.90x10^{-1}$ mg/mL by DPPH free radical scavenging assay method (Genest *et al*., 2008).

2.1.17 *Pyrus pashia*

Pyrus pashia Buch-ham. Ex.D. Don (Rosaceae) is a medium-sized tree having a strong thorny stem with dark green leaves (Figure 7d). White flowers with pinkish stamens give rise to spherical dotted fruits. The stem barks of the plant were collected from the Kaski district where it is known as 'Mel' and the local people use a decoction of the leaves and stem bark for the treatment of cough, fever, headache, and the common cold. Tsering *et al*. (2012) evaluated the antioxidant activity and total phenolic content of the leaf extracts of the plant by DPPH free radical scavenging and Folin-Ciocalteu's method respectively. The extract exhibited a significant antioxidant activity (IC_{50} = 10.81 ± 0.44 mg/mL) and the methanolic extract was found to contain the highest total phenolic content (351.16 ± 0.43 mg/g). The flowers of *P. pashia* were found to contain hydroquinone in a high proportion (10.31 ± 0.21 mg/g). The significant antioxidant property of the plant might be due to the presence of hydroquinone with the other 27 phenolic compounds (He *et al*., 2015).

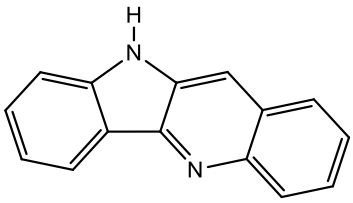
2.1.18 *Rubus ellipticus*

R. ellipticus Sm (Figure 8a) is an evergreen shrub belonging to the family Rosaceae. It grows up to 4 - 5 m tall with dark green spherical leaves. The leaves and stems are covered by hairy structures and spikes (Figure 8a). The white flowers give rise to spherical berries which are edible. Root barks of a mature plant were collected from Pokhara -7, Masbar for the study. It is known as 'Yensalu' and the locals of the Kaski district use the decoction of the roots of the plant for the treatment of fever, diarrhea, and dysentery. The root paste is applied for the treatment of wounds on the skin. The antioxidant and antiproliferative activities of the fruit extracts of *R. ellipticus* were studied using different solvents. The acidic acetone extract was found to contain the

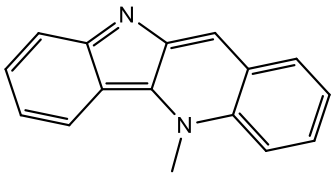
highest level of phenolics (899 mg GAE/100 g FW) and flavonoids (433.5 mg CE/100g FW). The DPPH free radical scavenging activity was found maximum in acetone extract (619.3 mg CE/100 g FW). Similarly, the extracts showed antiproliferative activity against cervical cancer cell lines (C33A) up to 40-60% without affecting normal peripheral blood monolayer (PBM) cells (Saini *et al*., 2012). Saklani *et al*. (2012) carried out the disc diffusion method to determine the antibacterial activity of the fruit extracts of *R. ellipticus* using different solvents. The ethanol extract was found to exhibit a significant zone of inhibition (ZOI) of 16 ± 1mm, 15 ± 1mm, and 15 ± 1mm against *Escherichia coli* (MTCC 729), *Streptococcus pyogenes* (MTCC 1925), and *Escherichia coli* (MTCC 443) respectively. The methanol leaf extracts of *R. ellipticus* were evaluated for wound healing and antitumor property in animal models. The extract was found to prolong the life span of mice with Ehrlich Ascites Carcinoma (EAC) by 45.76% at the dose of 250 mg/kg. It reduced the volume of Dalton's Lymphoma Ascites (DLA) tumors from 4.06 cm^3 to 2.56 cm^3 when fed with the dose of 250 mg/kg by body weight on the 38^{th} day. Similarly, substantial wound healing activity was observed on the incision, excision, and *Staphylococcus aureus*-induced wounds in rats (George *et al*, 2013).

2.1.19 *Sida acuta*

Sida acuta Bumr. J. (Malvaceae) grows with an erect, cylindrical green stem up to 1m in height. The leaves are alternate, simple, and lanceolate to ovate with a serrated margin. Small light-yellow flowers are actinomorphic and bisexual (Figure 8b). The plant is found in the Rupa rural municipality of Kaski district where it is called 'Ballujhar'. The local people use this plant for different medicinal purposes. The decoction of the leaves is used on fever and to wash wounds; the plant paste is applied to soften abscesses and release pus. The antimicrobial activity of the alkaloids extracted from *S. acuta* was evaluated by agar-disc diffusion. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were calculated by the broth-microdilution method for the effective samples. The MIC values ranging from 16 – 400 μg/mL and MBC from 80 – 400 μg/mL were obtained indicating the extract to have good antimicrobial activity. The GC-MS analysis revealed the presence of cryptolepine and quindoline alkaloids in the plant (Karou *et al*., 2006).



Quindoline



Cryptolepine

The ethanolic leaf extracts of *Sida acuta* from Nigeria were tested for antibacterial activity by the disc-diffusion method. The test was performed against *Staphylococcus faecalis, Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus aureus.* The extracts exhibited significant antibacterial activity with a zone of inhibition of 18 mm for *S. aureus*, 16 mm for *E. coli,* and 14 mm for *S. faecalis* and *P. aeruginosa*. The extracts showed the minimum inhibitory concentration of 0.0625 mg/mL for *S. aeruginosa* indicating the plant has an effective antimicrobial property (Stanley *et al*., 2014).

Figure 8: *(a) R. ellipticus (b) S. acuta (c) T. parvulum,* and *(d) Z. mauritiana*

2.1.20 *Taraxacum parvulum*

Taraxacum parvulum belongs to the family Asteraceae and is abundant in the Himalayan range of Nepal, Bhutan China, etc. The plant was collected from the agricultural waste fields of Jomsom. Locally it is known as Kungu or Dudhe. The plant is of Chinese origin compendium and has been used in the Chinese traditional medicinal system. It grows as a simple herb 2-10 cm tall. The leaves are grown up from the base which grows up into larger tap roots and beautiful yellow flowers grow from June-July (Figure 8c). The plant contains white latex from its roots and leaves. Leaves are winged with irregular margins. The yellow flowers directly grow from the base and leave a cotton-like hairy structure on maturity (Figure b). The local people use the plant as an

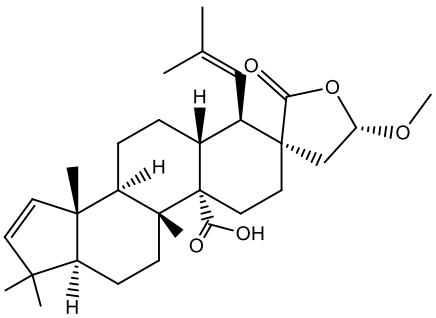
antimicrobial, liver protection, diuretic, etc. Different species of taraxacum are called 'Dandelion' and have been discovered to exhibit diverse medicinal values.

2.1.21 *Trichosenthes wallichiana*

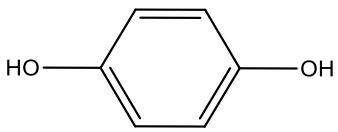
Trichosanthes wallichiana Lour is a climber vine growing into a large bush on larger trees with climbers. The plant belongs to the family Cucurbitaceae. It was spotted on the outskirts of Pokhara and is locally known as 'Indreni ko Laharo' and the paste is applied on wounds and older lesions. The plant was collected from the western part of Pokhara for the study.

2.1.22 *Ziziphus mauritiana*

Ziziphus mauritiana Lam (Rhamnaceae) grows as a medium-sized shrub that grows up to 10 m in the bush. The stem is armed with opposite pairs of straight and hooked spines. The leaves are elliptical with a serrate margin and have green color on the upper side and silvery underside (Figure 8d). Yellowish flowers grow up to give spherical fruits that are edible. The plant is called Bayer and the root paste is applied to old wounds, and the decoction is used on fever by the locals in Rupa village of Kaski. The methanol extracts of the leaves of *Ziziphus mauritiana* were evaluated for antimicrobial activity by the disc diffusion method. The extract was found effective against *Bacillus subtilis* with a zone of inhibition of 15.2 ± 0.05 mm (Sharma *et al*., 2017). Methanol root extracts of *Ziziphus mauritiana* were used to isolate three novel secondary metabolites zizimauritic acid A, zizimauritic acid B, and zizimauritic acid C.



Zizimauritic acid B



Benzene-1, 4-diol

The compounds were evaluated against different cancer cell lines. Zizimauritic acid B was found to show the highest activity with IC_{50} values of 5.05 (A549), 5.36 (HeLa), and 6.24 (BGC-823) μg/mL (Ji *et al*., 2012). The silver nanoparticles synthesized from the leaf extracts of *Z. mauritiana* having sizes 7 -22 nm were used for the colorimetric

detection of the Hg^{2+} ion in an aqueous medium (Memon *et al*., 2020). The biosynthesized silver nanoparticles from aqueous leaf extracts of *Z. mauritiana* exhibited significant antibacterial activity against *S. aureus* (MIC = 2.5 μg/mL) and *E. coli* (MIC = 5 μg/mL). The extract contained high total phenolic content which is believed to be responsible for the synthesis of the AgNPs (Asimuddin *et al*., 2020).

2.2 Synthesis of silver nanoparticles using plant extracts

Different physical and chemical methods have been patented to synthesize silver nanoparticles. Methods like mechanical /ball milling, laser ablation, sputtering, lithography, chemical etching, electrochemical precipitation, vapor deposition, spray pyrolysis, sol-gel process, etc. are toxic from the point of their application and safety concerns. The green methods of using plants, bacteria, fungi, yeast, etc. are better than the physical or chemical methods due to their biosafety, eco-friendly nature, the management of human ailments, and targeted delivery of drug candidates (Barkat *et al*., 2017). There are some reports of green synthesized nanoparticles from Nepalese medicinal plants exhibiting significant biological activities. The silver nanoparticles were synthesized from the methanolic extracts of *Berberis asiatica* (Chutro) and *Cassia fistula* (Rajbriksha) collected from Kaski and Kailali districts respectively. The synthesized AgNPs exhibited and enhanced antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* on the disc diffusion method. The AgNPs showed higher DPPH radical scavenging activity than that of corresponding extracts (Khadka *et al*., 2021). In another study, the aqueous extract of *B. asiatica* collected from the Salyan district of Nepal was used to fabricate AgNPs. The XRD analysis established the FCC structure with a crystallite size of ~14 nm. Transmission electron microscopy was used to establish the morphology and spherical shape of the particles. They exhibited good antibacterial activity against *E. coli, S. aureus, K. pneumoniae*, and *Salmonella typhimurium* (Dangi *et al*., 2020).

The aqueous extracts of *Camellia sinensis* (tea) collected from different altitudes of eastern Nepal were reacted with $AgNO_3$ solution to synthesize AgNPs. The FCC structure of the particles was established by comparing the XRD pattern of the AgNPs with JCPDS 04-0783. They had different sizes and exhibited varying antioxidant activity on the DPPH test method (Chandra *et al*., 2020). Similarly, the leaf extract of *Ageratina adenophora* (Kalo Banmara) of Nepalese origin was reacted with silver

nitrate solution to synthesize AgNPs. The XRD study showed a crystalline nature with a particle size of 24 nm. They were reported to exhibit moderate antibacterial activity against *Bacillus subtilis* and *Escherichia coli* on the disc diffusion method (Gautam *et al*., 2021). The fruit extract-mediated Ag/AgCl-NPs were synthesized and evaluated for their antimicrobial and antiproliferative activity against human cancer cell lines. The nanoparticles having an average size of 16 nm inhibited the growth of two pathogenic *Bacillus subtilis, Shigella boydii,* and *Escherichia coli.* Similarly, showed significant antifungal activity against *Aspergillus niger* and *Trichoderma* species. The Ag/AgCl-NPs also inhibited the growth of breast cancer (MCF-7) and Ehrlich Ascites Carcinoma (EAC) cells with IC_{50} values of 28 and 84 μg/mL respectively (Kabir *et al*., 2020). The green synthesized AgNPs from *R. ellipticus* leaves exhibited significant toxicity against mosquito larvae of *Anopheles stephensi, Aedes aegypti,* and *Culex quinquefasciatus* with LC_{50} values of 12.50, 13.83, and 15.09 μg/mL respectively. The AgNPs shortened the egg-laying capacity of gravid females as well as reduced the hatching ability of the mosquito eggs (AlQahtani *et al*., 2017). The AgNPs synthesized from the extracts of *Brassica oleracea* (Broccoli), *Capsicum annuum* (Chilli), and *Parthenium hysterophorus* (Carrot grass) collected from Kathmandu were spherical and had the size ranging from 10 – 40 nm. The AgNPs synthesized from *B. oleracea* and *P. hysterophorus* exhibited higher antioxidant and antibacterial activities respectively (Shahi *et al*., 2018).

2.2.1 Factors affecting the synthesis of AgNPs

The fabrication of AgNPs by reacting plant extracts with silver nitrate solution is greatly affected by various factors like types of phytochemicals in the extract, pH, temperature, concentration, and reaction time of the solution. These factors influence the shapes and sizes of the NPs. Synthesis of desired quality AgNPs for any specific purpose is imperative for their commercial production.

2.2.2 Effect of pH

The acidic or basic nature of the reaction mixture greatly influences the shape, size, and morphology of AgNPs. The reduction of silver ions is facilitated by increasing the pH. At low pH (~4), slow formation of larger-sized AgNPs occurs while alkaline (above 8) favors the formation of smaller-sized spherical NPs which get aggregated in solution. UV-visible spectra of AgNPs at high pH results the broad peaks indicating an unstable

and wider range of size of NPs (Alqadi *et al*., 2014). The effect of pH in the green synthesis of AgNPs by the action of *Emblica officinalis* extract and 1mM $AgNO_3$ was studied. They reported the acidic condition was not suitable and the alkaline condition produced less stable particles which undergo nucleation and form highly dispersed AgNPs and the optimal condition was found to be neutral (Andreia & Ivanescu, 2018). The phenomenon of aggregation and dissolution is greatly affected by the pH of the solution. In acidic to neutral solutions, NPs are destabilized by aggregation whereas in the alkaline condition they are agglomerated and form more stable suspensions (Fernando & Zhou, 2019).

2.2.3 Effect of temperature

The temperature of the reaction plays an important role in the shape and size of AgNPs. There are many reports in the literature that small-sized nanoparticles were formed at higher temperatures. A higher temperature generally increases the rate of reaction but above 60ºC may result in the denaturation of the compounds in the extract leading to the formation of larger particles with agglomeration (Kaabipour & Hemmati, 2021). Fayaz *et al*. (2009) observed sharp surface plasmon resonance (SPR) peaks in UV-visible spectra at 405 nm at 40°C and gradually the peaks were observed at higher frequencies indicating the formation of larger-sized NPs in the synthesis of AgNPs by using *Trichoderma viride*. The increase in temperature resulted in the formation of small-sized nanoparticles when synthesized from olive leaf extracts. With the increase in temperature, the rapid conversion of Ag^+ ions into neutral NPs undergoes subsequent nucleation (Khalil *et al*., 2014). Synthesis of AgNPs by reacting *Vitex agnus-castus* leaves extract and 1 mM $AgNO_3$ in the ratio of 1:9 also revealed the rapid formation of smaller-sized particles at a higher temperature of 60 - 80°C (Stavinskaya *et al*., 2019).

2.2.4 Effect of concentration

UV-visible spectra are used to find the effect of the concentration of reactant silver nitrate on the formation, shape, and size of NPs. Many reports show that the increase in the concentration of $AgNO_3$ shifts the SPR peaks to a higher wavelength and becomes narrower which is accompanied by a decrease in the size of the AgNPs in the solution. The UV-visible spectra of the AgNPs synthesized by using *Capparis moonii* fruit extracts showed the movement of SPR peaks to the higher frequency regions using

$AgNO_3$ of 1 mM, 2 mM, 3 mM, 4 mM, and 5 mM at 430 nm (Anigol *et al*., 2017). Different concentrations of silver ions such as 0.5 mM, 1 mm, 4 mm, 4 mm, and 6 mM were tried in the biosynthesis of AgNPs by using *Hagenia abyssinica* (Bruce) J.F leaf extract. The SPR peaks in the UV-visible spectra showed the maximum absorbance at 406 nm for the concentration of 4 mM (Melkamu & Bitew, 2021). The sharper SPR peak was observed at 416 nm when 1mM $AgNO_3$ was used for the synthesis of silver nanoparticles by using *Artemisia sieberi* leaf extract (Rousta & Ghasemi, 2019). The proportions of plant extract to Ag^+ ions also plays important role in the shape, size, and yield of AgNPs. The concentration of plant extract and silver ions in the reaction mixture was changed to get the optimized synthesis of AgNPs. Melkamu and Bitew (2021) observed the best formation of AgNPs in the ratio of 1:2 volumes of $AgNO_3$ (4 mM) and *Hagenia abyssinica* leaf extract with a maximum absorption SPR peak at 406 nm. Researchers prefer biological methods to synthesize AgNPs because of their incredible applications in healthcare, water treatment, food, textiles, and agriculture. Moreover, controlled synthesis of AgNPs can be achieved by optimizing the parameters like temperature, time of reaction, pH, the concentration of the metal ion, and biological system for the production of desired nanoparticles for specific purposes (Garg *et al*., 2020).

2.3 Synthetic mechanism of AgNPs

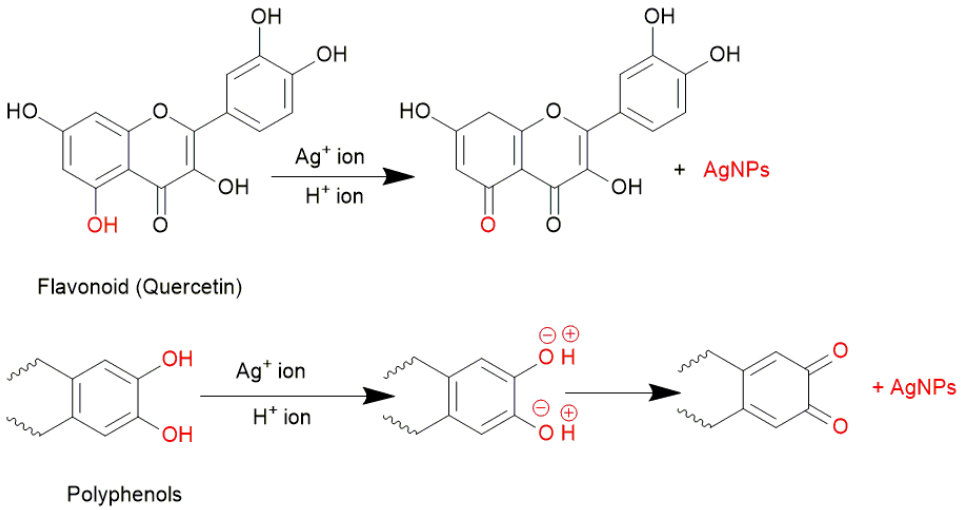
Green production of silver nanoparticles using plants, microbes, and biomolecules consists of an assembly of atoms in the range of 1-100 nm by the bottom approach.

Table 2: List of specific components involved in the green synthesis of AgNPs

Green source	Particular components involved
Plants	Phenolics, alcoholics, flavonoids, vitamins, terpenoids, alkaloids, antioxidants, etc.
Bacteria	NADH-dependent enzymes, quinones, actinorhodin pigments, biosurfactant, flagellin, exopolysaccharides, rhamnolipids, α-NADH-dependent nitrate reductase, Formation of H_2S gas, etc.
Fungi	Proteins, polypeptides, NADPH, and bio-micromolecules
Biopolymers	Chitosan, lignin, cellulose, alginate, etc.

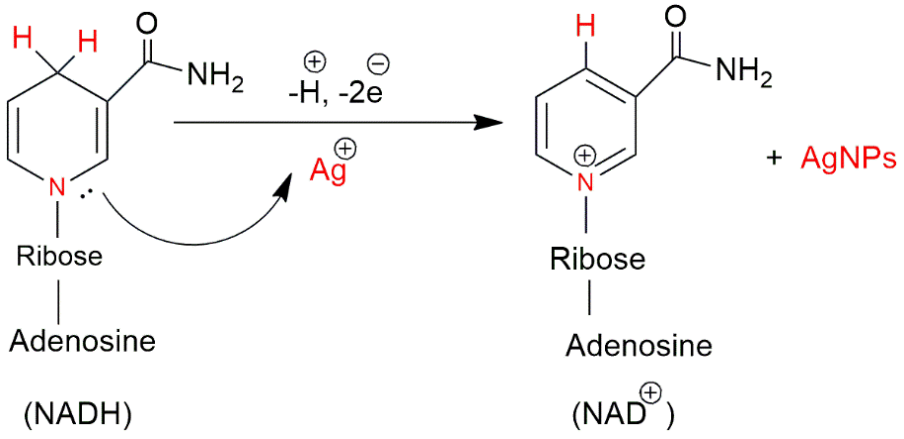
Source: (Rana *et al*., 2020; Singh *et al*., 2015)

Various types of molecules like phenolics, flavonoids, enzymes, amino, carboxylic, sulphadryl, etc. groups are directly or indirectly involved in the conversion of Ag^+ ions into neutral AgNPs (Thakkar *et al*., 2010). Different components in plants, fungi, bacteria, and biopolymers that assist to synthesize AgNPs can be summarized in Table 2. The involvement of polar –OH groups in plant extracts and synthesized AgNPs can be ascertained by the appearance of peaks around 3300 cm^{-1} in the FTIR spectra. Similar secondary metabolites in plants containing –OH groups such as phenolics, flavonoids, terpenoids, polyphenols, alcohols, etc. are converted into corresponding carbonyl groups by reducing Ag^+ ions into zerovalent Ag° which grow into AgNPs as shown in Figure 9**.**



Source: (Omidi *et al*., 2018)

Figure 9: Mechanism of synthesis of AgNPs from flavonoids and phenolics



Source: (Tarannum *et al*., 2019)

Figure 10: Mechanism of synthesis of AgNPs from NADH

In fungi and bacteria, several specific compounds like NADH, proteins, and other compounds are involved in the conversion of silver ions into Ag°. The lone pair of electrons on the nitrogen atom of NADH or NADPH molecules are used to reduce Ag^+ ions into Ag° that convert into AgNPs as shown in Figure 10 (Tarannum *et al*., 2019).

2.4 Applications of AgNPs

Silver nanoparticles exhibit special physical, chemical, and biological properties that commanded them in diverse applications. They have been used in multifarious applications in several disciplines including food storage, textile coatings, and several environmental uses. Since AgNPs have been used for decades, no sufficient proof of toxicity is established. Different products composed of AgNPs are approved to practice by several authorizing bodies of the US, FDA, EPA, Japan, and Korea (Tarannum *et al*., 2019). There are many industrial products used in clinical and human-safe appliances. AgNPs–coated bandages are reported to kill harmful microbes and allowed better remedial of the injured tissues. Biomedical products such as wound healing bandages, catheters, and endotracheal tubes manufactured by reputed industries in the world use nanocrystalline silver for long-term bactericidal and antifungal purposes (Gherasim *et al*., 2020). Silver nanoparticles have been applied against several types of cancer cells, including hepatocellular, lung, breast, and cervical cancers. Silver nanoparticles produce ROS in the cells which can break chromosomes, and DNA, disrupt Ca^+ ions, and inhibit cell cycles leading to apoptosis (Asharani, Hande, *et al*., 2009; Carlson *et al*., 2008). Dental and mouth-related problems are common in many people in the world. Development of biofilm, inflammatory lesions, pores, and peri-implant mucosa AgNPs laden substances are used in several dental practices. Similarly, AgNPs are widely used in bone healing materials to promote osteogenesis, and propagation of mesenchymal stem cells (Burdusel *et al*., 2018).

The fabrics functionalized with AgNPs are used in clothing like socks, bags, sportswear, surgical suits, etc. to prevent contagious infection. Fabrics are functionalized with AgNPs by various methods. Composite fabrics coated with silver nanoparticles in the outer part of the fabric are used (Benn & Westerhoff, 2008). AgNPs functionalized materials have been significantly used in food packaging to prevent bacterial contamination and long-term preservation. Many researchers have reported sonochemical, and ultrasonication methods to coat AgNPs on the surface of packaging

materials. These materials gradually release Ag^+ ions which kill or inhibit the growth of different pathogens like *E. coli*, and *S. aureus* (Samberg *et al*., 2011).

The adsorbed molecules on the surface of Ag or Au cause a Surface-enhanced Raman scattering (SERS) effect and produce amplification of Raman signals. This effect is used to detect certain biomolecules, proteins, early cancer biomarkers as well as drug levels in the body fluid. Silver nanoparticles having sharp SPR have been extensively used for targeting and imaging small molecules, DNA, and cellular tissues. Nanostructures comprising of AgNPs can be used to explore the location and size of tumors *in vivo* so that they can be destroyed by a photothermal route (Lee & Jun, 2019). Specific AgNPs have been used to prepare nanoprisms which are used in low-intensity light-emitting diodes (LEDs) for the emission of different wavelengths in combination with color filters. These devices in communication fibers reduce the loss of messages and contamination of signals (Bastys *et al*., 2006).

Silver nanoparticles are expansively used in agriculture to improve crop production, seed germination, quality of seedlings, and plant nutrition, and defend against bacterial and fungal infections. Mixing of optimized quantity AgNPs was reported to promote but higher concentrations inhibited the growth of plants. Nanosilver materials are efficiently applied in foliar spray against fungi, molds, and bacteria. Silver nanoparticle-based pesticides are non-toxic, safe, and target oriented for the management of pests in the crops (Kale *et al*., 2021). Green synthesized AgNPs have the potential application to split water and generate hydrogen as a sustainable and viable source of energy. A large surface area of AgNPs absorbs photons from sunlight that can be used to dissociate water molecules for the production of hydrogen (Rana *et al*., 2020). Several industries manufacture paper, plastic, leather, textiles, pharmaceutical, food, cosmetics, etc., and exhaust dyes and other synthetic compounds into the environment. Azo dyes in the food chain travel to the living organism and are highly carcinogenic. These compounds cause severe contamination of water, soil, and air and the management of this waste has become a challenge to the concerned authorities. The exopolysaccharide-stabilized silver nanoparticles efficiently catalyzed the degradation of methyl orange and Congo red by using $NaBH_4$ at room temperature (Saravanan *et al*., 2017).

2.5 Toxicity of silver nanoparticles

Green-synthesized AgNPs have unlocked new insights in a wide range of applications in several disciplines due to their size, shape, optical, biological, and catalytic properties. The increasing prevalence of AgNPs in several consumer products has forced authorities to conduct comprehensive assessments of the long-term effects on human physiology and the ecosystem. The unprecedented production and use of AgNPs have raised the fear of excessive concentration in the environment. The ever-increasing release of AgNPs by several industries in the ecosystem poses antagonistic influences on the physiological and biochemical processes of the living being in the food web (Tripathi *et al*., 2017). The commercial production of AgNPs has increased radically in the last decades, but intensive studies on toxicity have been the subject of concern only in recent times. The most important drawback of AgNPs is their nonselective toxicity to bacterial and eukaryotic cells (Burdușel *et al*., 2018). Lack of mandatory information, modeling schemes, and standard guidance for the test, fate, and effects are hindering the environmental risk assessments of nanomaterials (Voelker *et al*., 2015). Most of the silver contents will ultimately end up in the ecosystem from solid waste, effluents, wastewater, and incineration products like ash or slags.

Nanosilver undergoes aggregation, agglomeration, and transfer into stable silver compounds like silver chloride and silver sulphide in the environment and recycled into the living world. The presence of Ag and AgNPs especially targets the spleen, kidneys, liver, and brain (Hartemann *et al*., 2015). AgNPs are easily inhaled and reach the lungs and alveolar region. They are more harmful than particulate matter which is in the micrometer range. AgNPs are more cytotoxic than other NPs like TiO_2, Fe_2O_3, ZrO_2, and carbon nanotubes (Quadros & Marr, 2010). Some of the reports of the toxic effects of AgNPs on plants and animal subjects are listed in Table 3.

Table 3: Physiological toxicity of AgNPs on some organisms

No	Test subjects	Analysis	Results	References
1.	Rat models	Immune suppression,	Spleen swelling, an increase of IL-1b, a decrease of IL-6, IL-10, and TNF-α cells	(De Jong *et al*., 2013)

2	Japanese medaka (*Oryzais latipes*)	Toxicity on embryo by $AgNO_3$, Ag-colloids, and AgNPs at different pH	Reduced hatching capacity and eye size	(Kataoka *et al*., 2018)
3	White male mice	Biokinetics of AgNPs accumulation in the brain	Crossed the brain-blood barrier which can cause memory impairment	(Antsiferova *et al*., 2016)
4	Sea urchins	Embryonal development	Abnormalities	(Buric *et al*., 2015)
5	*Caprinus carpio*	Blood toxicology, histopathological study	Amassing of AgNPs in the gills and intestine caused necrosis	(Kakakhel, *et al*., 2021)
6	Wistar rats	Effect on reproductive health	Decrease in estrogen level, antioxidant, capacity, hyperplasia, and degeneration of ovarian follicle	(Mohamed *et al*., 2022)
7	*Ceriodaphnia cornuta*, *Paramecium* sp, and Guppy fish	Ecotoxicity of *Solanum nigrum* mediated AgNPs	Lethal at 30 mg/mL, and affected heart rate and swimming capacity	(Jenifer *et al*., 2020)
8	*Microcystis aeruginosa* (Algae)	Toxicity of citrate-coated AgNPs	Reduction of α-chlorophyll due to free Ag^+ ions from AgNPs	(Xiang *et al*., 2018)
9	*Caenorhabditis elegans* (Soil nematode)	Ecotoxicity	Reduction in reproduction potential	(Roh *et al*., 2009)
10	*Crassostrea virginica* (Oyster)	Impact on embryonic development	Adverse effect	(Ringwood *et al*., 2010)
11	*Labeo rohita*	Acute toxicity	Bioaccumulation of AgNPs in tissues,	(Rajkumar *et al*., 2016)

	(Freshwater fish)		blood vessels, and lamella in the liver and gills	
12	Rat	Toxicity of AgNPs on liver function	Increased the activity of serum liver enzymes	(Ramadhan & Ghareeb, 2021)

Waterborne or foodborne uptakes of AgNPs by the living organism are either exhausted in benign form or undergo bioaccumulation in specific organs like gills, skin, brain, liver, kidneys, etc. When the concentration exceeds the safety limit, various complications arise in the subject (Uddin *et al*., 2020). AgNPs induce the production of ROS which blocks cell division, inspires apoptosis, and causes cell death. The toxicity of AgNPs is governed by the size, pH, concentration, and duration of exposure (Saadh, 2021). Several studies have reported the toxic effects of AgNPs on lives and environmental factors. The toxicity of starch-coated AgNPs was accessed on human lung fibroblast (IMR-90), and glioblastoma (U251). The AgNPs having a size ranging from 6-20 nm induced mitochondrial dysfunction by ROS production leading to DNA damage, chromosomal irregularities, and seizure of cell cycle at the G2/M phase (Asharani, Mun, *et al*., 2009). Exposure of AgNPs to Wistar rat liver significantly decreased the mitochondrial membrane potential, respiratory control, and ADP-induced depolarization (Teodoro *et al*., 2011). The presence of AgNPs in a rat model increased alanine transaminase, phosphatase, and aspartate transaminase enzymes in the spleen, liver, and other parts significantly causing liver damage (De Jong *et al*., 2013).

The extensive manufacture and use of nanosilver in consumer products have created research attention globally. The potential health hazards of uncontrolled use and accidental and deliberate discharge of huge quantities of AgNPs in the environment cannot be overlooked. Several pieces of research are dedicated to the short and long-term implications of nanosilver on the biotic and abiotic factors in the ecosystem. The ionic silver (Ag^+) that is released from AgNPs is considered the most notorious for the toxicity of aquatic organisms as it can easily cross the cell barrier and accumulates in the organisms (Jiang *et al*., 2017).

In comparison to natural NPs, engineered AgNPs are creating the greatest concern for the environment and lives. The toxicological investigation of AgNPs from exposure sites should be accomplished to predict the probable adverse impacts on the health of living beings and their surroundings. The fate and risk of AgNPs on environmental and health issues should be estimated properly.

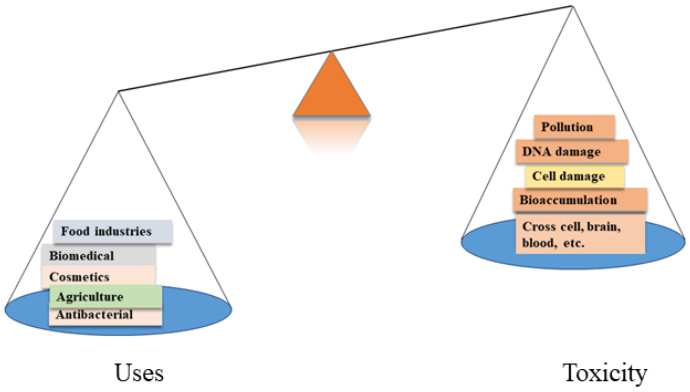


Figure 11: Appraisal of uses and toxicity AgNPs

At the time of incorporation of AgNPs in consumer products, adverse effects on the environment and the food chain must be investigated accurately. Most of the laboratory reports are based on the conclusions derived from the studies on the limited number of subjects exposed for some hours to days. The fate of AgNPs and their toxicity in the environment and the organisms are influenced by various factors including pH, dissolved oxygen, temperature, sunlight, shape, size, surface coatings, etc. However, the actual rate of transformation may vary in natural settings, the lab-based toxicological data can partially be used to extrapolate the risk assessment of AgNPs in the whole environment. (Zhang *et al*., 2019). The prerequisite data of AgNPs on their behavior and risk must be clarified for the optimized application in a particular field. Wide-scale and vigorous studies are needed to evaluate the risks and hazards associated with AgNPs for a safe and sustainable application for mankind which can be depicted in Figure 11.

The *in vivo* and risk assessment of nanoparticles can be achieved by toxicokinetic studies like absorption, metabolism, distribution, and elimination. There are limited data and knowledge gaps on the toxicokinetic properties, mainly metabolism, and clearance in the aquatic and terrestrial environments (Ferdous & Nemmar, 2020). Comprehensive studies are required on the physicochemical properties of AgNPs highlighting biodistribution, *in-vitro,* and *in vivo* toxicity, and, bioaccumulation from

various routes of entry into the living world. These research data should be considered before launching any consumer products embedded with AgNPs for commercial use. Stringent rules and guidelines should be enforced globally to reduce the hazardous effects of AgNPs.

2.6 Research gap

There are 1624 species of medicinal plants in Nepal that have been documented to exhibit biological effects like antibacterial, antiviral, antidiabetic, etc. (Pandey & Shrestha, 2018). Still, several plants are to be scientifically documented for their pharmacological and biological properties. Green synthesized silver nanoparticles are cost-effective and eco-friendly biomedical products that can be manufactured by using different plants under benign conditions. The proposed research will establish the green method to synthesize AgNPs and phytochemical and biological evaluation of some plant extracts. The results of this study will be supportive of the development of AgNPs-mediated biomedical products and natural drug development. This will facilitate the sustainable development of biodiversity and the economic growth of the nation.

2.7 Research hypothesis

- Medicinal plants contain different phytochemicals
- The secondary metabolites in the plants support the synthesis of biogenic silver nanoparticles
- The plant extracts/AgNPs exhibit significant biological properties like antioxidant, antimicrobial and antidiabetic activities
- The novel compounds having significant biological properties can be isolated from the active plant extract

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All the chemicals and reagents of the highest purity and distilled water (DW) were used throughout the lab work. The Folin-Ciocalteu reagent (FCR), $AlCl_3$, $AgNO_3$, HCl, Na_2CO_3, H_2SO_4, CH_3COOK, and dimethyl sulphoxide (DMSO), were purchased from Thermo-Fisher Scientific India, Pvt Ltd. Gallic acid, ascorbic acid, Muller Hinton Agar (MHA), Muller Hinton Broth (MHB) and quercetin of Himedia Laboratories Company Ltd. India, were used. Similarly, ethyl acetate, methanol, ethanol, chloroform, *n*-hexane, and dichloromethane from Merk Life Science Limited were used. Neomycin, acarbose, porcine pancreatic α-amylase (PPA), and 2-chloro-4-nitrophenyl-α-D-maltotrioside ($CNPG_3$) from Sigma-Aldrich (Germany) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Tokyo Chemical Industries Co. Ltd, (Japan) respectively.

3.1.2 Instruments

Most of the equipment and instruments of the branded company were used for the study. Borosilicate glassware was used to perform the chemical test and preparation of solutions. IKA RV 10 Rotary evaporator, Thermo-scientific centrifuging machine, Shimadzu IR 100 tracer, BioTek Synergy LX Multi-mode reader, Multichannel pipettes of Thermo Fisher Scientific, Samsung refrigerators, Stuart Scientific hotplate magnetic stirrer, etc. which are available in our lab were used for the study. Column chromatography was done on the Merck silica gel (60-120 mesh) and TLC was performed over the pre-coated Merck plates. The plates were viewed by using the Spectroline ENF-280C/F UV light at 254 nm. The X-ray diffraction (XRD) was taken by using D/Max 2500 V/PC; Rigaku Co. Japan. The SEM and TEM images of the synthesized AgNPs were taken by using Hitachi S-7400, Japan, and JEM-2100 plus at 200kV (JEOL Ltd., Japan) respectively. The GC-MS analysis of the essential oil of the plants and the extracts were performed by using an Agilent 5975 Series MSD.

3.1.3 Software

Software like MSWord, MS PowerPoint, MS Excel of the latest version, and Origin 2019 were used for data processing and analysis. Gen5 Microplate data collection and analysis software, Graph Pad Prism 9 to calculate IC_{50}, and Image J to find the sizes of the AgNPs from their TEM images were used. The chemical structures were drawn using ChemDraw Ultra 12 and Mendeley Reference Manager was used for the write-up.

3.1.4 Study area

Plant samples for the study were taken from Rupa rural municipality, Pokhra metropolitan area of Kaski, and Gharpajhong rural municipality of Mustang district of Gandaki province (Figure 12). The Kaski district lies between 83°40′ east to 84° 12′ east longitude and 28°06′ north to 28°36′ latitude. It covers diverse geography with high altitude ice-capped mountains to mid-hills and plain areas with temperate, cold, and alpine climates (Adhikari *et al*., 2019). Mustang district lies within the Annapurna Conservation Area which is one of the protected zones in the country. It lies within 28° 24′ to 29° 20′ of latitudes, and 83° 30’ to 84° 10′of longitudes (Rajchal & Meilby, 2013). The district comprises high peaks, valleys, and ice-capped mountains with semi-arid alpine and tundra climate (Ojha *et al*., 2021). People residing in the village areas of the Kaski and Mustang districts rely on different medicinal plants for their primary health care (Adhikari *et al*., 2021; Dwa, 2013; Pandey, 2006).

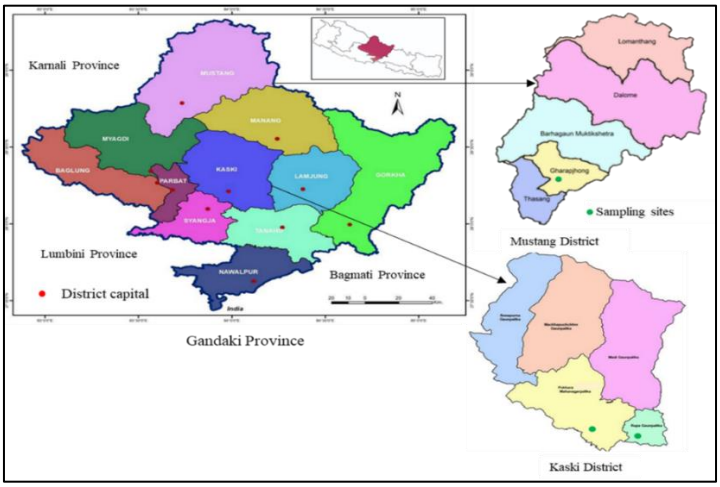


Figure 12: Location map of the sampling sites

3.2 Work Plan

The research work was conducted on a structured plan as shown in Figure 13. Medicinal plants were selected based on the ethnobotanical survey, key informant interviews, and group discussion in the study area. Methanol extracts of the plants were subjected to preliminary investigation of phytochemicals, antioxidant, antibacterial, and antidiabetic properties. Based on the preliminary results, active plants were identified that were used for the synthesis of silver nanoparticles. All of the AgNPs synthesized from the plants were characterized by using customary tools including UV-visible, FTIR spectroscopy, XRD, FESEM, TEM, and EDX analysis. Finally, the antibacterial and antioxidant activity of the synthesized AgNPs were compared with corresponding aqueous extracts.

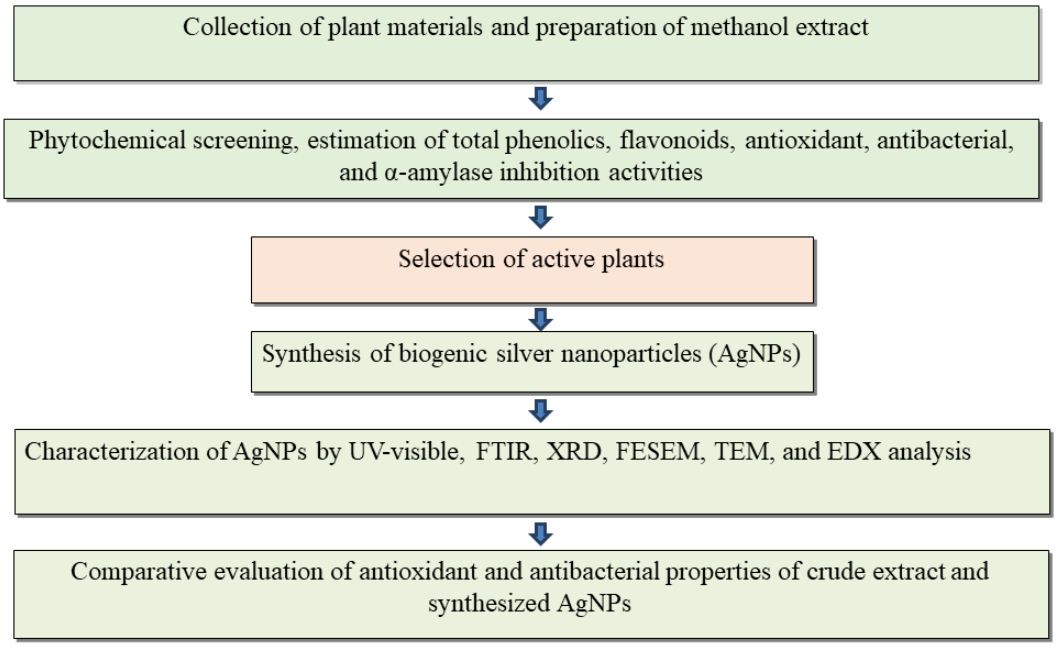


Figure 13: Workflow diagram

3.3 Methods

3.3.1 Collection of medicinal plants

Field visits, extensive surveys, and consultations were conducted with local people of Pokhara-7, Masbar, Rupa village of Kaski, Jomsom, and Thini villages of Mustang district. Suggestions and required information on the locally used medicinal plants were taken from the local healers as well. The evidence and survey of the related literature guided the collection of the plant samples. Altogether 22 plants (13 from the Kaski and 9 from the Mustang district) were taken for the study (Table 4).

Table 4: List of plants used in the study

S.No	Botanical/local names	Local use	Collection site	Collected part
1	*Angiopteris helferiana* (Gaikhure)	Tonic, backache	Kaski	Tuber
2	*Arctium lappa* (Chisung)	Burns, fever	Mustang	Aerial parts
3	*Artemisia roxburghiana* (Kalo pati)	Fever, cold	Mustang	”
4	*Artemisia stricta* (Seto- Pati)	Cold, cough	Mustang	Aerial parts
5	*Aster indamellus* (Metok)	Fever, headache	Mustang	”
6	*Ayenia grandifolia* (Kholebirale)	Cooling agent	Kaski	Stem bark, leaves
7	*Beilschmieida roxburghiana* (Hadchur)	Bone fracture	Kaski	Stem bark
8	*Bigonia megaptera* (Makarkanjay)	Fever, stomach ache	Kaski	Whole plant
9	*Clematis graveolens* (Baghjunghe)	Cough, and joit pain	Mustang	”
10	*Crataeva unilocularis* (Sipligan)	Wound healing, antiseptic	Kaski	Twigs
11	*Dischidia bengalensis* (Thirjo)	Tonic	Kaski	Aerial vine
12	*Drynaria coronans* (Kammari)	Tonic, backache	Kaski	Tuber
13	*Elaeagnus umbellate* (Gunyeli)	Pickling	Mustang	Aerial parts with fruits
14	*Ephedra pachyclada* (Somlata)	Asthma, pressure	Mustang	”
15	*Juniperus indica* (Dhupi)	Incense	Mustang	”
16	*Mimosa rubicaulis* (Areli)	Diarrhea, fever	Kaski	”
17	*Pyrus pashia* (Mel)	Fever, cough	Kaski	Stem bark
18	*Rubus ellipticus* (Yensalu)	Fever, wounds	Kaski	Rootbark

19	*Sida acuta* (Balujhar)	Wounds	Kaski	”
20	*Taraxacum parvulum* (Dudhe)	Diuretic	Mustang	Whole plant
21	*Trichosanthes wallichiana* (Indreni ko laharo)	Wounds	Kaski	Aerial vine
22	*Ziziphus mauritiana* (Bayer)	Fever, wounds	Kaski	Rootbarks

Legal permission to collect samples from the study area was taken from the National Trust for Nature Conservation (NTNC, Nepal). The plant samples were collected obeying common ethical guidelines such as not hurting the natural biodiversity and the existence of the species. The voucher samples of all the samples were deposited for the botanical authentication at the National Herbarium and Plant Laboratories, Godavari, Lalitpur.

3.3.2 Preparation of extracts

All the collected plant parts were washed with clean water followed by distilled water separately. Each of the collected parts was chopped into small pieces and dried in shade for three weeks. The dry parts were ground into fine powder using a mechanical grinder and stored in separate plastic cans for use. The extracts were prepared separately by using a Soxhlet apparatus with 80% methanol. The crude extracts were concentrated through a rotary evaporator and stored in the freezer at 4℃.

3.3.3 Phytochemical screenings

The methanol extracts of the plant were tested for the presence of different phytochemicals adopting standard protocols (Tiwari *et al*., 2020). Tests will be performed for polyphenols, alkaloids, flavonoids, terpenoids, reducing sugars, glycosides, tannins, carotene, coumarins, saponins, and anthraquinones.

3.3.3.1 Test for alkaloids

The presence of alkaloids was detected by the following tests:

Meyer’s test: The extract was treated with Meyer’s reagent (Potassium mercuric iodide). The formation of yellow precipitate indicated the presence of alkaloids.

Wagner's test: The formation of reddish-brown precipitate on the addition of the solid extract with Wagner's reagent (Iodine in KI).

Dragendorff's test: The extract was treated with Dragendorff's reagent (solution of potassium bismuth iodide) and the formation of a reddish precipitate was noticed.

Hager's test: The extract was dissolved in methanol and the filtrate is treated with Hager's reagent (saturated picric acid solution). A yellow precipitate was observed by the alkaloids.

3.3.3.2 Test for glycosides

The methanolic extract (1 mL) was shaken with three drops of Molisch's reagent and concentrated sulphuric acid (2 mL) was poured from the sides of the test tube slowly. The formation of a violet ring at the junction confirms the presence of glycosides.

3.3.3.3 Test for flavonoids

Alkaline test: Extract (~ 0.2 g) was treated with dilute NaOH. The formation of intense yellow color which disappears with the addition of dilute HCl confirms the presence of flavonoids.

Lead acetate test: The extract was treated with a few drops of lead acetate solution. The formation of a yellow precipitate indicated the presence of flavonoids.

3.3.3.4 Test of reducing sugars

The extract was boiled with distilled water and filtered. The filtrate was heated with Fehling's solution (A and B mixed) in a water bath for a few minutes. The formation of red precipitate confirmed the presence of reducing sugars.

3.3.3.5 Test of terpenoids

Nearly 0.3 g of the extract was shaken with chloroform and 3 mL of Conc. H_2SO_4 was added to the mixture. The formation of reddish-brown color at the junction of two liquid layers confirmed the presence of terpenoids.

3.3.3.6 Test for tannins

About 0.5 g of the extract was boiled with 2 mL of water in a test tube and filtered. A few drops of 0.1 M $FeCl_3$ solution were added. The formation of brownish-green to black coloration indicated the presence of tannins.

3.3.3.7 Test for saponins

A pinch of the extract was shaken vigorously with distilled water. The formation of froth indicated the presence of saponins.

3.3.3.8 Test for carotenoids

Nearly 1 g of the sample was extracted with chloroform in a test tube with vigorous shaking. The mixture was filtered and the filtrate was treated with 85% H_2SO_4. A blue color at the junction indicated the presence of carotenoids.

3.3.3.9 Test for coumarin

A single pellet of KOH was dissolved in 2 mL of ethanol. 1mL of the extract was added to the solution. The formation of a yellow color/precipitate indicated the presence of coumarin.

3.3.3.10 Test for anthraquinones

About 0.5 g of the extract was boiled with 10 mL H_2SO_4 and filtered while hot. The filtrate was shaken with 5 mL chloroform. The chloroform layer was separated and 1ml of dilute ammonia solution was added to get color.

3.3.3.11 Test of polyphenols

The extract (~ 0.5 g) was treated with distilled water and 2/3 drops of 1% ferric chloride solution were added. A greenish color indicated the presence of polyphenols

3.3.4 Fractionation of methanol extract of *A. grandifolia*

The active plant or fraction was identified by the preliminary screening of the phytochemical test, TPC, TFC, antioxidant, and antibacterial activities. The active plant extracts were subjected to fractionation using n-hexane, dichloromethane, and ethyl acetate serially using a separating funnel. Each of the fractions was evaluated for TPC, TFC, antioxidant and antimicrobial activities using the standard protocol. Based on

biological activities, *A. grandifolia* was found to be an active plant, so it was subjected to fractionation using different solvents and evaluated for antibacterial and antioxidant activity. The shed-dried stem bark of the plant (1.5 kg) was extracted with 90% methanol three times and concentrated by using a rotary evaporator (150 g). The concentrated extract was dissolved in distilled water. It was subjected to solvent-solvent extraction repeatedly by using *n*-hexane, DCM, and ethyl acetate in small lots. All of the extracts were concentrated by using a rotary evaporator and collected separately as in Figure 14. Each of the fractions was subjected to the estimation of TPC, TFC, antioxidant and antibacterial activities.

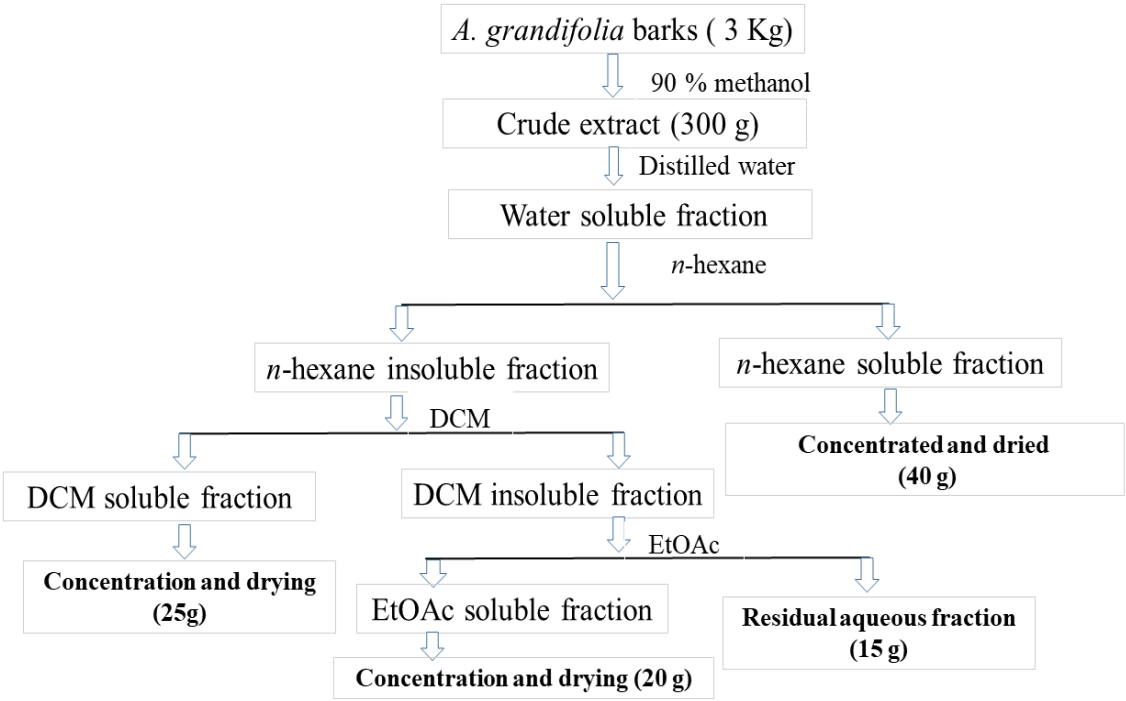


Figure 14: Flow sheet diagram of fractionation of A. grandifolia extract

3.3.5 Isolation and GC-MS analysis of essential oil

The essential oil was isolated from the aerial parts of the plant by the hydro-distillation method using a Clevenger apparatus. The plant powder (60 g) was put in a round bottom flask containing 400 mL of distilled water and boiled for about 3 hours. The light brown-colored oil having a sharp fragrant smell was collected, dehydrated by anhydrous Na_2SO_4, and stored in a refrigerator. The GC-MS analysis was performed by a Shimadzu QP-2010 GC-MS system comprising an AOC-20i auto-sampler. The injection temperature was 220 ℃. The oven temperature used was holding 80 ℃ for 2 minutes, heating at the rate of 6 ℃/min up to a constant temperature of 280 ℃ for 5

minutes. Helium was used as carrier gas at a constant flow of 1.03 mL/minute and an injection volume of 1 μL was employed. The mass spectra were taken at 70 eV at the scan interval of 0.5 sec. and the fragments from 50-500 m/z were analyzed. The components of the EO were identified by comparing their mass spectra with those in the NIST05 library.

3.3.6 Biosynthesis of silver nanoparticles

Every 10 g powdered material of the plant was gently heated with 100 mL of distilled water at about 80°C for about 20 minutes. The mixture was filtered by using a Whatman No 1 filter paper. The filtrate was stored in a refrigerator and used for the synthesis of AgNPs within a week. The green synthesized silver nanoparticles (AgNPs) were prepared by the reaction of fresh aqueous plant extract and $AgNO_3$ (1 mM) in the ratio of 1:9 with constant stirring over a magnetic stirrer at lab temperature and pH 7 (Adebayo-Tayo *et al*., 2019; Rautela *et al*., 2019). A gradual change of color of the solution within an hour indicates the formation of AgNPs in the solution. After the completion of the reaction, the nanoparticles were washed and separated by centrifuging at 1300 rpm repeatedly with double distilled water. Finally, they were recovered by dehydrating using ethanol, dehydrated by using a desiccator and stored for characterization and biological studies. The percentage yield of AgNPs was calculated by using the formula (Sila *et al*., 2019):

$$\% \text{ Yield} = \frac{Mass\ of\ AgNPs}{Mass\ of\ \ Ag^{+}\ ion}\ X\ 100$$

3.3.7 Characterization of AgNPs

The synthesized silver nanoparticles were characterized by using UV-visible, IR spectroscopy, Powder X-ray diffraction, scanning electron microscopy, and transmission electron microscopy.

3.3.7.1 UV- visible spectroscopy

The absorption of electromagnetic radiation of 200 - 800 nm brings the electronic transition from lower to higher energy levels. Silver nanoparticles show maximum absorption in the range of 400-500 nm due to their surface plasmon resonance (Ashraf *et al*., 2016).

Principle and working

When monochromatic light passes through a solution, the absorbance of light is expressed by the Beer-lambert law,

$$A = \log Io/I = \in cl$$

Where,

A = absorbance, I_0 = Intensity of incident radiation,

I = intensity of transmitted radiation, Ɛ = molar extinction coefficient,

c = Concentration of solute (moles/L), and l = path length of the sample (cm).

The molar absorptivity of a substance is constant for a given intensity and the wavelength of maximum absorbance is denoted as λ-max. A spectrometer consists of a radiation source, monochromator, detector, amplifiers, and recorder. A hydrogen discharge lamp is the most commonly used for 180-400 nm and a tungsten filament lamp is used for 400 - 800 nm. The monochromator disperses the incident light from the source into separate wavelengths. It is a prism or grating made up of quartz to be used in the UV region. The light from the monochromator is divided into two beams of equal intensities. One of them passes through the sample and the other through the reference. Detectors are photomultiplier tubes that generate a voltage proportional to the radiant energy which strikes. The amplifier automatically subtracts the absorption of the solvent from the solution and the recorder makes a plot of wavelengths of absorbed radiation against absorbance. The test sample in a suitable solvent and the reference are filled into the quartz cells up to the mark and loaded into the sample holder and the absorption is recorded.

3.3.7.2 Fourier-transform infrared (FTIR) spectroscopy

The potential role of particular functional groups in the synthesis of nanoparticles was revealed by the widening and shifting of the peaks of that group in the extract and the corresponding AgNPs. This technique is applied to verify the functional groups which are involved in the reduction, capping, and stabilizing of the AgNPs. FTIR spectroscopy is related to the rotational and vibrational energy levels of the atoms of the test sample. When electromagnetic radiation of the IR region passes through a sample of organic

compounds, suitable frequencies that cause electronic transition of the vibrational level of the molecule are absorbed. The analysis of the transmitted light is used to calculate the quantity of radiation absorbed. The particular bonds or functional groups having specific vibrations absorb infrared radiation of a particular frequency. The plot of absorbance against the wavenumbers of radiation is called an IR spectrum. The IR active molecules get change in their dipole moment after absorption of the radiation of proper wavelength. The presence of functional groups in the molecule is ascertained by comparing the vibrational frequency to an IR-stored data repository in the literature. The FTIR spectra of the solid plant extracts and the synthesized AgNPs were taken by a Shimadzu IR Tracer-100 from 4000 - 400 cm^{-1}. The sample was directly put in the sample holder and the spectra were recorded.

3.3.7.3 X-ray diffraction analysis

X-ray diffraction is one of the fundamental instrumental analyses that is used for measuring the crystallinity of the synthesized nanoparticles. The diffraction pattern of the AgNPs indicates the arrangement of atoms in a fixed crystalline plane. The crystal structure of the synthesized material is ascertained by comparing the XRD pattern with that of a computerized library in the literature. An X-ray diffractometer was used to confirm the crystallinity of the synthesized AgNPs. The diffractogram was obtained by standard protocol by using CuKα (λ = 1.5406 Å) radiation with 30 mA of current, and 40 kV of voltage in a scan rate of 10º/minute across the 2θ angle ranging from 0 to 90.

Principle

When a crystalline material is irradiated by an X-ray beam of comparable wavelength λ, they collide with electrons in atoms and are diffracted with constructive and destructive interferences to produce a diffraction pattern. When X-rays are incident on a crystal plane with an angle of incidence (θ), they are reflected with the same angle of reflection. A constructive interference occurs when the path difference is equal to the whole number multiple of the wavelength.

According to Bragg's law,

$$n\lambda = 2dSin\theta$$

Where,

N = integer, λ = wavelength, d = Interplannar spacing, θ = angle of diffraction

3.3.7.4 FESEM and TEM analysis

The SEM images, EDX spectrum, and TEM images were taken using the standard protocol and instruments. The TEM images and selected area electron diffraction (SAED) pattern were taken by using a JEM-2100 plus at 200 kV (JEOL Ltd, Japan). The TEM images were captured by spraying the suspension of AgNPs over a carbon-coated copper grid. The size of the synthesized AgNPs was measured with the help of Image J software from the TEM images.

Scanning electron microscopy uses a high-energy beam of electrons to obtain highly magnified 2D images of the sample. When a highly energized beam of electrons is irradiated on the surface of a solid sample. The impact produces ionization of surface atoms and liberates electrons. These secondary electrons and the backscattered electrons are used to produce the image of the sample. SEM images provide the morphology and quasi-three-dimensional views of the sample in the nano-range.

A heated tungsten filament is used as the electron source which directs the beam in a particular direction by the use of anode plates. The beam of electrons passes through the condenser, scan coil, and objective lens. When this beam hits the sample, they get reflected and scattered in all directions randomly. The secondary and backscattered electrons are detected which are later processed to ascertain the morphology and topography of the sample. The sample consisting of bumps and valleys reflects the electrons differently which helps to develop the image of the specimen at the micro-level.

The transmission microscope uses electrons to generate a high-magnification image of the internal structure of the sample under examination. A high-voltage electron beam is produced from a tungsten cathode by electrical heating and moved through an aperture by using a condenser lens by applying an anode (positive field). The beam is passed through a very thin film of the specimen that is loaded on a grid. Some of the electrons transmitted through the sample are collected by the objective lens. The image created by the objective lens is magnified by using a projector lens. This image is passed into an electron-sensitive camera to get the magnified image of the sample.

3.3.8 Determination of TPC and TFC

The total phenolic and total flavonoid contents in the plant extracts were evaluated by the Folin-Ciocalteu method (Ainsworth & Gillespie, 2007; Kamtekar *et al*., 2014). A standard gallic acid calibration curve was constructed and the concentration of phenolics was calculated from the equation of a straight line. Aliquots of 20 μL of the gallic acid and the test solutions (5 mg/mL) were filled in the bores of a microplate in triplicates. To each of the test solutions, 100 μL of FCR (1:10 in DW), and 80 μL of 7.5% Na_2CO_3 solution were added. The mixture was incubated for about 25 minutes in dark at room temperature and optical density was recorded at 765 nm using a microplate reader. Then, TPC was calculated from the standard calibration curve and expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of the dry extract.

The total phenolic content of different plant extracts and fractions was calculated by using the formula:

$$\mathbf{C} = \frac{CV}{m}$$

Where, **C** = total phenolic content in mg/g, in gallic acid equivalents (GAE)

C = concentration established from the calibration curve in mg/mL

V = Volume of the extract in mL, and

m = weight of the plant extract (g)

Data were recorded as the mean of three determinants of absorbance for each concentration, and a linear correlation coefficient (R^2) was calculated. The regression equation was used to calculate the concentration of phenolic content in the extract.

$$y = mx + C$$

Where,

y= absorbance, m = Slope of the calibration curve, x = concentration of the extract, and C = intercept

The TFC in the extracts was determined by the aluminum chloride colorimetric method with slight adjustments (Pawar & Dasgupta, 2018). A standard quercetin calibration

curve was constructed and the concentration of flavonoids was calculated from the equation of a straight line. The aliquots of 130 μL of quercetin, 5 μL of $AlCl_3$, 5 μL of CH_3COOK, and 60 μL of ethanol are loaded into a 96-well microplate in triplicates. Similarly, 20 μL of each of the extracts (5 mg/mL), 110 μL of double-distilled water, 5 μL of $AlCl_3$, 5 μL of CH_3COOK, and 60 μL of ethanol are also filled in triplicates. The microplate is incubated for 30 minutes in the dark and the absorbance is recorded at 415 nm against the blank. The TFC can be calculated from the standard curve and is expressed as milligrams of quercetin equivalent per gram (mg QE/g) of the dry extract.

The total flavonoid content of different plant extracts and fractions was calculated by using the formula:

$$\mathbf{C} = \frac{CV}{m}$$

Where, **C** = total flavonoid content in mg/g, in quercetin acid equivalents (QE)

C = concentration established from the calibration curve in mg/mL

V = Volume of the extract in mL, and

m = weight of the plant extract (g)

Data were recorded as the mean of three determinants of absorbance for each concentration, and a linear correlation coefficient (R^2) was determined. The regression equation was used to calculate the concentration of phenolic content in the extract.

$$y = mx + C$$

Were,

y = absorbance, m = Slope of the calibration curve, x = concentration of the extract, and

C = intercept

3.3.9 *In-vitro* antioxidant activity

The antioxidant activity of different extracts of the plant was evaluated by the DPPH) free radical scavenging method (Alabri *et al*., 2014; Brand-Williams *et al*., 1995) with slight modifications by taking ascorbic acid as a positive control. The test solutions of

the extracts, AgNPs, and ascorbic acid of 250, 125, 62.5, 31.25, 15.6, and 7.8 μg/mL are prepared in 50 % DMSO. Each 100 μL of 0.1 mM DPPH and test solutions/ascorbic acid are filled into a 96-well microplate in triplicates. The mixture is incubated for 30 minutes in dark at lab temperature and the optical density is recorded at 517 nm against blank. The data were processed and analyzed using Microsoft Excel 2016. The radical scavenging activity of different extracts and ascorbic acid is calculated by using the formula:

$$\text{Percentage scavenging} = \frac{\text{The absorbance of control} - \text{absorbance of the sample}}{\text{Absorbance of control}} \; X \; 100$$

The half-maximal inhibitory concentration (IC_{50}) of the extracts and the positive controls were calculated by using the Graph Pad Prism 9 software.

3.3.10 Antibacterial activity

The antibacterial activity of different extracts of the plant was evaluated by the agar well diffusion method (Ghosh *et al*., 2008; Murray *et al*., 2007). The pure samples of American Type Culture Collection (ATCC) of Gram-negative and Gram-positive bacteria were used for the test (Table 5).

Table 5: List of bacteria used in the study

Microorganisms	Type	ATCC
Klebsiella pneumonia	Gram-negative	700603
Escherichia coli	Gram-negative	25922
Salmonella typhi	Gram-negative	14028
Staphylococcus aureus	Gram-positive	25923
Shigella sonnei	Gram-negative	25931
Acinetobacter baumannii	Gram-negative	19606
Enterococcus faecalis	Gram-positive	29212

The microorganisms were grown up in the freshly prepared Muller Hinton Broth (MHB) solution overnight at 37 ℃ to equate turbidity to 0.5 McFarland's standard. The

bacterial suspension was coated on the sterile Muller Hinton Agar (MHA) media. The boreholes of 6 mm diameter were punched at equivalent distances on the surface by using a sterile cork- borer. The wells were fed with 50 μL of the plant extracts (50 mg/mL), neomycin (1 mg/mL), and 50 % DMSO as a control. The plates were incubated for 24 hours at 37℃. On the next day, the samples were taken out from the incubator and the clear zones around the holes that correspond to the respective inhibition zones were measured and recorded.

The plant extract (100 mg/mL) which showed a maximum zone of inhibition by agar well diffusion was evaluated for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by the resazurin microtiter assay (Serker *et al*., 2020). The bacterial suspension was grown up in MHB at 37℃ to equate the turbidity with that of 0.5 McFarland's standard. The cultures were diluted 1/100 times into the broth (1.5 x 10^6 CFU/mL). Each 100 μL of the broth was loaded into all the wells from (1, 2, 3), (4, 5, 6), and (7, 8, 9) for extracts, positive (neomycin 1 mg/mL), and negative controls in triplicates. Then, 1, 2, and 3 were filled with 100 μL of extract, 100 μL neomycin 4, 5, and 6, and the broth in 7, 8, and 9 with negative controls. Then, each of the wells except 7, 8, and 9 were serially two-fold diluted up to the eighth row. After this, 5 μL of the bacterial culture solution was added to each of the wells and allowed to incubate at 37˚C with the lid covered for about 18 to 24 hours. On the next day, the plate was taken out and 30 μL of 0.01% resazurin was added to each of the wells and incubated for about 2 hours at 37˚C. Finally, the microtiter plate was taken out and the visual change of color of the solutions was observed and the MIC is determined as the concentration that corresponds to the no development of bacteria. The solutions in the wells with a pink color corresponding to the MIC and stronger solutions were streaked onto the MHA plates at different compartments and incubated overnight at 37˚C.

3.3.11 α-Amylase inhibitory activity

Methanol extracts from all of the plant samples were evaluated for α-amylase inhibitory activity by using $CNPG_3$ as a substrate (Khadayat *et al*., 2020). 1.56 g of sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) and 1.779 g of disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) were separately dissolved in double distilled water to prepare 50 mL of solutions. They were mixed to get a phosphate buffer solution (50 mM), the pH was

adjusted to 7 and 0.9% NaCl was added. The stock solution of α-amylase (9 U/mL) was prepared by dissolving 1 mg/mL in the phosphate buffer. The solution was diluted to 3.75 U/mL so that it becomes 1.5 U/mL as the final concentration. Exactly 7.25 mg of $CNPG_3$ was dissolved in 5.5 mL of phosphate buffer (2 mM) so that the final concentration will be 1 mM in the reaction mixture. The bores of a microtiter plate were filled with 80 μL of acarbose, and 20 μL plant extracts (5 mg/mL in 50% DMSO) in triplicates and incubated for 10 minutes at 37°C. To the mixture, 100 μL of $CNPG_3$ was added and again incubated for 15 minutes. The development of the light yellow color of *p*-nitro aniline was measured by using a microplate reader at 405 nm using the blank (50% DMSO), acarbose (0.1 mg/mL) as the positive control. The percentage inhibitions of each of the plant extracts were calculated by using the formula:

$$\text{Percentage inhibition} = \frac{\text{The absorbance of control} - \text{absorbance of the sample}}{\text{Absorbance of control}} \; X \; 100$$

The percentage inhibitions of α-amylase at different concentrations (250 -7.8 μg/mL) for the standard acarbose, *P. pashia, M. rubicaulis,* and *A. grandifolia* extracts were determined. The concentration causing 50% inhibition of the enzyme was calculated by using the Graph Pad Prism 9 software.

3.3.12 Statistical analysis

All the experiments were performed in triplicates and calculated as Mean ± SD. The spectrophotometric results were processed by Gen5 Microplate data collection and analysis software and Microsoft Excel 2019. The regression analysis was done by using the equation of the straight line: y = mx + C. The half maximal concentration (IC_{50}) was calculated by using Graph Pad Prism 9 software.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Percentage yield

The research was conducted on the 22 medicinal plants collected from the Kaski and Mustang districts of Nepal. The methanol extracts of the dried samples were prepared by the cold percolation method. The percentage yields of methanol extracts of all the plants are shown in Figures 15 and 16. The results show that the studied plants have different proportions of extracts. The plants like *R. ellipticus, P. pashia, Z. mauritiana, and J. indica* contained a higher percentage of secondary metabolites. Plants that are rich in secondary metabolites are supposed to contain a relatively larger number of phytocompounds. The proportion of secondary metabolites in plants is affected by the season, collection time, maturity, etc. of the plant.

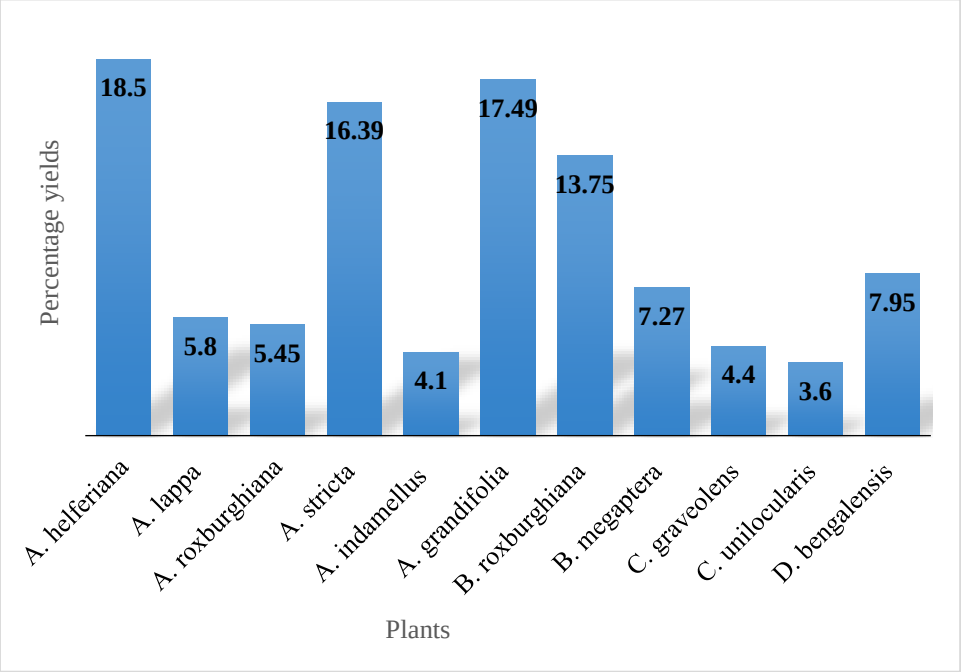


Figure 15: Percentage yields of methanol extracts of different plants

Plants synthesize a large number of complex molecules in response to various biotic abiotic stresses. These compounds support different tasks like attracting pollinators, establishing symbiosis, and enduring against climatic peripherals, and soil characteristics. The proper time of harvesting, application of suitable solvents and extraction methods are crucial to obtaining a higher level of natural products for

medicinal usage (Guerriero *et al*., 2018). The percentage yield of AgNPs synthesized by using four different plant extracts was calculated and presented in Table 6. In this study, *P. pashia*, mediated AgNPs were formed in the highest proportions (80.52 ± 2.01), followed by *Z. mauritiana*, and *R. ellipticus*, and the lowest percentage was obtained by *A. grandifolia* extract. The quantity of AgNPs in the synthesis depends on various factors like the nature of biomolecules in the extract, pH, temperature, reaction time, etc.

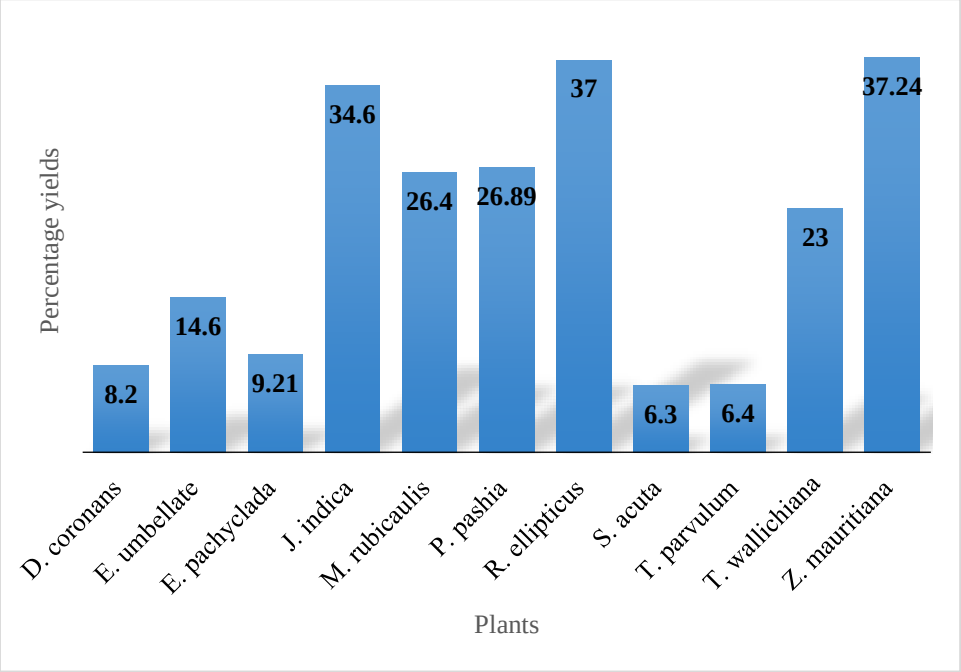


Figure 16: Percentage yields of methanol extracts of different plants

In this study, the reaction was optimized by mixing extract and $AgNO_3$ in the ratio of 1:9, pH = 7, at room temperature and allowed to react for 48 hours with constant stirring. These results are in agreement with the results of AgNPs synthesized by using the leaf and stem barks of *Grewia lasiocarpa.* They observed a maximum yield of 99% for the leaf extract prepared at 80°C and 72% for the stem bark extract (Akwu *et al*., 2021).

Table 6: Percentage yield of synthesized AgNPs by using different plant extracts

Plants used for AgNPs	Percentage (%) yield
Ayenia grandifolia	52.58 ± 1.03
Pyrus pashia	80.52 ± 2.01
Rubus ellipticus	72.57 ± 1.6
Ziziphus mauritiana	73.55 ± 0.92

Note: Values are the mean ± SD (n = 3)

4.2 Phytochemical screening

The extracts were evaluated for the presence of polyphenols, alkaloids, flavonoids, terpenoids, reducing sugars, glycosides, tannins, carotene, phytosterols, coumarins, saponins, and anthraquinones. Most of the plants contained polyphenols, flavonoids, terpenoids and glycosides, and tannins.

4.3 GC-MS analysis

4.3.1 Composition of essential oil of *A. grandifolia* and *E. pachyclada*

The mass spectra for each peak in the GC-MS analysis were compared with the NIST05 library to identify each of the compounds present in the essential oil of the plants. The detected compounds with their retention time and relative percentage of the EO of *A. grandifolia* and *E. pachyclada* are listed in Tables 7, and 8 respectively.

Table 7: List of compounds in *Ayenia grandifolia* essential oil

Peak	Retention time	Area%	Name of the compounds
1	5.642	3.49	Heptane, 3,3,5-trimethyl
2	6.408	7.07	Phenol, 2-methoxy-
3	6.52	5.29	Octane, 3,3-dimethyl-
4	10.34	4.29	Dodecane, 4,6-dimethyl-
5	10.45	6.40	Phenol, 4-ethyl-2-methoxy-
6	10.52	3.72	Dodecane, 2,6,10-trimethyl (Farnesane)
7	11.34	4.21	Dodecane, 4,6-dimethyl-
8	14.89	4.29	Dodecane, 4,6-dimethyl-
9	15.78	4.49	Dodecane, 2,6,11-trimethyl-
10	18.98	3.77	Dodecane, 2,6,11-trimethyl-
11	19.75	3.81	Dodecane, 2,6,11-trimethyl-
12	21.24	6.83	3-Ecosyne
13	22.70	6.19	Hexadecanoic acid, methyl ester
14	23.34	3.70	Dodecane,2,6,11-trimethyl-
15	26.06	4.06	Ledol
16	31.69	28.39	Di-n-octyl phthalate

The EO of *A. grandifolia* and *E. pachyclada* contained hydrocarbons, esters, and some derivatives of phenol. A sesquiterpene (ledol), two esters (methyl palmitate and di-n-octyl phthalate), and two derivatives of phenol (o-guaiacol and *p*-ethyl guaiacol) were detected in *A. grandifolia*. Among the identified compounds, Di-n-octyl phthalate (28.39%), 2, 6, 11-Trimethyl dodecane (15.77%), 4, 6-Dimethyl dodecane (12.79%), o-Guaiacol (7.07%), 3-Eicosyne (6.83%), p-Ethyl guaiacol (6.40%) and Methyl palmitate (6.19%) were observed (Table 7). Similarly, the GC-MS data of the EO of *E. pachyclada* showed the presence of Diisooctyl phthalate (46.90%), 2,6, 11- Trimethyl dodecane (16.35%), 4,6-Dimethyl dodecane (11.59%), tetrapentacontane (11.56%), and myrtenol (4.37%) as the major compounds (Table 8).

Table 8: List of compounds in *Ephedra pachyclada* essential oil

Peak	Retention time	Area%	Name of the compounds
1	6.521	2.31	Heptane, 3,3,5-trimethyl-
2	8.664	4.37	Bicyclo [3.1.1] hept-2-ene-methanol, 6,6-dir (Myrtenol)
3	10.347	2.73	Dodecane, 4,6-dimethyl-
4	11.340	2.27	Dodecane, 4,6-dimethyl-
5	14.893	2.69	Dodecane, 4,6-dimethyl-
6	15.785	3.90	Dodecane, 4,6-dimethyl-
7	18.983	3.79	Dodecane, 2,6,11-trimethyl-
8	19.757	4.10	Dodecane, 2,6,11-trimethyl-
9	22.659	3.78	Dodecane, 2,6,11-trimethyl-
10	23.347	4.68	Dodecane, 2,6,11-trimethyl-
11	23.608	2.67	1-Iodohexadecane
12	26.609	4.25	Hexadecane
13	29.589	3.61	Tetrapentacontane
14	29.683	2.36	Tetrapentacontane
15	30.031	3.61	Tetrapentacontane
16	31.690	46.90	1,2-Benzenedicarboxylic acid, diiosoctyl ester
17	32.424	1.98	Tetrapentacontane

The structures of the main compounds in the EOs of *A. grandifolia*, and *E. pachyclada* are listed in Figures 17 and 18 respectively. The chemical composition of essential oil of three Italian species of ephedra was evaluated by GC-MS analysis. The EO of *E. distachya* was reported to contain ethyl benzoate as a major component (46.9%) with benzaldehyde (8%), and cis-Calamene (3.6%). Similarly, the EO of *E. fragilis* contained phytol, pentacosane, and 6, 10, 14-Trimethyl-2-pentadecanone.

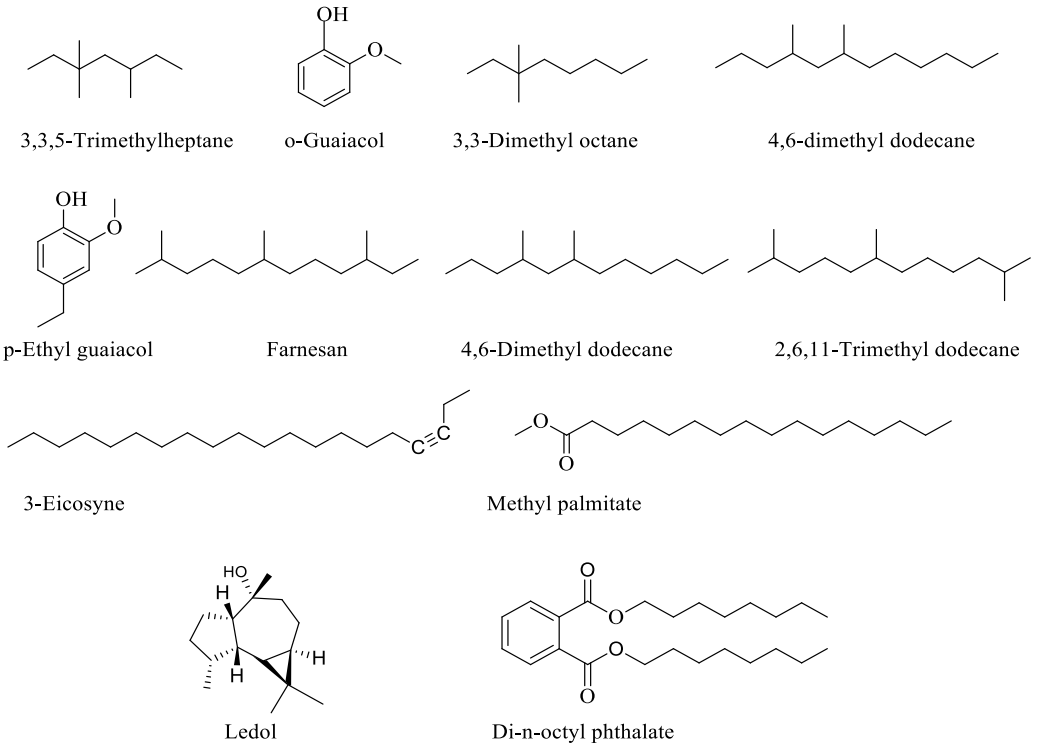


Figure 17: Structures of the molecules present in the essential oil of *A. grandifolia*

The third sample, *E. major* contained eugenol, methyl linoleate, and α-terpineol as the main components in the EO (Kobaisy *et al*., 2005). The EO isolated by the hydrodistillation of *E. intermedia* collected from Iran was assessed by GC-MS examination. The oil contained 2-Ethyl pyrazine (67.4%), Benzyl acetate (9.2%), a sesquiterpene γ-Elemene (9.2%), 2-Methyl butyl acetate (5.3%), and Z-isoeugenol (4.2%) (Ni *et al*., 2019).

In a separate study, EOs isolated from different species of ephedra were analyzed by using a Varian CP-3800 equipped with a flame ionization detector (FID). *E. foliata* EO was characterized by the highest percentage of limonene (28.5%), α-Terpeneol (16%), with ρ-Cymene, 1, 8-Cineol, camphor, and myrtenol. The EO of *E. sarcocarpa* contained carvone (53.3%), carvocrol (16.3%), piperitone, and thymol. They reported

limonene (35.7%), camphene (14.6%), with p-Cymene, 1,8-Cineol, camphor, α-Terpineol, and myrtenol in the EO of *E. intermedia*. The EO of *E. distachya* contained α-Terpineol (17.3%), myrtenol (14.6%), and hexadecanoic acid (14.4%). Similarly, the EO of *E. major* was characterized to have the presence of citronellol (21.4%) and (3Z)-3-hexenyl benzoate (17.6%) (Ehtesham-Gharaee *et al*., 2017). Essential oils are a complex mixture of more than 300 volatile organic compounds with low molecular weights. They are produced in the cytoplasm of the plants in epidermal, internal secretory cells, and secretory pockets. The qualitative and quantitative composition of essential oil in plants is affected by various factors like soil type, parts collected, degree of maturity, environment, geography, genetic variation, the season of harvesting, extraction method, etc. (Dhifi *et al*., 2016).

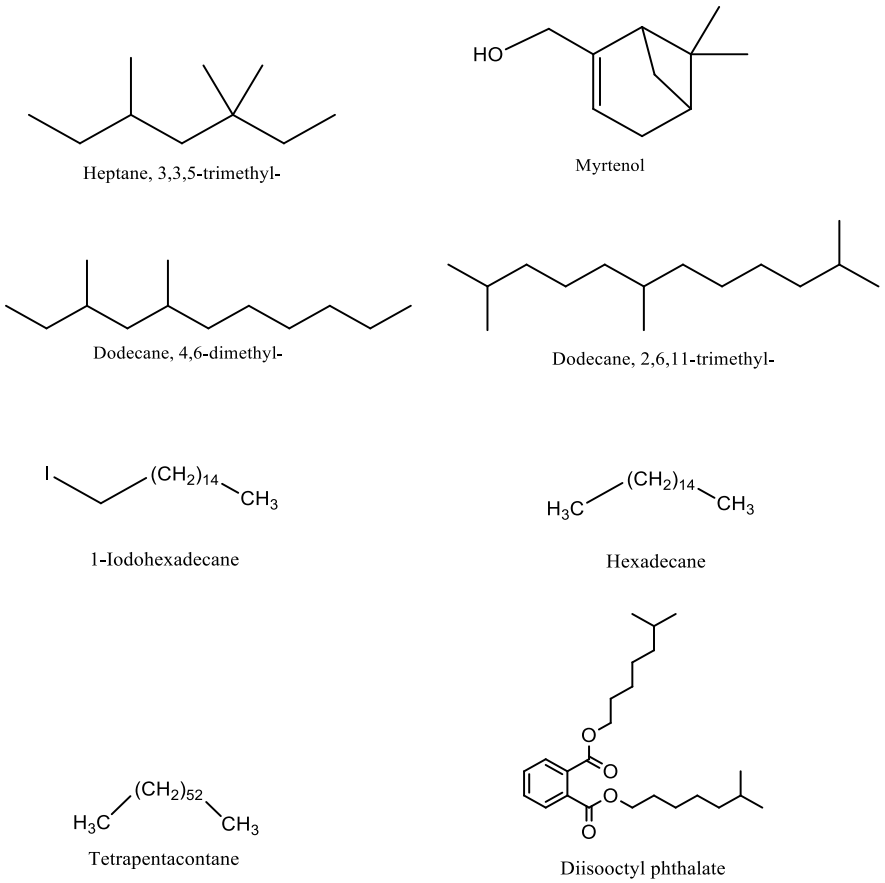


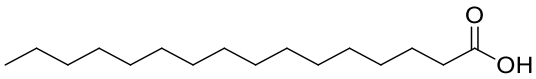
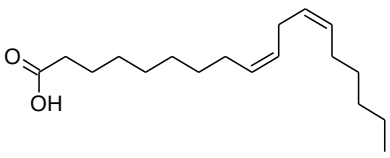
Figure 18: Structures of the molecules in the essential oil of *E. pachyclada*

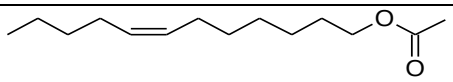
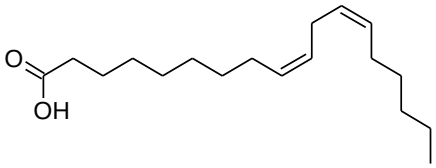
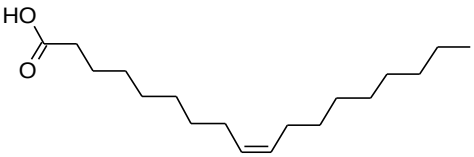
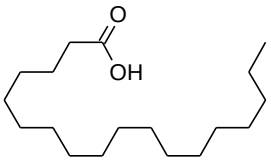
4.3.2 Composition of methanol extract of *Ayenia grandifolia*

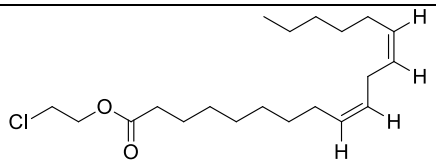
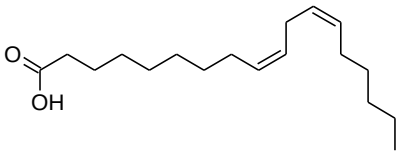
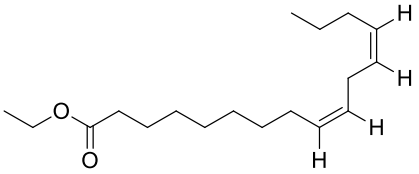
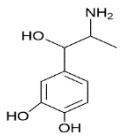
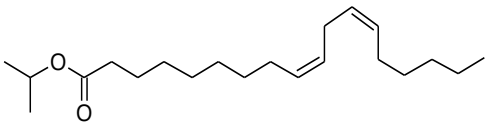
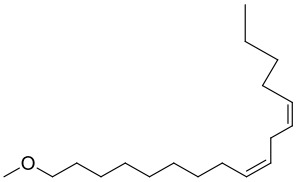
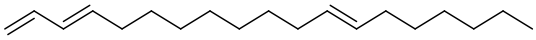
The spectra for each peak in the GC-MS analysis were compared with the NIST08 library to identify each of the compounds present in the methanol extract of the plant. The probable compounds in methanol extract with their retention time and relative

percentage of *A. grandifolia* are listed in Table 9. The compounds were passed through the GC column and the fractions were analyzed by mass spectrometry. The spectra were compared with that of the NIST08 library to find the compounds. Our results showed that the plant contained organic acids, esters, and compounds containing nitrogen. The extract contained 6-Octadecenoic acid, 9,12-Octadecadienoic acid (Z, Z), and oleic acid in the highest amount (27.7%) appeared at the retention time of 41.44 minutes followed by 9,12-Octadecadienoic acid (Z, Z)-, cis-7-Dodecen-1-yl acetate (19.25%) at 41.347 minutes, and 2-Chloroethyl linoleate, 9,12-Octadecadienoic acid (Z, Z)-, ethyl 9,12-Hexadecadienoate (12.32%) at 47.32 minutes. The extract contained a few nitrogen-containing compounds also. At the retention time of 47.69, acenaphtho (1,2b) pyridine, 9-Anthracenecarbonitrile, and naphtha (2, 1, 8, def) quinolone were observed in 0.60%. At 43.26 minutes, 5-Nitro-3-phenyl-1H-indazole, phenethylamine, p-Methoxy-alpha-methyl, ethyl isopropyl dimethylphosphramidate were detected. Similarly, terodiline, 1, 2-Benzenediol, 4-(2-amino-1-hydroxypropyl) (nordephrine), hexanedioic acid, 2-Amino, and fluoxetine were detected in small proportions. At the retention time of 45.23 minutes, cannabidiols were detected in a small proportion (1.29%).

Table 9: Compounds detected in the methanol extract of *Ayenia grandifolia*

Major peaks	Retention time	Area %	Probable compounds
1	9.056	0.49	3-methyl butanoic acid
2	37.711	1.25	trans-9-hexadecenoic acid, cis-9-hexadecenoic acid, cis-11-hexadecenoic acid
3	38.142	11.62	n-hexadecanoic acid 
4	40.699	0.60	Acenaphtho (1,2b) pyridine, 9-anthracenecarbonitrile, Naphthol (2,1,8, def) quinolone
5	41.347	19.25	9,12-octadecadienoic acid (Z, Z)-  cis-7-dodecen-1-yl actetate

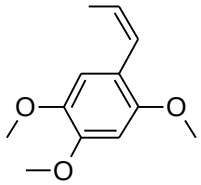
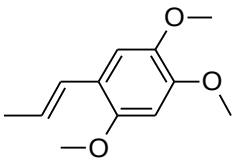
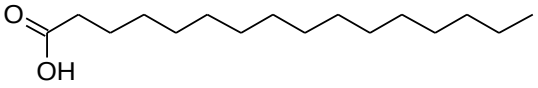
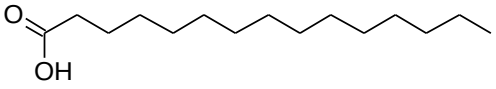
			
6	41.444	27.70	6-octadecenoic acid 9,12-octadecadienoic acid (Z, Z)  oleic acid 
7	41.78	4.96	Octadecanoic acid 
8	43.267	0.32	5-nitro-3-phenyl-1H-indazole, Phenethylamine,p-methoxy-alpha-methyl, ethyl isopropyl dimethylphosphramidate,
9	44.022	2.48	Hexanedioc acid, bis(2-methylpropyl) ester, Hexanedioic acid, bis(2-ethylhexyl) ester, Diisooctyl adipate
10	45.23	1.29	Resorcinol,2-p-mentha-1,8-dien-3-yl-5-pentyl (E) *trans* (Cannabidol), ,3-Benzenediol, 2-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-, (1R-trans) (Cannabidiol)
11	46.266	0.8	9,12-Octadecadienoic acid (Z, Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester
12	46.374	1.04	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester, 2,3-dihydroxypropyl elaidate, Benzenepropanamine, N-(1,1-dimethylethyl)-α-methyl-γ-phenyl (Terodiline)
13.	47.28	12.32	2-chloroethyl linoleate,

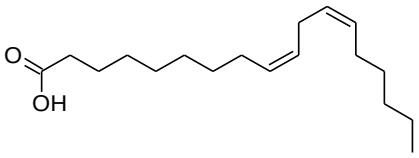
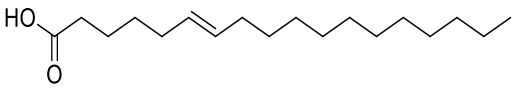
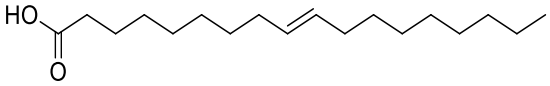
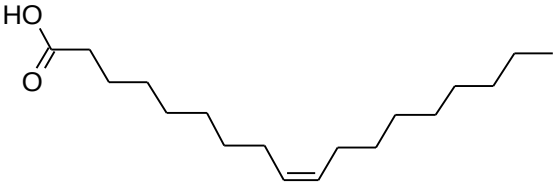
			 9,12-Octadecadienoic acid (Z, Z)-  Ethyl 9,12-hexadecadienoate 
14.	47.388	2.37	Cyclopropaneoctanal, 2-octyl, 9, 17-octadecadienal, R (-)-14-methyl-8-hexadecyn-1-ol
15.	47.982	0.94	1,2-benzenediol, 4-(2-amino-1-hydroxypropyl) (Nordephrine)  Hexanedioic acid, 2- amino fluoxetine
16.	48.187	4.90	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, Cyclohexane, 1-(1-tetradecylpentadecyl)-, 15-Hydroxypentadecanoic acid
17.	53.764	7.66	Isopropyl linoleate  Methyl 9,12-heptadecadienoate  1,3,12 nonadecatriene 

4.3.3 Composition of ethyl acetate fraction of *Ayenia grandifolia*

The spectra for each peak in the GC-MS analysis were compared with the NIST08 library to identify each of the compounds present in the ethyl acetate extract of the plant. The detected compounds with their retention time and relative percentage of the ethyl acetate extract of *A. grandifolia* are listed in Table 10. In this fraction, oleic acid was detected in the highest amount (50.67%) at a retention time of 41.358 minutes. The fraction contained 9, 12-Octadecadienoic acid (16.96%), α-Asarone and β-Asarone (8.84%), n-Hexadecanoic acid, and n-Pentadecanoic acid (13.32%) by the GC-MS analysis. Cannabidiols are an important group of compounds present in *Cannibas sativa* having different pharmacological properties. They can be used for neuroprotection, and against Alzheimer's disease (Li *et al*., 2020). Cannabinoids are reported to selectively attack the malignant cells over normal cells in glioma and acute lymphoblastic leukemia (Lal *et al*., 2021). 2-chloroethyl linoleate, 9,12-Octadecadienoic acid (z, z)-, and Isopropyl linoleate are important which are detected in different plants in GC-MS analysis (Berwal *et al*., 2019; Joshi & Prabhakar, 2020).

Table 10: Compounds detected in the ethyl acetate extract of *Ayenia grandifolia*

Major peaks	Retention time	Area (%)	Probable compounds
1	31.00	8.84	β-Asarone  α-Asarone 
2	38.078	13.32	n-Hexadecanoic acid,  Pentadecanoic acid 
3	41.25	16.96	9,12-Octadecadienoic acid

			
4	41.358	50.67	6-Octadecenoic acid,  9-Octadecenoic acid (E) trans  9-Octadecadecenoic acid (Z) cis (Oleic acid) 
5	41.746	8.14	N-Acetyl-O-trimethylsilyl kanamycin

Both α and β-asarone exhibit different biological activities including neuroprotective, analgesic, antipyretic, and anticonvulsant. They are effective against depression, anxiety, and epilepsy (Liu *et al*., 2020). McGaw *et al*. (2002) isolated β-asarone from the alcoholic rhizome extract of *Acorus calamus* L. and observed antibacterial and anthelmintic activity. Palmitic acid (*n*-Hexadecanoic acid) was isolated from the ethanolic extract of the leaves of *Canthium parviflorum*. The compound exhibited significant activity against both Gram-positive and Gram-negative bacteria (Krishnan *et al*., 2016). This compound was isolated from the ethyl acetate fraction of root barks of *Terminalia glaucescencs* (Bulama *et al*., 2014). Oleic acid was isolated with linoleic acid from the dichloromethane extracts of the leaves of *Helichrysum pedunculatum* by activity-guided fractionation. The compounds had mild antibacterial activity against Gram-positive and Gram-negative bacteria (Dilika *et al*., 2000).

4.4 Estimation of TPC and TFC and antioxidant activity

4.4.1 Estimation of TPC, TFC, and antioxidant activity of methanol extracts

The TPC of the extracts was determined by the Folin-Ciocalteu method using a 96-well plate reader taking gallic acid as standard. The absorbance values (optical density) of the test samples were recorded at 765 nm. The total phenolic content in different extracts, fractions, and the synthesized AgNPs was calculated with the help of a calibration curve (Figure 19) using regression equation y= 0.0459x, and R^2 = 0.9957. The concentration of gallic acid (C_1) established from the calibration curve in mg/mL was used to estimate the total phenolic content in mg/g, in GAE (Gallic Acid Equivalent) of the dry plant material.

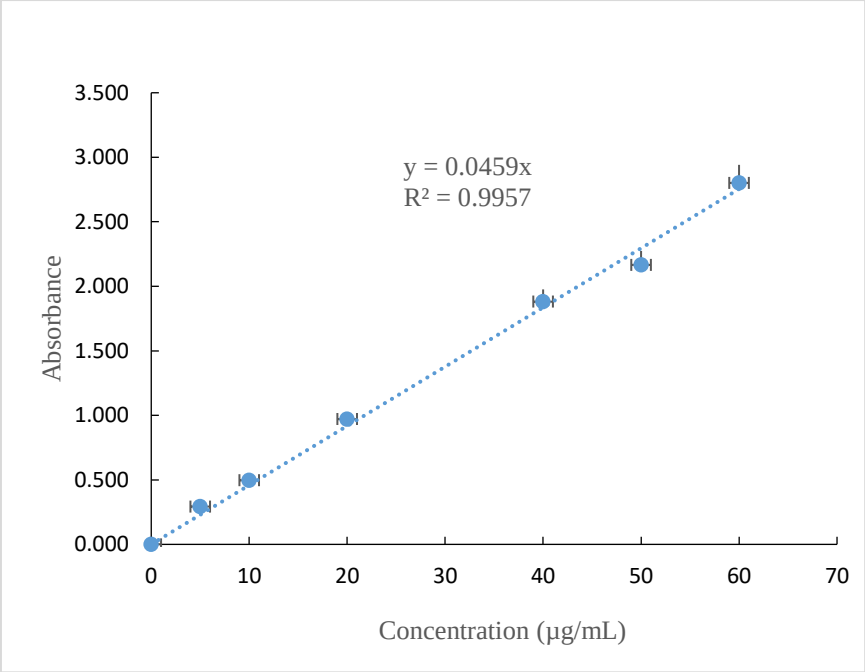


Figure 19: Calibration curve of gallic acid

Similarly, the total flavonoid was determined by using the quercetin calibration curve (Figure 20). The absorbance values (optical density) of the test samples were recorded at 415 nm.The concentration of flavonoid was calculated by using a regression equation y = 0.0239x, and R^2 = 0.9893. Then the TFC was calculated in mg QE/g of dry material.

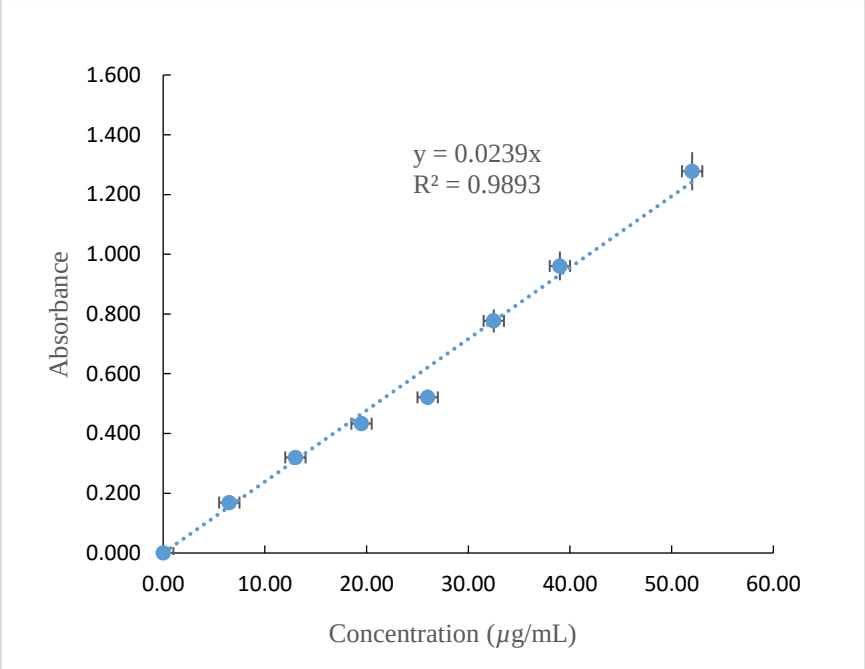


Figure 20: Quercetin calibration curve

The evaluation of TPC and TFC of methanol fractions of all of the plants studied is presented in Table 11. *R. ellipticus* extract contained the highest TPC (145.97 ± 0.62 mg GAE/g), and TFC (94.98 ± 0.73 mg QE/g) followed by *P. pashia* TPC (145.81 ± 0.60 mg GAE/g), and TFC (57.91 ± 0.74 mg QE/g) to the minimum of *S. acuta* with TPC (8.54 ± 0.57 mg GAE/g) and TFC (26.86 ± 0.77 mg QE/g). Eight medicinal plants collected from Eastern Nepal were evaluated for total phenolic and flavonoid contents by Folin-Ciocalteu and aluminum chloride methods respectively. The TPC value ranged from 1076.73 mg GAE/g of *Nephroleps auriculata* to 92.28 mg GAE/g of *Phytolocca acinosa*. Flavonoid content was maximum in the extract of *N. auriculata* (40.8 mg QE/g) followed by *Rumex nepalensis* (39.06 mg QE/g) and minimum TFC was found in *Heracleum nepalense* (7.47 mg QE/g) (Basnet & Kalauni, 2020).

Table 11: TPC and TFC of methanol extracts of medicinal plants

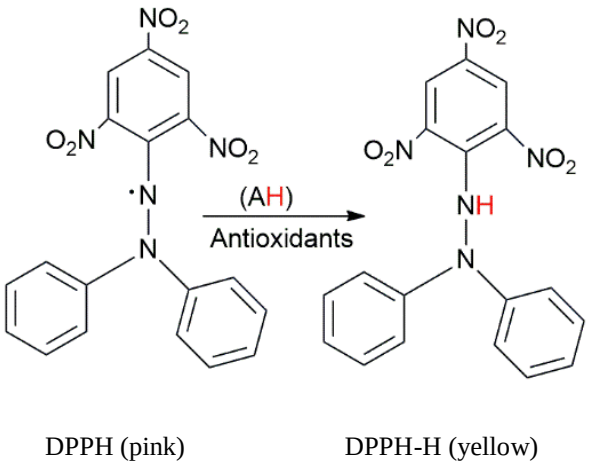
S. No	Name	TPC (mg GAE/g)	TFC (mg QE/g)
1	*A. grandifolia*	107.81 ± 0.55	52.30 ± 0.84
3	*A. helferiana*	45.10 ± 0.70	12.27 ± 0.59
4	*A. indamellus*	86.11 ± 0.38	67.39 ± 0.26
2	*A. lappa*	18.87 ± 0.61	45.94 ± 0.36
5	*A. roxburghiana*	36.56 ± 0.68	27.39 ± 0.35
6	*A. stricta*	23.18 ± 0.26	53.72 ± 0.25
8	*B. megaptera*	102.18 ± 0.97	37.46 ± 0.71
7	*B. roxburghiana*	98.23 ± 0.72	15.93 ± 0.41
10	*C. graveolens*	37.02 ± 0.69	67.25 ± 0.63
9	*C. unilocularis*	16.06 ± 0.74	42.29 ± 0.62
11	*D. bengalensis*	22.28 ± 0.42	31.13 ± 0.92
12	*D. coronans*	91.82 ± 0.34	23.91 ± 0.59
13	*E. pachyclada*	53.29 ± 0.31	31.30 ± 0.88
14	*E.umbellata*	49.96 ± 0.67	20.70 ± 0.76
15	*J. indica*	60.12 ± 0.92	94.06 ± 0.68
16	*M. rubicaulis*	139.20 ± 0.29	12.08 ± 0.46
17	*P. pashia*	145.81 ± 0.60	57.91 ± 0.74
18	*R. ellipticus*	145.97 ± 0.62	94.98 ± 0.73
19	*S. acuta*	8.54 ± 0.57	26.86 ± 0.77
21	*T. parvulum*	23.15 ± 0.42	38.69 ± 0.39
20	*T. wallichiana*	15.96 ± 0.59	67.36 ± 0.89
22	*Z. mauritiana*	97.89 ± 0.73	93.03 ± 0.57

Note: values are mean ± SD (n = 3)

Methanolic extracts of five leading medicinal plants collected from the Far-western region of Nepal were evaluated for the level of TPC and TFC. Among them, *Rheum australe* extract contained the highest TPC and TFC of 249.58 ± 7.73 mg GAE/g and 480.84 ± 8.81 mg RE/g respectively. *Bergenia ciliata* extract had the TPC and TFC of 213.47 ± 2.13 mg GAE/g and 188.01 ± 9.25 mg RE/g. among the five plants studied *Picrorhiza kurroa* contained the lowest amount of TPC and TFC of 59.37± 1.54 mg GAE/g and 39.08 ± 2.61 mg RE/g respectively (Neupane & Lamichhane, 2020a). These reports are consistent with our observations. Phenolics and flavonoids are active plant

secondary metabolites that are responsible for diverse biological activities such as antioxidants, anticancer, antibacterial, cardio-protective, anti-inflammation, immune promoting, UV protection, etc. So they have been considered an interesting candidate for pharmaceutical application and drug discovery (Tungmunnithum *et al*., 2018).

The DPPH radical scavenging assay is a rapid, easy, inexpensive, and one of the most frequently used methods for evaluating the antioxidant potential of plant extracts. This method is based on the principle of reduction of purple-colored DPPH solution to the yellow diphenyl picryl hydrazine (DPPH-H) in the presence of antioxidants (Figure 21).



DPPH (pink) DPPH-H (yellow)

Figure 21: Reduction of DPPH radical by antioxidants

The antioxidant potentials of the crude methanol extracts of all of the plants were screened by the DPPH assay method. Antioxidants in plant extracts convert the purple-colored DPPH solution into a light-yellow product which is detected by the spectrophotometer. Each of the extracts of 250 μg/mL was screened for their radical scavenging activity in a 96-well plate at 517 nm. The preliminary screening of the radical inhibiting activity of the methanol extracts of 22 medicinal plants is shown in Table 10. The result shows that 12 plants had a percentage scavenging of more than 50%. So, these plants were considered the active ones and were further evaluated for the determination of half-maximal concentration (IC_{50}) taking ascorbic acid as a positive control.

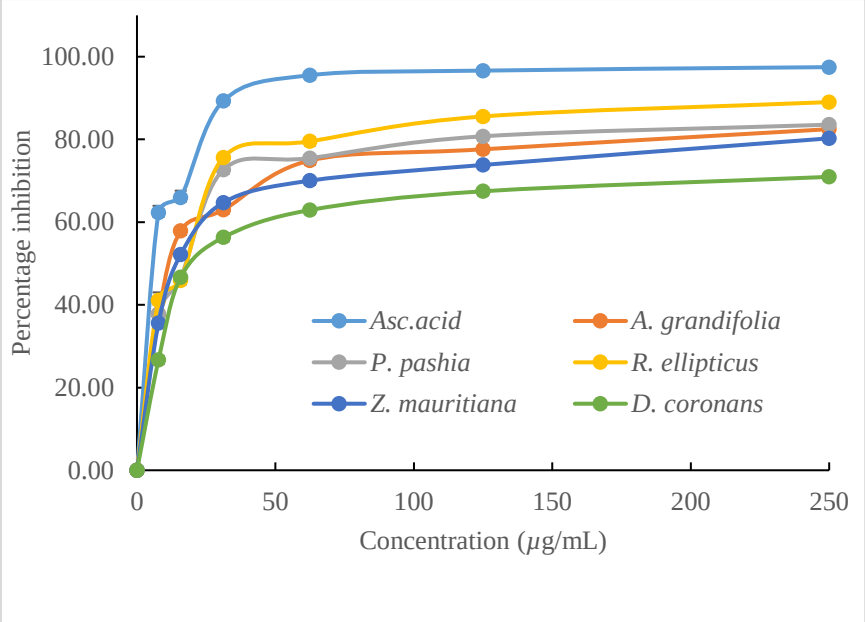


Figure 22: DPPH radical scavenging activity with concentrations of methanol extracts

The percentage inhibition of different extracts of the plants was compared with that of ascorbic acid (Figures 22 and 23).

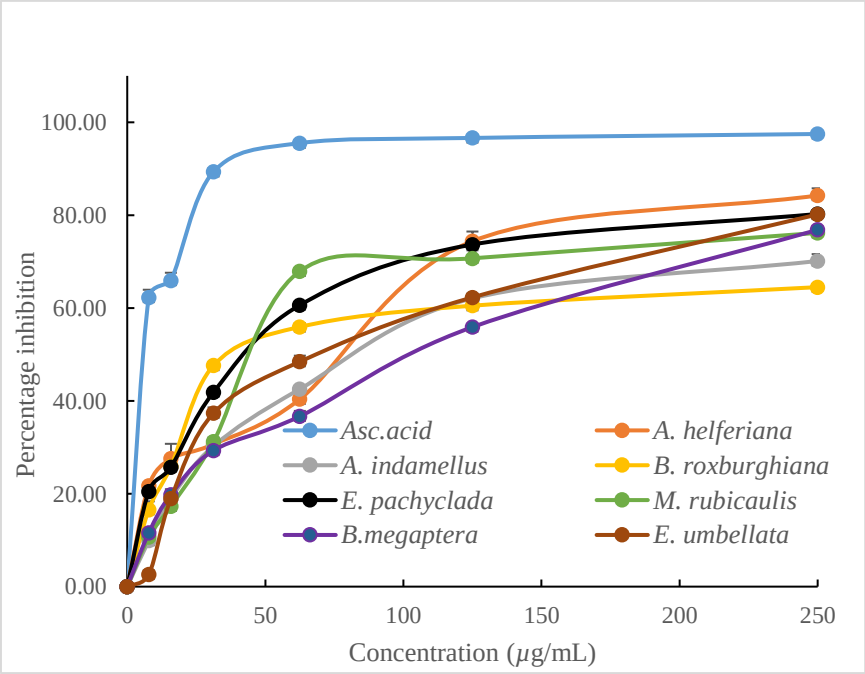


Figure 23: DPPH radical scavenging activity with concentrations of methanol extracts

They showed a dose-dependent variation of inhibition of DPPH free radicals. Most of the plants showed a dose-dependent variation of % scavenging with concentration with ascorbic acid as a standard. The extract of five plants namely *A. grandifolia, Z. mauritiana, R. ellipticus, P. pashia,* and *D. coronans* showed relatively higher

inhibition capacity than that of other extracts. The concentrations inhibiting 50% of the DPPH radical (IC_{50}) by the selected active plant extracts are presented in Table 12.

Based on their inhibitory capacities, the IC_{50} values of 12 plants were calculated which ranged from 12.87 ± 0.14 μg/mL of *A. grandifolia* to 88.62 ± 2.01 μg/mL of *B. megaptera*.

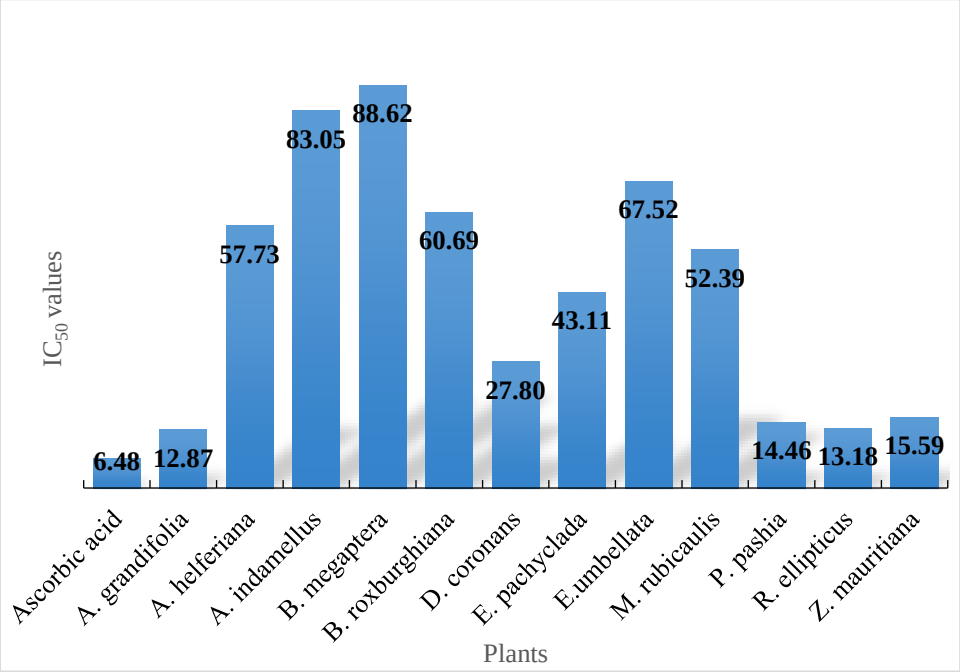
Table 12: Preliminary screening of DPPH inhibitory capacity of methanol extracts

S. No	Name of plants	% Scavenging
1	*A. grandifolia*	82.48 ± 0.31
3	*A. helferiana*	84.97 ± 0.84
4	*A. indamellus*	67.41 ± 0.82
2	*A. lappa*	25.16 ± 0.87
5	*A. roxburghiana*	33.27 ± 0.63
6	*A. stricta*	37.01 ± 0.94
8	*B. megaptera*	76.33 ± 0.92
7	*B. roxburghiana*	66.97 ± 0.71
10	*C. graveolens*	21.19 ± 1.20
9	*C. unilocularis*	16.57 ± 0.88
11	*D. bengalensis*	18.80 ± 0.56
12	*D. coronans*	70.94 ± 0.74
13	*E. pachyclada*	80.25 ± 0.79
14	*E. umbellata*	77.98 ± 1.15
15	*J. indica*	40.53 ± 0.34
16	*M. rubicaulis*	75.47 ± 0.72
17	*P. pashia*	83.56 ± 0.57
18	*R. ellipticus*	89.02 ± 0.59
19	*S. acuta*	34.04 ± 0.13
21	*T. parvulum*	47.97 ± 0.54
20	*T. wallichiana*	30.10 ± 2.27
22	*Z. mauritiana*	80.25 ± 0.99

Note: values are mean ± SD (n = 3), concentration = 250 μg/mL

Our results showed the four plants namely, *A. grandifolia, R. ellipticus, P. pashia*, and *Z. mauritiana* as active antioxidants with IC_{50} values less than 20 μg/mL (Figure 24). The study carried out on the fruit extracts of *Z. mauritiana* from different sites reported

the level of TPC ranging from 172.08 to 328.65 mg GAE/100 g of the extract. They found a significant variation in the antioxidant capacity with IC_{50} values ranging from 14.18 to 28.65 μmol Trolox/g in the DPPH method (Koley *et al*., 2016). Methanol extracts from 15 medicinal plants collected from different parts of Nepal were evaluated for the level of TPC, TFC, and antioxidant activities. Among them, the extracts of *Acacia catechu, Myrica esculenta, mangifera indica, Syzygium cumini,* and *Shorea robusta* were found to exhibit significant DPPH radical scavenging capacity.



Note: values are the means of n = 3

Figure 24: Antioxidant activity (IC_{50}) values in μg/mL) of different plant extracts

The content of TPC and TFC in the extracts was proportional to their antioxidant activity which is similar to our results (Shrestha *et al*., 2021). Similarly, methanol extracts of different parts of eight medicinal plants from of Kaski district of Nepal were evaluated for antioxidant activity by the DPPH method. The extract of *S. operculatus, Astilbe rivularis,* and *Mallotus philippnensis* which had relatively greater TPC and TFC exhibited higher antioxidant activity (Subedi *et al*., 2014). Parajuli *et al*. (2012) evaluated the quantity of TPC, TFC, and antioxidant capacity of methanol extracts from 12 common medicinal plants collected from the Kaski district of Nepal. Among the plants, TPC varied from 440.6 ± 0.4 mg GAE/g of *Diplazium stoliczkae* to 24.9 ± 0.0 mg GAE/g of *Amaranthus viridis.* Similarly, TFC values ranged from 625.5 ± 0.4 mg GAE/g of *D. stoliczkae* to 34.0 ± 0.0 mg GAE/g of *Pogostemon benghalensis.* The

extracts of *Stephania japonica*, *Mimosa rubicaulis himalayana*, *D. stoliczkae*, and *Drynaria propinqua* exhibited significant DPPH radical scavenging activity. The antioxidant activities of six medicinal plants collected from different parts of Nepal were evaluated by DPPH radical scavenging method. Ethanol extract of *Pogostemon cablin* exhibited the highest activity (IC_{50} = 32 μg/mL), followed by *Mallotus philippensis* (IC_{50} = 62.5 μg/mL), *Mussaenda macrophylla* (IC_{50} = 63.5 μg/mL), and *Colebrokea oppositifolia* (IC_{50} = 68 μg/mL) (Subba & Basnet, 2014). The comparative evaluation of the antioxidant potential of five medicinal plants from the farwestern part of Nepal was performed by the DPPH method. On the basis of IC_{50} measurements, *Rheum australe* (IC_{50} = 3.47 ± 0.09 μg/mL) and *Bergenia ciliata* (IC_{50} = 3.56 ± 0.01 μg/mL) respectively. The extract of *Picrorhiza kurroa* was found to be the least efficient DPPH scavenger with an IC_{50} value of 39.08 ± 2.61 μg/mL (Neupane & Lamichhane, 2020a). Seven medicinal plants collected from the Kaski district of Nepal were evaluated for their antioxidant potential by the DPPH method.

Ethanolic extracts of the plants showed varied scavenging capacity with IC_{50} values ranging from 6.27 μg/mL of *Melastoma malabathricum* followed by *Ficus glaberrima* Blume with an IC_{50} value of 11.7 μg/mL and the lowest activity was shown by *Cascabela thevetia.* The antioxidant activity had shown a good correlation with TPC in plants (Khatri & Chhetri, 2020). In a separate study, eight wild vegetables collected from the Rupandehi district of Nepal were analyzed for antioxidant activity. The methanolic extract of *Cassia tora* exhibited the maximum DPPH free radical scavenging capacity (IC_{50} = 9.898 μg/mL) followed by *Leucas cephalotes* (IC_{50} = 33.82 μg/mL), and the lowest activity was displayed by *Basella alba* ((IC_{50} = 45.68 μg/mL). The potential role of phenolic and flavonoids in the antioxidant activity of these plants was established by the correlation analysis (Aryal *et al*., 2019).

Methanolic extracts of *Rosa sericea, Fritillaria corrhosa, Neopicrorhiza scrophulariflora,* and *Juniperus squamata* collected from the Gorkha district of Central Nepal were evaluated for TPC, TFC, and antioxidant activity. The extract of *R. sericea* contained the highest TFC of 34.41 ± 0.47 mg QE/g and that of *J. squamata* highest TPC of 321.5 ± 0.7 mg GAE/g. These plants exhibited significant DPPH radical scavenging activity indicating the potential role of phenolics and flavonoids in the antioxidant property of the plant (Bhatt & Basukala, 2017). The extracts of four medicinal plants of Nepalese origin showed antioxidant capacity strongly correlated to

corresponding TPC and TFC values. The ethanolic extract of *Mangifera indica* contained the highest level of TPC and TFC of 353.81 ± 0.84 mg GAE/g and 310.54 ± 0.00 mg QE/g followed by *Magnolia grandifolia* with 150.20 ± 6.46 mg GAE/g and 231.72 ± 5.93 mg QE/g respectively. *M. indica* exhibited the highest antioxidant activity (IC_{50} = 5.91 μg/mL) followed by *M. grandifolia* (IC_{50} = 7.63 μg/mL) and the lowest activity was shown by *Nephrolepis cardifolia* (IC_{50} = 66.65 μg/mL) which contained the lowest level of phenolic and flavonoids (Ghimire *et al*., 2019).

4.4.2 TPC, TFC, and antioxidant activity of different solvent extracts of *E. pachyclada* and *B. roxburghiana*

The active plants namely *E. pachyclada* and *B. roxburghiana* were evaluated for phytochemical screening, estimation of TPC, TFC, and antioxidant and antibacterial activities. The extracts of polar solvents like water, ethyl acetate, and methanol have relatively higher TPC and TFC in comparison to the extracts of less polar solvents which is comparable to the results of Jaiswal *et al*. (2012). The BRM contained the highest TPC, TFC values, and antioxidant activity. Methanol extracts of *B. roxburghiana* and *E. pachyclada* contained the highest TPC (106.73 ± 1.62 and 54.42 ± 1.40 mg GAE/g) respectively. The hexane extracts of the plants contained the lowest values of TPC and TFC. The TFC of *n*-hexane extract of *B. roxburghiana* is 29.98 ± 0.9 mg QE/g) and that of *E. pachyclada* is 21.44 ± 2.91 mg QE/g. The dose-dependent variation of the radical inhibiting capacity of different extracts with concentrations of *B. roxburghiana* and *E. pachyclada* are shown in Figures 25 and 26 respectively. Methanol extracts of both of the plants showed higher rates of inhibition than other extracts.

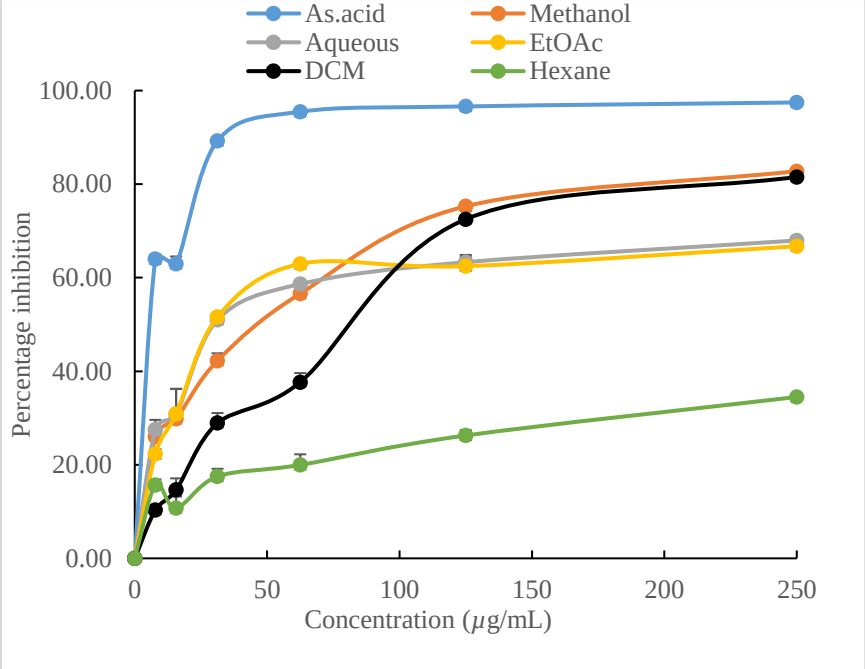


Figure 25: DPPH radical inhibitory capacity of different extracts of *B. roxburghiana*

Many investigation results reveal that the amount of TPC and TFC in the same plant extract was greatly influenced by the polarity of the extracting solvents. Elhadef *et al*. (2020) reported the trend of variation of TPC and TFC in different solvents as water <95% ethanol <80% ethanol in the sample of Tunisian *E. alata* which is comparable to our study methanol> EtOAc> DCM> *n*-hexane.

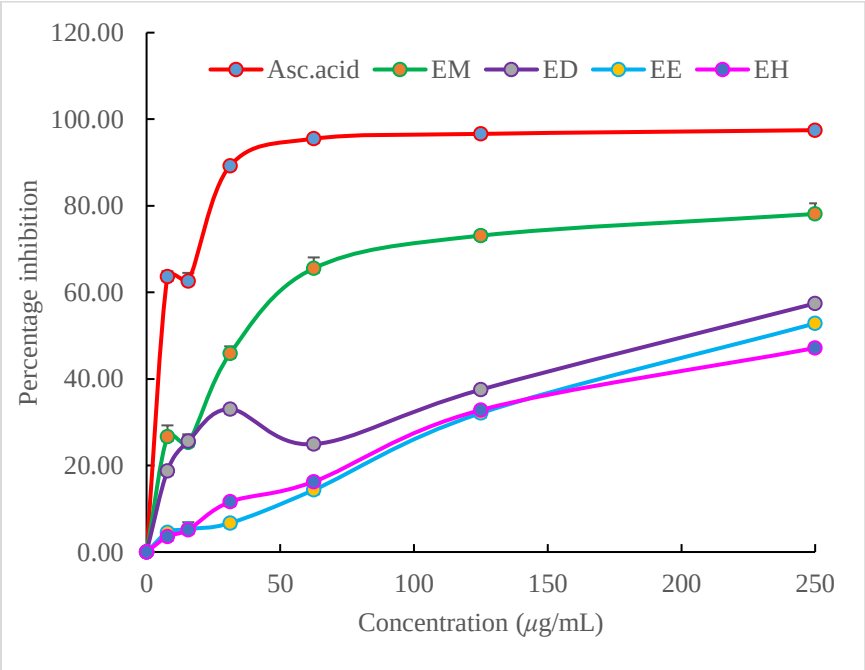


Figure 26: DPPH radical inhibitory capacity of different extracts of *E. pachyclada*

The same species of the plant collected from Palestine contained TPC in the order of water <95% ethanol <80% ethanol and TFC as Water <80% ethanol <95% ethanol (Al-Rimawi *et al*., 2017). The TPC and TFC contents in ephedra plants were evaluated by using different solvents and extraction protocols. The extracts were prepared by maceration and ultra-sound assisted methods using 80% ethanol, 80% methanol, and 50% methanol with 1% glacial acetic acid. The extract obtained by ultrasound-assisted technique contained higher TPC but no significant difference was found in the case of TFC. Irrespective of the extraction methods, *E. gerardiana*, and *E. ciliata* had the lower TPC whereas *E. chilensis* was reported the highest TPC followed by *E. equisetina*, *E. sinica*, and *E. distachya* (Ibragic *et al*., 2021). The antioxidant activities of different extracts of *B. roxburghiana* and *E. pachyclada* were evaluated by the DPPH method taking ascorbic acid as a positive control. The results of the study presented in Table 13 show that out of five samples of *B. roxburghiana* evaluated, BRM exhibited the highest activity (IC_{50} = 39.86 ± 3.69 μg/mL), followed by BRA (IC_{50} = 43.55 ± 6.16 μg/mL), and BRE (IC_{50} = 44.30 ± 5.88 μg/mL) with closely equivalent activity. The extracts of DCM and *n*-hexane had very low antioxidant activities. Similarly, methanol extract of *E. pachyclada* (EPM) showed the highest activity (IC_{50} = 37.81 ± 2.24 μg/mL), followed by EPE (IC_{50} = 230.30 ± 4.75 μg/mL), EPD (IC_{50} = 249.97 ± 17.65 μg/mL), and the lowest activity was exhibited by EPH (IC_{50} = 278.93 ± 23.52 μg/mL). The results of this study are in agreement with the results of other studies where the extracts of polar solvents exhibited relatively higher biological activities than that of less polar solvents. Crude methanol extract from the root and stem of *E. gerardiana* from Pakistan was fractionated into five different solvents. The fractions of ethyl acetate (IC_{50} = 2.96 ± 0.39 μg/mL) and *n*-butanol (IC_{50} = 2.73 ± 0.84 μg/mL) showed strong DPPH radical scavenging activity than the fractions of less polar solvents which is consistent to our findings (Khan *et al*., 2017). Similarly, Alam *et al*. (2017) reported analogous results of the antioxidant activity of methanol extract and its fractions of *Clinacanthus nutans* collected from Malaysia. The DPPH radical scavenging activity was found in the order of ethyl acetate > butanol > hexane > methanol > aqueous fraction. Phenolic and flavonoids are the chief constituents in plants that contribute the antioxidant activity. Portions of plants used, state of maturity, collection time, the polarity of solvents, season, etc., play a significant influence on the number of phytochemicals in the extract (Wakeel *et al*., 2019). Different parts of *Carica papaya* exhibited dissimilar antioxidant activities on the DPPH method but were positively correlated with TPC and TFC

(Maisarah *et al*., 2013). Here, we also observed the DPPH radical scavenging capacity of the fractions or the extract reliant upon corresponding TPC and TFC.

Table 13: TPC and TFC, and antioxidant capacity (IC_{50}) of different extracts of *E. pachyclada* and *B. roxburghiana*

Extracts	TPC (mg GAE/g)	TFC (mg QE/g)	IC_{50} (μg/mL)
BRA	78.41 ± 0.34	39.86 ± 0.90	43.55 ± 6.16
BRE	90.69 ± 2.71	49.79 ± 1.07	44.30 ± 5.88
BRM	106.73 ± 1.62	99.32 ± 0.66	39.86 ± 3.69
BRD	74.87 ± 0.93	35.59 ± 0.90	71.50 ± 4.70
BRH	65.59 ± 1.79	29.98 ± 0.90	1498.67 ± 62.13
EPE	46.84 ± 0.62	31.73 ± 0.52	230.30 ± 4.75
EPM	54.42 ± 1.40	33.28 ± 0.48	37.81 ± 2.24
EPD	19.58 ± 0.24	31.64 ± 0.56	249.97 ± 17.65
EPH	5.21 ± 1.49	21.44 ± 2.91	278.93 ± 23.52
*Ascorbic acid	-	-	6.40 ± 0.29

Note: *ascorbic acid= positive control, values are mean ± SD (n = 3)

BRA= *B. roxburghiana* aqueous extract, BRE = *B. roxburghiana* ethyl acetate extract, BRM = *B. roxburghiana* Methanol extract, BRD= *B. roxburghiana* DCM extract, BRH = *B. roxburghiana* n-hexane extract, EPA = *E. pachyclada* ethyl acetate extract, EPM = *E. pachyclada* methanol extract, EPD = *E. pachyclada* DCM extract and EPH = *E. pachyclada*, and *n*-hexane extract

4.4.3 TPC, TFC, and antioxidant activities of different fractions of *A. grandifolia*

The total phenolic and flavonoid contents in different fractions of *A. grandifolia* were evaluated by the Folin-Ciocalteu and aluminum chloride methods respectively. The results presented in Table 14 show that ethyl acetate extract contained the highest phenolic (128.53 ± 1.79 mg GAE/g) as well as flavonoid content (122.68 ± 2.74 mg QE/g). Methanol extract contained a TPC of 111.58 ± 1.18 mg GAE/g followed by a DCM fraction of 111.18 ± 1.75 mg GAE/g, aqueous fraction of 66.70 ± 1.79 mg GAE/g, and the lowest value was obtained for the *n*-hexane fraction 51.81 ± 1.32 mg GAE/g. The trend of TFC variation is a little bit different from that of TPC. Ethyl acetate fraction > Methanol extract > aqueous fraction > DCM fraction > *n*-hexane fraction. The solvent polarity and extraction method greatly influence the quality and quantity of phytochemicals and biological activities of the plant extracts (Rafińska *et al*., 2019).

Crude methanol extract of *Torilis leptophylla* was separated into *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and residual aqueous fractions.

Table 14: TPC, TFC, and IC_{50} of methanol extract and fractions of *A. grandifolia*

Fractions	TPC (mg GAE/g)	TFC (mg QE/g)	IC_{50} (μg/mL)
AM	111.58 ± 1.18	116.90 ± 1.01	30.93 ± 3.18
AH	51.81 ± 1.32	8.95 ± 0.65	451.17 ± 8.5
AD	111.18 ± 1.75	23.54 ± 0.80	74.78 ± 4.12
AE	128.53 ± 1.79	122.68 ± 2.74	12.044 ± 0.61
AW	66.70 ± 1.79	63.88 ± 2.07	243.80 ± 4.22
Ascorbic acid*	---	---	6.48 ± 0.43

Note: *ascorbic acid = positive control, values are mean ± SD (n = 3)

AM= *A. grandifolia* methanol extract, AH= *A. grandifolia n*-hexane fraction, AD = *A. grandifolia* DCM fraction, AE = *A. grandifolia* ethyl acetate fraction, AW = *A. grandifolia* water fraction, TPC = Total phenolic content, TFC = Total flavonoid content, GAE = Gallic acid equivalents, QE = Quercetin equivalents, DPPH = DPPH radical, n.d. = Not Determined

Each of the fractions was evaluated for antiradical activity by different methods. On the basis of EC_{50} (concentration required to reduce 50% of the radical) values, the antioxidant capacity followed the order of *n*-butanol > EtOAc > $CHCl_3$ > methanol > water fractions. The antioxidant capability of different fractions had a substantial correlation to the corresponding level of TPC and TFC which is in agreement with this study (Saeed *et al*., 2012). The antioxidant activity of all of the fractions was evaluated by different methods. The radical inhibiting activity of different fractions of the plant showed a linear variation with concentration (Figure 27). The ethyl acetate fraction showed the highest DPPH radical inhibition rate followed by methanol, aqueous, and DCM. The lowest inhibition rate was observed for the *n*-hexane fraction.

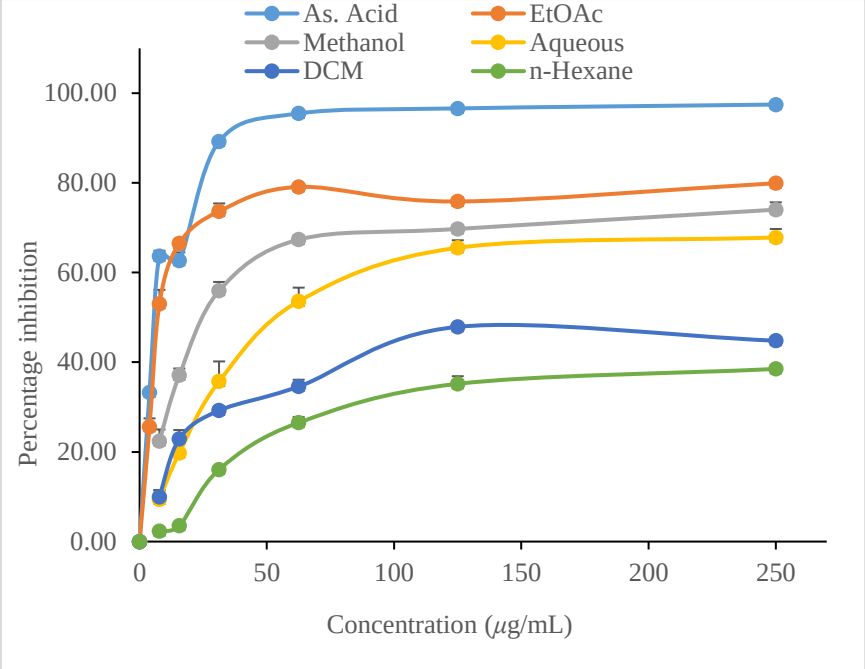


Figure 27: DPPH scavenging capacity of ascorbic acid and fractions of *A. grandifolia*

The relationship between TPC and TFC with antioxidant capacity was analyzed by using Pearson's correlation in Figure 28. The level of TPC in the fractions of *A. grandifolia* showed a strong correlation (R^2 = 0.922) with corresponding IC_{50} values but the TFC showed a feeble correlation (R^2 = 0.5301) which is comparable to the studies of (Lyu *et al*., 2022; Muflihah *et al*., 2021). The phytochemical and antioxidant properties of crude methanol extract and different fractions of *P. pashia* of Chinese origin were evaluated.

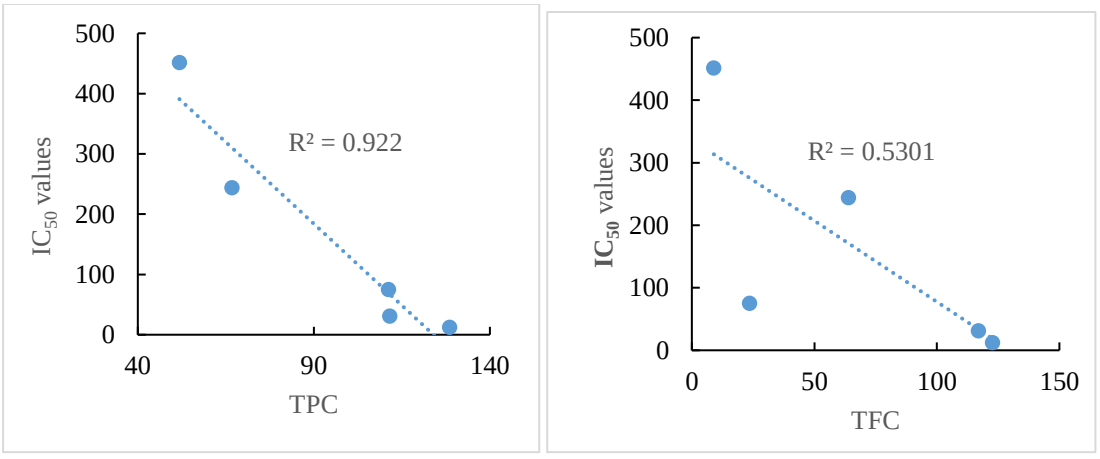


Figure 28: Correlation of TPC and TFC with antioxidant activity (IC_{50})

The TPC level ranged from 28.10 mg GAE/g for the aqueous extract to the maximum of 168.9 mg GAE/g for the EtOAc fraction. Similarly, flavonoid content was found to

be 51.2 mg RE/g for the aqueous fraction to the maximum of 278.8 mg RE/g for EtOAc extract. In the DPPH method, the IC_{50} values ranged from 2.47 μg/mL of EtOAc fraction to the maximum of 167.5 μg/mL for the aqueous fraction. The results indicated a good correlation between the level of TPC and TFC to antioxidant activity (He *et al*., 2015). Similar to our study, Ghimire *et al*. (2011) reported correlations of TPC and TFC of different medicinal plants collected from Nepal to corresponding DPPH radical scavenging activity.

4.4.4 TPC, TFC, and antioxidant activity of plant extracts and synthesized AgNPs

The involvement of the secondary metabolites especially phenolic and flavonoids in the synthesis, capping, and stabilizing of AgNPs can be ascertained by the estimation of TPC and TFC in corresponding extracts. These compounds reduce Ag^+ ions into silver nanoparticles. The quantity of TPC and TFC in the aqueous plant extracts and the corresponding AgNPs are shown in Table 15.

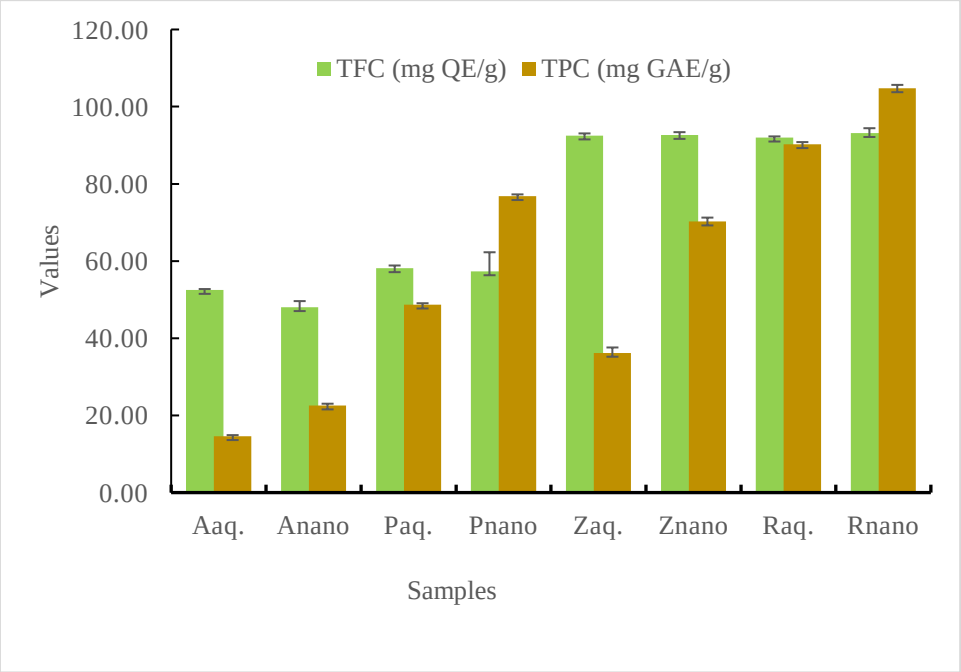
Table 15: TPC, TFC, and antioxidant capacity (IC_{50}) of plant extracts and synthesized AgNPs

Samples	IC_{50} (μg/mL)	TPC (mg GAE/g)	TFC (mg QE/g)
Ascorbic acid	8.19 ± 0.13	--	--
A.grandifolia (Aq.)	142.77 ± 10.75	14.61 ± 0.28	52.50 ± 0.25
Ag-AgNPs	63.76 ± 5.87	22.55 ± 0.48	48.03 ± 1.58
P. pashia (aq.)	13.66 ± 0.35	48.70 ± 0.36	58.11 ± 0.73
Pp-AgNPs	10.67 ± 0.05	76.81 ± 0.46	57.32 ± 4.95
Z. mauritiana (aq)	37.02 ± 1.00	36.20 ± 1.40	92.65 ± 0.74
Zm-AgNPs	17.55 ± 0.49	70.23 ± 1.02	92.51 ± 0.56
R. ellipticus (aq.)	15.86 ± 4.14	90.97 ± 0.55	91.97 ±0.33
Re-AgNPS	13.85 ± 0.34	104.75 ± 0.89	93.14 ± 1.27

Note: values are mean ± SD (n = 3)

Total phenolic contents of the aqueous extracts and corresponding AgNPs of *A. grandifolia*, *P. pashia, R. ellipticus,* and *Z. mauritiana* were 14.61 ± 0.28, 48.70 ± 0.36, 90.97 ± 0.55, and 36.20 ± 1.40 mg GAE/g and, 22.55 ± 0.48, 76.81 ± 0.46, 104.75 ± 0.89, and 70.23 ± 1.02 mg GAE/g respectively. Similarly, the TFC of aqueous extracts and the corresponding synthesized AgNPs were 52.50 ± 0.25, 58.11 ± 0.73, 91.97 ± 0.33, and 92.51 ± 0.56 mg QE/g and 48.03 ± 1.58, 57.32 ± 4.95, 93.14 ± 1.27 and 92.65

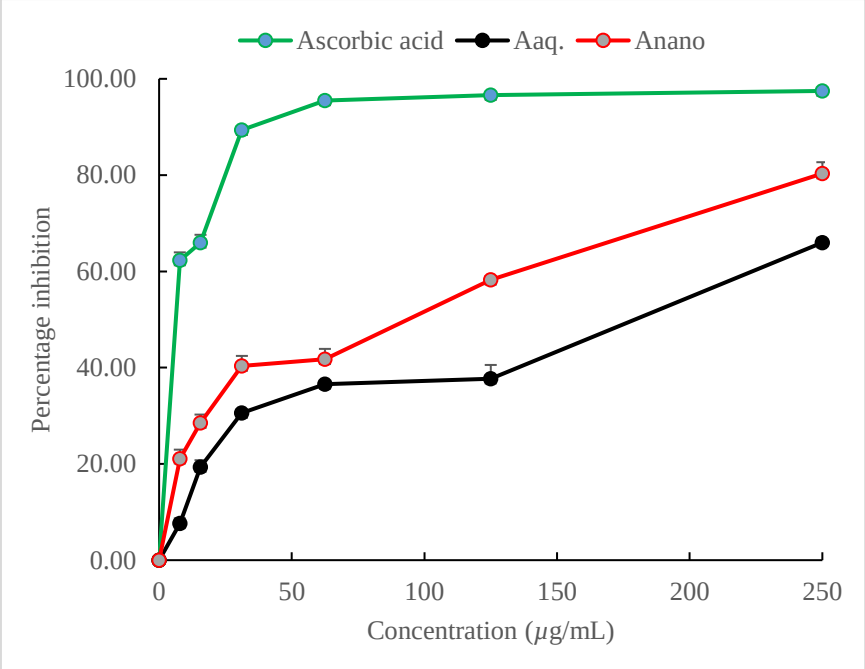
± 0.74 mg QE/g respectively. Here, values of TPC for AgNPs are higher, but TFC values for AgNPs are nearly analogous to those of corresponding extracts (Figure 29). The observation of higher quantity of TPC corroborates the possible role of the polar phenolic compounds in the reduction of $AgNO_3$, capping, and stabilizing AgNPs. It stipulates the major role of phenolics in the synthesis of AgNPs in comparison to flavonoids. A similar result was obtained in the synthesis of AgNPs using *Astragalus tribuloides* extract (Sharifi-Rad, Pohl, *et al*., 2020). The concentration of the extracts and AgNPs against relative percentage inhibition was plotted by taking ascorbic acid as a standard. In all cases, the percentage inhibition of DPPH varied with concentration. The synthesized AgNPs of all four plants exhibited higher rates of variation than their corresponding aqueous extracts (Figures 30-33).



Note: values are means of n = 3

Figure 29: TPC and TFC of aqueous extracts and AgNPs

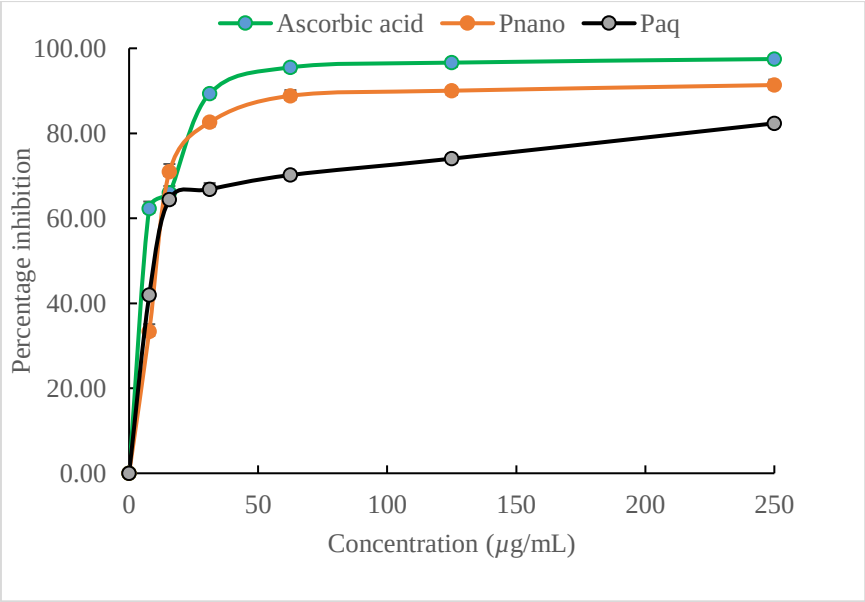
The antioxidant activity of the synthesized AgNPs is found to be proportional to the TPC and TFC. The results of our study show that all of the AgNPs had higher antioxidant activities than their extracts.



Note: Aaq = *A. grandifolia*-aqueous extract, Anano = Ag-AgNPs

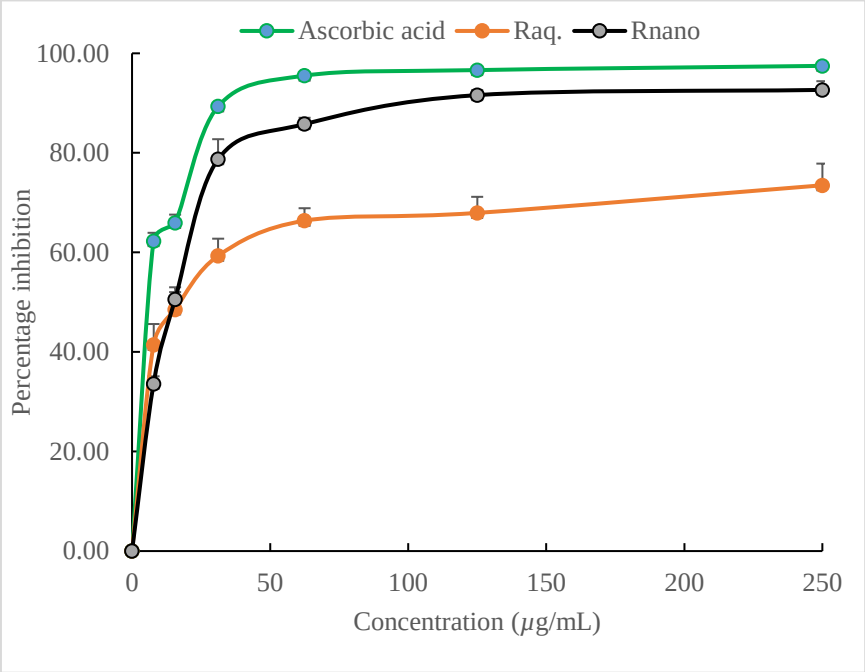
Figure 30: DPPH radical capacity of aqueous extract and Ag-AgNPs

A. grandifolia extract mediated AgNPs was found enhanced antioxidant property (IC_{50} = 142.77 ± 10.758 µg/mL) than its crude extract (IC_{50} = 63.76 ± 5.87 µg/mL)). Similarly, P. *pashia* mediated AgNPs ((IC_{50} = 17.55 ± 0.495 µg/mL), crude (IC_{50} = 13.66 ± 0.35 µg/mL), *Z. mauritiana* mediated AgNPs (IC_{50} = 36.20± 1.40 µg/mL) and crude (IC_{50} = 37.02 ± 1.00 µg/mL), *R. ellipticus* mediated AgNPs (IC_{50} = 13.85 ± 0.34 µg/mL) and crude extract (IC_{50} = 15.86 ± 4.14 µg/mL) were obtained (Table 15).



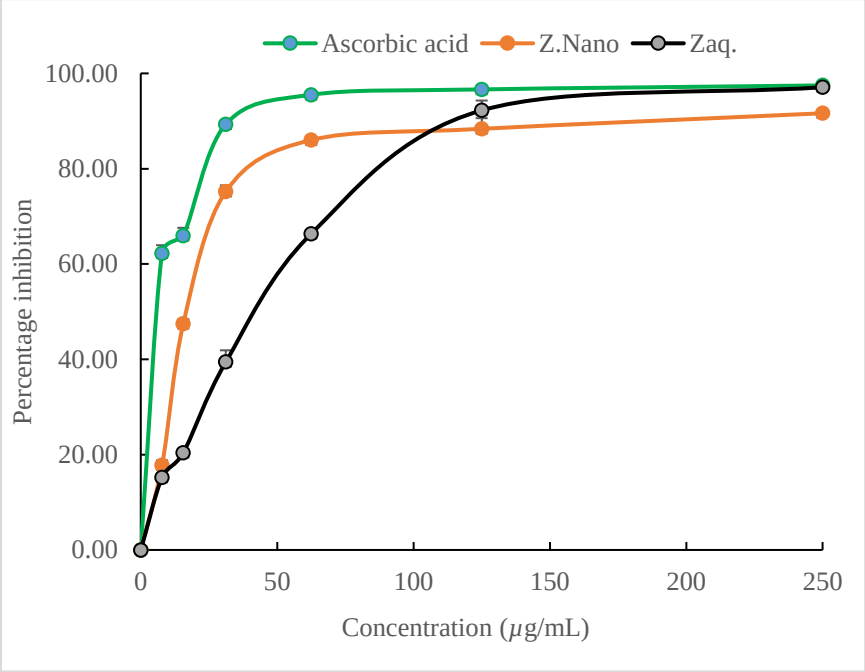
Note: Paq = *P. pashia*-aqueous extract, Pnano = Pp-AgNPs

Figure 31: DPPH radical scavenging capacity of aqueous extract and Pp-AgNPs



Note: Raq. = *R. ellipticus*-aqueous extract, Rnano = Re-AgNPs

Figure 32: DPPH radical scavenging capacity of aqueous extract and Re-AgNPs



Note: Zaq. = *Z. mauritiana*-aqueous extract, Z. Nano = Zm-AgNPs

Figure 33: DPPH radical scavenging capacity of aqueous extract and Zm-AgNPs

Singh and Dhaliwal (2018) also reported higher antioxidant activities of the *Nepeta leucophylla* root extract-mediated AgNPs than their aqueous extracts. The leaf extract of *Tridax procumbens* of Indian origin was used for the green synthesis of AgNPs. The

nanoparticles showed higher amounts of TPC and TFC with elevated DPPH inhibition activity (Rani *et al*., 2020). The AgNPs synthesized by using an aqueous fruit extract of *Prosopis farcta* of Iranian origin had TPC and TFC values higher than that of the crude extract. They reported relatively higher antioxidant activity of the AgNPs (IC_{50} = 0.70 ± 0.09 mg/mL) than that of the extract (IC_{50} = 1.64 ± 0.08 mg/mL) which are comparable to our results (Salari *et al*., 2018). In a separate study, the synthesized AgNPs of aerial parts of *Asphodelus aestivus* were reported to exhibit lower antioxidant activity than the extract. The antioxidant activity was proportional to TFC but not to TPC (Fafal *et al*., 2017). The electron-donating ability of the quinonoid compounds that are produced during the oxidation of phenolics is sufficiently adsorbed on the surface of nanoparticles. Different phytochemicals like glycosides, alkaloids, tannins, flavonoids, phenolics, phytosterols, proteins, saponins, etc. were reported in the extract of the plants. The reduction of $AgNO_3$ into AgNPs might be due to the formation of intermediate complexes with phenolic–OH groups of the hydrolyzable tannins that subsequently oxidize into quinine (Vivek *et al*., 2012). Flavonoids which are the major capping agents in the synthesis of nanoparticles increase the antioxidant activity of AgNPs (Singh *et al*., 2021). DPPH is a stable lipophilic free radical that can accept electrons or donate hydrogen from antioxidants to convert purple to yellow which is easily detected at 517 nm (Inbathamizh *et al*., 2013). Important phytochemicals including alkaloids, terpenoids, flavonoids, proteins, vitamins, and polyphenols in plant extracts reduce Ag^+ ions into Ag° and inhibit agglomeration of AgNPs by chelating on metals (Nikaeen *et al*., 2020).

4.5 Antibacterial activity

4.5.1 Preliminary screening

Bacteria have a vital role in humans and their activities. Some species of bacteria are very useful and some are and some are disadvantageous to human health. Several medicinal plants have been reported to exhibit significant antibacterial activities and have been used for the treatment of many health complications for many years (Izah, 2018). Different parts of medicinal plants and their active compounds have long been used by humans in traditional medicine against several bacterial infections. The improper or overuse of synthetic as well as plant-derived antibacterial agents has led to the development of multidrug resistance capability that is creating a major threat and a

stern challenge to the medical field (Swamy *et al*., 2017). The emergence of antimicrobial resistance has created pressure on conventional antimicrobial agents. There are a large number of lead compounds in medicinal plants that can be used for the development of novel drugs to combat antimicrobial resistance (Nigussie *et al*., 2021). The preliminary antibacterial susceptibility of the methanol extracts of all of the 22 plants was evaluated by the agar well diffusion method. Among the 22 plants tested, 14 plant extracts showed moderate to strong activity against the tested bacteria (Table 16).

Table 16: Zones of inhibition (mm) of methanol extracts from different plants:

S.No.	Plants	P.C.	*S. aureus*	P.C.	*E. coli*	P.C. *K. pneumonia*		P.C.	*S. typhi*
1	*A. stricta*	13.0	NS	15.0	NS				
2	*C. graveolens*	13.0	NS	15.0	NS				
3	*D. bengalensis*	13.0	NS	15.0	NS				
4	*E. pachyclada*	15.0	12.0	15.0	13.0	15.0			
5	*D. coronans*	15.0	8.0	15.0	NS				
6	*B. roxburghiana*	15.0	11.0	15.0	NS	15.0	12.0	15.0	12.0
7	*Z. mauritiana*	12.0	9.0	13.0	NS				
8	*A. indamellus*	12.0	NS	13.0	NS				
9	*T. parvulum*	12.0	NS	13.0	NS				
10	*J. indica*	18.0	NS	15.0	NS				
11	*R. ellipticus*	18.0	17.0	15.0	NS				
12	*E. umbellate*	18.0	NS	15.0	NS				
13	*A. lappa*	16.0	7.0	15.0	NS				
14	*P. pashia*	16.0	14.0	15.0	NS				
15	*M. rubicaulis*	16.0	6.0	15.0	NS				
16	*T. wallichiana*	17.0	NS	17.0	NS				
17	*A. roxburghiana*	17.0	NS	17.0	NS				
18	*A. helferiana*	17.0	8.0	17.0	NS				
19	*S. acuta*	16.0	NS	15.0	NS				
20	*B. Megaptera*	16.0	7.0	15.0	NS				
21	*C. unilocularis*	16.0	NS	15.0	NS				
22	*A. grandifolia*	16.0	14	15.0	13.0				12.0

Note: PC = neomycin, NS = non-significant

The results shows that *Rubus ellipticus* extracts had the strong activity (ZOI = 17.0 mm) against *S. aureus*, *A. grandifolia* showed moderate activity against *S. aureus* (ZOI = 14.0 mm), *E. coli* (ZOI = 13.0 mm), and *S. typhi* (ZOI = 12.0 mm).

The methanol extract of *B. roxburghiana* showed mild activity against all of the tested organisms. *E. pachyclada* was active against *E. coli* and *S. aureus* only. Methanol extracts of *A. stricta, C. graveolens, A. indamellus, J. indica, T. wallichiana, A. roxburghiana, S. acuta,* and *C. unilocularis* were found inactive against the tested bacteria. The antibacterial susceptibility of methanol extract of *Mahonia nepaulensis* collected from Kathmandu was evaluated by using the disc diffusion method. The extract was tested against five human pathogens named *Staphylococcus aureus, Escherichia coli, Shigella spps., Pseudomonas aeruginosa,* and *Salmonella typhi* by taking chloramphenicol as a standard. The extract showed maximum activity against *S. aureus* (ZOI = 27.3 mm), followed by *P. aeruginosa, Shigella spps,* and *E. coli*, and the least activity was shown against *S. typhi* (Paudel *et al*., 2020). Basnet and Kalauni (2020) evaluated the antibacterial activity of eight medicinal plants collected from the Ilam district of Nepal against *E. coli* and *S. aureus* by using the agar well diffusion method. At the concentration of 100 mg/mL, the extract of *Erythrina arborescens* exhibited the highest activity with ZOI values of 11 mm for *E. coli* and 14 mm for *S. aureus*. Seven medicinal plants collected from the Tanahun and Dhankuta districts of Nepal were tested for antibacterial activity against *E. coli, S aureus, K. pneumoniae,* and *Proteus vulgaris.* Among the plants, four had remarkable antibacterial activity (Subba & Basnet, 2014). Fifteen medicinal plants collected from different parts of Nepal were evaluated for antibacterial activity by agar well diffusion and broth dilution method. Among the plants tested, *Morus australis* exhibited the highest activity against *K. pneumoniae* (ZOI = 25 mm, and MIC = 0.012 mg/mL) followed by *S. aureus* (ZOI = 22 mm, MIC = 0.012 mg/mL). Moreover, *Morus australis, Eclipta prostate*, and *Hypercium cordifolium* extracts displayed significant activities against the tested microorganisms (Shrestha *et al*., 2021). The antibacterial activity of five medicinal plants from the Sagarmatha region was assessed by using hexane, dichloromethane, and methanol as the extracting solvents. They were tested against one Gram-positive (*S. aureus*) and three Gram-negative strains (*E. coli, K. pneumoniae, and P. aeruginosa*). Similar to the present study, the majority of the extracts were found active against *S. aureus* in comparison to Gram-negative strains (Bhattarai & Basukala, 2016). The

antibacterial activity of 40 medicinal plants which are extensively used by the local people of Manang, Mustang, and Nawalparasi districts against different ailments was evaluated by the disc diffusion method. Among the crude methanolic extracts examined, 21 extracts including *Artemisia caruifolia*, *Dicranostigma lacttucoides*, and *Rauvolfia serpentine* were active against all of the tested strains. They argued their finding of the majority of extracts showing activity against Gram-negative microbes was likely due to the selection bias of the plants. Most of the plants in the study were used against fever and diarrhea caused by Gram-negative bacteria (Bhattarai *et al*., 2009). Subba *et al.* (2016) studied the antibacterial activity of 30 medicinal plants which are commonly used by the *Yakkha* people of the Dhankuta district. Five potent plants were evaluated for the activity by using the paper disc-diffusion method. Among the extracts studied, *Clerodendrum trichotomum* and *Boehmeria platyphylla* showed moderate activity against *E. coli, S. aureus, S. typhi,* and *B. subtilis*. Different parts of medicinal plants including *Artemisia vulgaris, Azadirachta indica, Curcuma longa, Synzygium aromaticum, Elaeocarpus ganitrus, Psidium guajava*, and *Zanthoxylum armatum* collected from Bhaktapur, Lalitpur and Kabhrepalanchok districts of Nepal were studied for antibacterial activity. Zones of inhibition measurements revealed the plants to have diverse spectrum of activities against the tested organisms. The extracts of *S. aromaticum,* and *E. ganitrus* had MIC values in the range of 12.5 – 25 mg/mL which are similar to our results of *R. ellipticus* and *P. pashia* (Sakha *et al*., 2018).

4.5.2 Determination of MIC and MBC

The methanol extracts of *R. ellipticus* and *P. pashia* were found to exhibit maximum activity against *S. aureus* on the agar well diffusion method (Table 14). So, their relative efficacy against the bacteria was further assessed by measuring MIC and MBC by the resazurin microtiter technique. It is a very simple and efficient method that uses resazurin dye to indicate the growth of bacteria in extremely small volumes without the use of a spectrophotometer. The change of color of the dye indicates the living and dead cells in the solution. In living cells, mitochondrial NADH enzymes convert the purple color of resazurin into pink due to the formation of resorubin which can be explained by the reaction (Figure 34). The fluorescence production is proportional to the number of viable cells (Elshikh *et al*., 2016).

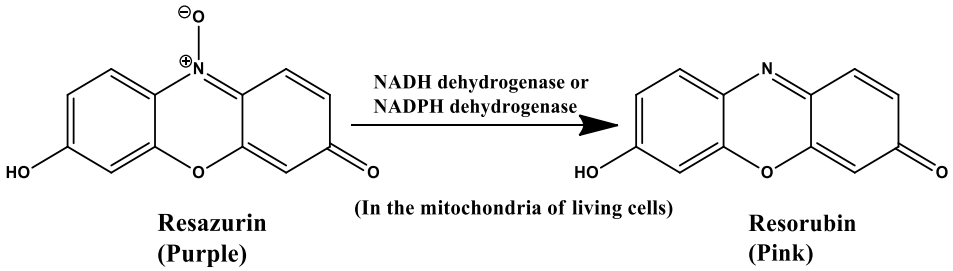


Figure 34: Enzymatic reduction of resazurin in the living cells

The antibacterial activity of the methanol leaf extract of *Z. mauritiana* was evaluated against both Gram-negative and Gram-positive bacteria. On preliminary investigation of ZOI measurement at the concentration of 400 mg/mL, *Proteus vulgaris* and *Bacillus cereus* were found to be the most active ones. They were further evaluated for the determination of MIC and MBC by broth dilution method. The MIC and MBC values of the plant extract against *B. cereus* were 25 and 100 mg/mL and that of *P. vulgaris* were 50 and 100 mg/mL respectively (Abdallah *et al*., 2016).

In the present study, the MIC and MBC of the methanol extracts of *R. ellipticus* and *P. pashia* against *S. aureus* were 3.12 mg/mL, 12.5 mg/mL, 12.5 mg/mL, and 25.0 mg/ml respectively (Figure 35). Five medicinal plants collected from the Rolpa and Bajura districts of Nepal were evaluated against pathogenic bacteria using the agar well diffusion method. Preliminary investigation showed considerable inhibition zones ranging from 10 - 17.5 mm at the concentration of 10 mg/mL. Broth dilution method revealed MIC of *R. australe* against *S. aureus* at 31.25 µg/mL, *Ragatsingey* against *E. coli* at 125 µg/mL, *Nirbikhi* against *Bacillus subtilis* at 250 µg/mL, *Ragatsingey* against *K. pneumoniae* at 250 µg/mL, and *R. australe* against *S. flexneri* at 250 µg/mL were observed (Neupane & Lamichhane, 2020b).

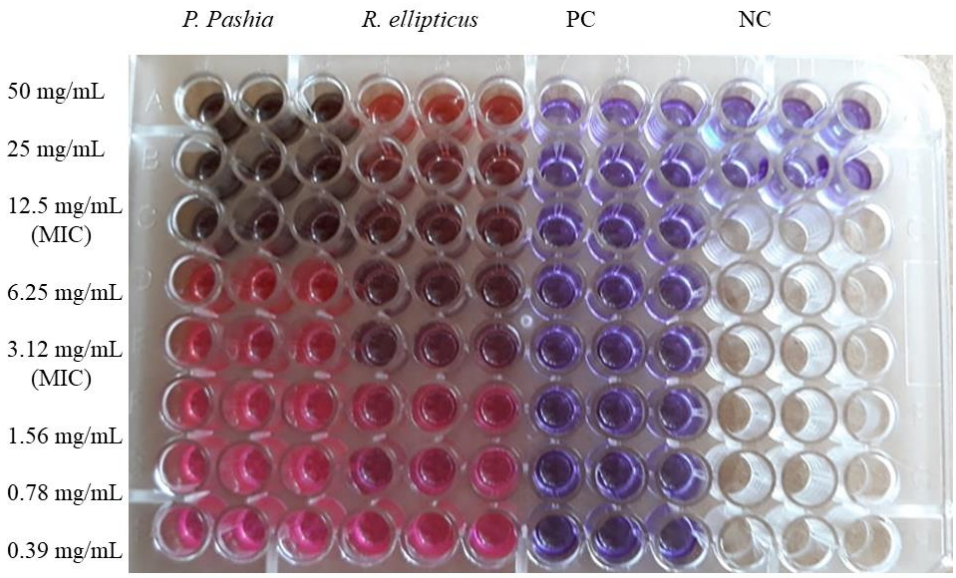


Figure 35: Resazurin microtiter assay to determine the MIC of plant extracts

4.5.3 Antibacterial activity of different solvent extracts of *E. pachyclada* and *B. roxburghiana*

The antibacterial activity of *E. pachyclada* extracts in solvents of different polarities was evaluated by the disc diffusion method. All the extracts were tested against *K. pneumonia*, *E. coli*, *S. typhi*, and *S. aureus*. Results of our study showed that the methanol extract had profound activity against *E. coli* (ZOI = 13 mm), and *S. aureus* (ZOI = 12 mm) at the concentration of 50 mg/mL as shown in Figure 42. Ethyl acetate extract showed slight activity against *S. aureus* (ZOI = 9 mm) but the *n*-hexane and DCM extracts were ineffective which is similar to the results of (Bakht *et al*., 2011; Chhetry *et al*., 2022; Omara *et al*., 2021). Methanol extract of *E. pachyclada* collected from Jiroft Heights, Iran exhibited significant antibacterial activity against some Gram-negative bacteria that cause nosocomial infections (Dosari *et al*., 2016). In similar research, methanol extract of *E. pachyclada* wild samples exhibited significant antibacterial activity against both Gram-negative and Gram-positive bacteria. At the concentration of 4 mg/mL, the plant showed the highest activity against *P. aeruginosa* (ZOI = 17.3 ± 1.15 mm), followed by *Bacillus subtilis*, and *S. aureus* (ZOI = 16 ± 2 mm), *Staphylococcus epidermis* (ZOI = 14 ± 1 mm), *K. pneumonia*e (ZOI = 14 ± 0.5 mm), *E. coli* (ZOI = 13 ± 1 mm), and *S. typhi* (ZOI = 12.3 ± 1.5 mm) (Parsaeimehr *et al*., 2010). Another active plant, *B. roxburghiana* which is locally known as Hadchur was collected from the Kaski district of Nepal. The bark has been used against different

ailments, especially bone fractures. The stem powder was used to prepare extracts in *n*-hexane, DCM, methanol, ethyl acetate, and water by cold percolation method. Each of the extracts was evaluated for antibacterial activity against *K. pneumoniae, E. coli, S. typhi,* and *S. aureus. S. sonnie,* and *A. baumannii* by disc diffusion method. The plant exhibited meager to moderate activity against the tested microorganisms (Figure 36).

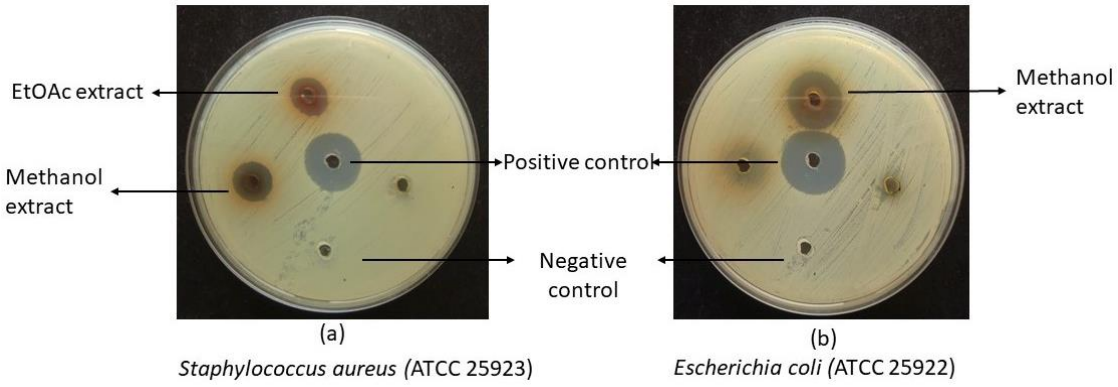


Figure 36: Antibacterial test slides of *E. pachyclada* extracts

All of the extracts were ineffective for *E. coli.* The extracts of less polar solvents like BRH and BRD were less active whereas the extracts of polar solvents like BRE, BRM, and BRA were more active towards the tested bacteria. The extract of DCM was active towards *K. pneumoniae* (ZOI = 7.0 mm) only and the *n*-hexane extract was susceptible to *S. typhi* (ZOI = 7.0 mm) and *S. aureus* (ZOI = 9.0 mm). The methanol extract was the most active against *S. sonnie* (ZOI = 13.0 mm), *S. aureus* (ZOI=12.0 mm), *S. typhi* (ZOI = 11.0 mm), *K. pneumoniae* (ZOI = 10.0 mm), and least active against *A. baumannii* (ZOI = 9.0 mm). Ethyl acetate extract of the plant exhibited higher activity against S. *typhi* (ZOI=13.0 mm), and similar activities against other bacteria (ZOI = 12.0 mm). The aqueous extract of the plant was active toward five microorganisms. Our results in Table 17 show that *K. pneumoniae* (ZOI = 8.0 mm), *S. aureus* (ZOI = 10.0 mm), *S. sonnie* (ZO I = 11.0 mm), *S. typhi*, and *A. baumannii* (ZOI = 12.0 mm). Pandey *et al*. (2017) evaluated the antibacterial capability of methanol extracts of *Artemisia vulgaris* and *Gaultheria fragratissima* collected from the Dhulikhel area of the Bagmati province of Nepal by disc diffusion method. They reported the significant activity of the plants against *S. aureus*, *Bacillus substilis, K. pneumoniae,* and *Enterococcus* species with ZOI values ranging from 11.16 ± 0.16 mm for *S. aureus* by *A. vulgaris* to 12.48 ± 0.04 mm for *B. substilis*. Phuyal *et al*. (2020) reported significant

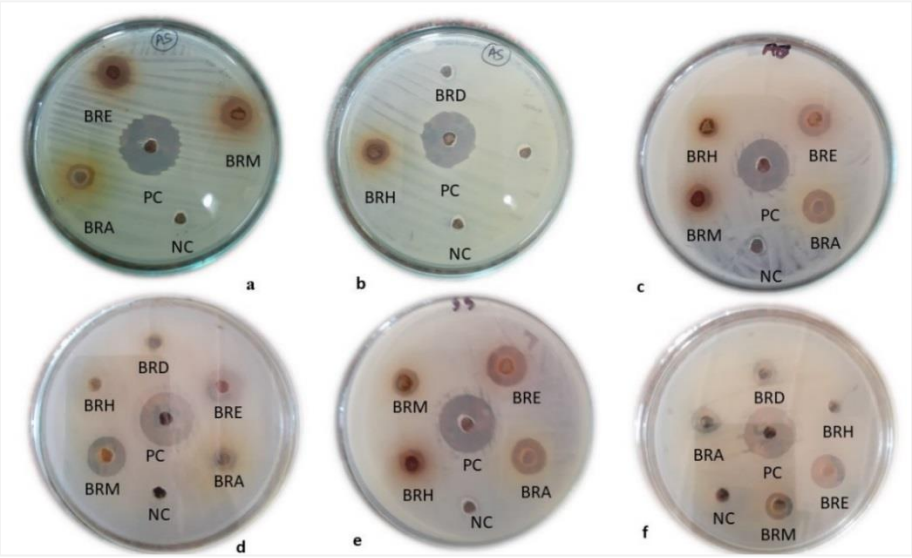
antibacterial activity against 9 pathogenic bacteria by the methanolic extracts of fruits, seeds, and barks of *Zanthoxylum armatum* collected from the Salyan district.

Table 17: Antibacterial activity of different extracts of *B. roxburghiana*

	Zone of Inhibition (ZOI) in mm					
Microorganisms	BRH	BRD	BRE	BRM	BRA	*Neomycin
Klebsiella pneumonia	-	7.0	12.0	10.0	8.0	18.0
Escherichia coli	-	-	-	-	-	17.0
Salmonella typhi	7.0	nd	13.0	11.0	12.0	18.0
Staphylococcus aureus	9.0	-	12.0	12.0	10.0	22.0
Shigella sonnei	-	-	12.0	13.0	11.0	19.0
Acinetobacter baumannii	-	nd	12.0	9.0	12.0	18.0

Note: nd = not done, Neomycin = positive control

The results validated the potential application of this plant for several bacterial infections. Methanol extract of *A. grandifolia* was subjected to fractionation and the fractions were evaluated against *Escherichia coli*, *Salmonella typhi*, *Shigella sonnei*, and *Staphylococcus aureus*. The current study revealed that the plant exhibited antibacterial activity against most of the tested bacteria (Figure 37).

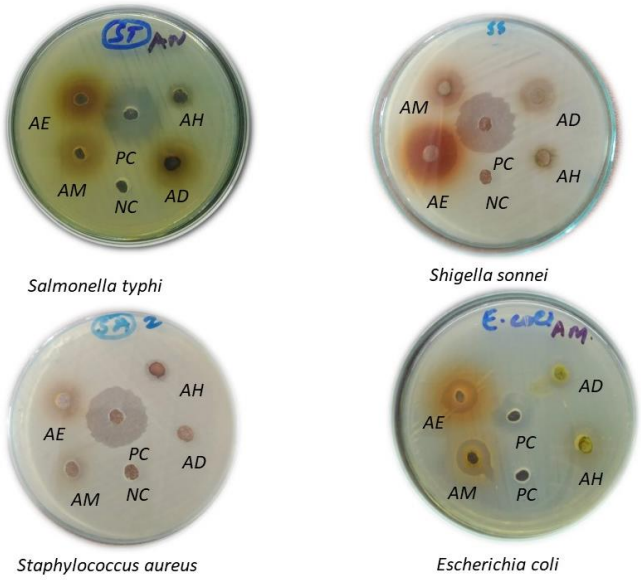


Note: a and b = *S. aureus*, c = *A. baumannii*, d = *S. sonnie*, e = *S. typhi*, and f = *K. pneumonia*, PC = positive control, NC = negative control, BRA = aqueous extract, BRE = ethyl acetate extract, BRH = *n*-hexane extract, BRD = DCM extract, and BRM = methanol extract

Figure 37: Antibacterial test slides of different extracts of *B. roxburghiana*

4.5.4 Antibacterial activity of different fractions of *A. grandifolia*

The ZOI measurements showed *S. sonnei* exhibited the maximum ZOI with all extracts (Figure 38). The *n*-hexane fraction showed the same activity (ZOI = 8.0 mm) towards all of the bacteria except *S. aureus*. The DCM fraction exhibited the maximum activity against *S. sonnei* (ZOI = 13.0 mm) followed by *E. coli* (ZOI = 12.0 mm), *S. aureus,* and *S. typhi* (ZOI = 9.0 mm). Ethyl acetate fraction of *A. grandifolia* exhibited the maximum activity against *S. sonnei* (ZOI = 19.0 mm) followed by *S. typhi* (ZOI = 15.0 mm), *E. coli*, and *S. aureus* (ZOI = 13.0 mm). Similarly, the methanol extract of the plant exhibited the highest activity against *S. sonnei* and *S. aureus* (ZOI = 16.0 mm) followed by *E. coli*, and *S. typhi* (ZOI = 14.0 mm) respectively (Table 18). The fractions of DCM and *n*-hexane exhibited relatively lower activity in comparison to the methanol and ethyl acetate fractions which is comparable to the results of Martins *et al*. (2013) and Mogana *et al*. (2020). The different solvent fractions of *Diploknema butyracea* collected from the Pyuthan district of Nepal were screened against *S. aureus* and *E. coli* by disc diffusion method. The fractions exhibited dissimilar modes of susceptibility against the tested microorganisms. Butanol and acetone fractions resulted in larger zones of inhibitions against the tested bacteria (Tiwari *et al*., 2020). Different fractions of *Nardostachys jatamansi* obtained from crude alcoholic root extracts were tested for antibacterial activity by the agar well diffusion method. All of the fractions were active against *E. coli*. ZOI values showed that the *n*-butanol and hexane fractions were active and the aqueous fraction was the least active against all of the pathogens tested (Sharma *et al*., 2016). Leaf and root extracts of *Mallotus philippensis* collected from the Banke district of Nepal were separated into different fractions and evaluated for antibacterial activity against seven bacteria by disc diffusion method.



Note: AD = DCM fraction, AH = *n*-hexane fraction, AE = ethyl acetate fraction, and AM = methanol extract

Figure 38: Antibacterial test slides of *A. grandifolia* fractions

Hexane, chloroform, ethyl acetate, aqueous, and *n*-butanol fractions of the plant exhibited significant activities against *E. coli*, *S. typhi*, *Enterobacter cloacae*, *K. pneumoniae*, *Serratia marcescens*, *Bacillus subtilis*, and *Micrococcus luteus* (Sharma *et al*., 2017).

Table 18: Zones of inhibition of different fractions of *A. grandifolia*

Name of bacteria/ATCC	Zones of inhibition (mm)				
	AD	AH	AE	AM	Control (Neomycin)
Escherichia coli (25922)	12.0	8.0	13.0	14.0	16.0
Salmonella typhi (14028)	9.0	8.0	15.0	14.0	18.0
Shigella sonnei (25931)	13.0	8.0	19.0	16.0	22.0
Staphylococcus aureus (25923)	9.0	-	13.0	16.0	22.0

Plants have an immense ability to synthesize a myriad of aromatic substances most of them are phenol and their oxygen-substituted derivatives. These compounds assist plants in the defense mechanism against microbes, and predators, or give odor and pigmentation (Cowan, 1999). Polyphenols cause morphological changes in the microbes, damage cell walls, disrupt the metabolic process, and inhibit DNA protein. The most important polyphenols present in many plants are the chief entities having biological activities. The antibacterial activity of plant polyphenols and specific

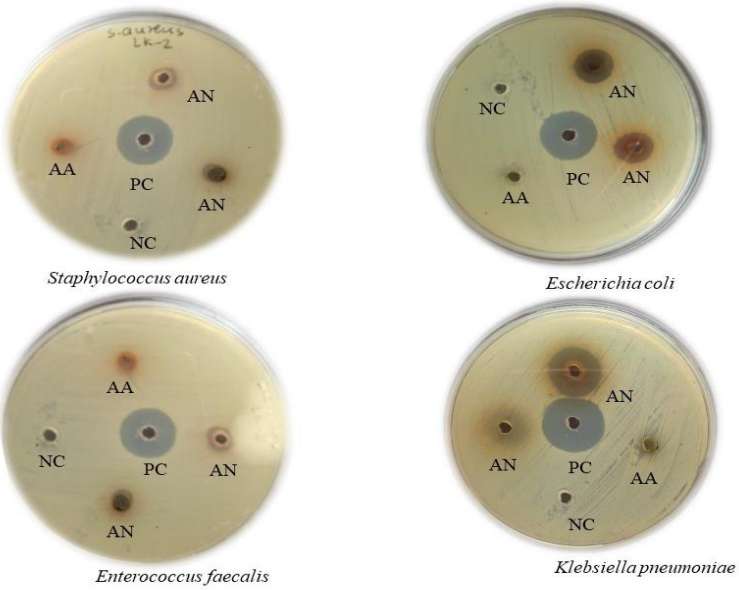
compounds depends on the bacterial strain, molecular structure, the concentration of the extract, etc. (Efenberger-Szmechtyk *et al*., 2021).

This study indicated that the plant extracts or the fractions containing a higher amount of phenolic and flavonoids exhibited higher antibacterial activity. The DCM and *n*-hexane extracts which had relatively lower TPC and TFC showed weaker susceptibility. Phenolics, flavonoids, and alkaloids are the major bioactive compounds in plants having profound antimicrobial properties. These compounds can alter the biochemical reactions, and inhibit the bacterial life process by binding their protein molecules. The polar phenolic compounds interact with enzymes, adsorb on the cell membrane and cause inflammation of the cells (Metrouh-Amir *et al*., 2015). Polyphenols and flavonoids interact with lipid bilayers to disturb the membrane function of bacterial cells. These compounds can damage plasma membrane by creating pores, outflowing the cellular material, fluctuating electrical charge and polarity, disrupting membrane permeability, delocalizing membrane protein, and other many absurd damages responsible for the antibacterial property (Álvarez-Martínez *et al*., 2021).

4.5.5 Antibacterial activity of the synthesized nanoparticles

Silver and silver-centered compounds are toxic to microorganisms and have been used as antibacterial agents from primeval periods. Several researchers have reported that AgNPs inhibit the growth or kill several pathogenic and MDR bacteria. Nowadays AgNPs are being extensively used for the treatment of bacterial infections as well as in surgical, dental, paramedical, burns, wounds, etc. (Bindhu *et al*., 2020). Here, we tested the antibacterial capacity of synthesized AgNPs by using aqueous extracts of *A. grandifolia*, *P. pashia*, *R. ellipticus*, and *Z. mauritiana* were separately tested against some Gram-negative and Gram-positive bacteria by agar well diffusion method. The result of the antibacterial activity of the plant extracts and the biosynthesized silver nanoparticles is presented in Table 19. The AA was inactive towards the tested microorganisms but Ag-AgNPs (AN) showed significant activity. It displayed the highest activity towards *K. pneumoniae* (ZOI = 13.5 ± 0.5 mm) and moderate activity against other bacteria (Figure 39). This study showed lower activities of AgNPs synthesized from the stem bark extracts of *A. grandifolia* against Gram-positive than Gram-negative strains. The zone of inhibition (ZOI) against Gram-positive *E. faecalis* (6.5 ± 0.5 mm), and *S. aureus* (10.5 ± 0.5 mm) are quite lower than that of Gram-

negative strains *E. coli* (12.5 ± 0.71 mm), and *K. pneumoniae* (13.5 ± 0.5 mm) respectively (Table 19).



Note**:** AA = Aqueous extract, AN = Ag-AgNPs, PC = positive control, and NC = negative control

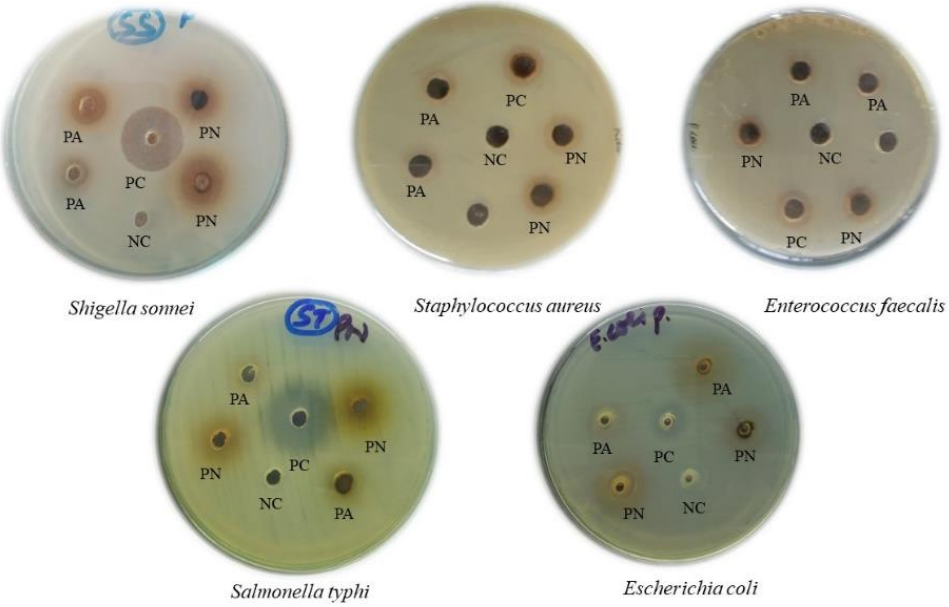
Figure 39: Antibacterial test slides of AA and Aa-AgNPs

In the agar well diffusion method, ZOI of Pp-AgNPs increased from 8.5 ± 0.5 to 11.5 ± 0.5 mm for *E. coli*, 7.5 ± 0.5 to 11.5 ± 0.5 mm for *S. aureus*, 6.5 ± 0.5 to 9.5 ± 0.5 for *E. faecalis*, 7.5 ± 0.5 mm to 7.5 ± 0.5 to 12.5 ± 0.5 for S. *typhi*, and 8.0 ± 0.5 mm to 8.5 ± 0.5 to 12.5 ± 0.5 mm for *S. sonnei*. The highest increment was observed for *S. sonnei* in the test (Figure 40). Similar results of higher activity were observed for the synthesized AgNPs of *R. ellipticus* root extracts. The AgNPs synthesized by using RERE had higher ZOI values than the crude aqueous extract. In the case of *S. aureus, K. pneumoniae*, and *E. coli*, ZOI was increased by 3 mm and by 2 mm for *E. faecalis*. The highest activity of AgNPs (ZOI=14 mm) was observed for *K. pneumoniae* (Figure 41). In our experiments, all of the synthesized AgNPs exhibited enhanced antibacterial activities than the crude extract against all of the tested bacteria. The results in Table 19 show the synthesized Ag NPs had higher activity than that of the aqueous extract of stem bark of *P. pashia*.

Table 19: Antibacterial test results of aqueous extracts and AgNPs

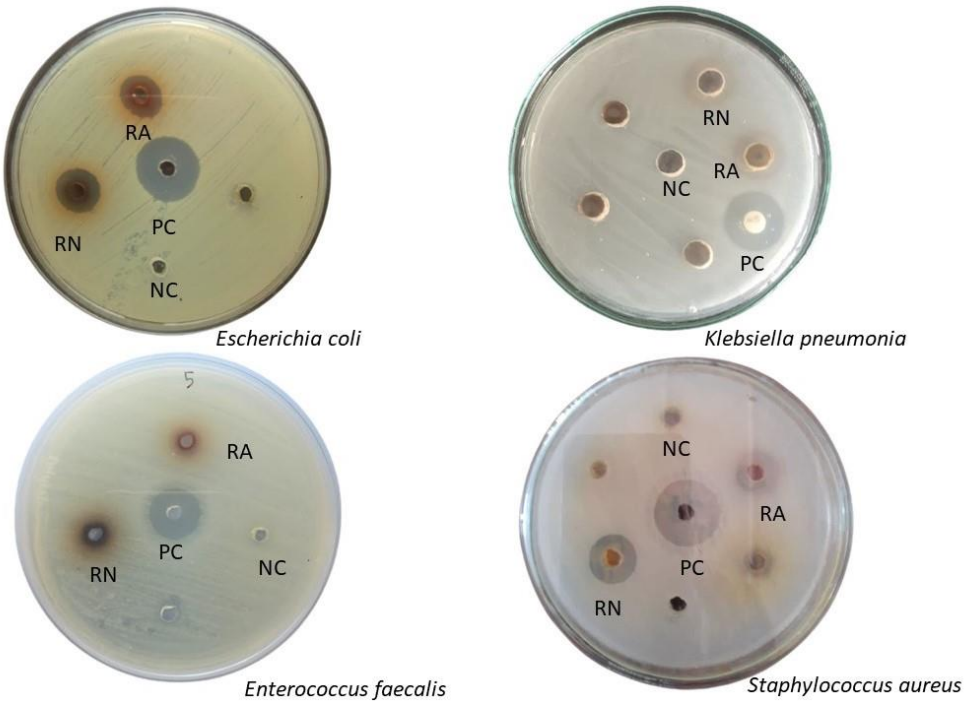
Sample (10 mg/mL)	Zone of inhibition (mm)											
	E. coli (ATCC 25922)	PC	*S. aureus* (ATCC 25923)	PC	*E. faecalis* (ATCC 29212)	PC	*K. pneumoniae* (ATCC 700603)	PC	*S. typhi* (14028)	PC	*S. sonnei* (25931)	PC
AA	-		-		-		-		-	--	- -	--
AN	12.5 ± 0.71	18.0	10.5 ± 0.5	18.0	6.5 ± 0.5	18.0	13.5 ± 0.5	18.0	-		-	
PA	8.5 ± 0.5		7.5 ± 0.5		6.0 ± 0.0		--	--	7.5 ± 0.5		8.0 ± 1.0	18.0
PN	11.5 ± 0.5	18.0	11.5 ± 0.5	15.0	9.5 ± 0.5	12.0	--	--	12.5 ± 0.5	18	12.5 ± 0.5	
RA	10.0		9.0	16.0	10.0		11.0		--		--	
RN	13.0	16.0	12.0		12.0	16.0	14.0	15.0	--		--	
ZA	--		6.0		--		--		--		9.5 ± 0.5	18.0
ZN	11.0 ± 1.0	14.0	12.5 ± 0.5	18.0	--		--		10.0 ± 1.0		12.5 ± 0.5	

Note: AA = *A. grandifolia* aqueous extract, AN = Ag-AgNPs, PA = *P. pashia* aqueous extract, PN = Pp-AgNPs, RA = *R. ellipticus* aqueous extract, RN = Re-AgNPs, ZA = *Z. mauritiana* aqueous extract, and ZN = Zm-AgNPs, and PC = positive control, concentration = 5 mg/mL



Note**:** PA= Aqueous extract, PN= AgNPs, and, PC= positive control, and NC= negative control

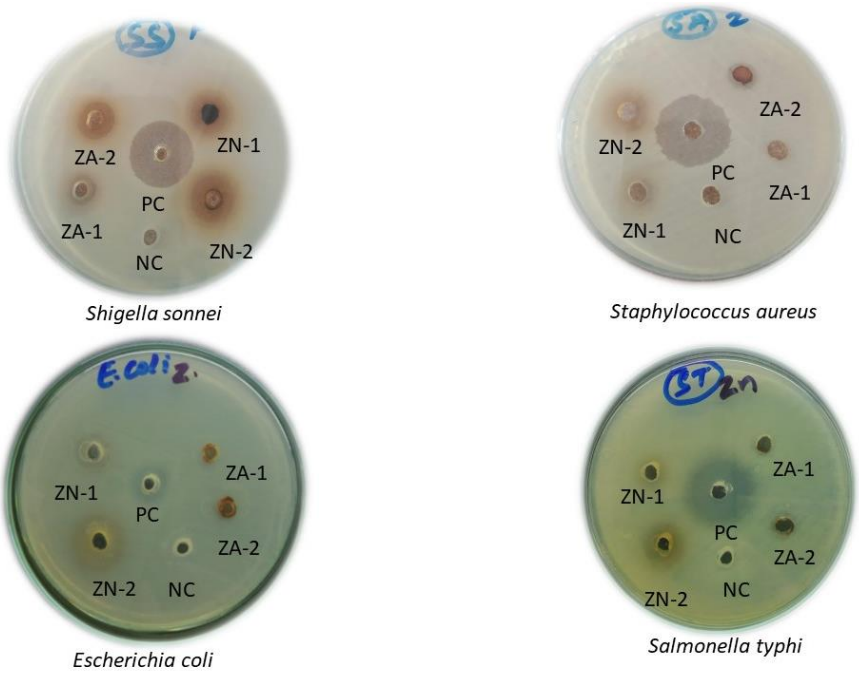
Figure 40: Antibacterial test slides of PA and synthesized Pp-AgNPs



Note**:** RA = *R. ellipticus* aqueous extract and RN = AgNPs, and, PC= positive control, NC = negative control

Figure 41: Antibacterial test slides of RA and Re-AgNPs

Different parts of *Ziziphus mauritiana* have been reported for traditional medicine, culinary, and health maintenance purposes. Stem, roots, leaves, and fruits were found to exhibit significant antioxidant, antimicrobial, and anti-inflammatory activities (Butt *et al*., 2021). Here, an aqueous extract of the stem bark of *Z. mauritiana* was used to synthesize silver nanoparticles. The antibacterial activity of the extract was compared with the AgNPs by the agar well diffusion method (Figure 42). The crude extract showed weak activity against *S. aureus* (ZOI = 6.0 mm), and moderate against *S. sonnei* (ZOI = 9.5 ± 0.5 mm). The ZME-mediated AgNPs showed significant activities against *E. coli* (ZOI = 11.0 ± 1.0 mm), *S. aureus* (ZOI = 12.5 ± 0.5 mm), *S. typhi* (ZOI = 10.0 ± 1.0 mm), and *S. sonnei* (ZOI = 12.5 ± 0.5 mm) as shown in Table 19. The crude extract as well as the synthesized AgNPs of ZME exhibited the highest activity against *S. sonnei* among the tested microorganisms (Figure 48). Biosynthesized AgNPs prepared by using methanol leaf extracts of *Cassia didymobotyra* had an average size of 18 nm and were characterized by XRD, TEM, SEM, and UV-visible spectroscopy.



Note**:** ZA = *Z. mauritiana* aqueous extract, and ZN = Zm-AgNPs, PC = positive control, NC = negative control

Figure 42: Antibacetrial test slides of ZA and Zm-AgNPs

The synthesized AgNPs were found more potent than the crude extract against *E. Coli, K. pneumoniae, S. aureus, Micrococcus luteus, Pseudomonas aeruginosa,* and *Streptococcus pneumoniae* on the disc diffusion method which is analogous to our results (Akhtar *et al*., 2015). In a recent study, green synthesized AgNPs of leaf extracts of *Eucalyptus globulus* and *Salvia officinalis* exhibited relatively higher antibacterial activity against both Gram-negative and Gram-positive bacteria on the agar well diffusion method (Balciunaitiene *et al*., 2022). The results of Kambale *et al*. (2020) stated a higher antibacterial activity of AgNPs prepared from three Congolese plants, *Brillantaisia patula, Crossopteryx febrifuga,* and *Senna siamea* corroborate the findings of this study. They obtained lower MIC values for the synthesized AgNPs than corresponding aqueous extracts on broth micro-dilution methods against *E. coli, P. aeruginosa*, and *S. aureus.* Consistent results were obtained by the AgNPs of *Plumbago auriculata* leaf and calyx extracts against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa, S. typhi,* and *K. pneumoniae* (Singh *et al*., 2018).

The type of microorganisms, pH, temperature, zeta potential, shape, size, concentration of AgNPs, etc. are the key factors affecting the biological activity of nanoparticles. The active phytochemicals in the plant extract which are involved in the fabrication of

AgNPs would have preferentially assisted in the greater antibacterial activity (Wahab *et al*., 2021). The different rates of enhancement of the antibacterial activity of AgNPs against Gram-positive and Gram-negative bacteria are attributed to the thickness of the cells of the organisms. The Gram-positive bacteria have a relatively thick peptidoglycan layer that is less susceptible to attack by AgNPs (Pazos-Ortiz *et al*., 2017).

The green synthesis of plant-based AgNPs has become a dominant research area in contemporary years. There are several documents in the literature explaining this as a simple, eco-friendly, and cost-effective technique for producing AgNPs. None of the studies have concluded the exact phytochemical or functional group in the plant responsible for the reduction of Ag^{+} ions into AgNPs. Although several kinds of research hypothesized many systematic pathways of biological activity of AgNPs there is a radical necessity for extensive research to fulfill the gap of mechanistic study of the plant-based AgNPs for human welfare (Rahuman *et al*., 2022). Various mechanisms are proposed and reported for the antibacterial activities of the AgNPs but none of them is fully validated. The plausible mechanism of the antibacterial action of AgNPs is depicted in Figure 43.

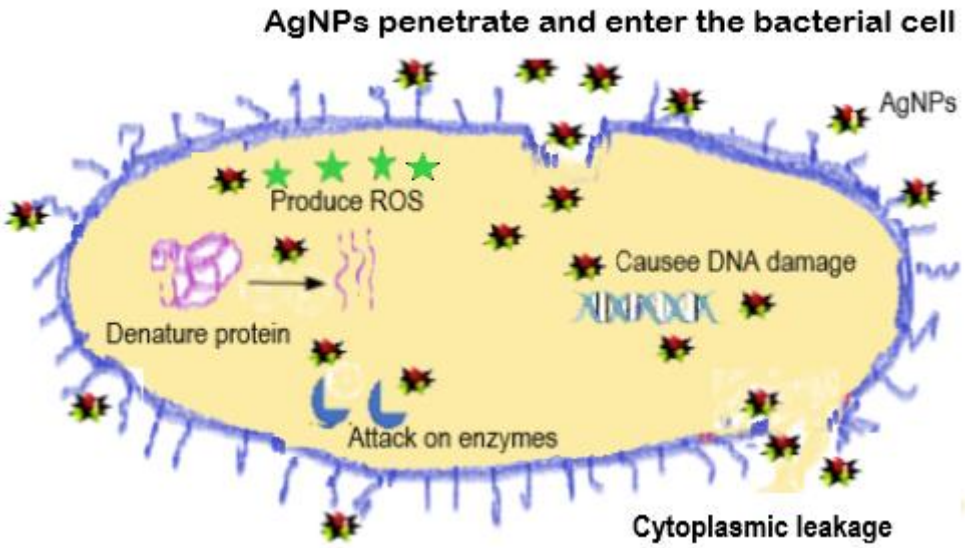


Figure 43: Schematic diagram of antibacterial activity of AgNPs

The negatively charged sulphur or phosphorus-containing biomolecules like proteins and nucleic acid get attached to the positively charged silver ions and disrupt the membrane permeability. Some reports explain that the reactive free radicals derived on the surface of AgNPs damage the cell membrane leading to apoptosis (Huq, 2021).

Silver ions have a unique capacity to detain the replication of bacteria. They enter the cell membrane and attack cellular protein by reacting with the thiol group and denaturing DNA leading to cell death (Das *et al*., 2020). In comparison to larger-sized AgNPs, smaller ones (< 10 nm) release excess Ag^+ ions which show better antibacterial activity so they have been used against several multidrug-resistant bacteria viruses and many eukaryotic microorganisms (Rai *et al*., 2012).

4.6 *α*-Amylase inhibitory activity

The antidiabetic potential of the methanol extracts of different plant extracts was estimated in a 96-well plate reader using CNPG3 as substrate and acarbose as the positive control. The results of the preliminary screening of the *α*-amylase inhibitory activities are presented in Table 20. Out of 22 plant extracts evaluated, only seven showed significant inhibitions of more than 50%. The plot of the values (Figure 39) of different plant extracts shows that the methanol extract of stem barks of *P. pashia* exhibited the highest inhibition (89.03 ± 2.04 %) followed by *M. rubicaulis* (80.11 ± 0.74%), *A. grandifolia* (75.73 ± 0.67%), *E. pachyclada* (66.76 ± 0.55%), *R. ellipticus* (63.81 ± 3.00%), and *D. coronans* (58.42 ± 3.84%). Acarbose taken as the positive control exhibited a maximum inhibition (98.41 ± 0.26 %) at the concentration of 0.1 mg/mL.

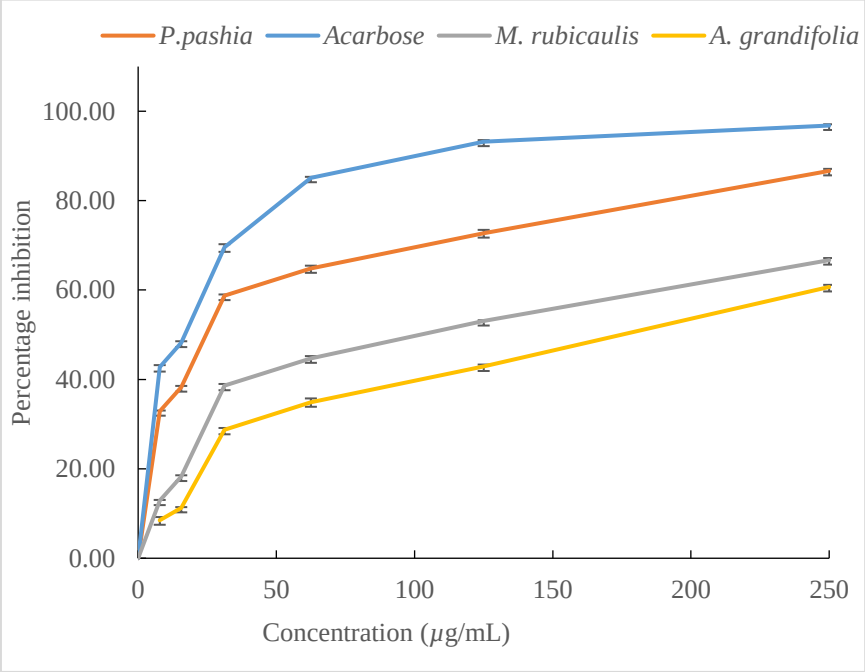
Table 20: α-Amylase inhibitory activities of methanol extracts

Name	% Scavenging
E. pachyclada	66.76 ± 0.55
A. grandifolia	75.73 ± 0.67
D. coronans	58.42 ± 3.84
M. rubicaulis	80.11 ± 0.74
B. megaptera	61.11 ± 2.95
P. pashia	89.03 ± 2.04
R. ellipticus	63.81 ± 3.00
Acarbose*	98.41 ± 0.26

Note: * = Positive control, values are mean ± SD, n = 3, the concentration of extract = 5 mg/mL

The fruit extracts of *P. pashia* collected from India were evaluated for alpha-amylase inhibitory activity by using dinitrosalicylic acid (DNS) and starch. The plant exhibited significant activity which is comparable to our results where we used the stem barks of

the plant (Prakash *et al*., 2021). The α-amylase inhibitory activity of different extracts of *Cinnamomum tamala* collected from Kathmandu was evaluated by the CNPG3 method. The methanolic young leaf extract showed maximum inhibition of 70.33 ± 0.47% and other extracts were less active. The extract was further assessed for IC_{50} value which was 224.6 ± 2.76 μg/mL (Maharjan *et al*., 2021). The α-amylase inhibitory property of ten medicinal plants from Nepal was assessed by a starch–iodine assay method. The methanol root extract of *Abrus precatorius* has reported the highest activity with an IC_{50} value of 70.29 ± 0.14 μg/mL (Rai *et al*., 2020). The dose-dependent relation of the % inhibition capacity of different extracts with corresponding concentrations is shown in Figure 44. Our study shows that all of the extracts show a dose-dependent inhibition comparable to that of acarbose. The plot shows that *P. pashia* had the highest inhibition action followed by *M. rubicaulis* and *A. grandifolia* extract.

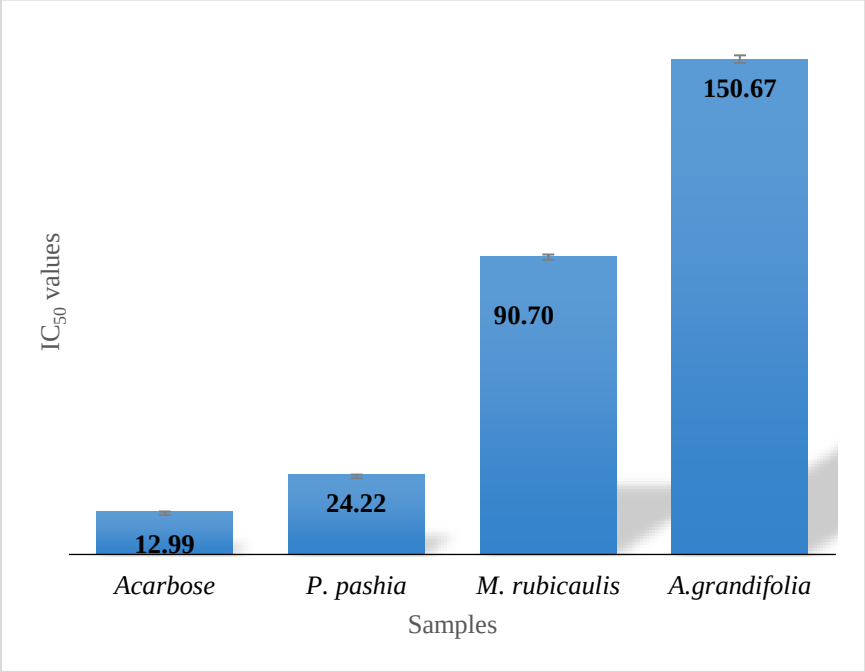


Note: values are means of n = 3

Figure 44: α -Amylase inhibitory activity of plant extracts and acarbose

The extracts of *P. pashia, M. rubicaulis,* and *A. grandifolia* which showed higher % inhibitions were assessed for the determination of IC_{50} values. Figure 45 shows the methanol leaf/bark extract of *P. pashia* exhibited the highest enzyme inhibition activity (IC_{50} = 24.22 ± 0.10 μg/mL) followed by the root extracts of *M. rubicaulis* (IC_{50} = 90.70 ± 0.63 μg/mL), and stem barks of *A. grandifolia* (IC_{50} = 150.67 ± 1.30 μg/mL). Khadayat *et al.* (2020) evaluated the methanolic extracts of 32 medicinal plants

collected from Nepal for *in vitro* α-amylase inhibitory activity by taking acarbose as a standard. They reported the extracts of *Acacia catechu*, *Swertia chirata*, and *Dioscorea bulbifera* to be the potent inhibitors with IC_{50} values of 49.9 ± 0.4, 296.1 ± 8.7, and 413.5 ± 11.1 μg/mL respectively.



Note: values are means of n =3

Figure 45: α-Amylase inhibitory activity (IC_{50} values in μg/mL)

Similarly, the *Carica papaya fruits*, *Buddleja asiatica*, and *Spondias pinnata* leaf extracts were reported to exhibit moderate α-amylase inhibitory activity on the starch-iodine method (Sai *et al*., 2019). Based on preliminary evaluation of phytochemical screening, estimation of total phenolics and flavonoids, antioxidant activity, antibacterial activity, and α-amylase inhibitory activity, and contemporary literature, the following plants were selected for additional studies (Table 21).

Table 21: List of active plants for further analysis

S. N	Name of the plant	Further experiments
1	*R. ellipticus*	Green synthesis, characterization, and evaluation of biological properties of AgNPs
2	*P. pashia*	Green synthesis, characterization, and evaluation of biological properties of AgNPs
3	*Z. mauritiana*	Green synthesis, characterization, and evaluation of biological properties of AgNPs

4	*B. roxburghiana*	Comparative evaluation of biological properties using different solvents
5	*E. pachyclada*	1. Comparative evaluation of biological properties using different solvents 2. GC-MS analysis of essential oil
6	*A. grandifolia*	1. Green synthesis, characterization, and evaluation of biological properties of AgNPs 2. Fractionation and evaluation of biological activities 3. GC-MS analysis of essential oil

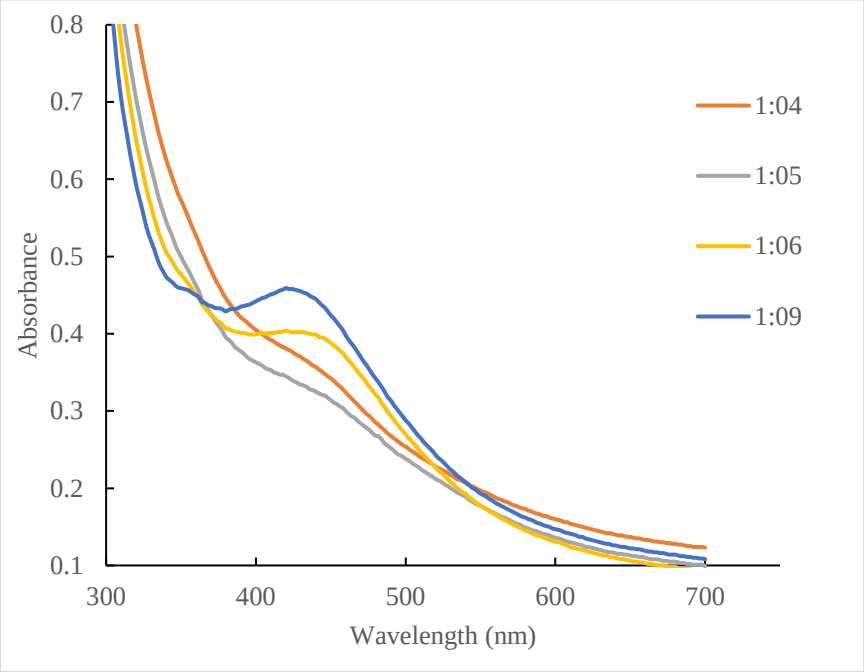
4.7 Characterization of biogenic AgNPs

The functional groups, surface morphology, crystallinity, elemental composition, size, and shape of AgNPs were characterized by using UV-visible, FTIR spectroscopy, powder XRD, FESEM, EDX, and TEM.

4.7.1 UV-visible spectroscopy

The concentration of the plant extracts, pH value of the solution, and reaction temperature influence the controlled synthesis of stable AgNPs. The color intensity of the reaction mixture changes to stable reddish brown which can be monitored by the steep broadening of the surface plasmon resonance (SPR) band at a particular time. The resonance of concomitant vibrations of free electrons of metallic silver with light waves of particular frequencies results in the brown color of the AgNPs. This phenomenon describes the origin of SPR absorption of the metallic nanoparticles, which is often verified by UV-visible spectroscopy as well as visual observation (Kambale *et al*., 2020). The simultaneous increase in concentration and time of the reaction mixture leads to the shifting of the SPR band towards a higher frequency and absorbance. The plant extract was added into 1mM $AgNO_3$ solutions were mixed up separately in the ratio of 1:4, 1:5, 1:6, and 1:9 drop wise with constant stirring over a magnetic stirrer at laboratory temperature at the pH of 7. Similarly, the formation of AgNPs was observed at different pH of the solutions. The UV-visible spectra of the reaction mixtures were taken after one hour within the range of 300 – 700 nm. An observation of a sharp absorption peak in the mixture of 1:9 in a solution at 7 pH was confirmed (Figure 46). The reaction was allowed to take place in the ratio of 1:9 at different pH values of the mixture for each plant extract. Each sample of a reaction mixture comprising plant

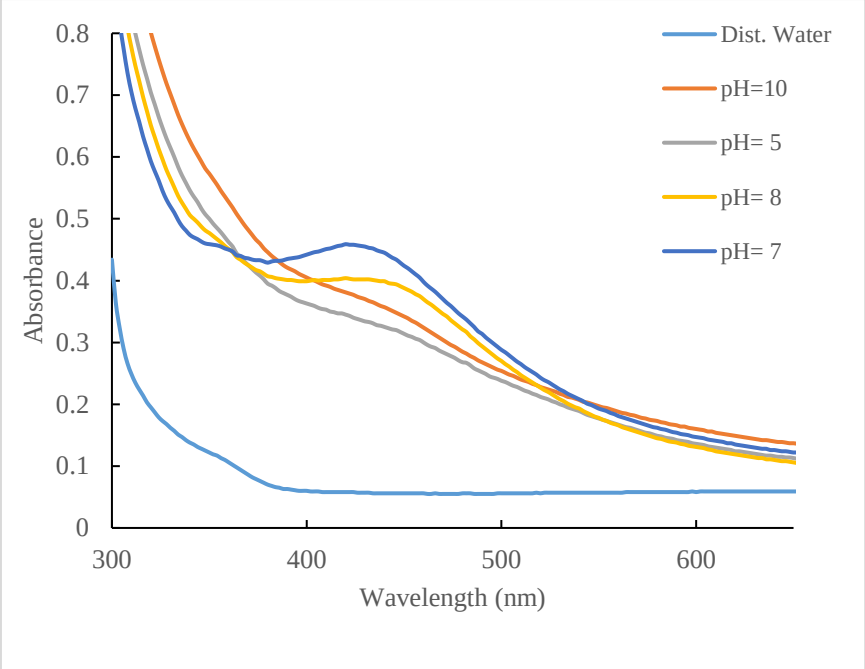
extract and 1 mM $AgNO_3$ was maintained at the pH values of 5, 7, 8, and 10 separately. The UV-visible spectra of the reaction mixtures were taken after one hour within the range of 300 – 700 nm. An absorption of 0.458 at 424 nm by the mixture of 1:9 in a solution at pH 7 was observed.



(Extract: $AgNO_3$ and pH = 7)

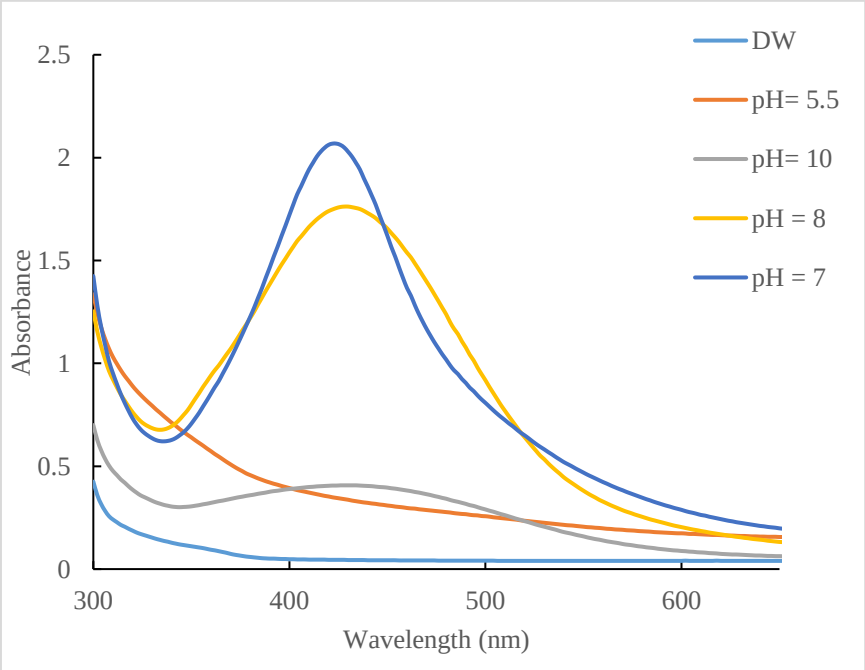
Figure 46: UV-visible spectra of AgNPs at different proportions

The synthesis of AgNPs from *A. grandifolia* was optimized by the appearance of a maximum peak at 432 nm when the mixture (1:9) was maintained at pH 7 (Figure 47). The same reaction condition was used for the synthesis of Pp-AgNPs which gave the maximum absorbance of 2.068 at 424 nm (Figure 48). Synthesis of Re-AgNPs was maximum at the condition of pH 7 and ratio of the reactants 1:9 (Figure 49). The synthesis of Zm-AgNPs was optimized by mixing the plant extract and 1mM $AgNO_3$ (1:9) at neutral pH and lab temperature. It was confirmed by the appearance of maximum absorbance of 1.727 at 420 nm (Figure 50).



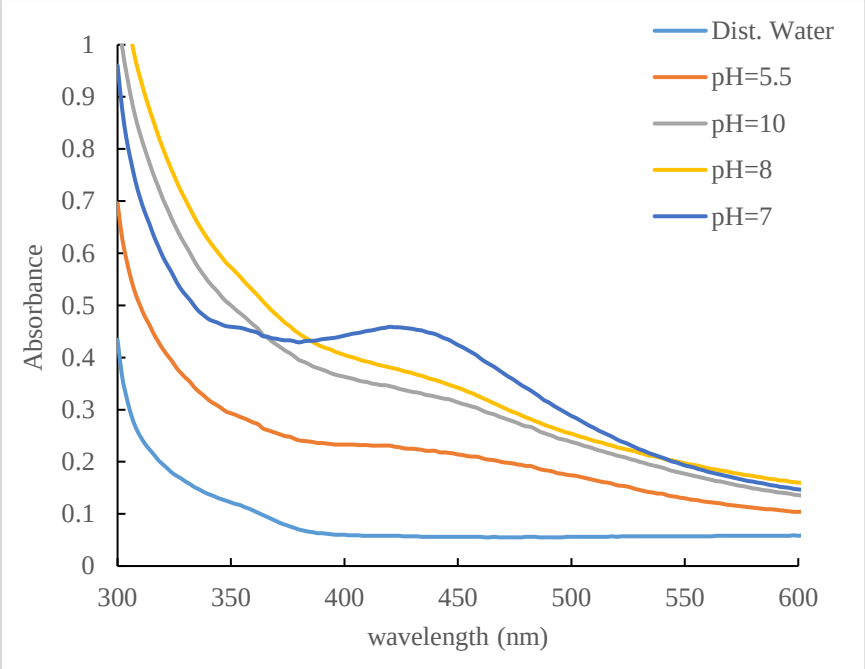
Ratio = Extract: $AgNO_3$ (1:9)

Figure 47: UV-visible spectra of Ag-AgNPs at different pH values



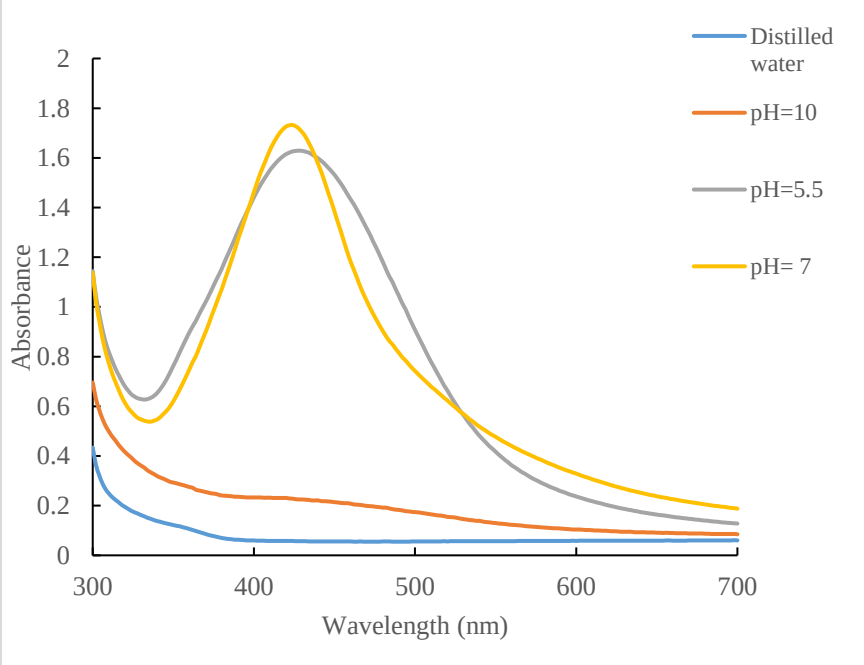
Ratio = Extract: $AgNO_3$ (1:9)

Figure 48: UV-visible spectra of Pp-AgNPs at different pH values



Ratio = Extract: $AgNO_3$ (1:9)

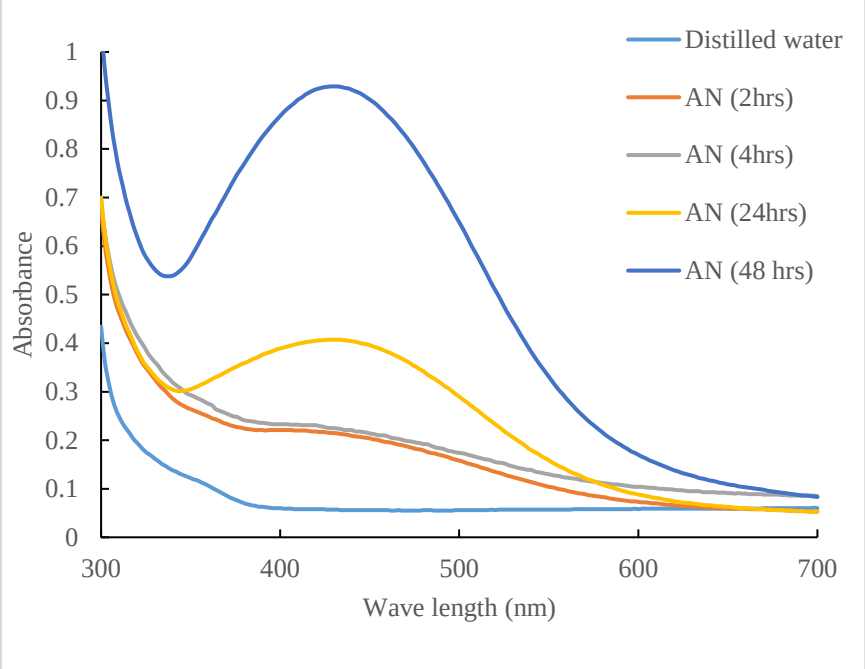
Figure 49: UV-visible spectra of Re-AgNPs at different pH values



Ratio = Extract: $AgNO_3$ (1:9)

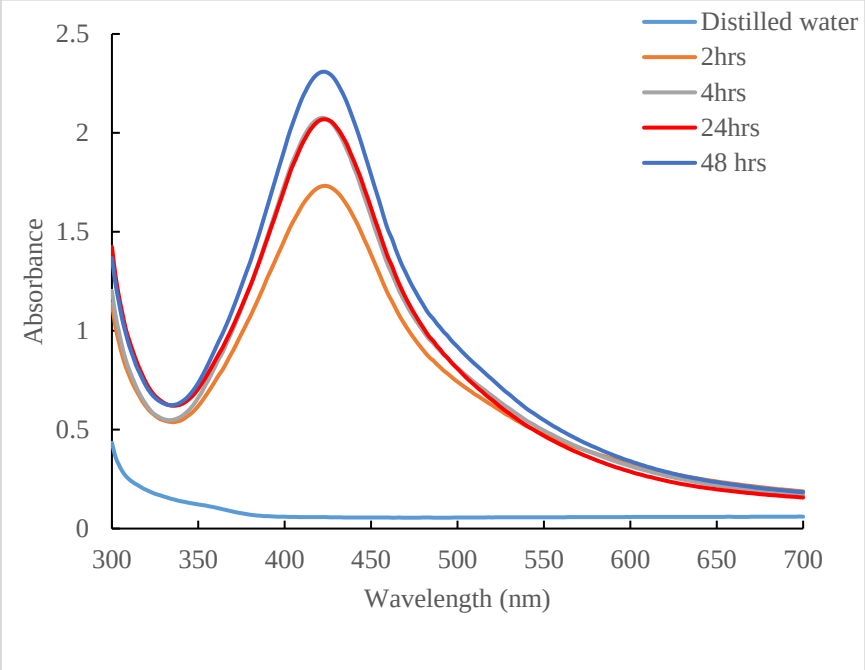
Figure 50: UV-visible spectra of Zm-AgNPs at different pH values

The synthesis of AgNPs depends on reaction time also. Here, the UV-visible spectra of the reaction mixture at different time intervals were used to find the completion of the reaction. In all the cases, the maximum absorbance observed at 48 hours of formation was taken as an indication of a complete reaction (Figures 51 to 54).



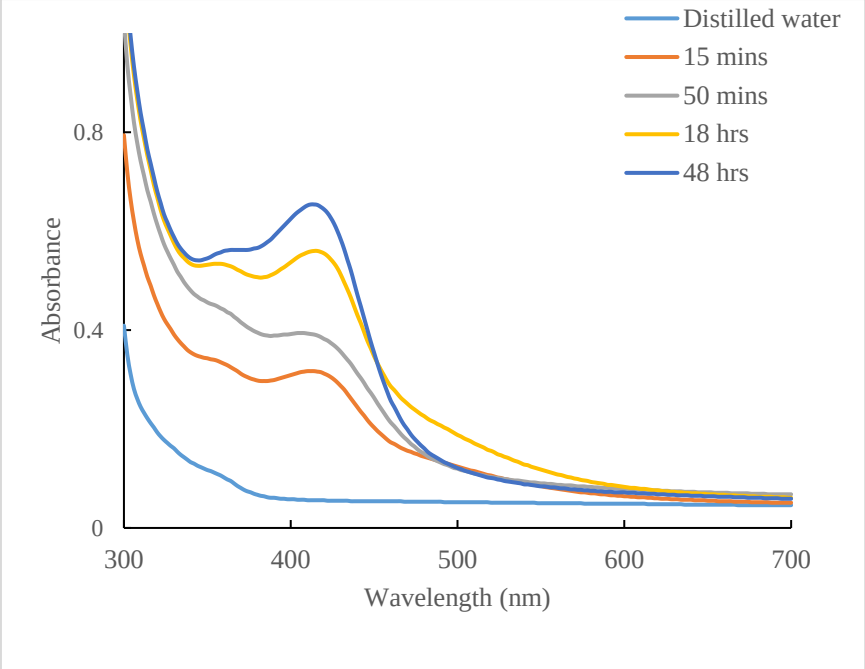
pH = 7, extract: $AgNO_3$ (1:9)

Figure 51: UV-visible spectra of Ag-AgNPs at different times



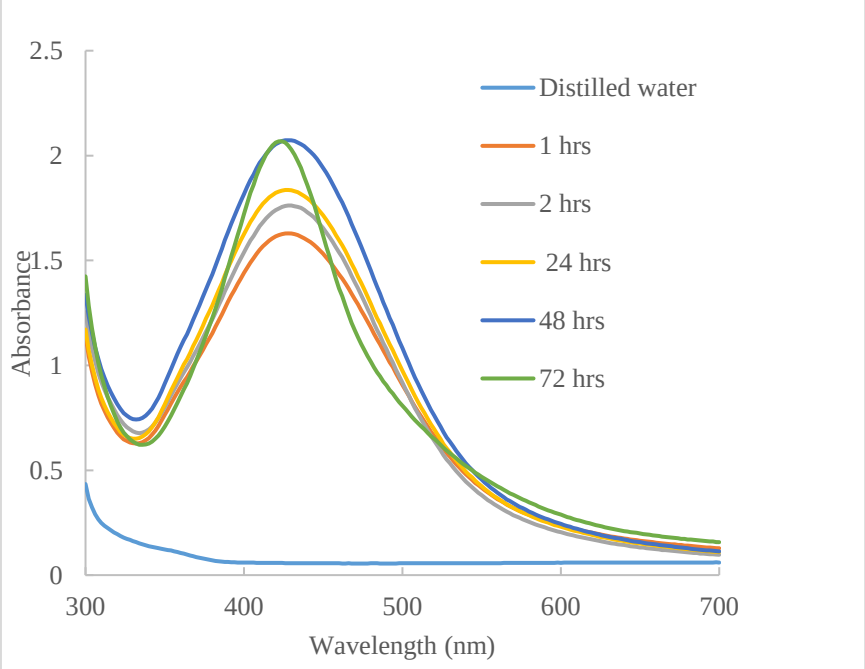
pH = 7, extract: $AgNO_3$ (1:9)

Figure 52: UV-visible spectra of Pp-AgNPs at different times



pH = 7, extract: $AgNO_3$ (1:9)

Figure 53: UV-visible spectra of Re-AgNPs at different times



pH = 7, extract: $AgNO_3$ (1:9)

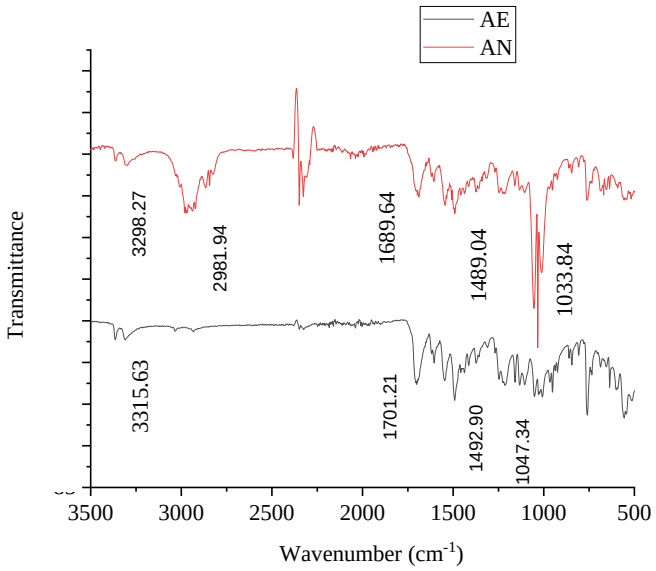
Figure 54: UV-visible spectra of Zm-AgNPs at different times

The UV-visible spectra of the reaction mixture of AgNPs were taken at different time intervals such as 15 minutes, 60 minutes, 24 hours, 48 hours, and 72 hours. Observation in the same range after 48 hours of reaction indicated the completion of reaction. The observation of the sharp peak at around 400 - 460 nm indicates the formation of

biogenic AgNPs. The less variation of absorbance with increasing time in this case proves easier and fast formation of AgNPs with *Z. mauritiana* extract.

4.7.2 FTIR spectroscopy

FTIR spectroscopy was performed to detect the functional groups which are involved in the synthesis of AgNPs. The comparative evaluation of the FTIR spectra of plant extract and the synthesized AgNPs revealed the role of certain functional groups in the formation of the nanoparticles. The shifting of certain peaks of functional groups in the extracts and the AgNPs indicates the potential roles of these groups in the synthesis, capping, and stabilizing of nanoparticles (Pirtarighat *et al*., 2019). In our synthesis, mostly the –OH groups of polyphenols, C-O stretching of ether linkages of flavones, C-H stretching of SP^3 carbons of alkanes or aldehydes, aromatic and aliphatic C=C and C=O bonds, and C-N or N-H stretching were involved in the synthesis of AgNPs. The involvement of active functional groups in the plant material which are involved in the synthesis of AgNPs is detected by the comparative study of FTIR spectra of the extract and the synthesized AgNPs.



AE = aqueous extract, AN = Ag-AgNps

Figure 55: FTIR spectra of aqueous extract and Ag-AgNPs

The important plant constituents including polyphenols, flavonoids, ascorbic acid, sterols, alkaloids, steroids, triterpenes, saponins, polysaccharides, proteins, enzymes, etc. serve as reductants, capping and stabilizing agents for the Phyto-fabrication of

AgNPs in the solution. The reduced biosynthetic cofactors play a crucial role in the reduction of $AgNO_3$ (salt) to nanoparticles (Prasad, 2014).

The bands appearing in Figures 55 and 58 around 3315.63, 2932.6, 1749, 1637.6, 1386.5, 1146.5, 1077, 829.5, and 642.4 cm^{-1} represent the stretching vibration of O-H of alcohol or N-H of amines, C-H of alkanes, -C=O of carboxylic acids, ester, -N-C=O bond of proteins, CH_2 of alkanes, C-O of carboxylic acid, ester, or ether, C-N of aliphatic amines, alcohols/phenols, N-H deformation of amines and C-C bending respectively (Socrates, 2004).

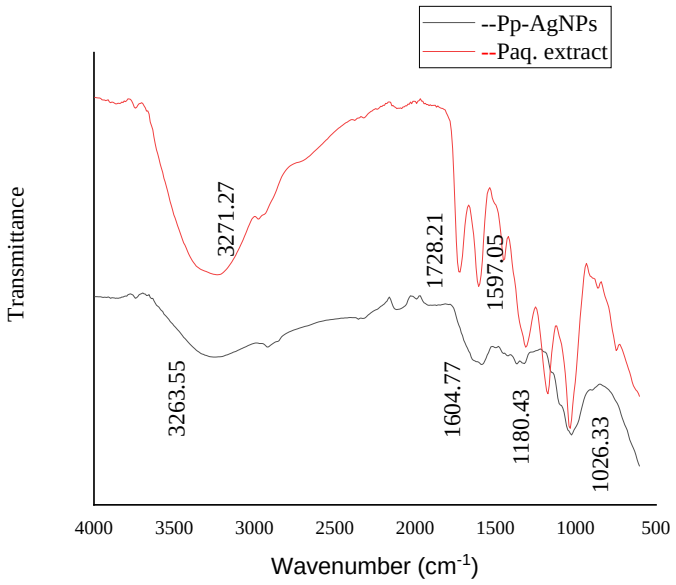


Figure 56: FTIR spectra of aqueous extract and Pp-AgNPs

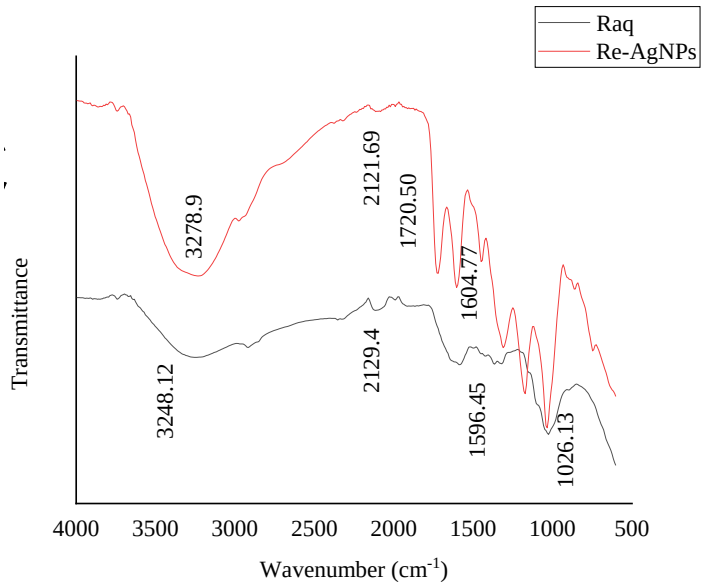


Figure 57: FTIR spectra of aqueous extract and Re-AgNPs

In our AgNPs, mostly the peaks corresponding to the above-mentioned bands were shifted indicating their role in the synthesis, capping, and stabilizing.

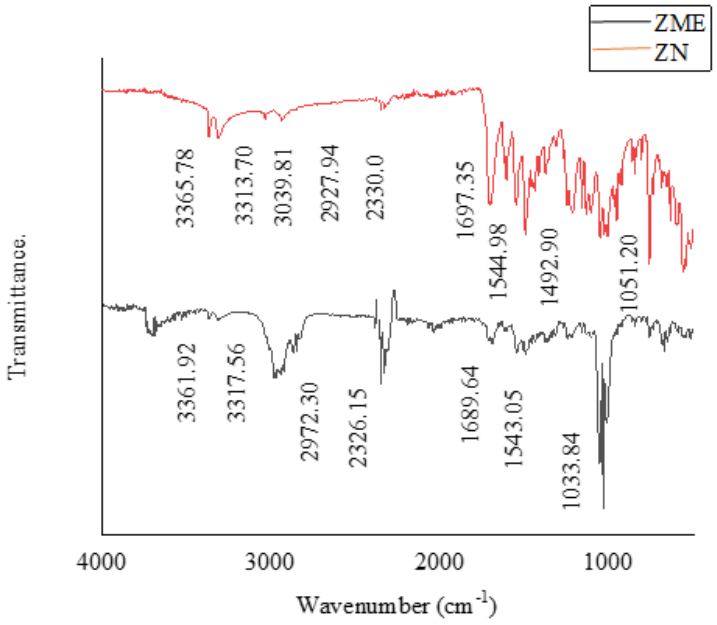


Figure 58: FTIR spectra of aqueous extract and Zm-AgNPs

4.7.3 XRD analysis

The X-ray diffraction analysis was performed to find the crystalline nature of the synthesized nanoparticles. The diffractograms showed Bragg's reflections indexed on the face-centered cubic structure of silver. X-ray diffractogram of Ag-AgNPs in Figure

59 shows the specific peaks at 2θ angles of 38.1°, 44.57°, 64.66°, and 77.51° corresponding to the crystal planes of (111), (200), 9220), and (311) of Ag nanoparticles respectively. Similarly, the XRD pattern of *P. pashia* bark extract mediated AgNPs in Figure 60 showed diffraction peaks at 2θ = 38.09º, 44.53 º, 64.61 º, and 77.50 º corresponding to the crystal planes (111), (200), (220), and (311) of Ag nanoparticles respectively. There are four clear peaks in the XRD pattern of AgNPs synthesized by using RERE at 2θ angles of 37.87°, 44.02°, 64.24°, and 77.24° respectively Figure 61. The crystal structure of AgNPs synthesized by using ZME also showed an analogous structure. The diffractogram in Figure 62 shows four clear signals at the points of 2θ of 38.12°, 44.58°, 64.65°, and 77.52° which correspond to the Miller indices of (111), (200), (220), and (311) respectively. These peaks indicate the characteristic metallic FCC crystalline structure of Ag matching the database of standard (JCPDS Card no. 03-0921) of the silver nanoparticles (Balaji *et al*., 2009). Many plant-based AgNPs have FCC crystalline structures confirmed by XRD. *Ficus benghalensis* and *Azadirachta indica* extract-mediated AgNPs had analogous crystal structures established by XRD (Nayak *et al*., 2016). XRD peaks of the AgNPs synthesized by using the seed of *Plantago major* showed peaks at 38.1°, 44.2°, 64.5°, and 77.4° which is similar to our observation of FCC crystalline geometry (Nikaeen *et al*., 2020).

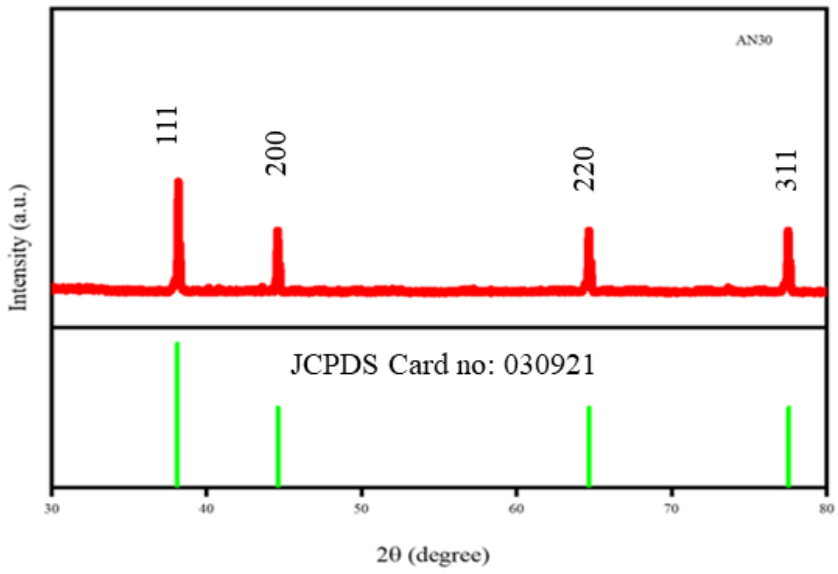


Figure 59: XRD diffractogram of Ag-AgNPs

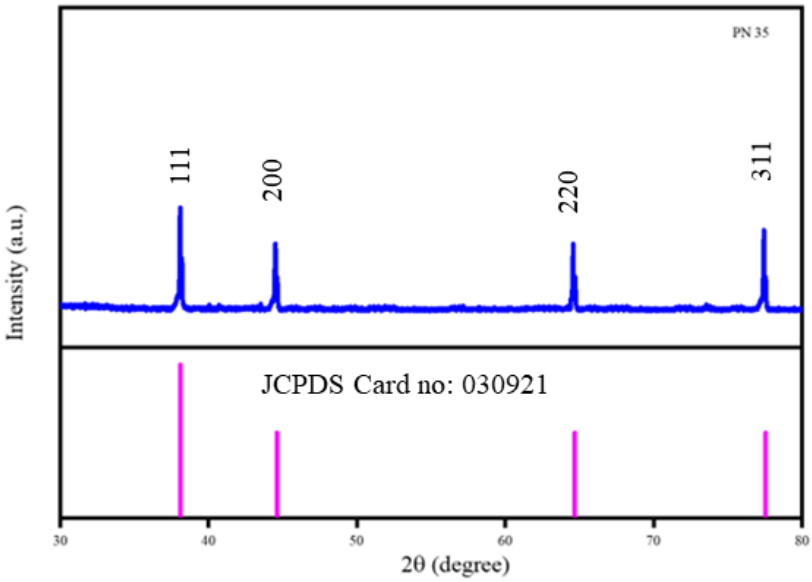


Figure 60: XRD diffractogram of Pp-AgNPs

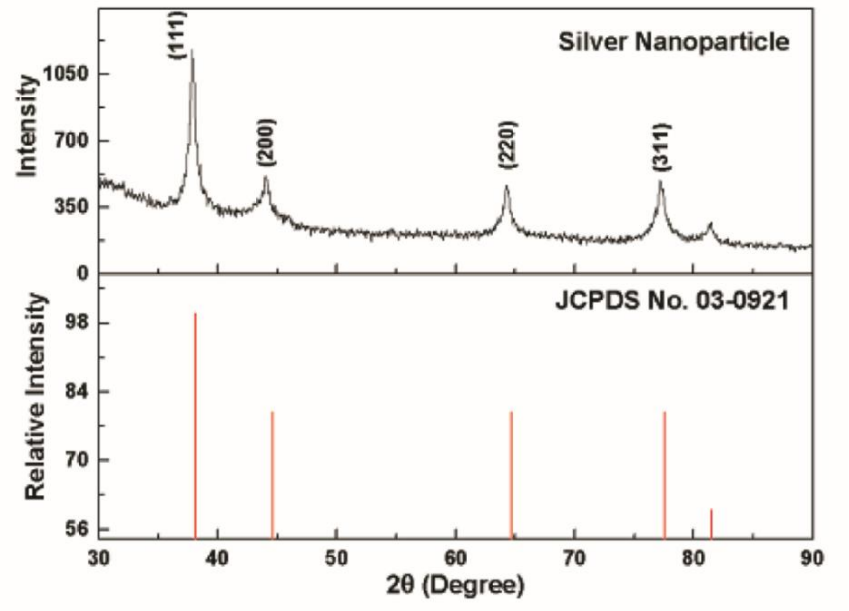


Figure 61: XRD diffractogram of Re-AgNPs

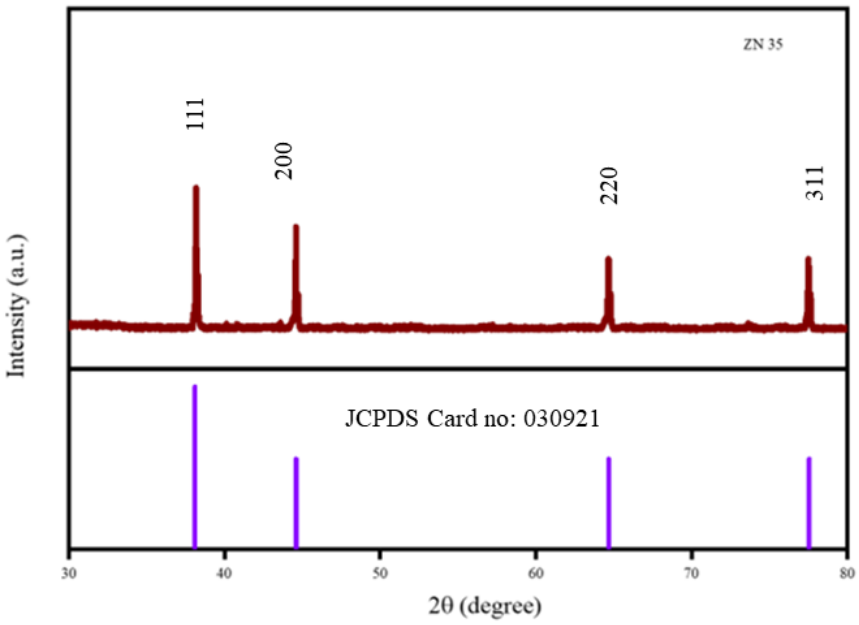
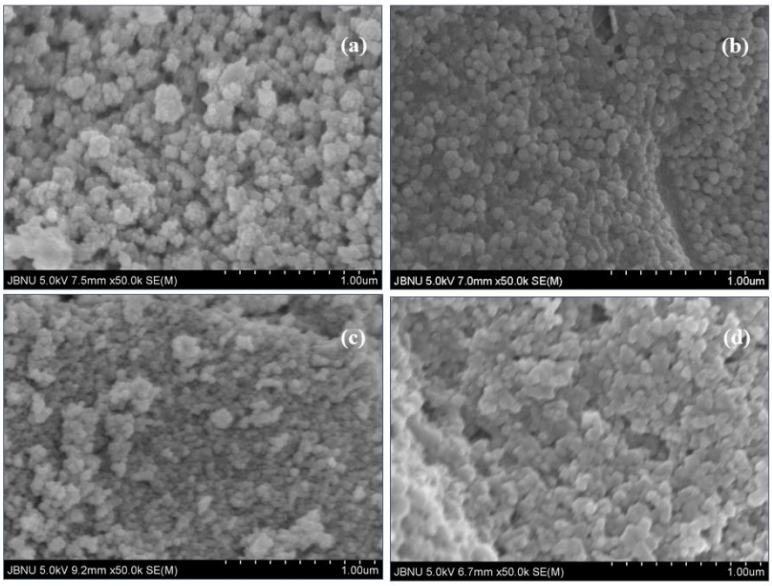


Figure 62: XRD diffractogram of Zm-AgNPs

The specific peaks that appeared in XRD are due to different phytochemicals in the plant extract which are used to reduce $AgNO_3$ into AgNPs and stabilize (Narayanan *et al*., 2021). The appearance of these peaks indicates the presence of certain organic compounds in the extract which are responsible for the reduction, capping, and stabilization of nanoparticles (Ibrahim, 2015).

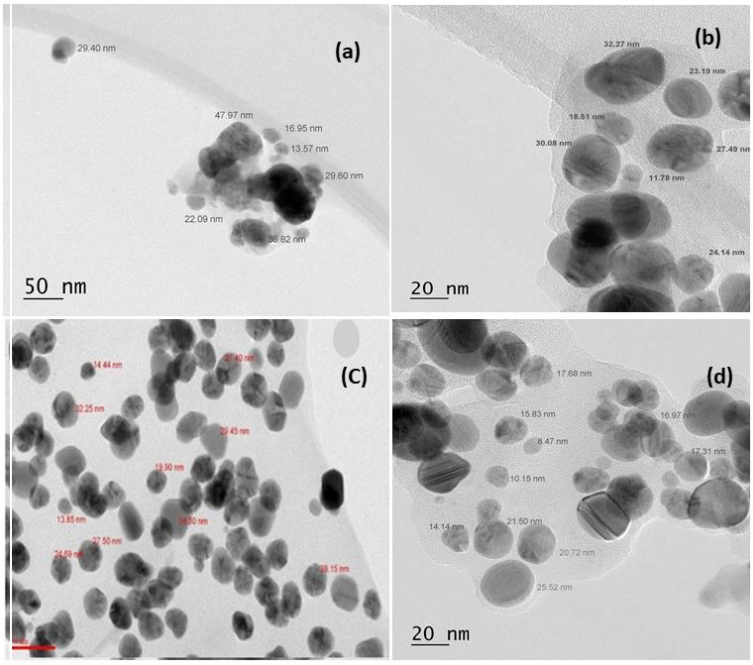
4.7.4 FESEM and TEM analysis

The surface morphology of the synthesized AgNPs was studied by field emission scanning electron microscopy. The SEM micrographs taken at different resolutions were observed. At the low resolution (7.4 mm x 5.0k) the particles were observed as broken lumps of irregular shapes and different sizes (Figure 63). The transmission electron microscopy revealed the presence of nearly spherical particles with varying shapes and sizes as shown in Figure 64. The size of the particles was measured by using Image J software from the TEM micrographs.



(a) Ag-AgNPs at the magnification of 7.5 x50.0k (b) Pp-AgNPs at the magnification of 7.0 x 50.0k (c) Re-AgNPs at the magnification of 9.2x 50.0k (d) Zm-AgNPs at the magnification of 6.7 x 50k

Figure 63: SEM images of AgNPs



Note: (a) Ag-AgNPs (b) Pp-AgNPs (c) Re-AgNPs (d) Zm-AgNPs

Figure 64: TEM images of synthesized AgNPs

The average dimensions of nanoparticles synthesized from *A. grandifolia*, *Pyrus pashia*, *R. ellipticus*, and *Z. mauritiana* extract were 28.05 ± 11.8, 23.92 ± 7.04, 25.20 ± 7.01, and 16.8 ± 5.51 nm respectively. The size of AgNPs synthesized from *Z. mauritiana* extracts was found to have the smallest size with less variation while that of *A. grandifolia* resulted in the largest sized AgNPs ranging from 13.57 to 47.97 nm. The size distribution of the synthesized AgNPs is shown in the histograms in Figure 66.

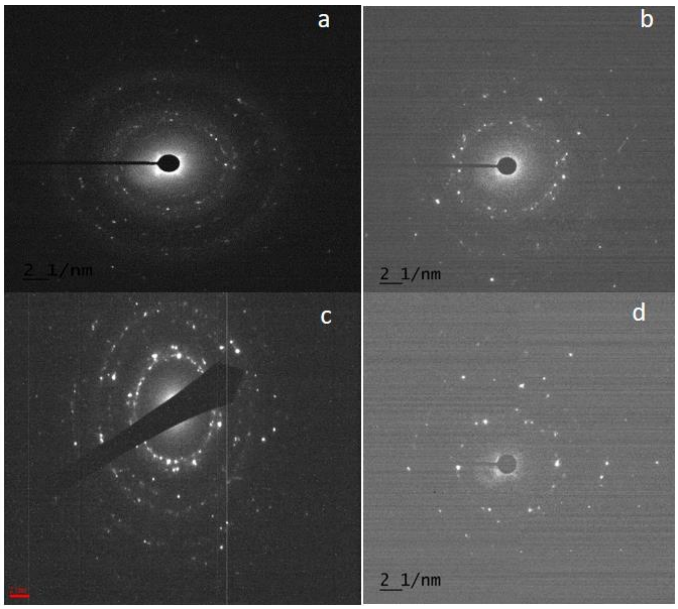
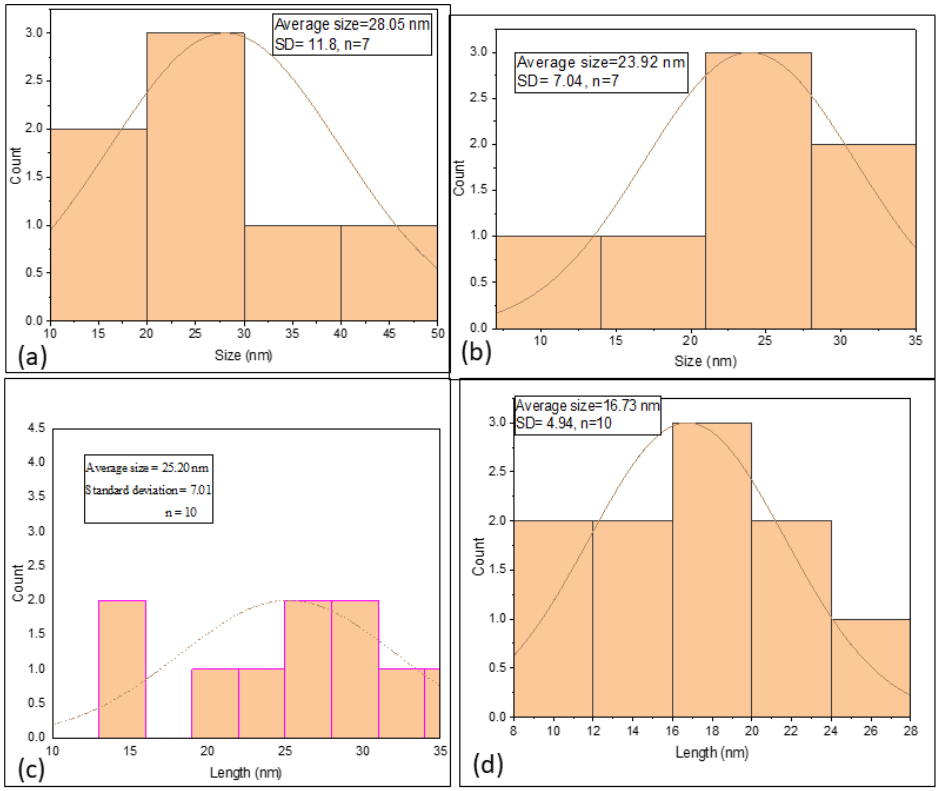


Figure 65: SAED pattern of synthesized AgNPs

(a) Ag-AgNPs (b) Pp-AgNPs (c) Re-AgNPs (d) Zm-AgNPs

The crystallinity of the synthesized AgNPs was further detected by the clear rings observed in the selected area electron diffraction (SAED) pattern (Figure 65). Four discrete ring patterns prove that the majority of the particles are single and oriented along their lattice planes of (111), (200), (220), and (311) confirming the fcc structure (Kharat & Mendhulkar, 2016). The size of crystalline AgNPs synthesized by using the extracts of *Berberis asiatica* and *Cassia fistula* collected from Nepal were 13 and 15 nm respectively (Khadka *et al*., 2021). Atomic force microscopy (AFM) investigation of biogenic AgNPs synthesized by using leaf extract of *Z. mauritiana* of Pakistani origin had a size of 7-22 nm which is analogous to our report (Memon *et al*., 2020). The presence of different elements in the AgNPs was recognized by the EDX spectrum in Figures 67 to 70. The appearance of an intense peak at ~3 keV in all of the spectra established the presence of Ag° that approves the formation of AgNPs (Fouda *et al*., 2020).



(a) Ag-AgNPs (b) Pp-AgNPs (c) Re-AgNPs and (d) Zm-AgNPs

Figure 66: Histograms showing size-distribution of AgNPs

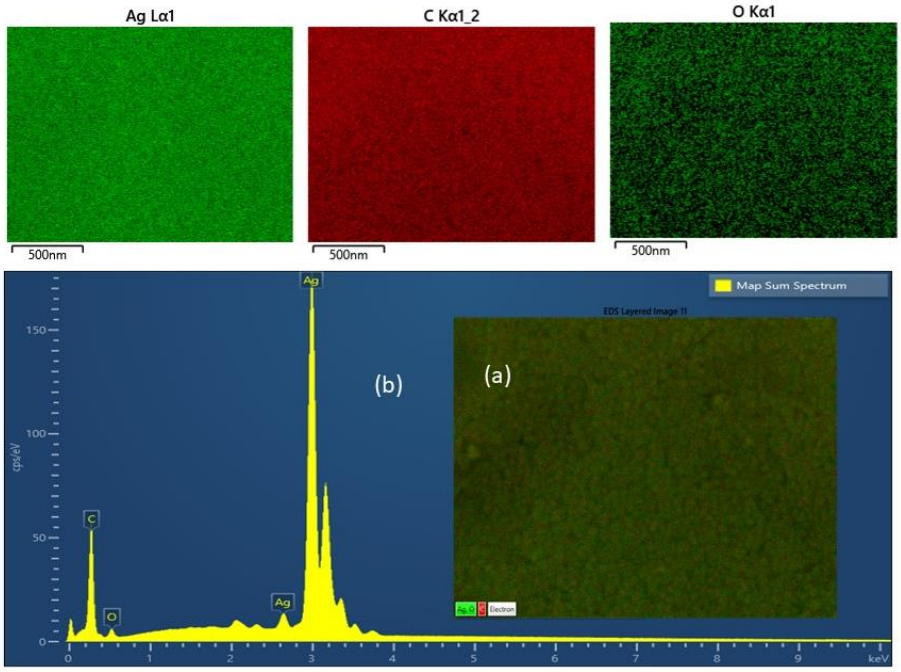
In addition to Ag, the EDX profile showed the peaks corresponding to carbon, oxygen, chlorine, and calcium which are likely due to the interaction of X-ray on proteins/enzymes existing in the biomass (Jayaraman *et al*., 2011; Menon *et al*., 2017). Table 22 shows the abundance of different elements in silver nanoparticles synthesized by using aqueous extracts of *A. grandifolia*, *P. pashia*, *R. ellipticus*, and *Z. mauritiana.* All of the Ag-AgNPs of contained silver, carbon, and oxygen with silver in the highest quantity.

Table 22: Elemental composition of different AgNPs by EDX analysis

Elements	Ag-AgNPs	Pp-AgNPs	Re-AgNPs	Zm-AgNPs
Silver	78.27%	66.75%	56.59%	67.30%
Carbon	17.99%	22.02%	30.74%	20.96%
Oxygen	3.37%	11.23	4.81%	11.74
Chlorine	-	-	7.11%	-
Calcium	-	-	0.74%	-

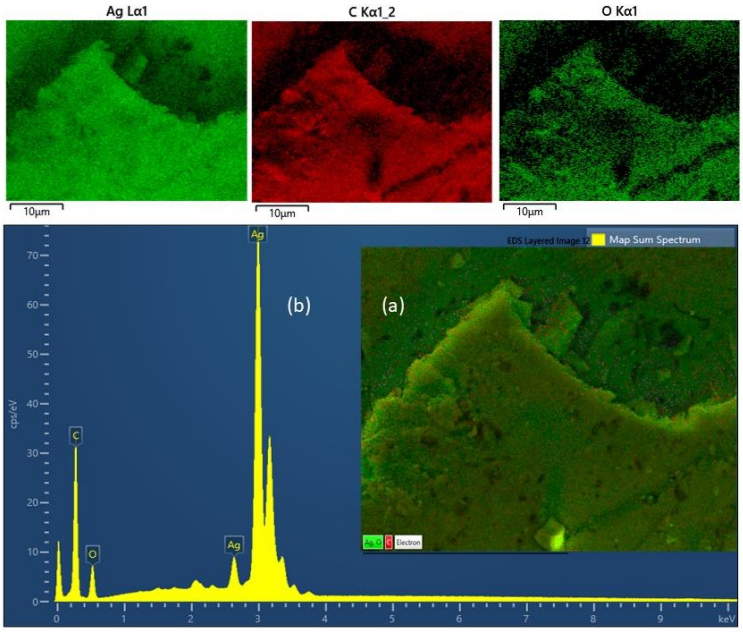
In addition to carbon and oxygen, AgNPs synthesized by using *R. ellipticus* root extract contained chlorine and calcium in small amounts which are similar to the presence of Cl obtained almost at 0.5 keV in the AgNPs synthesized by using the extract of *Phyllanthus emblica* (Renuka *et al*., 2020).

The higher proportions of silver might be attributed to the presence of Ag_2O formed by partial oxidation, and the occurrence of other elements is associated with the presence of biomolecules capped with AgNPs (Phuyal *et al*., 2022).



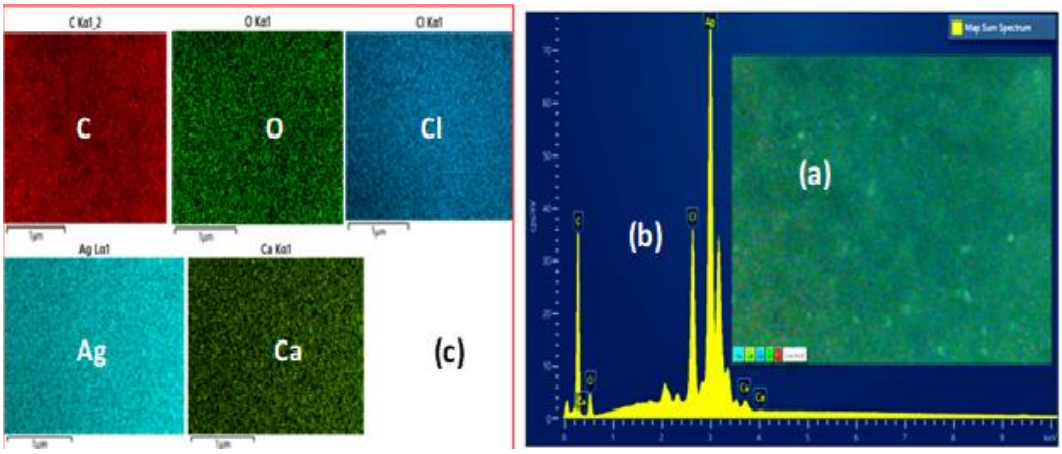
(a) Total elemental mapping (b) EDX spectrum of synthesized nanoparticles

Figure 67: EDX elemental composition of Ag-AgNPs



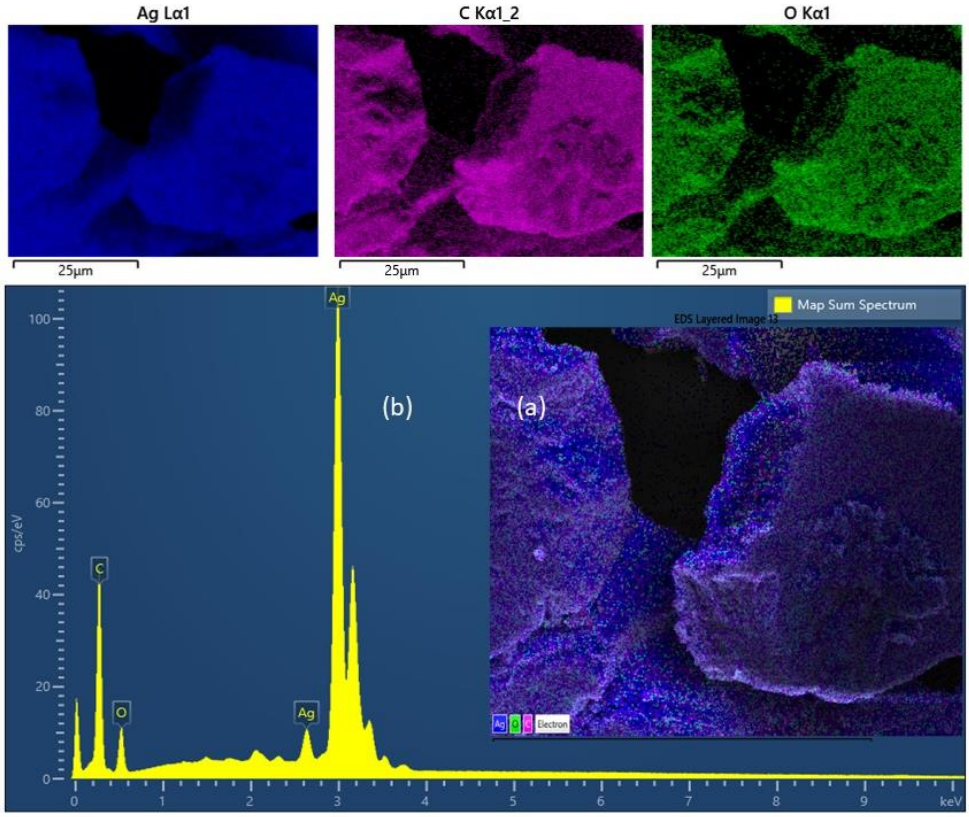
(a) Total elemental mapping (b) EDX spectrum of synthesized nanoparticles

Figure 68: EDX elemental composition of Pp-AgNPs



(a) Total elemental and color mapping for all elements (b) EDX spectrum (c) component elements

Figure 69: EDX elemental composition of Re-AgNPs



(a) Total elemental mapping (b) EDX spectrum of synthesized nanoparticles

Figure 70: EDX elemental composition of Zm-AgNPs

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

Phytochemical screening of twenty-two medicinal plants from Mustang and Kaski districts of Nepal showed the presence of important secondary metabolites like phenolics, alkaloids, flavonoids, tannins, and terpenoids.

The phytochemical and bilological studies of the *Ayenia grandifolia* was accomplished for the first time. The methanol extract of the plant exhibited a strong DPPH radical scavenging activity (IC_{50} = 12.87 ± 0.14 μg/mL), and moderate antibacterial and α-amylase inhibitory activity. Among the identified compounds by GC-MS analysis, Di-n-octyl phthalate (28.39%), 2, 6, 11-Trimethyl dodecane (15.77%), 4, 6-Dimethyl dodecane (12.79%), o-Guaiacol (7.07%), 3-Eicosyne (6.83%), p-Ethyl guaiacol (6.40%) and Methyl palmitate (6.19%) were detected in the essential oil of *A. grandifolia* aerial parts. The results of the phytochemical and biological studies of *A. grandifolia* have been found vital to recommend the isolation of active compounds from the plant.

The crude methanolic extracts of *Rubus ellipticus,* and *Pyrus pashia* exhibited significant antimicrobial activity against *S. aureus* with the highest MIC and MBC values. Among the plants tested, methanol extracts of A. *grandifolia, B. roxburghiana, D. coronans, E. pachyclada, P. pashia, R. ellipticus,* and *Z. mauritiana* exhibited significant antioxidant activity. The extracts of polar solvents e.g. methanol and ethyl acetate of aerial parts of *E. pachyclada,* and barks of *B. roxburghiana* exhibited considerably higher antioxidant and antibacterial activities against the tested bacteria. Essential oils isolated from *E. pachyclada* and *B. roxburghiana* were found to contain different compounds on GC-MS analysis.

Based on preliminary phytochemical screening, estimation of TPC, TFC, antioxidant, antibacterial, and antidiabetic activities, *A. grandifolia, P. pashia, R. ellipticus, and Z. mauritiana* were selected for the synthesis, characterization, and evaluation of biological activities of AgNPs. The potential role of functional groups in the synthesis of AgNPs was revealed by FTIR spectra. All of the biosynthesized AgNPs were nearly spherical shaped with some irregular particles revealed by SEM micrograph. EDX analysis showed the presence of silver in the highest proportions and trace quantities of

oxygen, chlorine, calcium, and carbon. When compared to JCPDS file 03-0921, the XRD pattern of all AgNPs were found to have FCC crystalline structure. It was further confirmed by the SAED pattern having four discrete rings corresponding to the crystal planes at 110, 200, 220, and 311. The size of the synthesized AgNPs ranged from 28.05 ± 11.8 nm (Ag-AgNPs) to 16.73 ± 4.94 nm (Zm-AgNPs). All of the silver nanoparticles exhibited enhanced DPPH scavenging and antibacterial activities. The synthesized nanoparticles exhibited enhanced antibacterial activity against both Gram-negative and Gram-positive bacteria.

From these results, it is concluded that the aqueous extracts of active medicinal plants of the Himalayan region of Nepal can act as prominent sources for the synthesis of AgNPs. Further works on the synthesis of size-controlled AgNPs by optimizing various parameters like pH, concentration, temperature, plant parts, collecting season, etc. are crucial to recommend for the application of biogenic AgNPs in other significant grounds. Comprehensive research is needed to design safety use and disposal of Ag and AgNPs without creating new threats to the environment. Plenty of works are necessary to explore the chemistry and mechanisms behind the compatibility of AgNPs to the biological, chemical or drug molecules. These green synthesized silver nanoparticles from the sustainable resource of Nepal may open the doors to the use of Nepalese medicinal plants in nanomedicine. The study highlights the space for the additional comprehensive studies on other plants to synthesize size-controlled AgNPs with better biological assets that are essential for the benefit of mankind.

CHAPTER 6

6. SUMMARY

Plants are considered ample sources of medicines and several drugs have been isolated from them. Medicinal plants contain a broad spectrum of several bioactive molecules which exhibit vital therapeutic possessions. Many people in Nepal especially those residing in rural areas are inspired by Ayurveda and apply different plants for their primary healthcare for a long time. There are many indigenous medicinal plants gaining research interest due to their unique constituents and versatile applicability in different fields of research and development. The plants selected based on an ethnobotanical survey from high-altitude hilly areas of Nepal have been used against various ailments including arthritis, asthma, stress, fever, cancer, diabetes, etc. Moreover, they are the leading sources of antioxidants and immune regulators.

Nanotechnology is gaining considerable attention from researchers owing to its multipurpose applications in diverse fields. Nanoparticles (NPS) of noble metals such as silver, gold, and platinum are recognized for their extensive applications in electronics, magnetic, biomedical, etc. Silver is one of the important naturally occurring metals has been widely used in electrical and thermal devices due to its high conductivity and inertness. Metallic silver is insoluble in water and inert but soluble silver salts have been used in treating epilepsy, mental disorders, gastroenteritis, syphilis, and gonorrhea. Nano formulation of silver is gaining consideration due to its unique antimicrobial assets leading to the manufacture of food packaging materials, food supplements, electronics, cosmetics, textiles, water purifications, biomedical applications, etc.

Chapter 1 of the thesis introduces the historical background of the use and importance of medicinal plants by different communities in the world. It highlights the progress of the application and documentation of herbal medications by various civilizations. It explains the diverse biochemical and metabolic processes in the living organisms responsible for the production of ROS and RNS that result in several health complications. The secondary metabolites in plants that are isolated and developed as important drugs against many diseases induced by oxidative stress are highlighted. It explains the green synthesis of silver nanoparticles by using plant extracts in benign

conditions. Green synthesis is a cost effective, eco-friendly, and easier technique that can be developed to manufacture several consumer products for biomedical applications. Moreover, this chapter opens up the motivation and objectives of this study. It provides an adequate background to conduct this research in Nepal for the integration of sustainable plant resources into nano-products having multifaceted applications.

A review of relevant literature from previous works on different plants that have been used by different communities in Nepal is included in Chapter 2 of the thesis. The documentation of some of the important biological activities of the crude extracts and isolated compounds is included, as are the morphological, geographical, and ethnobotanical applications of the plants. The synthesis and characterization of plant mediated silver nanoparticles as well as their activities are appraised. The plausible mechanisms of reduction, capping, and stabilization of AgNPs as well as their various parameters like pH, temperature, concentrations, etc. that influence their size, shape, morphology, etc., are reviewed. The urgency of toxicological investigation for the safe and economic use of AgNPs is explained in this section of the thesis. It provides the context for filling the research gap as well as the hypotheses required to carry out this work in Nepal.

In Chapter 3, the thesis enumerates the materials and methods used to establish the selection measures for the synthesis of AgNPs using the active plants selected for the study. It entails calculating the total phenolic and flavonoid content as well as their antioxidant, α-amylase inhibitory, and antibacterial activities. The shape, size, and morphology of the synthesized AgNPs were determined by using the FESEM, TEM images. The FCC crystallinity was established by the analysis of the XRD patterns of the synthesized materials.

The results and discussion of entire work is included in the Chapter 4 of the thesis. Methanol extracts most of the plants contained flavonoids, polyphenols, alkaloids, terpenoids, saponins, reducing sugars, reducing sugars, etc. Based on the results of biological activities, four plants viz. *A. grandifolia, P. pashia, R. ellipticus,* and *Z. mauritiana* were found to be the most potent and were selected for the synthesis of AgNPs. The presence of sharp peaks at 425-435 nm in UV-visible spectra was due to the surface plasmon resonance of the metal nanoparticles. FTIR analysis of the dried

AgNPs revealed the presence of functional groups responsible for the formation and stabilization of AgNPs. The size of AgNPs determined from TEM images ranged from 28.05 ± 11.8 nm (Ag-AgNPs) to 16.73 ± 4.94 nm (Zm-AgNPs). XRD patterns of the synthesized AgNPs were compared to the JCPDS 03-0921 to establish the FCC crystalline shape. EDX studies of the AgNPs revealed the presence of pure silver in the highest percentage and trace proportions of carbon, oxygen, chlorine, and calcium. Studies of the SAED pattern of the AgNPs showed the spherical concentric discrete lines indicating the crystal planes of the hkl values of (111), (200), (220), and (311).

The synthesized AgNPs exhibited higher DPPH radical scavenging capacity than that of the crude extract of all of the plants**.** The synthesized nanoparticles exhibited enhanced antibacterial activity against both Gram-negative and Gram-positive bacteria.

Chapter 5 of the thesis gives the conclusive statements of the overall findings of the study. The biosynthesizing of the plant material from the study area into AgNPs increases the antioxidant and antibacterial activities, demonstrating the potential relevance in biomedical applications. The work emphasizes the necessity for further thorough research on other plants in order to synthesize size-controlled AgNPs with superior biological properties that are crucial for the welfare of people.

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Hindawi
Journal of Nanomaterials
Volume 2022, Article ID 1832587, 11 pages
https://doi.org/10.1155/2022/1832587

Research Article

Green Synthesis of Silver Nanoparticles from Root Extracts of *Rubus ellipticus* Sm. and Comparison of Antioxidant and Antibacterial Activity

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Received 12 January 2022; Revised 23 February 2022; Accepted 1 March 2022; Published 11 March 2022

Academic Editor: Thathan Premkumar

The fabrication of metal nanoparticles through green synthetic pathways using plant extracts has increased attention due to low cost, benevolent methods, fewer hazardous byproducts, and applications. Silver nanoparticles (AgNPs) were synthesized by reacting to aqueous root extracts of *Rubus ellipticus* Sm. (RERE) with $AgNO_3$ solution (1 mM) at an ambient condition. The visual change of color from light yellow to reddish brown and the absorption peak at 416-420 nm in the UV-visible spectra indicated the formation of AgNPs in the solution. The shifting of the positions in the FTIR spectra indicated the potential role of the functional groups as capping and stabilizing agents. The powder XRD diffractogram exposed the crystalline nature of the nanoparticles. The surface morphology and the elemental composition of the AgNPs were established by the FESEM and EDX analysis. The TEM images revealed the spherical and monodispersed nanoparticles of size ranging from 13.85 to 34.30 nm with an average of 25.20 ± 7.01 nm ($n = 10$). The biogenic AgNPs showed a better 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity with lower IC_{50} ($13.83 \pm 0.33\ \mu g/mL$) as compared to that of the RERE with IC_{50} ($15.86 \pm 4.14\ \mu g/mL$). The synthesized AgNPs showed higher zones of inhibition (ZOI) on the agar well diffusion method against *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Klebsiella pneumoniae* (ATCC 700603). The result of this study highlights the potential benefits of *R. ellipticus* root extract-based AgNPs for biomedical practices.

1. Introduction

Nanotechnology is emerging as an extensive interdisciplinary area of research all over the world for a few decades. The design, manufacturing, characterization, and application of nanoparticles have increased attention due to their unique physical and chemical properties [1]. Generally, nanoparticles are extremely small particles with their size ranging from 1 to 100 nm and exhibit completely new properties compared to that of bulk materials. They have a higher surface area to volume ratio, which brings the variation in the other specific parameters like size, distribution, and morphology. The higher surface area of the AgNPs is responsible for their increased catalytic and biological properties [2]. The nanoparticles of noble metals like gold, silver, palladium, and platinum are extensively used in various industrial and pharmaceutical practices due to their incredible physicochemical, optical, and biological properties [3].

Silver is a safe antibacterial metal that is reported to kill more than 650 pathogenic bacteria, and many researchers are involved in the synthesis of silver nanoparticles (AgNPs) because of their higher antimicrobial nature

[4]. Nowadays, silver nanoparticles are used in diverse fields such as electronics, optics, photography, clothing, catalysis, dentistry, and food industries. Besides, it has immense application in biomedical fields such as biomolecular detection, biosensors, and antifungal, antibacterial, and antiangiogenetic agents [5, 6].

Conventionally, AgNPs are synthesized by various physical methods, e.g., evaporation-condensation, arc discharge, and spray pyrolysis, and chemical methods, e.g., photochemical and electrochemical reduction, Tollens's method, and sonochemical methods. These approaches suffer from the complications like the use of expensive and hazardous chemicals as well as the formation of toxic byproducts [7, 8]. The investigates are focused on the "green synthesis" methods as a substitute for orthodox procedures by using biological extracts such as plants, fungi, algae, and microbes [1]. The extract of a brown algae *Sargassum longifolium* was used for the synthesis of AgNPs which significantly inhibited the growth of pathogenic fungi *Aspergillus fumigatus*, *Candida albicans*, and *Fusarium* species by the agar well diffusion method [9]. *The mycogenic Penicillium chrysogenum*-derived AgNPs having an average size of 48.2 nm inhibited the process of biofilm formation by 90% against *Acinetobacter baumannii* (ATCC 19606) at the concentration of 2 μg/mL [10]. The AgNPs biosynthesized from the cultures of *Candida albicans* and 1.5 mM $AgNO_3$ with the size range of 20-80 nm exhibited significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [11]. The biogenic AgNPs synthesized having an average size of 15 nm from the cultures of *Corynebacterium glutamicum* were reported to exhibit enhanced antibacterial activity against *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus flexus*, and *Bacillus subtilis* by the agar well diffusion method [12]. The commercial production of nanoparticles using microbes is less manageable as it needs highly aseptic conditions and proper maintenance. The use of plants for this purpose is more advantageous due to lesser biohazards and benign methods as well as no risk of chemical contamination [5, 13]. In comparison to other green methods, plant-based approaches are easy, quick, safe and result in the formation of stable nanoparticles having fewer side effects [14]. Diverse secondary metabolites in plants like flavonoids, terpenoids, ketones, carboxylic acids, amides, proteins, and enzymes act as reducing, capping, and stabilizing agents and therefore facilitate the bioreduction and precipitation of AgNPs, which are safe and cost-effective [15–17]. The increase in antimicrobial effectiveness of green synthesized AgNPs is due to the presence of antimicrobial materials in the extract. The contemporary literature comprises many reports of synthesis, characterization, and applications of plant-mediated AgNPs. The green synthesized AgNPs from the leaf extract of *Pongamia pinnata* were reported to exhibit substantial antioxidant activity [18]. The biosynthesized AgNPs from the leaf extract of *Chenopodium murale* with sizes ranging from 30 to 50 nm exhibited elevated antioxidant and antimicrobial activities [19]. A medicinal plant, *Eriobotrya japonica*, collected from Iraq was used for the green synthesis of AgNPs. The crystalline nanoparticles having a size ranging from 17 to 35 nm were reported to inhibit the cancer cell proliferation and induced apoptosis. They decreased the IL-beta and IL-6 levels *in vivo* as well as *in vitro* models [20]. Similarly, biogenic AgNPs synthesized from the fruit extract of *Emblica officinalis*, leaves of *Citrus limon*, *Camellia sinensis* (green tea), *Coffea arabica* (coffee), etc. were characterized by proper procedures and accessed for their biological activities [13]. Moreover, the green synthesized AgNPs from the stem and root extracts of *Lysiloma acapulcensis* exhibited higher antimicrobial activity than chemically synthesized AgNPs. The values of the zone of inhibition (ZOI), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* of green AgNPs are higher than those of chemically synthesized AgNPs [21].

Rubus ellipticus Sm. (Rosaceae) is abundant in the wide areas of forest edges in the elevations of 548-1700 m in various parts of Asia and other continents. Different parts of the plant are reported to show various biological activities such as antidiabetic, antioxidant, antimicrobial, wound healing, and antitumor [22, 23]. It is also known as Himalayan raspberry and is locally named "Yensalu." The methanol root extract of the plant exhibited significant antioxidant and antimicrobial properties [24]. The synthesis and characterization of AgNPs from *R. ellipticus*roots have not been reported to our information. Therefore, this study is aimed at synthesizing and comparing the antioxidant and antimicrobial activities of the biogenic AgNPs with that of aqueous extract of *R. ellipticus.*

In this research, a new and green route is explored for the synthesis of AgNPs in which the phytochemicals present in the root extract of *R. ellipticus* reduced Ag^+ ion into elemental AgNPs. The UV-visible and Fourier-transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and powdered X-ray diffraction (XRD) methods were used for the characterization of the AgNPs. The synthesized AgNPs were analyzed for the comparative antioxidant and antibacterial activities.

2. Materials and Methods

2.1. Materials and Reagents. The root barks of the plant were collected from the outskirts of Pokhara city in the Gandaki province of Nepal in July 2020. The proof of identity of the plant was taken from the National Herbarium and Plant Laboratories, Godawari, Nepal. The root barks were cleaned and rinsed with distilled water properly. The barks were shade-dried for four weeks and converted into a fine powder using a mechanical mill. Analytical grade reagents and deionized water were used throughout the experiment. Silver nitrate, dimethyl sulphoxide, and methanol were purchased from Thermo Fisher Scientific India Pvt. Ltd.; neomycin, Muller Hinton Agar (MHA), and Muller Hinton Broth (MHB) of Himedia Pvt. Ltd. India and DPPH of Tokyo Chemical Industries Co. Ltd. Japan were used.

2.2. Preparation of Plant Extract. Five grams of root powder was added to 50 mL of distilled water in an Erlenmeyer's flask of 250 mL capacity. The mixture was heated carefully for about 15-20 minutes and was filtered through a Whatman filter paper (no. 1). The prepared aqueous extract was stored at 4°C and used within a week.

2.3. Biosynthesis of Silver Nanoparticles. 5 mL of the fresh RERE was dropped into 45 mL of $AgNO_3$ (1 mM) solution with constant stirring on a magnetic stirrer at 25 ± 2°C [25–27]. The light yellow color of the solution started to change gradually and turned reddish brown within one hour. It was considered as a visual sign of the growth of AgNPs. After the completion of the reaction, the solution was centrifuged at 8000 rpm for 40 minutes at 20°C and repeatedly washed three times. It was collected and further centrifuged at 14000 rpm with double-distilled water for 20 minutes. Finally, it was dehydrated by ethanol, dried using a desiccator, and stored for the necessary characterization and biological studies.

2.4. Characterization of AgNPs

2.4.1. UV-Visible and FTIR Spectroscopy. The absorption spectra of synthesized AgNPs were recorded by using UV-visible spectroscopy from 300 to 600 nm (BioTek, Synergy LX multimode reader) at the intervals of 15 minutes, 50 minutes, 18 hours, and 48 hours by taking distilled water as blank. The FTIR spectra of the solid RERE and AgNPs were taken by a Shimadzu IRTracer-100 from 4000 to 400 cm^{-1}. It helped to detect the functional groups in the extract and the AgNPs that might be accountable for synthesizing the nanoparticles.

2.4.2. X-Ray Diffraction (XRD) Analysis. An X-ray diffractometer (Rigaku Co., Japan) was used to confirm the crystallinity of the synthesized AgNPs. The diffractogram was obtained from the dried layer of the sample over a sample holder by using CuKα ($\lambda = 1.5406$ Å) radiation with 30 mA of current and 40 kV of voltage in a scan rate of 10°/minute across the 2θ angle ranging from 0 to 90 [20].

2.4.3. FESEM, EDX, and TEM Analysis. The suspension of AgNPs was spread uniformly and dehydrated over a cover glass and placed on a copper stub attached with carbon tape. The TEM images and selected area electron diffraction (SAED) pattern were taken by using a JEM-2100 plus at 200 kV (JEOL Ltd., Japan). The images were taken by coating the suspension of AgNPs over a carbon-coated copper grid. The size of the synthesized AgNPs was measured with the help of ImageJ software from the TEM images.

2.5. In Vitro Antioxidant Activity. The DPPH free radical method was used to assess the antioxidant activity of the RERE and the AgNPs with minor adjustments [28, 29]. Briefly, the solutions of 500, 250, 125, 62.5, 31.25, and 15.62 μg/mL were prepared by serial dilution in 50% dimethyl sulphoxide (DMSO). Aliquots of 100 : 100 μL of the test solutions and DPPH (0.1 mM) were filled in the bores of a 96-well microplate with negative and positive controls in triplicates. Then, the microplate was put in the dark at room temperature for 30 minutes. The absorbance of the solutions was recorded by using a microplate reader at 517 nm. The percentage scavenging (%Sc) of the samples was calculated by applying

$$\%\mathrm{Sc} = \frac{A_b - A_t}{A_b} \times 100, \quad (1)$$

where A_b and A_t are the absorbances of the blank and test sample, respectively. The DPPH free radical inhibition activity of the RERE and the synthesized AgNPs at different concentrations was plotted and compared with that of ascorbic acid. The concentration corresponding to 50% inhibition (IC_{50}) was calculated.

2.6. Antimicrobial Activity

2.6.1. Microorganisms. The antibacterial tests were made by taking the pure cultures of bacteria from the American Type Culture Collection (ATCC). They were subcultured and stored into the Muller Hinton Agar (MHA) media at 4°C. The organisms used in the test are listed in Table 1.

2.6.2. Agar Well Diffusion Assay. The agar well diffusion method was adopted to observe the antibacterial susceptibility [30]. The test bacteria were grown in the Muller Hinton Broth (MHB) so that the turbidity equals that of 0.5 McFarland's standard (1.5×10^8 CFU/mL). Aliquots of 30 μL of the RERE, synthesized AgNPs, negative and positive controls were loaded into the wells of 6 mm diameter and incubated for 24 hours at 37°C. On the next day, the plates were taken out from the incubator, and the clear regions formed around the wells were recorded as the corresponding zones of inhibition (ZOI).

2.6.3. Statistical Analysis. The experiments were performed in triplicates, and the results were presented as mean ± standard deviation. The raw data of antioxidant activities were processed by using Gen5 Microplate Data Collection and Analysis Software and then by Microsoft Excel. The FTIR data of AgNPs and the plant extract were plotted in "Origin 19b." The concentration corresponding to 50% inhibition (IC_{50}) was determined with the help of GraphPad Prism 9 software.

3. Results and Discussion

3.1. UV-Visible and FTIR Spectroscopy. Various methods have been used for the synthesis of metallic nanoparticles. The biosynthetic method is safe and ecofriendly and uses secondary metabolites in the plant extract for the reduction. The addition of RERE to 1 mM of silver nitrate solution with constant stirring led to the change of color of the solution from yellowish to reddish brown. It is due to the excitation of surface plasmon resonance (SPR) variations in the biosynthesized nanoparticles [31].

The synthesis of AgNPs was monitored at different time intervals by the change of color and absorption spectra appeared at around 416-420 nm by UV-visible spectroscopy

Table 1: List of bacteria used for the test.

Bacteria	Type	ATCC
Escherichia coli	Gram-negative	25922
Enterococcus faecalis	Gram-positive	29212
Staphylococcus aureus	Gram-positive	25923
Klebsiella pneumoniae	Gram-negative	700603

as shown in Figure 1. The biosynthesized silver nanoparticles of *Rheum australe* extract of Indian origin showed solid absorption bands between 410 and 420 nm indicating the presence of polydispersed particles which agrees with our result [32]. Change of color from golden yellow to pinkish brown was observed in the reduction of Ag^{+} to Ag° in the colloidal solution. A sharp absorption peak at 420 nm was observed in the UV-visible spectroscopy during the biosynthesis of AgNPs from the *Tagetes erecta* aqueous leaf extracts [33]. The strong broad absorption band obtained between 405 and 430 nm for silver nanoparticles indicates that the spherical particles were dispersed without aggregation [34, 35]. Venkatesan et al. [36] reported the SPR peak of the biosynthesized silver nanoparticles at 418 nm upon 18 hours of formation by UV-visible spectroscopy. They prepared the AgNPs from the aqueous extract of marine algae *Ecklonia cava* and the intensity of the peak increased with time which is analogous to our results. The positions of the peaks in the FTIR spectra of RERE and the AgNPs were compared to check the functional groups involved in the capping and stabilizing actions. The IR spectra of RERE and the AgNPs were recorded for different functional groups in the range of 400–4000 cm^{-1} shown in Figure 2. In the spectra, a broad peak at 3240.31 cm^{-1} in the extract is shifted to 3302.76 cm^{-1} in the AgNPs. It is due to the stretching frequency of a hydrogen-bonded -OH group of polyphenols present in the plant [15].

Similarly, the shifting of vibrations at 1039.63 to 1029.98 cm^{-1} corresponds to the C-O stretching of ether linkages present in the flavones which are adsorbed on the surface of the biogenic AgNPs [17, 37]. The peak at 2927.27 cm^{-1} in the RERE is moved to a higher wavenumber of 2939.92 cm^{-1} in the AgNPs corresponding to the stretching of SP^3-hybridized C-H of alkanes or aldehydes [38]. The FTIR peaks which are observed at 1724.90 cm^{-1} and 1600.00 cm^{-1} correspond to the presence of C=O and C=C or aromatic C=C bonds. The change of the position of the peaks of RERE from 1312.25 to 1321.23 cm^{-1} AgNPs corresponds to the stretching of a carbon-nitrogen bond of aromatic amines. The peaks of vibrations at 1174.64 cm^{-1} are observed.The shifting of the peaks of O-H or N-H stretching and double and triple bonds of hydrocarbons in the FTIR spectra were observed in the green synthesis of AgNPs [37, 39].

3.2. X-Ray Diffraction Analysis. Based on Bragg's reflections for a face-centered cubic (fcc) structure of silver, the diffractogram indicated the AgNPs to have a crystalline geometry. The nature of the XRD plot of our AgNPs was matched to the pattern of JCPDS (Joint Committee on Powder Diffraction Standards) file number 03-0921 (Figure 3). There are four intense peaks at the 2θ angles of 37.87, 44.02, 64.24, and 77.24 corresponding to the hkl values of 111, 200, 220, and 311 planes matching to the fcc crystal. This structure of our AgNPs is parallel to the XRD pattern of the synthesized silver nanoparticles from aqueous extracts of *Lysiloma acapulcensis* [21]. The relative position of peaks at the 2θ angles in the XRD pattern of the biosynthesized AgNPs of ethanol extracts of Indian Sandalwood (*Solanum album*) is like to our result indicating the formation of nanoparticles having equivalent geometry [40]. The organic molecules in the RERE that are tangled in the reduction and stabilization of AgNPs are responsible for the peaks in the XRD analysis [26].

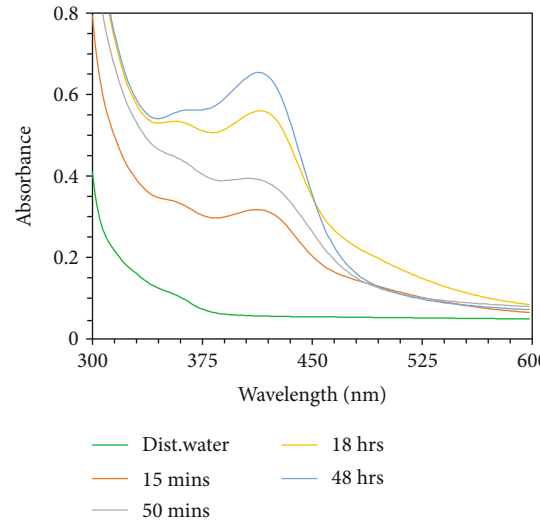

Figure 1: UV-visible spectra of AgNPs at different times.

3.3. FESEM, TEM, and EDX Analysis. The surface morphology of the nanoparticles was observed by scanning electron microscopy with different resolutions. The FESEM images show the particles like broken lumps at the low resolution of 15.2 mm × 100 to clear agglomerates of spherical, oval, or bead-like units of the nearly same size at a higher magnification of 9.2 mm × 150 k as shown in Figures 4(a)–4(d). The single SPR peak observed in the UV-visible spectra of biosynthesized AgNPs suggests the formation of spherical particles which is further confirmed by SEM micrograph [25]. The qualitative and quantitative status of the component elements in AgNPs was recognized by the EDX spectrum. Figures 5(a) and 5(b) show the elemental mapping of synthesized AgNPs that demonstrates the existence of silver, carbon, oxygen, chlorine, and calcium. The existence of an intense peak at 3 keV of Ag° with the highest counts confirms the formation of silver particles [41].

The EDX result shows different peaks of oxygen (0.52 keV), carbon (0.28 keV), calcium (3.69 and 4.12 keV), and chlorine (2.62 keV) in trace amounts (Figure 5(b)). These peaks might be attributed to the contamination of organic materials and carbon tape used in the measurement process. The TEM images (Figure 6) were observed to find the shape and size of the synthesized AgNPs. Most of the nanoparticles are spherical; some are irregular and dispersed

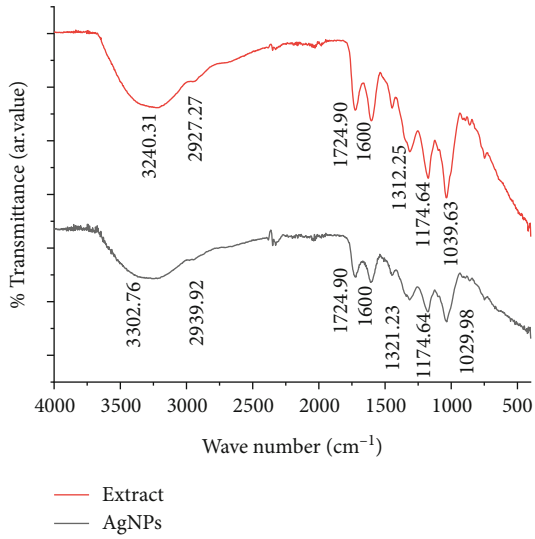

Figure 2: Comparative study of FTIR spectra of RERE and AgNPs.

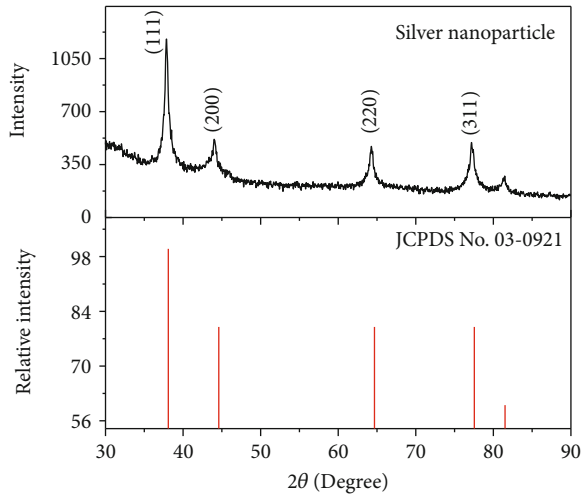

Figure 3: X-ray diffraction (XRD) patterns of synthesized AgNPs and JCPDS no. 03-0921.

with minor agglomeration (Figure 6(a)). The nanoparticles had a size variation from 13.85 to 34.30 nm with an average of 25.20 ± 7.01 nm ($n = 10$) which is shown in the histogram (Figure 6(b)).

The spherical shape and coarse exterior are seen in the TEM image of a single typical AgNPs which have a diameter of 23 nm (Figure 6(d)). There are four diffraction rings equivalent to the fcc planes of (1 1 1), (2 0 0), (2 2 0), and (3 1 1) in the selected area electron diffraction (SAED) diagram of the biogenic AgNPs (Figure 6(c)). Comparable to our observation, the TEM images of the plant-mediated AgNPs prepared by using aqueous leaf extract of *Artocarpus altilis* were also reported an fcc structure and size extending from 20 to 50 nm [42]. Suman et al. [43] reported the size of AgNPs ranging from 32 to 55 nm synthesized by using the root extracts of *Morinda citrifolia* collected from Mangalore, India, is comparable to our result.

3.4. In Vitro Antioxidant Activity. The results of *in vitro* radical inhibiting potential of the AgNPs and RERE against DPPH are presented in Table 2. We observed that the biosynthesized AgNPs were found to be more potent antioxidants than the aqueous root extract of *Rubus ellipticus*. Figure 7 illustrates that the synthesized AgNPs had a higher percentage inhibition in comparison to that of the crude extract at different concentrations. The AgNPs were found to be more powerful antioxidant ($IC_{50} = 13.83 \pm 0.33\,\mu g/mL$), than the RERE ($IC_{50} = 15.86 \pm 4.14\,\mu g/mL$). Ascorbic acid taken as positive control exhibited the highest activity ($IC_{50} = 6.40 \pm 0.29\,\mu g/mL$) as shown in Table 3. A separate study reported that biochemically synthesized AgNPs at an ambient temperature had relatively higher antioxidant activity over aqueous extract of the rhizome of *Rheum australe* collected from Jammu and Kashmir, India [32].

The radical scavenging activity of the synthesized AgNPs was higher than that of *Prosopis farcta* aqueous fruit extract at different concentrations. The higher activity of the biogenic nanoparticles was described due to the abundance of phenolic and flavonoid contents in the extract in comparison to the AgNPs [6]. The AgNPs synthesized using aqueous leaf extracts of *Costus afer* collected from Nigeria also showed higher radical scavenging activity. The advanced activity of the synthesized AgNPs is explained due to the simultaneous mechanisms of hydrogen atom transfer (HAT) and single electron transfer (SET) of flavonoids and silver ions present in the AgNPs [44]. Similarly, the DPPH radical scavenging capacities of AgNPs were increased in comparison to the corresponding extracts which are analogous to our results [36, 45]. The AgNPs synthesized from the rhizome extract of *Bergenia ciliata* of Pakistani origin exhibited an elevated DPPH radical scavenging activity and cytotoxicity against Brine shrimp nauplii ($LD_{50} = 33.92\,\mu g/mL$) than the crude extracts [46].

The secondary metabolites such as phenolics, flavonoids, terpenoids, and soluble proteins act as capping agents for the synthesis of nanoparticles [47]. The electron-donating potential of polyphenols in the plant extracts facilitated the bioreduction of Ag^{+} to Ag° and stabilized the AgNPs. Similarly, the water-soluble flavonoids in plants are also involved in the reduction of silver ions for the synthesis of AgNPs [38]. Important phytochemicals like flavonoids, polyphenols, saponins, terpenoids, and vitamins are responsible for the antioxidant activity of the plant extracts. We have also reported the abundance of these phytochemicals in the roots of *R. ellipticus* collected from the same site [24]. The compounds may have adsorbed on the larger surface area of the spherical AgNPs and amplified the antioxidant potential. The negatively charged phytochemicals exhibit an electrostatic attraction to the positively charged or neutral AgNPs which also synergically improved the bioactivity [48].

3.5. Antibacterial Activity. Most of the AgNPs exhibit significant antimicrobial actions against various bacteria, fungi, and viruses. So, they have been extensively used in many common products like bandages, plasters, catheters, blades, cosmetics, toothbrushes, and cellphones [49]. Here, both

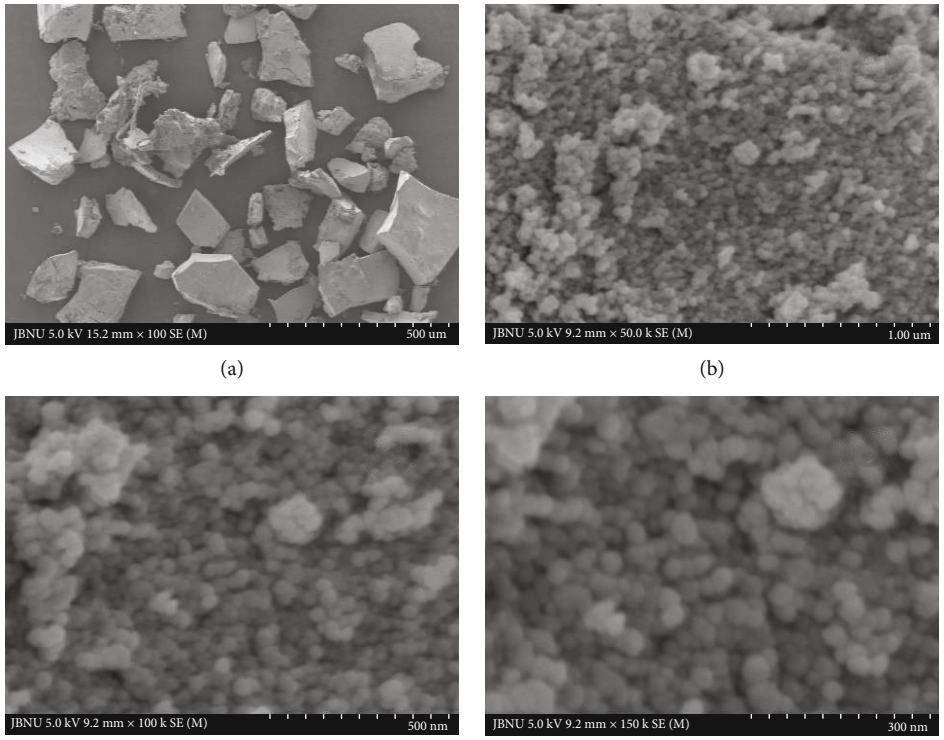

Figure 4: SEM images of the synthesized AgNPs.

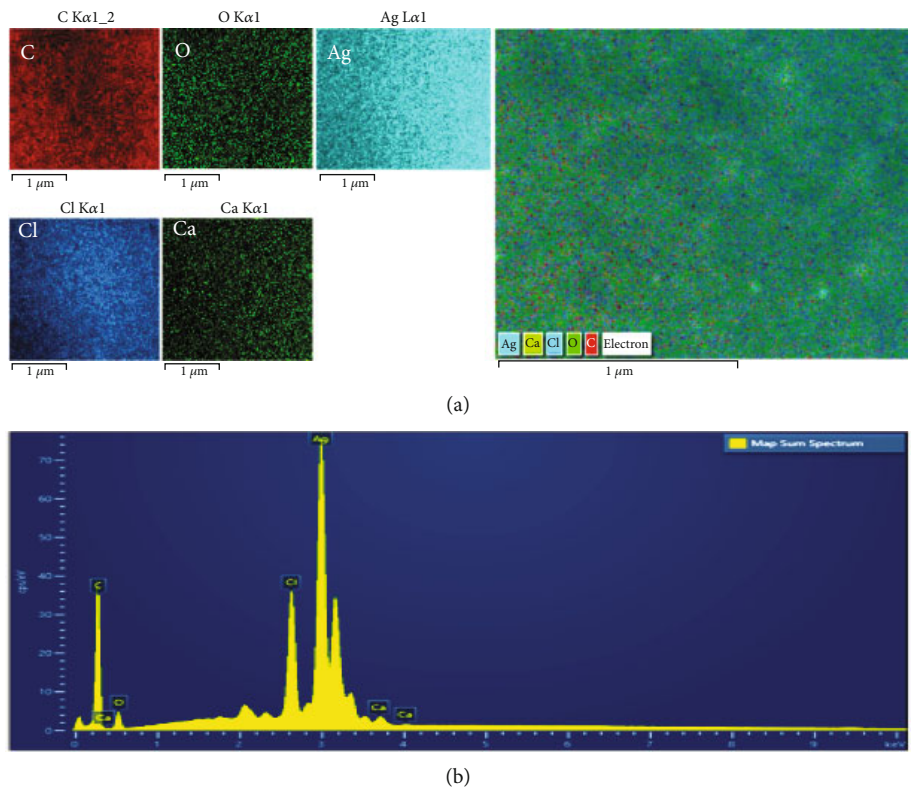

Figure 5: An energy-dispersive X-ray (EDX) spectrum of AgNPs with elemental mapping. (a) Total elemental mapping and color mapping for individual elements existing in synthesized AgNPs. (b) EDX spectrum of synthesized AgNPs.

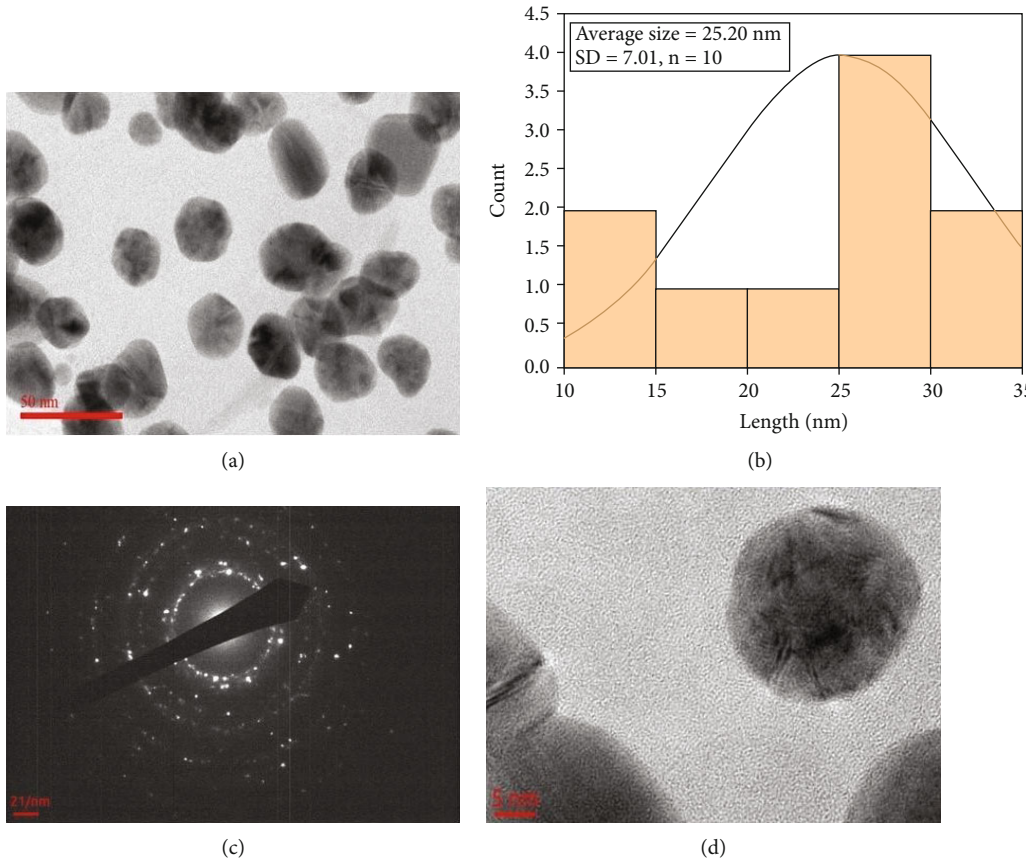

Figure 6: TEM images of the synthesized AgNPs. (a) Synthesized nanoparticles. (b) Mean size of the particles in the histogram. (c) SAED of synthesized AgNPs. (d) A single nanoparticle.

Table 2: Results of antioxidant activities of RERE and AgNPs.

Samples	IC_{50} (μg/mL)
R. ellipticus (aqueous extract)	15.86 ± 4.14
R. ellipticus (AgNPs)	13.83 ± 0.33
*Ascorbic acid	6.40 ± 0.29

Note: values are mean ± SD ($n = 3$); *ascorbic acid is a positive control.

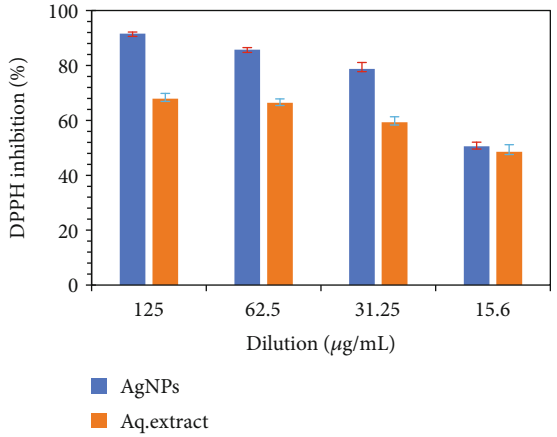

Figure 7: DPPH radical scavenging activity of RERE and AgNPs.

the Gram-positive and Gram-negative bacteria were evaluated for antibacterial susceptibility and the results are shown in Figure 8. Our results obtained from the agar well diffusion method had shown that the AgNPs displayed greater activity against all the tested bacteria. Table 3 shows that the ZOI values of RERE and AgNPs against *E. coli* increased from 10 mm to 13 mm, *S. aureus* increased from 9 mm to 12 mm, *E. faecalis* increased from 10 mm to12 mm, and *K. pneumoniae* increased from 11 mm to 14 mm. The maximum activity was exhibited against *K. pneumoniae*.

The AgNPs synthesized from *Areca catechu* showed dose-dependent zones of inhibition against antibiotic-resistant bacteria on the agar well diffusion method. The values of MIC and MBC of AgNPs revealed the substantial action on *Enterococcus faecalis* and Vancomycin-resistant *E. faecalis* (MIC = 11.25 μg/mL and MBC = 22.5 μg/mL), *Pseudomonas aeruginosa* and multidrug-resistant *P. aeruginosa* (MIC = 5.6 μg/mL and MBC = 22.5 μg/mL), and *Acinetobacter baumannii* and multidrug-resistant *A. baumannii* (MIC = 5.6 μg/mL, MBC = 22.5 μg/mL, and 11.25 μg/mL), respectively [50]. Rashid et al. [51] reported the increased antibacterial activity of AgNPs synthesized from the water extracts of the roots of *Bergenia ciliata*, *Bergenia stracheyi*, *Rumex dentatus*, and *Rumex hastatus* from Pakistani origin against six pathogenic bacteria including *E. coli*, *S. aureus*, *S. haemolyticus*, *Bacillus cereus*, *Salmonella typhi*, and

Table 3: Zones of inhibition (ZOI) of the tested bacteria against RERE and AgNPs.

Samples	Microorganisms			
	Escherichia coli	*Staphylococcus aureus*	*Enterococcus* faecalis	*Klebsiella pneumoniae*
RERE	10 mm	9 mm	10 mm	11 mm
AgNPs	13 mm	12 mm	12 mm	14 mm
PC	16 mm	16 mm	16 mm	15 mm

Note: PC = positive control (neomycin).

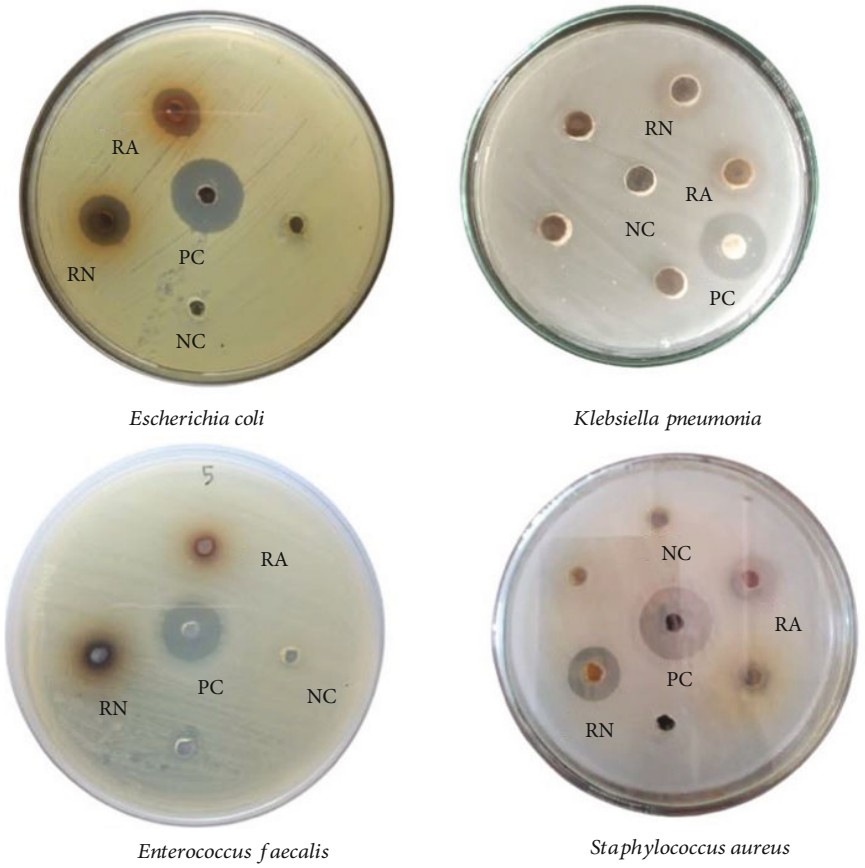

Figure 8: Antibacterial activity of RERE and synthesized AgNPs. Note: RA = RERE; RN = AgNPs; PC = positive control; NC = negative control.

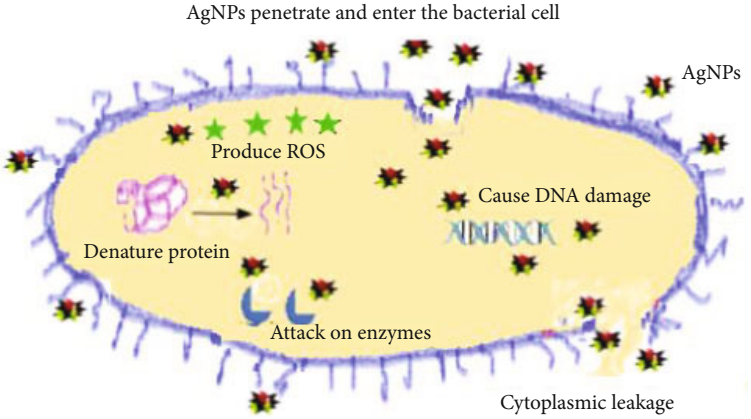

Figure 9: Schematic diagram of antibacterial activity of AgNPs.

Pseudomonas aeruginosa. In the agar well diffusion method, the AgNPs showed the inhibition zones ranging 7-15 mm, 10-16 mm, 8-16 mm, and 7-18 mm, respectively. The aqueous extracts of the plants were found inactive even at the doses of 1200 μg/well against all the bacteria used in the study compared to the positive control ($p < 0.05$). The crystalline AgNPs having an average size of 20 nm were synthesized by using the extracts of *Cestrum nocturnum* from India. They were reported to exhibit strong antibacterial activity against *Citrobacter*, *S. typhi*, and *E. coli* (MIC = 16 μg/mL); *Vibrio cholerae* and *Proteus vulgaris* (MIC = 8 μg/mL); and *E. faecalis* (MIC = 4 μg/mL) by the broth microdilution method. The plant extract was inactive towards all the bacteria in the disc diffusion method [52]. The aqueous extract of *Zataria multiflora* from Iran was used for the fabrication of AgNPs. The nanoparticles were monodispersed with an average size of 25.5 nm. They exhibited an elevated antibacterial activity in comparison to the commercial AgNPs against *S. aureus* with the minimum inhibitory concentrations of 4 and 8 μg/mL [53]. In a separate study, the AgNPs synthesized from the aqueous rhizome extracts of *Bergenia ciliata* of Pakistani origin were reported slightly higher antibacterial activities. The zones of inhibition were measured by the disc diffusion method. The AgNPs were inactive against *Micrococcus luteus* (ATCC 10240) but showed enhanced activities against *Bordetella bronchiseptica* (ATCC 4617), *S. aureus* (ATCC 6538), and *Enterobacter aerogenes* (ATCC 13048) which supports our results [46]. In this study, we found that our AgNPs showed greater activity against the Gram-negative (*E. coli* and *K. pneumoniae*) than Gram-positive (*E. faecalis* and *S. aureus*) bacteria. Urnukhsaikhan et al. [54] reported contradictory results in which the AgNPs synthesized from the aqueous extracts of *Carduus crispus* collected from Mongolia exhibited higher potency against Gram-positive bacteria *Micrococcus luteus* (ZOI = 7 ± 0.4 mm to 7.7 ± 0.5 mm) in comparison to the Gram-negative *E. coli* (ZOI = 5.5 ± 0.2 mm to 6.5 ± 0.3 mm). They had concluded that the antibacterial activity of different plant-based AgNPs does not depend on the thickness and texture of the bacterial cell wall only.

The antimicrobial potency of AgNPs depends upon various factors like shape, size, morphology, colloidal state, and the nature of bacteria. The AgNPs which have smaller sizes, irregular shapes, and larger surface areas show relatively higher antibacterial activities. The relatively thin lipid layer of Gram-negative bacteria is easily penetrated by the smaller AgNPs leading to the damage of the bacterial cells [55].

The accurate antimicrobial mechanism of AgNPs has been studied extensively but is not hitherto authenticated. Many propositions explain the antibacterial activity of AgNPs which can be depicted in Figure 9. The silver nanoparticles destroy the bacteria via the generation of reactive oxygen species (ROS), the release of Ag^+ ions from AgNPs that denaturize proteins bonding sulfhydryl group, and the gripping of AgNPs on bacteria resulting in cell damage [54]. Similarly, it is hypothesized that the AgNPs get stuck to the bacteria, interact with the cell wall, and kill them by interrupting the membrane permeability. It is also explained that the AgNPs break down the cells or disturb cell functions by interacting with amino acids and enzymes and generating reactive oxygen species. Silver nanoparticles interact with the soft bases like sulphur and phosphorus present in the nucleus, damage DNA, and ultimately cause cell death[56, 57].

4. Conclusion

This study established a simple, easier, and eco-friendly technique of synthesizing AgNPs from the aqueous extracts of root barks of a commonly used medicinal plant, *R. ellipticus* Sm. The observation of shifting of the peaks in the FTIR spectra indicated the potential role of different functional groups of plant secondary metabolites as capping and stabilizing agents. The fcc crystallinity and spherical morphology of the particles were established by the XRD and FESEM measurements. The relative abundance of silver, oxygen, carbon, calcium, and chlorine was revealed by the EDX analysis. The TEM image analysis shows the size of the nanoparticles ranging from 13.85 to 34.30 nm with an average size of 25.20 ± 7.01 nm. The synthesized AgNPs were found to exhibit higher antibacterial and antioxidant activity as compared to the aqueous extract. Further works on the possible effects of the size, morphology, and phytochemicals on physicochemical parameters are warranted to enable greater optimization of the biological activities of the AgNPs. Hence, the biosynthetic technique might be an alternative to chemical and physical methods indicating the possibility of the plant for the development of different products for biological and medical applications.

Data Availability

The data obtained in this study are available from the corresponding author upon request.

Conflicts of Interest

All the authors declare no conflicts of interest.

Acknowledgments

Prithvi Narayan Campus, Pokhara provided Ph.D. study leave to the first author. The Jeonbuk National University, Jeonju, South Korea, is acknowledged for the XRD, FESEM, EDX, and TEM analysis of the synthesized nanoparticles.

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Hindawi
The Scientific World Journal
Volume 2022, Article ID 6717012, 7 pages
https://doi.org/10.1155/2022/6717012

Research Article

Phytochemical Analysis and *In Vitro* Antioxidant and Antibacterial Activity of Different Solvent Extracts of *Beilschmiedia roxburghiana* Nees Stem Barks

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Received 19 January 2022; Accepted 12 March 2022; Published 26 March 2022

Academic Editor: Ghadir A. El-Chaghaby

Plants have long been considered as a basis of medicines for different indigenous cultures around the globe. They continue as a prominent source of important phytoconstituents which exhibit significant biological activities. In this study, we performed the phytochemical screening, estimation of total phenolic and flavonoids, antioxidants, and antimicrobial activities of the stem bark of *Beilschmiedia roxburghiana* Nees using different solvents. The total phenolic and total flavonoid contents ranged from 106.73 ± 1.62 mg GAE/g and 99.32 ± 0.66 mg QE/g (methanol extract) to 65.59 ± 1.79 mg GAE/g and 29.98 ± 0.90 mg QE/g (n-hexane extract), respectively. The maximum 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity with a half-maximal inhibitory concentration (IC_{50}) of 39.86 ± 3.69 μg/mL was observed for methanol extract followed by aqueous (IC_{50} = 43.55 ± 6.16 μg/mL), ethyl acetate (IC_{50} = 44.30 ± 5.88 μg/mL), dichloromethane (IC_{50} = 71.50 ± 4.70 μg/mL), and the lowest activity was observed for n-hexane extract. The disc diffusion method revealed that the ethyl acetate extract exhibited relatively higher activity against *Salmonella typhi* (ZOI = 13 mm), and moderate activities against *Shigella sonnei, Acinetobacter baumannii*, *Klebsiella pneumoniae,* and *Staphylococcus aureus* (ZOI = 12 mm). The methanol and aqueous extracts showed nearly parallel and the n-hexane and dichloromethane extracts exhibited mild antibacterial activities. The results indicated that the polarity index of the extracting solvents amplified the biological activities of the extract. The study is helpful to support the validity of the traditional application of the plant as natural medicine.

1. Introduction

Medicinal plants, microorganisms, and animals are the leading sources of traditional medicines against different illnesses over many centuries. The plant-derived compounds share a major portion of medicines that have been used by humans against several diseases. The global market of medicine that worth about 1.1 trillion US dollars, comprises of plant-based medicines about 25%, microorganisms 13%, and animals 3% [1]. Especially, people residing in developing countries rely on natural products as a major source of medicines due to their low cost, fewer side effects, and availability [2]. The use of herbal medicines continuously plays a vital role in primary health care, and about 80% of the world's population is estimated to depend on traditional medicine for their primary healthcare [3]. The development of knowledge of the exercise and consciousness of medicinal plants by different human civilizations in the past has augmented the notions and capacity of pharmacists and physicians to tackle the challenges of spreading professional services to assist human health [4].

Under certain conditions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the living cells during mitochondrial respiration. These free

radicals which are more reactive than molecular oxygen cause oxidative injuries on biomacromolecules such as proteins, nucleic acids, and lipids [5]. When our natural antioxidant defense and repair system is incompetent to alleviate them, a state of oxidative stress is set up leading to adverse effects such as DNA damage, cellular degeneration, carcinogenesis, and aging. This condition is responsible for various ailments such as Alzheimer's disease, Parkinson's disease, cancer, epilepsy, inflammation, retrolental fibroplasia, atherosclerosis, ischemia-reperfusion, lung injury, and other disorders [6]. The concentrations of the free radicals in our body are controlled by the endogenous antioxidant arrangement as well as exogenous nutrient supplements. They protect from apparent intracellular and extracellular oxidative stress. Antioxidants in the diet can delay or hinder lipid oxidation, react with reactive oxygen and nitrogen species and alleviate cellular damage by terminating the detrimental chain reactions [7].

Many valuable secondary metabolites such as alkaloids, flavonoids, phenolics, terpenoids, tannins, essential oils, and coumarins, etc. exhibit awesome antibacterial activities. These compounds are used as precursors for the synthesis of valuable antibiotics to treat several infections in the skin, urinary, gastrointestinal, and respiratory systems [8, 9]. Despite the development of a large number of antibiotics against several infectious diseases, the microbial resistance is continuously increasing all over the world [10]. The speedy rise of multidrug-resistant (MDR) bacteria is constantly pressurizing the global healthcare system for a few decades. Plants contribute to a large chemodiversity of active compounds which provide a hopeful source of antibacterial lead compounds to develop effective drugs against antibiotic-resistant bacteria [11].

Plants are the big reservoirs of important secondary metabolites but they need proper solvents of matching polarity for efficient extraction. The documentation of the active extracts or fractions is crucial for future research on the isolation and pharmaceutical application of significant compounds [12]. There are several noteworthy compounds isolated from plants such as cocaine, digitoxin, quinine, morphine, and codeine which are still used as drugs. The exploration of new compounds from a natural source is exciting in present and future times for the sustainable conservation and utilization of biodiversity. The progress in plant tissue culture, sophisticated extraction, isolation and characterization tools, and modern biotechnological approaches have simplified the production and standardization of herbal medicines, to elucidate analytical marker compounds [13, 14]. Natural products such as prostaglandins, steroids, and peptides hormones play a vital role in the pharmaceutical industries to boost the inspiration for drug discovery during the 20^{th} century [15].

Beilschmiedia is a pantropical genus of the Lauraceae family that contains about 250 species distributed in Asia and Africa. *B. roxburghiana* Nees is a medium-sized evergreen tree growing in tropical forests of China, India, and Myanmar. It bears alternate elongated leaves which are petiolate, and pinnately veined. Flowers are small, bisexual, and greenish with six tepals that develop into an ellipsoid, pyriform, or spherical fruits [16, 17]. In Southeast Asia, the plant is abundant in the subtropical forest at the altitudes of 200–400 m and is commonly used for treating bone-related problems like arthritis, renal problems, rheumatism, and as timber in Bhutan [18, 19]. The plants of this genus have been extensively studied in the past decades because of their widespread application in traditional medicine. These investigations had led to the isolation of important secondary metabolites like endiandric acid derivatives, epoxyfuranoid lignans, kingianins, and alkaloids having significant antibacterial, anti-inflammatory, enzyme inhibitory, and anticancer activities [20].

In the Kaski district of Nepal, the plant is known as "Hadchur" and local people use its stem bark in bone injuries and fractures. To the best of our knowledge, it is the first scientific study of the plant for phytochemical and biological activities. This research is aimed at the phytochemical screening, estimation of total phenolics and flavonoids, antioxidants, and antibacterial activities of extracts of the plant using water, methanol, ethyl acetate, dichloromethane, and *n*-hexane as solvents. The results of the study might be supportive to validate the traditional application of the plant as natural medicine.

2. Materials and Methods

2.1. Chemicals. All the chemicals and reagents of the highest purity and distilled water (DW) were used throughout the lab works. The Folin–Ciocalteu reagent (FCR), $AlCl_3$, HCl, Na_2CO_3, H_2SO_4, CH_3COOK, and dimethyl sulphoxide (DMSO) were purchased from Thermo Fisher Scientific India, Pvt. Ltd. Gallic acid, ascorbic acid, Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), and quercetin of Himedia Laboratories Company Ltd., India, were used. Similarly, ethyl acetate, methanol, ethanol, chloroform, n-hexane, and dichloromethane from Merck Life Science Limited were used. Neomycin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich and Tokyo Chemical industries Co. Ltd, respectively.

2.2. Plant Collection and Extraction. The stem barks of the plant were collected from Syastri, village of Kaski district of Nepal, in July 2020. The herbarium was submitted to the National Herbarium and Plant Laboratories, Lalitpur, Nepal, to get the botanical identification (voucher No L20). The shade-dried samples were pulverized using a mechanical grinder. The dry powder (50 gm in 500 mL) was dipped for 7 days into methanol, ethyl acetate, dichloromethane, n-hexane, and water separately. The extracts were filtered, concentrated using a rotary evaporator (IKA RV10), and stored in a refrigerator for use.

2.3. Phytochemical Screening. The presence of diverse phytochemicals in different extracts of the bark of *B. roxburghiana* was assessed by adopting standard protocols [21–23]. Tests were performed for the presence of alkaloids, anthracenes, carotenes, coumarin, flavonoids, glycosides, phytosterols, polyphenols, reducing sugars, saponins, tannins, and terpenoids.

2.4. Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The quantification of TPC in different extracts of *B. roxburghiana* was performed by the Folin–Ciocalteu method with a slight change [24, 25]. A set of standard gallic acid solutions of 5, 10, 20, 40, 50, and 60 μg/mL were prepared. Aliquots of 20 μL of the gallic acid and the test solutions (5 mg/mL) were filled in the bores of a microplate in triplicates. To each of the solutions, 100 μL of FCR (1 : 10 in DW) and 80 μL of 7.5% Na_2CO_3 solution were added. The mixture was incubated for 25 minutes in dark at room temperature, and optical density was recorded at 765 nm using a microplate reader (Biotek Multimode reader). The TPC was calculated from the standard calibration curve and expressed as milligrams gallic acid equivalents per gram (mg GAE/g) of the dry extract.

The TFC in the extracts of different solvents of *B. roxburghiana* stem bark was determined by the aluminum chloride colorimetric method [25, 26] with slight adjustments. The standard quercetin solutions of 10, 20, 30, 40, 50, 60, and 80 μg/mL were prepared in methanol. The aliquots of 130 μL of quercetin, 5 μL of $AlCl_3$, 5 μL of CH_3COOK, and 60 μL of ethanol were loaded into a 96-well microplate in triplicates. Similarly, 20 μL of each of the extracts (5 mg/mL), 110 μL of double-distilled water, 5 μL of $AlCl_3$, 5 μL of CH_3COOK, and 60 μL of ethanol were also filled in triplicates. The microplate was incubated for 30 minutes in the dark, and the absorbance was recorded at 415 nm against the blank. The TFC was calculated from the standard curve and expressed as milligrams quercetin equivalent per gram (mg QE/g) of the dry extract.

2.5. In Vitro Antioxidant Activity. The antioxidant activity of different extracts of the plant was evaluated by the DPPH free radical scavenging method [27, 28] with slight modifications. The test solutions of the extracts and ascorbic acid of 250, 125, 62.5, 31.25, 15.6, and 7.8 μg/mL were prepared in 50% DMSO. Each of 100 μL 0.1 mM DPPH and test solutions were loaded into a 96-well microplate in triplicates. Similarly, ascorbic acid was loaded as a positive control. The mixture was incubated for 30 minutes in dark at lab temperature, and the optical density was recorded at 517 nm against blank. The data were processed and analyzed by using Microsoft Excel 2016. The radical scavenging activity of different extracts and ascorbic acid was calculated:

$$\text{Percentage scavenging} = \frac{\text{The absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100. \quad (1)$$

The half-maximal inhibitory concentration (IC_{50}) of the extracts and the positive controls were calculated by using the Graph Pad Prism 9 software.

2.6. Antimicrobial activity. The antibacterial activity of different extracts of *B. roxburghiana* was evaluated by the agar well diffusion method [29, 30]. The test was performed against five Gram-negative and one Gram-positive bacteria. Six samples of the American Type Culture Collection (ATCC) bacteria were subcultured on the Muller Hinton Agar (MHA) from the pure samples and stored at 4°C. Table 1 shows the list of bacteria used in the study. The microorganisms were grown up in the freshly prepared Muller Hinton Broth (MHB) solution overnight at 37°C to equate turbidity to 0.5 McFarland's standard. The bacterial suspension was carpet-cultured on the sterile Muller Hinton Agar (MHA) media with cotton swabs. The boreholes of 6 mm diameter were punched at equivalent distances on the surface by using a sterile cork-borer.

The wells were fed with 50 μL of the plant extracts (50 mg/mL), neomycin (1 mg/mL), and 50% DMSO as a control. The plates were incubated for 24 hours at 37°C. On the next day, the samples were taken out from the incubator and the clear zones around the holes that correspond to the respective inhibition zones were measured and recorded.

Table 1: List of bacterial strains used for the study.

Microorganisms	Type	ATCC
Acinetobacter baumannii	Gram-negative	19606
Shigella sonnei	Gram-negative	25931
Klebsiella pneumoniae	Gram-negative	700603
Staphylococcus aureus	Gram-positive	25923
Escherichia coli	Gram-negative	25922
Salmonella typhi	Gram-negative	14028

Table 2: Results of phytochemical screening.

S. No	Secondary metabolites	BRM	BRE	BRA	BRD	BRH
1	Alkaloids	++	+	++	+	++
2	Flavonoids	+	++	++	+	+
3	Carotene	+	+	+	+	−
4	Polyphenols	++	++	++	++	+
5	Glycosides	+	+	+	−	−
6	Terpenoids	++	+	+	−	−
7	Saponins	++	+	+	−	−
8	Tannins	++	++	+	+	+
9	Coumarins	+	−	−	−	−
10	Anthracene	+	+	−	+	+
11	Reducing sugar	−	−	+	−	−
12	Phytosterol	++	++	++	+	−

BRM, methanol extract; BRE, ethyl acetate extract; BRA, aqueous extract; BRD, dichloromethane extract; BRH, n-hexane extract; +, present; –, absent.

3. Results and Discussion

3.1. Phytochemical Screening. The different extracts of the plant showed the presence of phytochemicals. Out of the 12 phytochemicals screened, BRM showed the abundance of 11

Table 3: TPC, TFC, and antioxidant activity (IC_{50}) of different extracts of *B. roxburghiana*.

Extracts	TPC (mg GAE/g)	TFC (mg QE/g)	IC_{50} (μg/mL)
BRA	78.41 ± 0.34	39.86 ± 0.90	43.55 ± 6.16
BRE	90.69 ± 2.71	49.79 ± 1.07	44.30 ± 5.88
BRM	106.73 ± 1.62	99.32 ± 0.66	39.86 ± 3.69
BRD	74.87 ± 0.93	35.59 ± 0.90	71.50 ± 4.70
BRH	65.59 ± 1.79	29.98 ± 0.90	1498.67 ± 62.13
*Ascorbic acid	—	—	6.40 ± 0.29

Values are the mean ± SD ($n = 3$), *Positive control.

phytochemicals followed by BRE and BRA with 10 phytochemicals. Six and five phytochemicals were detected in BRD and BRH, respectively. All the extracts contained alkaloids, flavonoids, polyphenols, and tannins (Table 2). The polarity of solvents is found proportional to the presence of phytochemicals. The more polar solvents such as methanol, ethyl acetate, and water extracts picked up the phytochemicals more efficiently than DCM and n-hexane. Thilagavathi et al. [31] also reported the same trend of the abundance of phytochemicals in different extracting solvents. The polarity index of the solvents, time, and method of extraction greatly influence the quality and quantity of phytochemicals in plants [12]. The secondary metabolites of plants are significant compounds that exhibit specific biological and pharmacological activities. The phytochemicals such as alkaloids, flavonoids, carbohydrates, polyphenols, steroids, and terpenoids have diverse physiological actions in the human and animal body.

So, they have a substantial role in the development of natural drugs against different health complications [32].

3.2. Total Phenolic and Total Flavonoid Content. The results of TPC and TFC contents among different extracts are presented in Table 3. The BRM showed the highest phenolic (106.73 ± 1.62 mg GAE/g) and flavonoid (99.32 ± 0.66 mg·QE/g) contents, and the lowest values were obtained for BRH (TPC = 65.59 ± 1.79 mg·GAE/g, TFC = 29.98 ± 0.90 mg·QE/g). Similarly, the BRA (TPC = 78.41 ± 0.34 mg·GAE/g, TFC = 39.86 ± 0.90 mg·QE/g), BRE (TPC = 90.69 ± 2.71 mg·GAE/g, TFC = 49.79 ± 1.07 mg·QE/g), and BRD (TPC = 74.87 ± 0.93 mg·GAE/g), TFC = 35.59 ± 0.90 mg·QE/g) were obtained. The TPC and TFC values are influenced by the composition and polarity of the solvent system. Similar types of variation of TPC and TFC in the extracts of different solvents was reported by Do et al. [33] in *Limnophila aromatica*. An Indian traditional plant, *Meyna spinosa* Roxb. Ex Link, was reported to have TPC and TFC values in the increasing order of polarity of the solvents in the order of methanol > ethyl acetate > petroleum ether [34].

3.3. In Vitro Antioxidant Activity. The DPPH method is short, efficient, and used extensively to predict the antioxidant capacity of plant extracts. In this technique, the violet color of the DPPH solution is reduced to yellow on the addition of the extract in a dose-dependent manner. The polyphenols and tocopherols in the extract scavenge the DPPH radical due to their hydrogen donating ability [35]. *B. roxburghiana* stem bark extracts of different solvents showed a linear concentration-response relationship to DPPH radical inhibition capacity. The increase in the concentration of the extracts was proportional to the corresponding scavenging capacity as shown in Figure 1. Ascorbic acid showed the highest scavenging capacity with an IC_{50} value of 6.40 ± 0.29 μg/mL. Table 3 shows that the IC_{50} value of BRM (39.86 ± 3.69 μg/mL) was followed by the BRA (43.55 ± 6.16 μg/mL), BRE (44.30 ± 5.88 μg/mL), and BRD (71.50 ± 4.70 μg/mL). The lowest antioxidant activity was shown by the BRH with a very high IC_{50} value of (1498.67 ± 62.13 μg/mL). Our results showed that the extracts having relatively high TPC and TFC showed better scavenging power. Like the results of Do et al. [33]; we observed that the methanol, ethyl acetate, and water which can catch up with the phytoconstituents efficiently had relatively lower IC_{50} values than that of less polar BRH and BRD. Sen et al. [34] also reported a similar trend of variation of antioxidant capacity which was linearly dependent upon the TPC and TPC of the extracts.

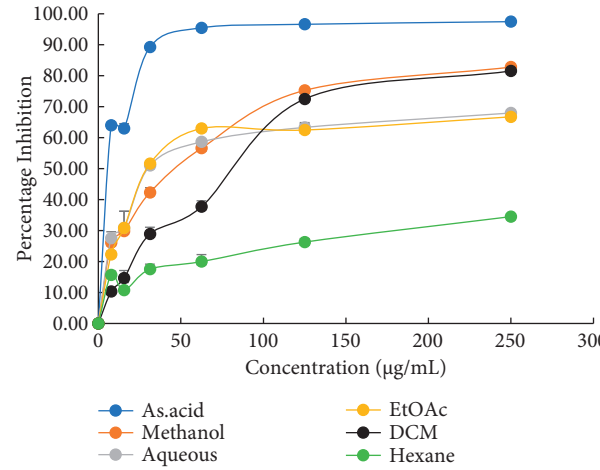

Figure 1: DPPH radical inhibiting capacity of different extracts of *B. roxburghiana*.

Table 4: Antibacterial activity of different extracts of *B. roxburghiana*.

	Zone of inhibition (ZOI) in mm					
Microorganisms	BRH	BRD	BRE	BRM	BRA	*Neomycin
Klebsiella pneumoniae	—	7	12	10	8	18
Escherichia coli	—	—	—	—	—	17
Salmonella typhi	7	Nd	13	11	12	18
Staphylococcus aureus	9	—	12	12	10	22
Shigella sonnei	—	—	12	13	11	19
Acinetobacter baumannii	—	Nd	12	9	12	18

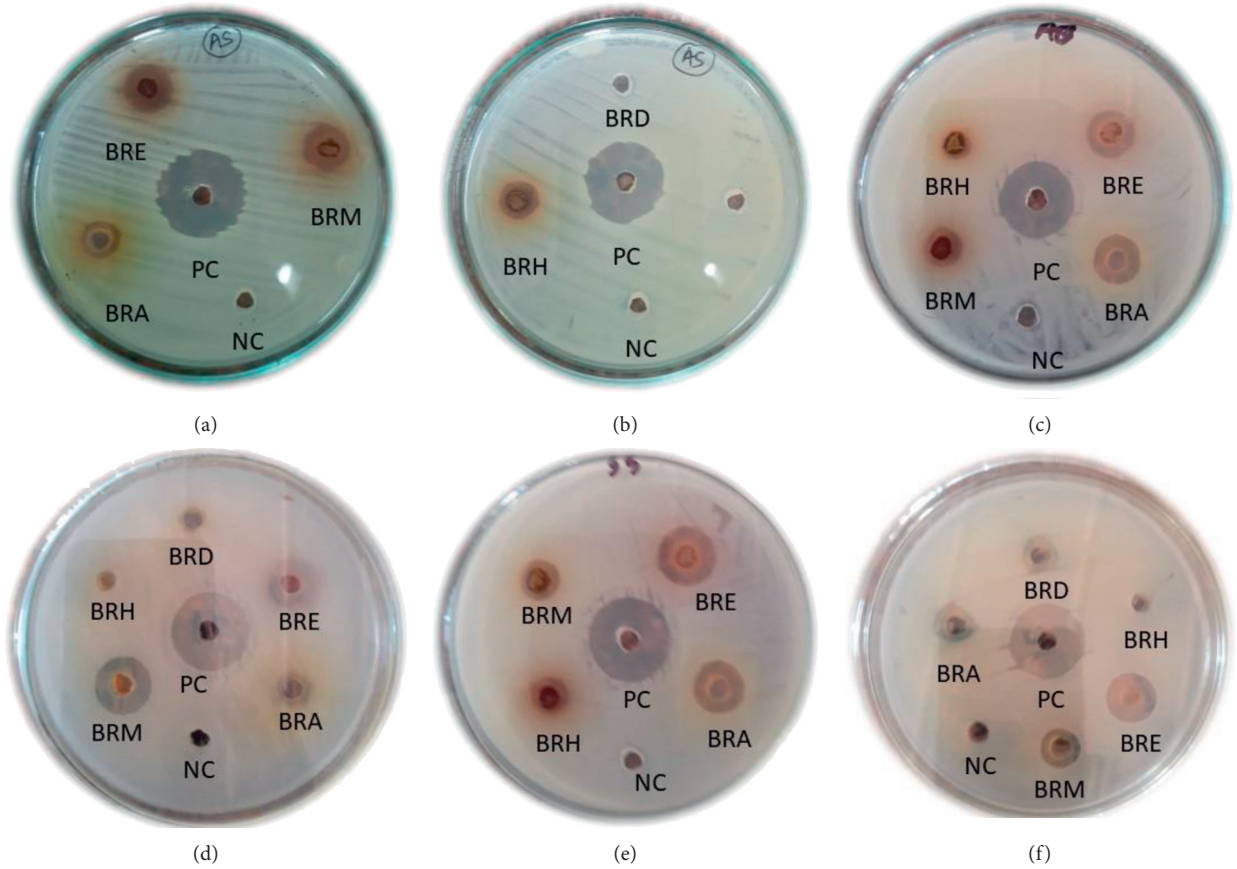

Figure 2: Antibacterial slides: (a, b) *S. aureus*, (c) *A.* baumannii, (d) *S. sonnei*, (e) *S. typhi*, and (f) *K. pneumoniae*. PC, positive control; NC, negative control; BRA, aqueous extract; BRE, ethyl acetate extract; BRH, n-hexane extract; BRD, DCM extract; and BRM, methanol extract.

Antioxidants are the key constituents that defend our bodies from the damages caused by free radical-induced oxidative stress. Plants provide many important compounds which offer resistance against oxidative stress by scavenging free radicals, preventing lipid peroxidation, and other mechanisms. The micronutrients such as vitamin C and E, β-carotene, and other important ingredients such as phenolic and flavonoids from plants are helpful to reduce oxidative stress [34]. The present study was undertaken to compare the antioxidant activity of *B. roxburghiana* stem bark by using solvents of different polarities. The results of this study indicate that the extracts of the plant showed radical scavenging activity due to their electron transfer or hydrogen donating ability because the total phenolic and total flavonoid contents in plants are proportional to the radical scavenging activity.

3.4. Antibacterial Activity. The antibacterial activity of different extracts of the *B. roxburghiana* against the tested microorganisms is given in Table 4.

The plant showed a diverse susceptibility towards the tested bacteria (Figure 2). All the extracts were found ineffective towards *E. coli* and moderate towards other organisms. The BRH against *K. pneumoniae*, *S. sonnei*, and *A. baumannii* as well as BRD against *S. aureus* and *S. sonnei* were ineffective. The BRE exhibited the highest activity against *S. typhi* (ZOI = 13 mm), followed by *K. pneumoniae*, *S. aureus, S. sonnei*, and *A. baumannii* (ZOI = 12 mm). The methanol and aqueous extracts showed a parallel activity towards the tested bacteria. The BRM showed the highest activity against *S. sonnie* (ZOI = 13 mm) followed by *S. aureus* (ZOI = 12), *S. typhi* (ZOI = 11 mm), and *K. pneumonia* (ZOI = 10), and minor activity against *A. baumannii* (ZOI = 9 mm). Similarly, the BRA exhibited the highest activity against *S. typhi* and *A. baumannii* (ZOI = 12 mm) followed by *S. sonnei* (ZOI = 11 mm) and *S. aureus* (ZOI = 10 mm), and the lowest activity was shown against *K. pneumoniae* (ZOI = 8 mm).

The antibacterial activity of endiandric acid derivatives (beilschmiedic acids A, B, and C) isolated from the stem bark of *B. anacardioides* from Cameroon was evaluated by the agar well diffusion method. The compounds were inactive against *E. coli* and *Pseudomonas palida* but active against *Bacillus subtilis, Micrococcus luteus,* and *Streptococcus faecalis* in terms of the zone of inhibition (ZOI) and minimum inhibitory concentration (MIC) values. Beilschmiedic acid C showed the highest activity against *M. luteus* (ZOI = 30 mm, MIC = <0.7) followed by *B. subtilis* (ZOI = 13 mm, MIC = 5.6 μM) and *S. faecalis* (ZOI = 18 mm, MIC = 22.7 μM). This result is comparable to our results of inactivity against *E. coli* [36]. The endiandric acid derivatives

might be present in the stem bark of *B. roxburghiana* which are passive towards some of the bacteria including *E. coli* and *P. palida*. The methanol stem bark extracts of *B. acuta* were found active against the 26 tested Gram-negative bacteria with MIC values ranging from below 8 to 256 μg/mL [37]. Salleh et al. [38] determined the antibacterial activities of the leaves and stem bark extracts of ethyl acetate, methanol, and n-hexane of *B. madang*, *B. glabra,* and *B. pulverulenta* from Malaysia. The extracts were evaluated for the MIC and minimum bactericidal concentration (MBC) against three Gram-positive: *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 29737), and *Enterococcus faecalis* (ATCC 19433), and three Gram-negative: *Pseudomonas aeruginosa* (ATCC 9027), *E. coli* (ATCC 10536), and *K. pneumoniae* (ATCC 13883) bacteria by microdilution method. The extracts exhibited weak to moderate activity with MIC and MBC values ranging from 125 to 1000 μg/mL. Most of the extracts showed weak activities against the Gram-negative bacteria in comparison to that of Gram-positive bacteria which might be due to their thick cell wall. Extensive research is necessary to identify and isolate the active compounds from *B. roxburghiana* responsible for substantial biological properties. So, the plant might be a source of natural antioxidants and antibiotics for the food industries to replace the synthetic chemicals.

4. Conclusions

This paper is the first-time report of a comprehensive study on phytochemical screening, estimation of total phenolics and flavonoids, antioxidants, and antimicrobial activities of *B. roxburghiana* of Nepalese origin. The secondary metabolites in plants require suitable solvents of the specific polarity index for the extraction. The identification of active extracts or fractions is helpful for the fundamental knowledge of pharmaceutical application and the isolation of active compounds. The antioxidant and antibacterial activities of the extracts were dependent upon the relative proportion of TPC and TFC. The polar solvents such as methanol, water, and ethyl acetate efficiently picked up the polar phenolic and flavonoid compounds from the plant material. Extensive research is warranted for the evaluation of different biological properties, bioassay-guided isolation of important secondary metabolites, their pharmacology, safety, clinical trials, and product development. It can strengthen the use of the plant as a vital resource of natural biodiversity for the sustainable development of novel potential therapeutic drugs or lead compounds.

Data Availability

The data used to support the findings of the study are available from the corresponding author.

Conflicts of Interest

All authors declare no conflicts of interest.

Acknowledgments

The authors would like to acknowledge Prithvi Narayan Campus, Tribhuvan University, Pokhara, for providing PhD leave to Mr. Lekha Nath Khanal and National Herbarium and Plant Laboratories, Godavari, Lalitpur, Nepal, for taxonomic identification of the plant.

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Journal of Institute of Science and Technology, 27(1), 27-35 (2022)
ISSN: 2467-9062 (print), e-ISSN: 2467-9240
https://doi.org/10.3126/jist.v27i1.40590

Research Article

CHARACTERIZATION OF ESSENTIAL OIL, ESTIMATION OF PHENOLIC AND FLAVONOID CONTENT AND BIOLOGICAL ACTIVITIES OF *Ephedra pachyclada* BOISS

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*(**Received**: November 14, 2021; **Revised**: April 05, 2022; **Accepted**: April 15, 2022)*

ABSTRACT

The northern areas of Nepal are rich in biodiversity and contain a large number of medicinal plant species including the Genus *Ephedra* of evergreen gymnosperm, belonging to the family Ephedraceae. The plants have been used by the peoples of the Himalayan region for the treatment of asthma, blood pressure, and gastritis for many years. This study was aimed for the evaluation of phytochemical, antioxidant, and antimicrobial activities of methanol, ethyl acetate, dichloromethane, and *n*-hexane extracts, and gas chromatography-mass spectrometry (GCMS) profiling of the essential oil of the aerial parts of *Ephedra pachyclada* Boiss from Mustang district of Nepal. Antioxidant activity was evaluated by 2,2-dipheny-1-picrylhydrazyl (DPPH) free radical scavenging assay and antimicrobial activity by the agar well diffusion method. Total phenolics and total flavonoid content were determined by Folin-Ciocalteu and aluminum chloride colorimetric method, respectively. The methanol extract contained the highest total phenolic content of 54.42 ± 1.40 mg GAE/g followed by the ethyl acetate (46.84 ± 0.62 mg GAE/g), DCM extract (19.58 ± 0.24 mg GAE/g), and the lowest TPC was shown for *n*-hexane extract (5.21 ± 1.40 mg GAE/g) of the dry weight. The methanol extract showed the maximum TFC of 33.28 ± 0.48 mg QE/g, followed by ethyl acetate extract (31.73 ± 0.52 mg QE/g), DCM extract (31.64 ± 0.56 mg QE/g), and the least value was obtained for the *n*-hexane extract (21.44 ± 2.91 mg QE/g). The methanol extract showed the highest antioxidant activity with a half-maximal inhibitory concentration (IC_{50}) of 37.81 ± 2.24 µg/mL. The methanol extract showed potent activity against *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) with zones of inhibition of 13 mm and 12 mm respectively. Ethyl acetate extract showed a slight potency against *Staphylococcus aureus* (ATCC 25923) with a zone of inhibition of 9 mm. The essential oil contained diisooctyl phthalate (46.90%), dodecane, 2,6,11-trimethyl-(16.35%), dodecane, 4,6-dimethyl- (11.59%), tetrapentacontane (11.56%), and myrtenol (4.37) as the major compounds. The plant exhibited significant antioxidant and antimicrobial activities which could be used as the source to isolate the active natural antioxidant and antimicrobial agent as the drug candidate in the future drug discovery process.

Keywords: Antimicrobial activity, antioxidant activity, *Ephedra pachyclada,* Mustang district

INTRODUCTION

Medicinal plants have long been used by different indigenous cultures for the treatment of a myriad of health complications around the world for many years. Many modern drugs have been established from the valuable biomedical information after the trial and error of plant-based compounds on human subjects over thousands of years (Li & Weng, 2017). Plants synthesize structurally specialized secondary metabolites containing highly active functional groups like aldehyde, sulfhydryl, epoxides, hydroxyl, and carboxyl which target enzymes, cell receptors, and transporters. They can mediate many cellular chain reactions which are useful in treating many diseases like cancer, diabetes, neurogenetic disorders, etc. Such compounds exhibit pharmacological properties like antiviral, antimicrobial, analgesic, antihypertensive, antitumor, psychoactive, etc. to the host (Mawalagedera et al., 2019). Alkaloids, flavonoids, phenolics, glycosides, tannins, gums, resins, etc. are the important secondary metabolites that are reported in the root, stem, flower, leaf, and barks of several plants of this genus that exhibit numerous pharmacological functions in the human body (Singh et al., 2016). Phenolics and flavonoids have a significant role in defense mechanisms and scavenging various types of free radicals produced in the cells by oxidative stress. They are interesting candidates for pharmaceutical and medical applications due to their substantial antioxidant, antibacterial, cardio-protective, immune defensive, UV-protective, and anti-inflammatory activities (Eugenio et al., 2017; Tungmunnithum et al., 2018).

Ephedra is a genus of gymnosperm seed-bearing plants of the family Ephedraceae that contains about 67 species distributed in the desert areas of Asia, America, North Africa, and Europe (Zhang et al., 2018). *Ephedra* (ephedra major host) known as 'Haoma' was considered a sacred plant in Zoroastrianism in Iran (Jamshidi-Kia et al., 2018).

The different plants of this genus have been used in traditional Chinese medicine against allergies, asthma, colds, coughs, fever, edema, flu, headache, and nasal congestions (Pirbalouti et al., 2013). The ephedra plants are valuable due to the presence of ephedrine and pseudoephedrine alkaloids, which are effective in the treatment of headaches, nasal inflammation, common cold, and bronchial asthma. Moreover, these alkaloids serve as marker compounds in the quality control of the ephedra herb (Minami et al., 2021).

The hydroalcoholic extract of *E. pachyclada* from Iran at the dose of 1000 mg/kg was effective in healing artificially induced ulcers in Wistar rats (Pirbalouti et al., 2013). Quinoline-2- carboxylic acid is an active compound isolated from the plant that exhibited potent antidiabetic activity. The compound was found to have significant activity with an IC_{50} of 9.1 ± 2.3 μg/mL and 15.5 ± 1.9 μg/mL in the α-glucosidase and α-amylase inhibitory activity respectively (Lee et al., 2014).

Essential oils are concentrated hydrophobic liquids containing volatile aromatic compounds isolated from various plants. They are composed of complex mixtures of different compounds like alcohols, hydrocarbons, phenols, aldehydes, ketones, esters, etc., (Rassem et al., 2016). Essential oils provide a benign defense mechanism against pests, pathogens and attract pollinators of the plant. Although the pharmacological properties of ephedra plants are due to the ephedrine-like alkaloids, essential oil compounds also exhibit certain medicinal properties. For example, 2,3,5,6-tetramethylpyrazine present in the essential oil of *E. sinica* is a cardiovascular drug (Wang et al., 2006).

Nepal lies in a unique position of the central Himalayan region having varied geography, high variation in altitude, and climate. It bears an excessive floral variety with more than 7000 and 4000 species of flowering and non-flowering plants respectively. Most of the people residing in remote areas depend on medicinal plants for primary health care as well as annual family income (Gurung & Pyakurel, 2017). *Ephedra pachyclada* Boiss is a perennial, dioecious plant distributed in the mountains and alpine dry zones in the Himalayan region between Iran and Nepal (Motomura et al., 2007). In the Mustang district of Nepal, the plant is known as '**Somlata'** that grows with bluish-green tough and cylindrical leaves of 5 – 50 cm height from big rootstalk. The local medical workers called *Amchis* use the plant against different health complications like asthma, blood pressure, eye problems, and gastric disorder (Chhetri et al., 2006). The increasing global demands of herbal products and subsequent uncontrolled exploitation, lack of conservation, and proper documentation have threatened the future of the country's important medicinal plants. The information of the biological activities and chemical constituents of plants is supportive for the identification of sources of important plant materials. It is valuable to the cultivators, producers, policymakers, exporters, processors, and academicians for the development of natural drugs (Kunwar et al., 2011; Shrestha et al., 2015). The present study is aimed at chemical profiling of essential oil, evaluating the phytochemical screening, estimation of total phenolics, total flavonoids, antioxidant, and antimicrobial activities of *E. pachyclada*. To the best of our knowledge, it is the first scientific study on phytochemical and biological activities which might be helpful to ascertain the traditional knowledge of the plant from the remote area of Nepal.

MATERIALS AND METHODS

Collection of plant and preparation of extracts

The aerial parts of the plant were collected from the Thini village of Mustang district in July 2018. The plant was taxonomically authenticated in the National Herbarium and Plant Laboratories, Lalitpur, Nepal (voucher no: L3 (2075). The shade-dried parts were ground into a fine powder using a mechanical grinder. Extraction of phytochemicals was performed by cold-percolation method from different solvents of different polarities namely, methanol, ethyl acetate, dichloromethane, and *n*-hexane separately. The different extracts were concentrated by using a rotary evaporator (IKA) and stored at 4℃ for use.

Isolation and GCMS analysis of essential oil

The essential oil was isolated from the aerial parts of the plant by using a Clevenger's apparatus. The powdered plant material (70 g) was put in a round bottom flask containing 500 mL of distilled water and heated for about 3 hours. A light brown colored oil having a sharp smell was obtained in the Clevenger's apparatus. The oil was collected, dehydrated over anhydrous Na_2SO_4, and stored in an airtight container in the refrigerator at 4℃ (Baharum et al., 2010). The GCMS analysis of the essential oil was performed by using a Shimadzu QP-2010 GCMS system with an AOC-20i autosampler. The sample was injected at 220 ℃, the oven temperature was 80℃ for two minutes, heated at the rate of 6℃/minute up to a constant temperature of 280℃ for 5 minutes. Helium was used as the carrier gas that flowed at a constant rate of 1.03 mL/minutes for an injection volume of 1 μL. The mass spectra were recorded by 70 eV at the scan interval of 0.5 seconds. The fragments with m/z of 50 -500 were analyzed. The compounds in the oil were recognized by comparing the mass spectra with those from the NIST05 library.

Phytochemical screening

The presence of different phytochemicals in the methanol extract of the plant was assessed by standard protocols (Bora et al., 2019; Tiwari et al., 2021). Tests were accomplished for alkaloids, polyphenols, flavonoids, terpenoids, saponins, coumarin, reducing sugars,

glycosides, carotene, tannins, anthracenes, and phytosterols.

Estimation of total phenolic and total flavonoids

The total phenolic content (TPC) in different extracts of the plant was determined by the Folin-Ciocalteu method (Ainsworth & Gillespie, 2007; Pawar & Dasgupta, 2018) with slight modifications. Gallic acid solutions of 20, 40, 50, 60, and 80 µg/mL were prepared from a stock solution of 1 mg/mL. The microplate bores were filled with 20 µL of gallic acid or the extract (5 mg/mL), 100 µL of Folin-Ciocalteu reagent (FCR),1:10 diluted with distilled water, and 80 µL of 1N sodium carbonate in triplicates. Distilled water with FCR and Na_2CO_3 solution was taken as a blank. The mixture was incubated for about 25 minutes in dark at lab temperature and the optical density was recorded at 765 nm using a microplate reader (Bio Tek Multimode reader). The TPC was calculated and expressed as milligrams gallic acid equivalents per gram (mg GAE/g) of the dry plant material from the standard calibration curve.

The total flavonoid content (TFC) in different extracts of the plant was determined by the aluminum chloride colorimetric method (Makhubu et al., 2019; Pawar & Dasgupta, 2018). Quercetin standard solutions of 10, 20, 30, 40, 50, 60, and 80 µg/mL were prepared from the stock solution (1 mg/mL) in methanol. An aliquot of 130 µL of quercetin solution of each concentration, 5 µL of $AlCl_3$, 5 µL of potassium acetate, and 60 µL of ethanol was filled into the wells of 96-well plate microplate reader in triplicates. Similarly, 20 µL of extract (5 mg/mL), 110 µL of distilled water, 5 µL of $AlCl_3$, 5µL of potassium acetate, and 60 µL of ethanol are fed into the wells of microplate reader in triplicates. The mixture was incubated for 30 minutes at dark, and absorbance was taken at 415 nm with a microplate reader against the blank containing all except the quercetin or the extract. The TFC was calculated and expressed as milligrams quercetin equivalent per gram (mg QE/g) of the dry extract from the standard curve.

***In vitro* antioxidant activity**

The extracts of the plant in different solvents were evaluated for their antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay method (Brand-Williams et al., 1995; Khadayat et al., 2020; Liu et al., 2009) with slight modifications. Each of the plant extracts was dissolved in 50% dimethyl sulphoxide (DMSO) to prepare the test solutions of 500, 250, 125, 62.5, 31.25, 15.62, and 7.8 µg/mL. The aliquots of 100 µL of 0.1 mM of DPPH in methanol were added into 100 µL of test solutions in a 96-well plate in triplicates. Ascorbic acid was taken as a positive control. The microplate was incubated for 30 minutes in dark at lab temperature and the absorbance was recorded at 517 nm against blank using a microplate reader. The results were processed by using Gen5 Microplate data collection and analysis software and then by Microsoft Excel 2016. The free radical scavenging activity of the extracts was calculated by using the formula,

$$\text{Percentage scavenging} = \frac{\text{The absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} X\ 100$$

The half-maximal inhibitory concentration (IC_{50}) values were calculated by using the Graph Pad Prism 8 software.

Antimicrobial activity

Microorganisms

The American Type Culture Collection (ATCC) bacteria were used for the test. The pure cultures from the Muller Hinton Agar (MHA) were sub-cultured and stored at 4°C. Both Gram-negative and Gram-positive bacteria were used (Table 1).

Table 1 List of bacterial strains used

Microorganisms	Type	ATCC
Klebsiella pneumonia	Gram-negative	700603
Escherichia coli	Gram-negative	25922
Salmonella typhi	Gram-negative	14028
Staphylococcus aureus	Gram-positive	25923

Agar well diffusion assay

The bacterial susceptibility of the extracts of the plant was assessed by the agar well diffusion method (Murray et al., 2007; Nasir et al., 2015). The bacteria were incubated in Muller Hinton Broth (MHB) overnight to adjust the turbidity equal to 0.5 McFarland's standard. The test bacteria were grown on the surface of a sterile agar medium at 37°C. The wells of 6 mm diameter were bored at equivalent distances on the surface of MHA plates with a sterile cork-borer. The wells were fed with 20 µL of the plant extracts (50 mg/mL) in 30% DMSO, neomycin (1 mg/mL), and the solvent as a negative control carefully, and incubated for 24 hours at 37°C. On the next day, the plates were taken out, and the clear region around the wells that corresponds to the zone of inhibition (ZOI) was measured with a scale and recorded.

RESULTS AND DISCUSSION

Chemical constituents identified by GCMS analysis

The mass spectra data for each peak of GC analysis were compared with the NIST05 library and the components in the essential oil of *E. pachyclada* were identified for each of the chromatograms. The compounds in the essential oil with their retention time and relative percentage are presented in Table 2. The molecular structures of the major compounds are shown in Figure1.

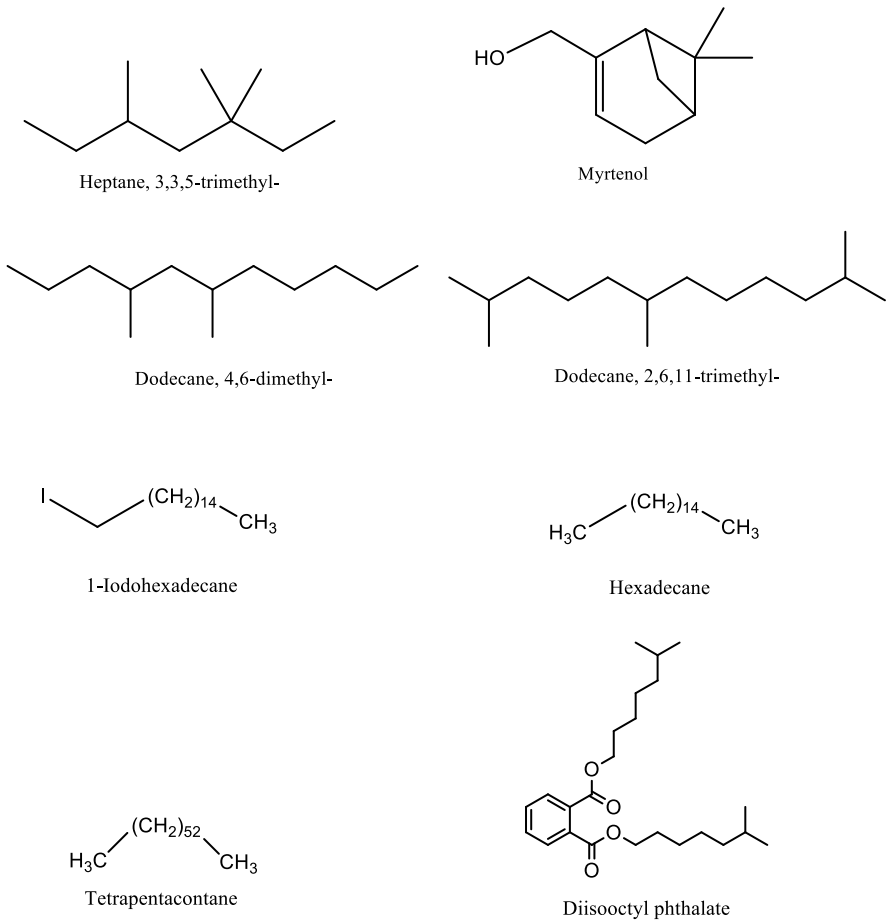


Figure 1 Molecular structures of the identified compounds in the essential oil of *E. pachyclada*

Our study shows that the essential oil contained diisooctyl phthalate (46.90%), dodecane, 2,6,11-trimethyl- (16.35%), dodecane, 4,6-dimethyl- (11.59%), tetrapentacontane (11.56%), and myrtenol (4.37) as the major compounds. We detected ester (46.90%) and hydrocarbons (39.5%) as the major compounds in the essential oil of *E. pachyclada*. Kobaisy et al., (2005) reported the presence of different compounds in the essential oil of three species of ephedra from Italy. *E. distachya* sample contained an ester, ethyl benzoate as a major component (46.9%) together with benzaldehyde (8%), and cis-calamene (3.6%). *E. fragilis* contained (E) phytol (10.1%), pentacosane (5.2%), and 6,10,14-trimethyl-2-pentadecanone (5.3%). Similarly, the oil of *E. major* contained eugenol (4.3%), α-terpineol (3.7%) and methyl linoleate (3.5%) as the main constituents. The essential oil of *E. intermedia* collected from Iran contained 2-ethyl-pyrazine (67.37%), γ-elemene (9.21%), benzyl acetate (9.10%) and 2-methyl-butyl acetate (5.28%) (Ni et al., 2019).

Table 2 List of compounds detected in the essential oil of *E. pachyclada*

Peak	Retention time	Area%	Names/Molecular formulae
1	6.521	2.31	Heptane, 3,3,5-trimethyl-/($C_{10}H_{22}$)
2	8.664	4.37	Bicyclo[3.1.1]hept-2-ene-methanol,6,6-dir (Myrtenol)/ ($C_{10}H_{16}O$)
3	10.347	11.59	Dodecane, 4,6-dimethyl- /$C_{14}H_{30}$
4	18.983	16.35	Dodecane, 2,6,11-trimethyl-/($C_{15}H_{32}$)
5	23.608	2.67	1-Iodohexadecane/$C_{16}H_{33}I$)
6	26.609	4.25	Hexadecane/($C_{16}H_{34}$)
7	29.589	11.56	Tetrapentacontane /($C_{54}H_{110}$)
8	31.690	46.90	1,2-Benzenedicarboxylic acid, diiosoctyl ester/($C_{24}H_{38}O_4$)

Phytochemical screening

The methanol extract of the plant was verified for the presence of different phytochemicals. The extract showed the presence of 11 phytochemicals out of 12 tested. Polyphenols, glycosides, reducing sugars, and phytosterol was found in excess whereas terpenes were absent as shown in Table3. These phytochemicals play a significant role in the biological activities of the plant. Most of the plant species contain a variety of phytochemicals like steroids, terpenoids, flavonoids, carbohydrates, etc. which have diverse physiological activities in the human body and, consequently, they can be used for the development of natural drugs for different health complications (L. Zhang et al., 2020). In addition to ephedrine alkaloids, ephedra plants contain polysaccharides, flavonoids, tannins, and other important phytochemicals. These compounds exhibit antioxidant, antimicrobial, anti-inflammatory, anticancer, and hepatoprotective properties (Zhang et al., 2018).

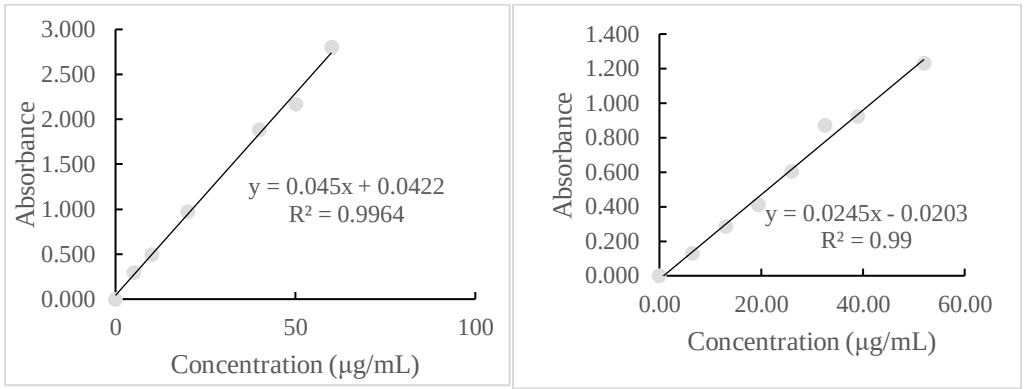
Table 3 Results of phytochemical screening

S. No	Phytochemical	Abundance	S. No	Phytochemical	Abundance
1	Polyphenols	++	7	Tannins	+
2	Alkaloids	++	8	Coumarin	+
3	Glycosides	++	9	Saponins	+
4	Reducing sugars	++	10	Carotene	+
5	Flavonoids	+	11	Phytosterol	++
6	Terpenes	-	12	Anthracene	+

Note: (++) = present, (+) = slightly present, and (-) = absent

Total phenolic and flavonoid contents

The TPC was determined from the linear equation of the standard curve (R^2 = 0.9964) prepared by plotting concentration against absorbance as Figure 2(a). TPC was expressed in terms of mg GAE/g of the dry weight. Similarly, TFC was calculated from the quercetin standard curve (R^2 = 0.99) and expressed as mg QE/g of the dry extract as Figure 2(b). The TPC and TFC of different extracts of *E. pachyclada* are shown in Table 4. Methanol extract shows the maximum phenolic content of 54.42 ± 1.40 mg GAE/g, followed by the ethyl acetate (46.84 ± 0.62 mg GAE/g), and DCM extract (19.58 ± 0.24 mg GAE/g), and the lowest TPC was shown for *n*-hexane extract (5.21 ± 1.40 mg GAE/g) of the dry weight. A similar trend of variation was observed for TFC also. The methanol extract showed the maximum TFC of 33.28 ± 0.48 mg QE/g, followed by ethyl acetate extract (31.73 ± 0.52 mg QE/g), DCM extract (31.64 ± 0.56 mg QE/g), and the least value was obtained for the n-hexane extract (21.44 ± 2.91 mg QE/g).



(a) (b)

Figure 2 (a) Gallic acid calibration curve (b) Quercetin calibration curve

Similarly, the estimation of TPC and TFC of seven ephedra species showed a similar variation to our results. The sample, which contained higher phenolics, was found to contain higher flavonoids also. In the tested species, *E. alata* contained the highest amount of TPC of 53.3 ± 0.1 mg GAE/g and TFC 2.8 ± 0.0 mg QE/g of dry weight (Ibragic & Sofić, 2015). The TPC of methanolic extract and aqueous extracts of *E. alata* were 47.62 and 19.175 mg GAE/g of the dry weight respectively which is comparable to that of our result (Al-Snafi, 2017).

Table 4 TPC and TFC of different extracts of *E. pachyclada*

Extracting solvents	TPC (mg GAE/g)	TFC (mg QE/g)
Dichloromethane	19.58 ± 0.24	31.64 ± 0.56
Ethyl acetate	46.84 ± 0.62	31.73 ± 0.52
n-Hexane	5.21 ± 1.49	21.44 ± 2.91
Methanol	54.42 ± 1.40	33.28 ± 0.48

Note: Vales are mean ± SD, (n = 3)

The TPC and TFC of the extracts of *E. alata* from Palestine prepared using different solvents were dissimilar. The extract of 80% methanol was reported the (TPC = 101.2 ± 0.9 mg/g, TFC = 9.8 ± 0.5 mg/g), 100% ethanol extract (TPC = 40.9 ± 0.2 mg/g, TFC = 19.5 ± 0.3 mg/g), and the water extract (TPC = 30.9 ± 0.5 mg/g, TFC = 4.2 ± 0.1 mg/g) (Al-Rimawi et al., 2017). These extracts contained the nearly same quantity of phenolics and lower flavonoid contents than our results. The polar solvents were more effective for the extraction of phenolic and flavonoids that are also polar, and the amount of the phytochemicals varies with species, collected part, solvents, geographical location, season, maturity of the plant, etc. The TPC of the methanolic extracts of the wild and callus culture of *E. pachyclada* was evaluated by the Folin-Ciocalteu reagent method. The wild sample was found to contain 454.5 ± 62.24 µmol eq catechin/g of dry material which is higher than that of callus culture with 108.77 ± 17.15 µmol eq catechin/g of dry material (Parsaeimehr et al., 2010).

In vitro antioxidant activity

The plant was found to exhibit a moderate antioxidant activity on examination with DPPH radical scavenging assay. Only the methanol extract showed the highest antioxidant activity with a half-maximal inhibitory concentration (IC_{50}) of 37.81 ± 2.24 µg/mL, while the others exhibited low activity as shown in Table 5. Ascorbic acid was taken as a positive control had the highest activity with an IC_{50} value of 6.39 ± 0.29 µg/mL. The extracts of *n*-hexane, DCM, and ethyl acetate showed weak scavenging power in comparison to that of methanol extract.

Table 5 DPPH radical scavenging (IC_{50} values) of different extracts of *E. pachyclada*

S. No	Extracts	IC_{50} (µg/mL)
1	Methanol extract	37.81 ± 2.24
2	Ethyl acetate extract	230.30 ± 4.75
3	Dichloromethane extract	249.97 ± 17.65
4	*n*-Hexane extract	278.93 ± 23.52
5	*Ascorbic acid	6.39 ± 0.29

Note: Values are mean ± SD, (n = 3), * Positive control

Our result agrees with the result obtained by Ghasemi et al., (2014). The aqueous extract of the plant showed significant antioxidant activity with an IC_{50} value of 55.53 ± 0.05 µg/mL. Similarly, the ethanol extract of *E. procera* from Iran also exhibited a substantial activity on DPPH scavenging assay with an IC_{50} of 56 µg/mL (Dehkordi et al., 2015). The antioxidant activity of different extracts was correlated with the polarity of the extracting solvent. The polar solvent, methanol can pick up the polar phenolic and flavonoid compounds showed relatively higher radical scavenging activity than the extracts of less polar solvents. The antioxidant activity of plants can be enzymatic and nonenzymatic. The enzymes and different compounds as well as polar secondary metabolites synergically comport the antioxidant defense system in the plants (Pisoschi et al., 2016). The plot of percentage scavenging activity of different extracts and ascorbic acid shows a proportionate variation with concentration in Figure 3.

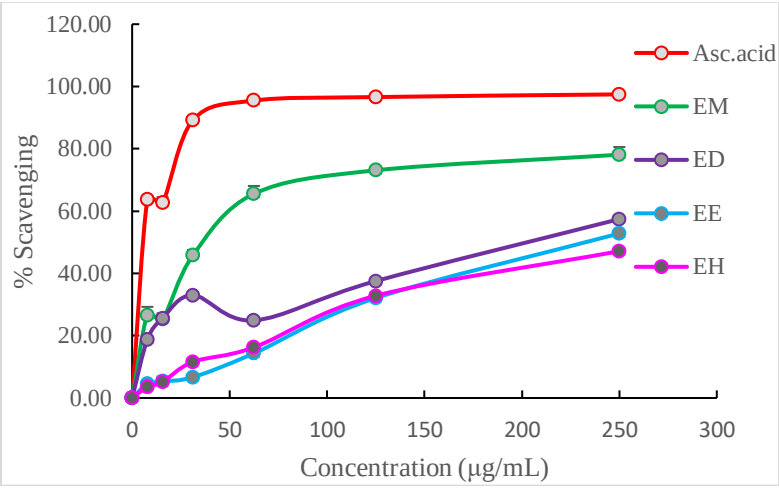


Figure 3 Percentage inhibition of ascorbic acid and different extracts of *E. pachyclada* (Note: EM= methanol extract, ED= DCM extract, EE= ethyl acetate extract, EH= n- hexane extract)

The antioxidant power of phenolic compounds depends on the structure of the aromatic ring, position, and number of -OH groups. The OH- group forms the free radical which is resonance stabilized by the aromatic ring of the molecule (Zeb, 2020). Flavonoids transfer electrons or hydrogen atoms to reactive oxygen and nitrogen species, chelate metal catalysts that generate free radicals, activate antioxidant enzymes as well as inhibit the actions of free radical generating enzymes (D'Amelia et al., 2018).

Antibacterial activity

The *E. pachyclada* plant collected from the Mustang district of Nepal exhibited a mild antibacterial activity towards some of the bacterial strains on the Agar well diffusion method. The Figure 4 shows that the plant was found effective against the two bacteria only. The methanol extract showed potent activity against *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) with the zone of inhibitions of 13 and 12 mm respectively. Ethyl acetate extract showed a slight potency against *Staphylococcus aureus* (ATCC 25923) with a zone of inhibition of 9 mm as shown in Table 6.

Table 6 Results of antibacterial test of different extracts of *E. pachyclada*

Microorganisms	Zone of Inhibition (ZOI) in mm				
	n-hexane extract	Dichloromethane extract	Ethyl acetate extract	Methanol extract	*Neomycin
Klebsiella pneumonia	-	-	-	-	15
Escherichia coli	-	-	-	13	15
Salmonella typhi	-	-	-	-	16
Staphylococcus aureus	-	-	9	12	17

Note: *Positive control (1mg/mL), concentration of the extracts = 50 mg/mL

Our result is in the agreement with the antibacterial activities exhibited by the plant against different nosocomial bacteria. The methanol extract at different concentrations was active against *Escherichia coli* (PTCC 0157), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (PTCC 1053), *Serratia marcesens* (PTCC 1111), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella dysenteriae* (PTCC 1118) (Dosari et al., 2016). The quinaldic acid isolated from the plant exhibited potent activity against *C. difficile* ATCC 9689, and *Clostridium perfringens,* and but was inactive against the tested *Lactobacillus acidophilus* (ATCC 4356), *Bifidobacterium bifidum* (ATCC 29521), and *L. casei* (ATCC 393) (C.-H. Lee & Lee, 2009). A. Parsaeimehr et al., (2010), evaluated the antimicrobial activity of methanol extracts of the wild and callus cultures of the plants from Iran. The test was performed against five Gram-negative, three Gram-positive, and two fungal strains by the disc diffusion method. *Pseudomonas aeruginosa* (PTCC 1074), *Staphylococcus aureus* (PTCC 1112), and *Candida albicans* (PTCC 5027) showed the maximum activity at the concentrations of 0.5 to 4 mg/mL.

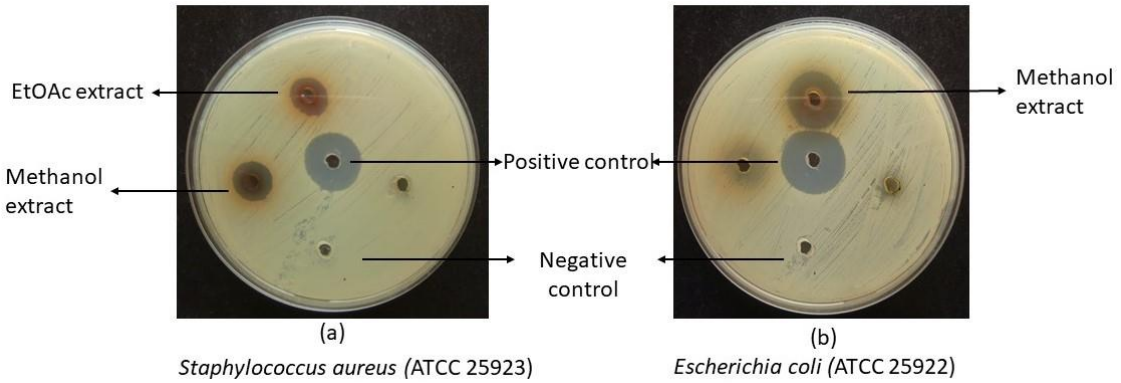


Figure 4 Antibacterial test slides of *E. pachyclada* extracts

CONCLUSIONS

This research attempts a comprehensive study of the phytochemical, antioxidant, and antimicrobial activities of *E. pachyclada* collected from the Mustang district of Nepal for the first time. The essential oil of the plant contained diisooctyl phthalate, dodecane, 2,6,11-trimethyl-, dodecane, 4,6-dimethyl-, tetrapentacontane, and myrtenol as the major compounds. The methanol extract of the plant which contained a relatively higher proportion of TPC and TFC exhibited higher antioxidant activities in comparison to other extracts. It exhibited a significant antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli*. The ethyl acetate extract showed moderate activity against *Staphylococcus aureus* whereas dichloromethane and *n*-hexane extracts were inert towards the tested bacteria. Our study highlights the use of suitable solvents for the extraction of phytoconstituents that exhibit better pharmacological activity. Our results indicated the possibility of isolating important bioactive molecules that could be developed for natural medicines. This study authenticates the traditional use of the plant by the local people against different ailments.

ACKNOWLEDGEMENTS

We are thankful to National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal, for the identification of the plant, National Food & Feed Reference Laboratory, DFTQC, Nepal for GC-MS analysis, and Prithvi Narayan Campus, Pokhara for providing Ph.D. study leave to the first author.

AUTHOR CONTRIBUTIONS

SKK conceptualized the research project; LNK performed the experiments, analyzed the data, and prepared the manuscript. YRP and KRS. reviewed the work and the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Scientific Research Publishing

American Journal of Plant Sciences, 2020, 11, 1383-1397
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Assessment of Phytochemical, Antioxidant and Antimicrobial Activities of Some Medicinal Plants from Kaski District of Nepal

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How to cite this paper: Khanal, L.N., Sharma, K.R., Pokharel, Y.R. and Kalauni, S.K. (2020) Assessment of Phytochemical, Antioxidant and Antimicrobial Activities of Some Medicinal Plants from Kaski District of Nepal. *American Journal of Plant Sciences*, **11**, 1383-1397.
https://doi.org/10.4236/ajps.2020.119099

Received: July 29, 2020
Accepted: September 15, 2020
Published: September 18, 2020

Abstract

Medicinal plants have been known to treat simple to life-threatening diseases in different communities of Nepal for many years. This study aims to analyze the phytochemicals as plant secondary metabolites, evaluate the antioxidant and antibacterial activities of *Rubus ellipticus*, *Ziziphus mauritiana*, *Pyrus pashia*, and *Drynaria coronans* extracts that are commonly being used as traditional medicine. Phytochemical analysis was performed to investigate the plant's secondary metabolites such as polyphenols, alkaloids, flavonoids, terpenoids, reducing sugar, glycosides, tannins, carotene, phytosterols, coumarins, saponins, and anthracenes. The methanol extracts of the plants were used to evaluate the *in vitro* antioxidant activity by using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical and the antibacterial activity against *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 700603), *Escherichia coli* (ATCC 25922) and *Salmonella typhi* (ATCC 14028) by the agar well diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by the Resazurin Microtiter Assay (REMA) method. Root extract of *R. ellipticus* was observed to have the highest antioxidant activity with IC_{50} of 42.40 ± 1.5 µg/ml followed by the root extract of *Z. mauritiana* (IC_{50} 55.67 ± 7.41 µg/ml), leaf and bark extract of *P. pashia* (IC_{50} 58.33 ± 2.9 µg/ml) and tuber extract of *D. coronans* (IC_{50} 93.30 ± 5.19 µg/ml) as compared to the standard ascorbic acid with IC_{50} of 28.44 ± 0.97 µg/ml. The plants were found active against Gram-positive *Staphylococcus aureus* with Zone of Inhibition (ZOI) for *R. ellipicus* 17 mm, *P. pashia* 12 mm, *Z. mauritiana* 9 mm and *D. coronans* 8 mm. The extracts exhibited no effect on all of the tested Gram-negative bacteria. The MIC and MBC of *R. ellipticus* and *P. pashia* were 3.125 mg/ml, 12.5 mg/ml, and 12.5 mg/ml, 25 mg/ml respectively.

Keywords

Medicinal Plant, Phytochemical, Antioxidant, Antibacterial Activity, Resazurin

1. Introduction

Different plants and plant-derived products are being used as medicine since the dawn of human civilization. Around 75% - 80% of the world's population residing in the developing countries rely on herbal remedies for primary healthcare because of their cultural acceptability and fewer side effects [1]. The application of plants as medicine had been enlightened in the holy book of Hindus "RIGVEDA". This book is considered as the earliest repository which was written nearly in 4500 BC [2]. Traditional Chinese Medicine (TCM), Tibetan Amchi Medicine (TAC), and other alternative medicinal systems are common in Nepal. These practices have been utilized and transferred from generations for hundreds to thousands of years and are still in practice today [3].

The extreme variation in topography, climate, and soil in a small geographic area has led Nepal to have diverse biodiversity with more than 7000 species of medicinal plants [4]. The secondary metabolites such as glycosides, tannins, phenolics, lipids, alkaloids, terpenoids, etc. are responsible for the pharmacological activities of the plants which have significant importance to mankind [5]. Diverse biochemical reactions in our body generate an unbalanced quantity of free radicals creating abnormal physiological conditions leading to oxidative damage of lipids, proteins, and other important biomolecules. This damage plays a key role in the overexpression of oncogenes, mutagens formation, induction of atherogenic activity, etc. Oxidative stress is responsible for the development of many chronic illnesses such as cancers, diabetics, cardiovascular disorder, neurodegeneration, aging, and others. Natural antioxidants repair the damage caused by the reactive oxygen species (ROS). They constantly convert the free radical into less harmful molecules by radical chain reactions [6].

With the progress of science and technology, scientists have established different kinds of antibiotics to control several pathogens in the 20th century. The emergence of multidrug resistance bacteria, pan-drug resistance bacteria, as well as misuse of antibiotics, had posed a serious challenge to the synthetic compounds. Researchers are now dedicated to the progress of benign biologically active plant-derived compounds to use for the development of novel drugs [3]. The drugs from plant sources are relatively non-toxic, safe, and free from serious side effects [7]. Therefore, there is an urgent need to search of active natural compounds from medicinal plants which can potentially be effective in the treatment of the awkward bacterial infections.

The local people of Kaski district of Nepal use different medicinal plants for their primary healthcare purposes. For this study, the most common plants

namely *R. ellipticus*, *P. pashia*, *D. coronans* and *Z. mauritiana* were taken (**Figure 1**). *R. ellipticus* SM. belongs to family Rosaceae and is commonly known as Himalayan Raspberry (**Figure 1a**). It comprises of more than 750 species in 12 subgenera and found in forest edges and mountains on all continents except Antarctica. The root juice is used for fever, gastric, diarrhea, dysentery, and the root-paste is applied to wounds [8]. In western Nepal, the root juice is drunk against urinary tract infection and the fruits are listed in the top-ten edible medicinal plants in Tanahun District [9]. *P. pashia* Buch, Ham. ex D. Don (**Figure 1b**) belonging to family Rosaceae is abundant in Nepal, China, India, Bhutan, Pakistan, Myanmar, Thailand, Vietnam, Laos, and Afghanistan [10]. The fruits are used in the treatment of dyspepsia and dysmenorrhea. Flowers are used to lowering blood lipids and branches for diarrhea in Chinese folk medicine [11]. Local people of Kaski district use the decoction of leaves and stem bark for the treatment of fever and headache. *Z. mauritiana* Lam (**Figure 1c**) is a medium-sized plant of the Rhamnaceae family. It is distributed in Bangladesh, India, Sri Lanka, and other South-East Asian countries [12]. The leaves had been used in the treatment of liver disease, asthma, and fever. Fruits are used in hyperdipsia, vomiting, nausea, dyspepsia, wounds, and ulcers [13]. In the Parbat district of central Nepal, a decoction of root bark is taken for the menstrual disorder, fever, dysentery, and diarrhea [14]. *D. coronans* (**Figure 1d**) is an epiphytic fern belonging to family polypodiaceae. It is abundant in different parts of Nepal and has been used against different health problems. Its roots are used for diarrhea and constipation [15].

Figure 1. Collected medicinal plants of Kaski district of Nepal: a. *R. ellipticus,* b. *P. pashia,* c. *Z. mauritiana,* d. *D. coronans.*

The plants were found to be extensively used by the locals and were readily available in the region. The study is aimed to assess the *in-vitro* antioxidant and antibacterial activities of some of the most commonly used plants as traditional medicine. This research provides a scientific background of the traditional use of plants by the local people.

2. Materials and Methods

2.1. Collection of Plant Materials

The plant materials were collected from the outskirts of Pokhara and Rupa village of Kaski district. The samples were collected from July 2018 to April 2019. The plants were authenticated at the National Herbarium and Plant Laboratories, Lalitpur, and the voucher specimen has been submitted to the same department. The list of plants with local names, parts used, collected site, and traditional uses are shown in Table 1.

2.2. Preparation of Extract

The collected samples were washed with clean water and dried in the shed for about three weeks. The dry samples were chopped into pieces and ground into powder by using a mechanical grinder. The powdered materials were stored in clean plastic bottles until the use. The materials were subjected to Soxhlet's extraction using 80% methanol as a solvent. The extracts were concentrated in a rotary evaporator. The extracts were stored at 4˚C before performing the biological activities.

2.3. Phytochemical Screening

The methanol extracts of all the plant extracts were tested for the presence of different phytochemicals. The tests were performed for the presence of polyphenols, alkaloids, flavonoids, terpenoids, reducing sugar, glycosides, tannins, carotene, phytosterols, coumarins, saponins, and anthracenes by adopting standard protocols [17] [18].

Table 1. List of plants with local names, parts used, collected site and traditional uses.

Plant samples	Local name	Parts used	Collected from	Traditional use
Ziziphus mauritiana Lam.	Bayer	Root bark	Pokhara, Kaski	Menstrual disorder, fever [14]
Rubus ellipticus Sm.	Yensalu	Root bark	Pokhara, Kaski	Dysentery, gastric and fever [8]
Pyrus pashia Buch. Ham. Ex D. Don.	Mel	Fruit, bark, leaves	Rupa, Kaski	dyspepsia and dysmenorrhea [11]
Drynaria Coronans (Wall. Ex. Mett.) T. Moore	Kammaru	Tuber	Rupa, Kaski	Bone injury [16]

2.4. Antibacterial Activity

2.4.1. Microorganisms

Pure cultures of all the tested bacteria were obtained from the American Type Culture Collection (ATCC) of the Central Department of Microbiology, Tribhuvan University, Kirtipur. The pure cultures were maintained on the Muller Hinton Agar (MHA) media at 4˚C and sub-cultured. The list of organisms used is shown in Table 2.

2.4.2. Agar Well Diffusion Assay

The bacterial susceptibility of the plants' extract was assessed by the agar well diffusion method in Mueller Hinton Agar plates [19]. The agar-well diffusion method is a very simple and reliable method of determination of antibacterial susceptibility. The test bacteria are grown on the surface of an agar medium. The test samples are loaded into the sterile wells on the agar surface bored aseptically. The antibacterial agent of a certain quantity is allowed to diffuse through the medium at 35˚C - 37˚C for 24 - 48 hours depending upon the test organism. The inhibition of bacterial growth corresponds to the diameter of the no-growth region on the medium [20].

The test organisms were incubated in Mueller Hinton Agar Broth (MHB) overnight at 37˚C to adjust the turbidity equivalent to 0.5 McFarland's standards (1.5×10^8 CFU/ml). The plates were carpet-cultured on the surface of agar by a sterile cotton swab and five wells of 6 mm diameter were bored at equal distances with the help of a sterile cork-borer. Plant extract of 50 mg/ml concentration were prepared in 50% dimethyl sulphoxide (DMSO). Neomycin (1 mg/ml) in autoclaved distilled water was taken as a positive control and 50% DMSO as a negative control. Each well was filled with 20 µl extracts, positive, and negative controls were incubated for 24 hours at 37˚C. After incubation, the plates were observed for the formation of a clear zone around the well which corresponds to the antibacterial activity of the sample. The zone of inhibition (ZOI) of each sample was measured in mm and recorded.

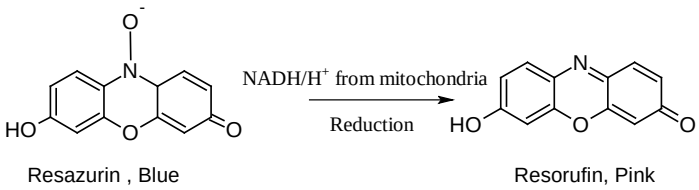
2.4.3. Resazurin Microtiter Assay (REMA)

The extracts of *Rubus ellipticus* and *Pyrus pashia* exhibiting maximum ZOI against *Staphylococcus aureus* were selected for the determination of MIC and MBC by resazurin microtiter assay. This method is a simple, rapid, sensitive, and reliable method to assess the antibacterial properties of natural products. It uses

Table 2. List of bacterial cultures.

Bacteria	Type	ATCC
Staphylococcus aureus	Gram-positive	25923
Klebsiella pneumoniae	Gram-negative	700603
Escherichia coli	Gram-negative	25922
Salmonella typhi	Gram-negative	14028

an indicator, resazurin that allows the detection of microbial growth in extremely small volume in the microtiter plate without using a spectrophotometer. In a 96-well microtiter plate, test materials are loaded in a sequence of serial dilution and the development of bacteria in a particular solution can be noticed by the change of color of the indicator. The use of resazurin is to detect visually the living and dead cells in the system. The dye is converted into pink color due to the NADH enzymes produced by the living cells [21].



Plant extracts were dissolved in 50% DMSO into a concentration of 100 mg/ml. The bacteria were cultured into the Muller Hinton Broth (MHB) overnight at 37˚C by using a sterile loop. The next day, the colony was sub-cultured into the broth at 37˚C for about two hours to match with 0.5 McFarland's standard (1.5×10^8 CFU/ml) solution. The culture was diluted 1/100 times into the broth so that bacterial concentration converted into 1.5×10^6 CFU/ml. 50 µl of the broth is loaded into all well from 1 to 6 (for two sets of triplicates) plant extracts) and 7, 8, and 9 are set up for neomycin (1 mg/ml in distilled water and syringe filtered) as a positive control. Similarly, 10, 11, and 12^{th} are filled with 100 µl of the broth to set up the negative control. Each of the well from 1 to 9 is filled by 100 µl of the broth using a multichannel pipette. The solutions from the topmost row are serially subjected to twofold diluted using a multichannel pipette up to the eighth row. Then each of the wells is loaded by 5 µl of the bacterial culture solution and allowed to incubate at 37˚C with lid covered for about 18 to 24 hours. The plate was taken out and 30 µl of 0.01% resazurin was added into each of the wells and again incubated for about 2 hours in the incubator at 37˚C. Finally, the microtiter plate is taken out and the visual change of color of the solutions observes and the MIC is determined as the concentration that corresponds to the no development of bacteria as shown in **Figure 2**. The solutions in the wells with a pink color corresponding to the MIC and stronger solutions were streaked onto the MHA plates at different compartments and incubated overnight at 37˚C.

The next day, the solutions of the compartments corresponding to MIC to higher concentrations were seeded on Muller-Hinton Agar media. The minimum concentration corresponding to no growth of bacteria was noted as the MBC of the corresponding plant extract.

2.5. Antioxidant Activity

The antioxidant activity of the plant extracts was determined by 2-2-Diphenyl-1-picrylhydrazyl free radical scavenging assay using a 96-well plate reader with slight modifications [22] [23].

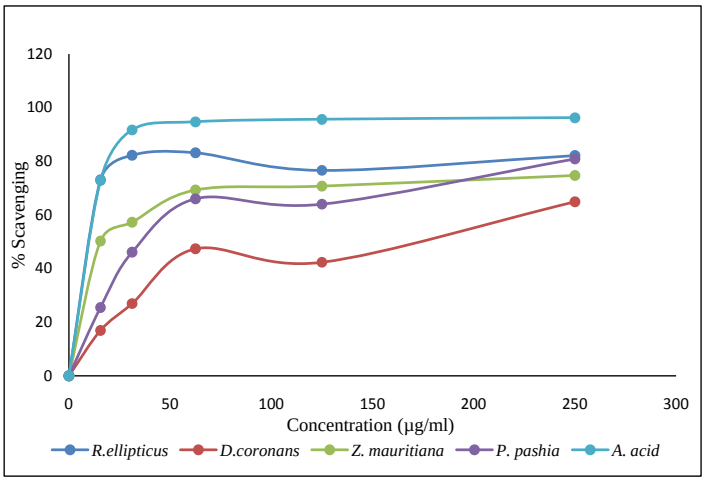


Figure 2. DPPH scavenging activities of different plant extracts against the concentration.

2,2-Diphenyl-1-picrylhydrazyl (DPPH·) has a molecular weight of 394.32 g/mol. Thus, 0.1 mM solution of the compound was prepared by dissolving 1 mg in methanol to adjust 25 ml in a volumetric flask. The solution was stored by covering with black paper and aluminum foil in a frost-free refrigerator at 4˚C.

First of all, 10 mg of the extract is dissolved in 10 ml of dimethyl sulphoxide (DMSO) to get the stock solution (1000 µg/ml) and two-fold diluted using 50% DMSO. The microplate wells are vertically labeled serially for the solutions as 500, 250, 125, 62.5, 31.25, 15.62 µg/ml in triplicate. 100 µl of the solutions of each concentration and 100 µl of DPPH is filled. The same setting was adjusted for the standard ascorbic acid as well as a negative control. The microplate was allowed to incubate at room temperature for about 30 minutes in dark. Then, the plate was loaded into the microplate reader (BioTek, Synergy LX multimode reader) at 517 nm and the data was recorded. The percentage scavenging was calculated by using the formula:

$$\%\ \text{Scavenging} = \frac{\text{Absorbance of Control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The percentage scavenging of the samples at different concentrations are used to calculate the concentration inhibiting 50% of the radical by using linear regression analysis.

2.6. Data Processing

Data obtained from the experiments were processed carefully. Mean, standard deviation, and standard error of means was calculated by using Microsoft Excel 2016. Concentration causing 50% inhibition (IC_{50}) was calculated by using probit analysis table.

3. Results and Discussions

The result of the phytochemical study is shown in **Table 3**. The phytochemical

investigation showed the presence of polyphenols, glycosides, reducing sugar, flavonoids, and tannins. Saponins were present in *P. pashia* which did not show the presence of carotenoids. Carotenoids were detected in *R. ellipticus* only. A trace presence of anthracene was detected on *P. pashia* and *D. coronans*. Alkaloids saponins and phytosterols were detected in trace amounts. Alkaloids are the special group of nitrogenous compounds used during middle ages against various human and animal ailments. Tannins are a group of high molecular weight polyphenols that protect plants from microorganisms. In animals, they cause indigestion of protein leading to a retardation of growth [24]. Flavonoids and polyphenols are important human dietary compounds that could be used for the prevention of radiation damage, treatment of cancer, cardiovascular and inflammatory diseases [25].

3.1. Antioxidant Activity

The DPPH antioxidant assay provides information on the reactivity of the plant extracts with the stable free-radical. It is a relatively stable nitrogen-containing radical having pink color. It is easily reduced by accepting an electron or a hydrogen atom from the antioxidants and loses pink color. The loss of color depends on the number of electrons accepted and it can be quantitatively measured by the changes of absorption of light of 517 nm [26]. The graph of the concentration against the corresponding percentage scavenging of different extrats and ascorbic acid is shown in **Figure 1**. The percentage scavenging of *R. ellipticus*, *P. pashia*, and *Z. Mauritiana* show an analogous mode of variation with that of ascorbic acid standard but that of *D. coronans* moves somewhat below from them.

Table 3. Result of preliminary qualitative phytochemical analysis.

Phytochemicals	*Ziziphus mauritiana*	*Pyrus pashia*	*Rubus ellipticus*	*Drynaria coronans*
Polyphenols	++	++	++	++
Alkaloids	++	+	+	+
Glycosides	++	+	++	+
Reducing sugar	++	++	++	+
Flavonoids	++	+	++	+
Terpenoids	+	+	++	+
Tannins	++	+	+	+
Coumarins	-	++	+	+
Saponins	+	++	+	+
Carotenoids	-	-	+	-
Phytosterol	+	+	-	-
Anthracene	-	+	-	+

+ = Present, - = Absent.

The concentrations causing 50% inhibition of the free radical (IC_{50}) values were calculated graphically.

The result presented in Table 4 reveals that the extracts of *R. ellipticus* had the maximum antioxidant activity with the lowest IC_{50} value (42.40 ± 1.5 µg/ml) followed by *Z. mauritiana* (55.67 ± 7.4 µg/ml) *and P. pashia* (58.33 ± 2.9 µg/ml). The extract of *D. coronans* exhibited the minimum antioxidant activity with maximum IC_{50} of 93.30 ± 5.19 µg/ml. The higher antioxidant activity of the root extracts of *R. ellipticus* is in agreement with the IC_{50} value of 33.41 µg/ml exhibited by the methanolic extracts of the leaves of *R. ellipticus* from the Arghakhanchi district of Nepal [27]. The antioxidant activity of the leaf extracts of *R. ellipticus* in different solvents was evaluated by DPPH scavenging assay. The methanol extract was found to exhibit the maximum antioxidant property with IC_{50} of 6.96 ± 2.32 µg/ml while that of standard butylated hydroxytoluene (BHT) was 13.18 ± 1.43 µg/ml. The extract at the dose of 250 mg/Kg was found to prolong the life span of Swiss albino mice with Ehrlich ascites carcinoma by 46.76% and reduced the volume of solid tumor of Dalton's lymphoma ascites by 2.56 cm^3 [28]. The higher antioxidant activity of *R. ellipticus* may be due to the abundance of important phytochemicals like polyphenols, flavonoids, glycosides which are known for the antioxidant activity [29]. The antioxidant activities of leaf extracts of *P. pashia* of Indian origin were evaluated by DPPH scavenging assay taking ascorbic acid as a standard. The methanol and water extracts were found to exhibit a significant activity with IC_{50} values of 10.81 ± 0.44 µg/ml and 11.57 ± 0.36 µg/ml respectively [30]. The ethyl acetate fraction of the fruits of *P. pashia* from China was found to have strong antioxidant activity (IC_{50} = 2.47 ± 0.08 µg/ml) followed by butyl alcohol fraction (5.23 ± 0.21 µg/ml), crude extract (7.78 ± 0.13 µg/ml), petroleum ether fraction (68.88 ± 4.80 µg/ml) and aqueous fraction (167.48 ± 5.6 µg/ml) by DPPH method. The chemical investigation of the extract led to the isolation of 28 important phenolic compounds from the plant. The compound hydroquinone, which is present in the highest proportion may play an important role in the strong antioxidant activity [31].

The antioxidant activity of the leaf extracts in different solvents of *Z. mauritiana* was assessed by different methods. The ethanol extract exhibited the maximum activity with IC_{50} of 19.44 ± 0.79 µg/ml as compared to standard ascorbic acid with IC_{50} of 6.44 ± 0.20 µg/ml by DPPH assay. The result demonstrated that the radical scavenging activity was due to the flavonoid and phenolic content in the plant [12].

Table 4. IC_{50} (µg/ml) of the plant extracts and ascorbic acid (Mean ± SD, n = 3).

Plant extracts	IC_{50} (µg/ml)
Rubus ellipticus	42.40 ± 1.5
Ziziphus mauritiana	55.67 ± 7.41
Pyrus pashia	58.33 ± 2.9
Drynaria coronans	93.30 ± 5.19
Ascorbic acid (Standard)	28.44 ± 0.97

3.2. Antibacterial Activity

Evaluation of the antibacterial susceptibility of the plant extracts was initially determined by the agar-well diffusion method. The results of the antibacterial susceptibility test are shown in Table 5. The diameter of the zone of inhibition (ZOI) reflects the relative antibacterial activity of the extract. The extracts exhibited no effect on all of the Gram-negative and a significant effect against Gram-positive bacteria, *S. aureus*. The extracts of *R. ellipticus* and *P. pashia* were found to exhibit the highest activities with the zone of inhibition (ZOI) of 17 mm and 12 mm respectively. The extracts of *Z. mauritiana* and *D. coronans* showed lower activities with ZOI of 9 mm and 8 mm respectively. The effectiveness of the extracts in the tested microorganisms was determined by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The test was performed for the extracts of *R. ellipticus* and *P. pashia* which showed high antibacterial activity. The MIC and MBC of *R. ellipticus* and *P. pashia* were 3.125 mg/ml, 12.5 mg/ml, and 12.5 mg/ml, 25 mg/ml respectively are shown in Table 6 and Figure 3.

Table 5. Diameter of ZOI in Agar-well diffusion method.

Plant samples	Bacteria	Zone of Inhibition (ZOI)	
		Extracts	Control (Neomycin)
R. ellipticus	*S. aureus*	17 mm	18.5 mm
Z. mauritiana	*S. aureus*	9 mm	12 mm
P. pashia	*S. aureus*	12 mm	16.5 mm
D. coronans	*S.aureus*	8 mm	15.5 mm

Table 6. MIC and MBC values of plant extracts against *S. aureus* (mg/ml).

Plant samples	MIC	MBC
Rubus ellipticus	3.125 mg/ml	12.5 mg/ml
Pyrus pashia	12.5 mg/ml	25 mg/ml

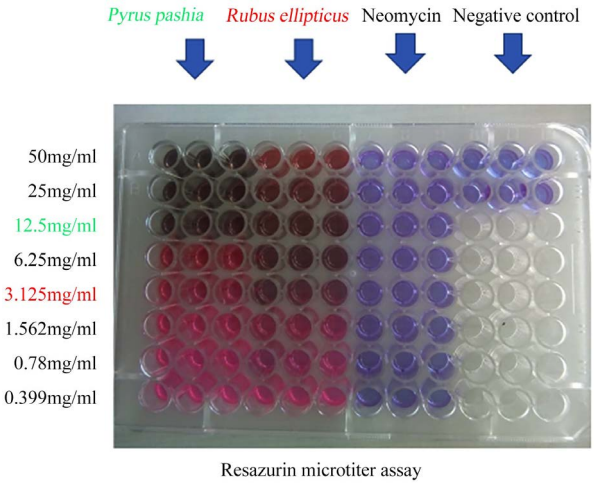


Figure 3. Results of MIC of plant extracts against *S. aureus* (mg/ml).

The results of the present study were found comparable to the previously reported results. The antibacterial activity leaf extracts of *R. ellipticus* of Tamil Nadu, India exhibited significant activity against *S. aureus* (MTCC 96). The methanol extracts were evaluated by the disc diffusion method using streptomycin as a control and 16 mm, 20 mm, and 22 mm ZOI was observed for the concentrations of 1.25, 2.5, and 5 mg/ml respectively. The MIC was determined by the broth microdilution method as 31.25 µg/ml and 250 µg/ml for methanol and ethyl acetate extracts respectively [32]. The ethanolic fruit extract of *R. ellipticus* from Uttarakhand, India was evaluated for the antibacterial activity by a disc diffusion method. The extract exhibited significant activity with ZOI of 16 ± 1, 15 ± 1 and 15 ± 1 mm for *E. coli* (MTCC 729), *Streptococcus pyogenes* (MTCC 1925) and *E. coli* (MTCC 443) at the concentration of 50 mg/ml respectively [33]. Five cyclopeptide alkaloids were isolated from the methanol root extracts of *Z. mauritiana* from Thailand. The isolated compounds exhibited potent *in vitro* antiplasmodial activity against *Plasmodium falciparum* (K1, multidrug-resistant strain) with IC_{50} values ranging from 3.7 to 10.3 µM. The alkaloids mauritine M and nummularine H exhibited significant antimycobacterial activity against *Mycobacterium tuberculosis* with the MIC values of 72.8 and 4.5 µM respectively [34]. Zizimauritic acid (A-C), ceanothenic acid, betulinic acid, and ceanothic acid were isolated from the roots of *Z. mauritiana*. All the compounds were found inactive against *Candida albicans*. Zizimauritic acid-A showed the maximum antibacterial effect against *Staphylococcus aureus* with IC_{50} of 2.17 µg/ml [35]. A broad spectrum of antibacterial activity was observed on different bacterial strains of leaf extracts of *Z. mauritiana*. The test was performed against both gram-negative and gram-positive bacteria by a disc diffusion method. The ethyl acetate extract was found to have a strong activity with ZOI, MIC, and MBC of 22.33 ± 0.58 mm, 2.5 mg/ml, and 4 mg/ml respectively for *Vibrio parahaemolyticus* (ATCC 17802). Ethyl alcohol extract was effective against *Salmonella typhi* (ATCC 13311) with ZOI, MIC, and MBC of 19.0 ± 0.10 mm, 4.5 mg/ml, and 8 mg/ml respectively. Kanamycin was taken as a standard that had the ZOI ranging from 26.67 ± 1.15 mm to 34.00 ± 0.00 mm [12]. The ethanolic extract of the bark of *P. pashia* was found to exhibit a significant antibacterial activity. The disc diffusion method showed the ZOI of 17.0 ± 1 mm, 15.0 ± 1 mm, and 14 ± 1 mm against *K. pneumonia*, *Shigella flexneri*, and *E. coli* respectively [36]. The low activity of *D. coronans* is supported by a low antibacterial property of the plant from central Nepal against some of the Gram-positive and Gram-negative bacteria [15].

The plant extracts investigated were found to contain important phytochemicals such as alkaloids, polyphenols, flavonoids, cardiac glycosides, tannins, etc. The presence of alkaloids, phenols, and flavonoids are responsible for different kinds of biological properties [32]. Flavonoids are very important secondary metabolites reported to possess antibacterial, antiviral anti-inflammatory, enzyme inhibition, cytotoxic, antitumor, antioxidant properties [37]. Glycosides support the defense mechanism against microbes, insects, and herbivores. Sapo-

nins exhibit significant hypo cholesterol, hypertensive, and cardiac depressant properties [38].

The evaluation of the extract in different fractions would give better insights into their biological properties. The antioxidant activities could be evaluated by using different methods. Multidrug resistance bacterial strains could also be used. The plants were collected in only one season from August to July. That's why the fluctuations in the results of the biological activities due to seasonal variation could not be addressed

4. Conclusion

This study suggests the crude methanol extracts of *R. ellipticus* and *P. pashia* are found to exhibit potent antioxidant and antibacterial activity that provides the partial scientific validation for using these plants against infectious diseases in different communities of Kaski district Nepal. The results indicated that plants are the good sources of secondary metabolites from which pure compound can be isolated and perform *in-vivo* biological activities with the mechanism of action to develop products for the herbal remedy against infectious diseases.

Acknowledgements

Authors are grateful to the Central Department of Chemistry, Tribhuvan University, for providing laboratory facility and Prithvi Narayan Multiple Campus, Pokhara for granting the study leave to Lekha Nath Khanal. We are thankful to National Herbarium and Plant Resources, Ministry of Forest and Soil Conservation, Godawari, Nepal for the identification of the plants.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Paper presented in national/international seminar/workshop

Lekha Nath Khanal, Khaga Raj Sharma, Yuba Raj Pokharel, Surya Kant Kalauni, Estimation of Antioxidant, and Antibacterial Activities of Some Medicinal Plants from Kaski District of Nepal, Himalayan Knowledge Conclave, 7th Graduate Conference on Environment and Sustainable Development, organized by Resources Himalaya, May 24-25, 2021 **(online conference).**

Lekha Nath Khanal, Khaga Raj Sharma, Yuba Raj Pokharel, Surya Kant Kalauni. Fabrication of silver nanoparticles from *Rubus ellipticus* Sm. roots and assessment of antioxidant and antibacterial activity, National Conference on Recent Trends in Science, Technology and Innovation (RTSTI)-2022, Organized by Research Management Unit (RMU), Paschimanchal Campus, Pokhara-16, Nepal.

Lekha Nath Khanal, Khaga Raj Sharma, Yuba Raj Pokharel, Surya Kant Kalauni, Synthesis of Silver Nanoparticles Using Stem Barks of *Pyrus pashia* and Evaluation of Antioxidant and Antibacterial Activity**,** International Chemical Congress-2023 (ICC-2023), Kathmandu, May 25-27.

HIMALAYAN KNOWLEDGE CONCLAVE

7TH GRADUATE CONFERENCE ON ENVIRONMENT AND SUSTAINABLE DEVELOPMENT

Sustainability beyond Pandemic: Youth Innovation for Environment and Spirituality

Himalayan Knowledge Conclave

CERTIFICATE OF APPRECIATION

This certificate is awarded to

Mr LEKHA NATH KHANAL

in recognition as a PAPER PRESENTER in the Seventh Graduate Conference held on May 24-25, 2021.

Mr Ukesh Raj Bhuju
Dean, School of Development Studies & Applied Sciences
Lumbini Buddhist University

Sharada Poudel, PhD
Conference Secretary
Assistant Professor, Lumbini Buddhist University

National Conference on Recent Trends in Science, Technology and Innovation

RTSTI-2022 | Pokhara, Nepal

Certificate of Participation

This is to certify that Mr./Mrs. **LEKHA NATH KHANAL** has participated as an **ORAL PRESENTER**

entitled **Fabrication of silver nanoparticles from Rubus ellipticus Sm. roots and assessment of antioxidant and antibacterial activity (CHO-02)**

in the **National Conference on Recent Trends in Science, Technology and Innovation, RTSTI-2022**

organized by Research Management Unit, Pashchimanchal Campus, Pokhara on 29th - 30th May, 2022.

Asst. Prof. Nirmal Prasad Baral
Chairman, Organizing Committee, RTSTI
Pashchimanchal Campus, Pokhara

Assoc. Prof. Dr. Krishna Raj Adhikari
Chairman, Technical Committee, RTSTI
Pashchimanchal Campus, Pokhara

Prof. Dr. Ganesh Man Gurung
Chief Guest
Chancellor, Gandaki University

Organizer: Research Management Unit, RMU, Pashchimanchal Campus, Pokhara-16, Lamachaur

Co-organizer: Gandaki Province Academy of Science and Technology (GPAST), Pokhara, Nepal

In association with: Pokhara University; Gandaki University; Prithvi Narayan Campus; Tribhuvan University Institute of Forestry, Pokhara Campus; Tribhuvan University Institute of Medicine, Pokhara Nursing Campus; College of Natural Resource Management; Gandaki College of Engineering and Science

International Chemical Congress (ICC-2023)

Chemistry for Sustainable Development

May 25-27, 2023 | Kathmandu, Nepal

Organized by
Nepal Chemical Society
in association with
Central Department of Chemistry
Tribhuvan University

Certificate of Participation

This is to certified that
Prof./Dr./Mr./Ms. .....Lekha Nath Khanal.....
.....
has participated and delivered oral lecture in International Chemical Congress (ICC-2023) held in Park Village Hotel, Kathmandu, Nepal during May 25-27, 2023.

Dr. Surendra K. Gautam
Conference Convener
(President NCS)

Dr. Mahesh K. Joshi
Conference Secretary
(General Secretary NCS)

Prof. Dr. Jagadeesh Bhattrai
Conference Co-convener
(HoD, CDC, TU)

Date: May 27, 2023

Research Permit

NATIONAL TRUST FOR URE CONSERVATION
ANNAPURNA CON ON AREA PROJECT
Head Quarters, Pokhara

Ref: 408 /074/075

Date: May 29, 2018

Mr. Lekh Nath Khanal
PhD Scholar
Tribhuvan University, Central Department of Chemistry
Kritipur, Kathmandu

Re: Permission to conduct research in Annapurna Conservation Area.

We received your request letter regarding permission to conduct research on **"Phytochemical and biological studies on some selected medicinal plants of Mustang District and identification of the active constituents"**. You have been given permission to carry out your field research in ACA with the following terms and conditions.

1. The research must be for scientific and academic purpose with the aim of making contribution in conservation and development of conservation area.
2. This permission will be valid up to 15 June, 2019.
3. You have to follow the ACAP Minimum Impact Code and the Conservation Area Management Regulation 2053 during your entire stay in the Annapurna Conservation Area.
4. You have to follow the mentioned condition in the research permit provided by Department of National Park and Wildlife Reserve.
5. You will have access to the NTNC-ACAP Resource Library in Pokhara.
6. Upon the completion of the research, **you must submit a hard copy and digital copy of your report to the NTNC-ACAP/HQ**.
7. You will maintain communication with the Unit Conservation Offices.
8. Any dispute arose during the execution period will be solved by mutual understanding.
9. Any unsolved disputes will be handled as per the existing law of Nepalese government.

Thank you and wish you all the best.

Binod Basnet
Project Manager

cc: NTNC/ ACAP- Unit Conservation Office - Jomsom

Central Office : P.O. Box 3712
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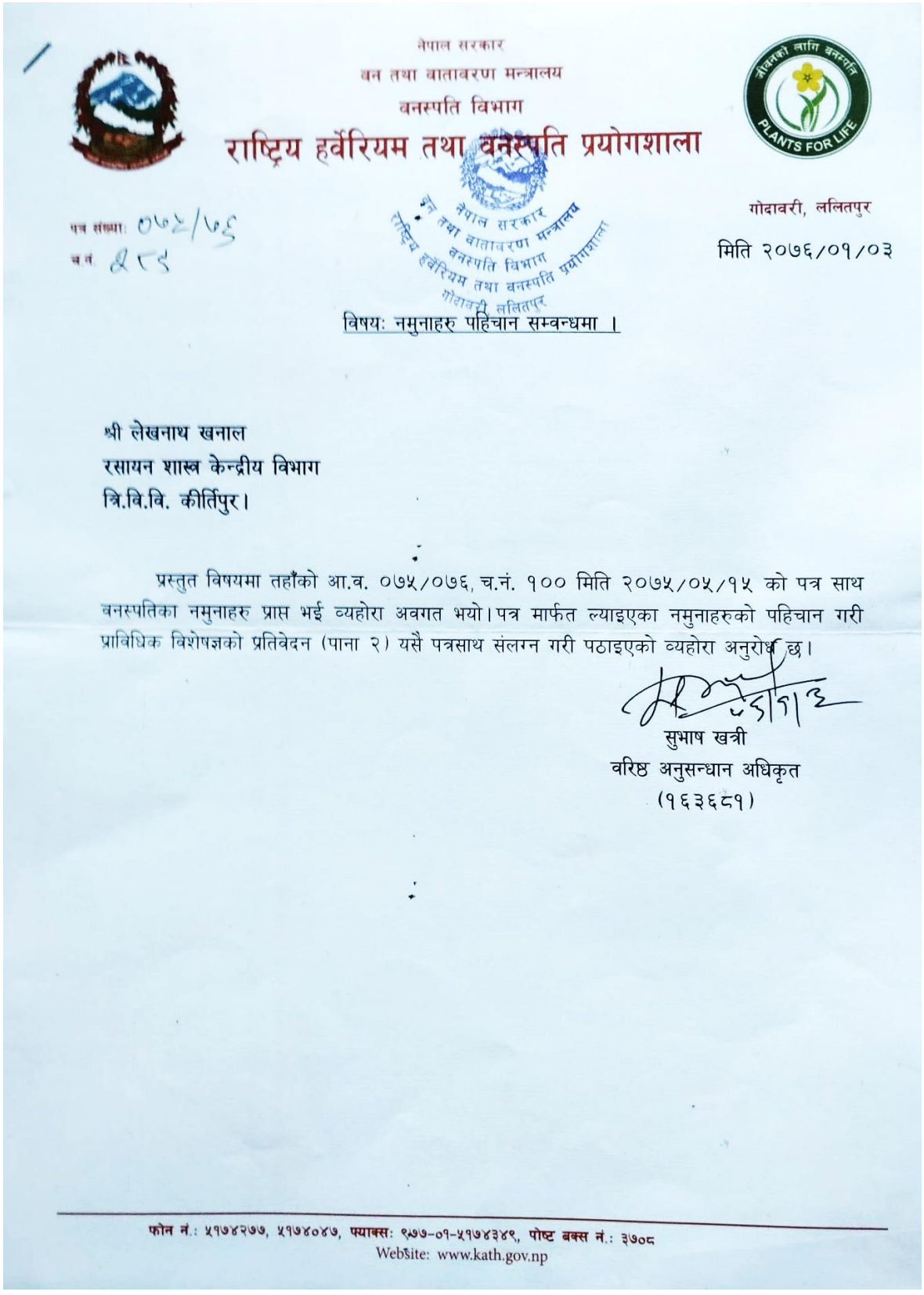
Fax No. : 00977-61-431203
E-mail : info@acap.org.np

List of plants authenticated



नेपाल सरकार
वन तथा वातावरण मन्त्रालय
वनस्पति विभाग
राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशाला

नेपाल सरकार वन तथा वातावरण मन्त्रालय वनस्पति विभाग राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशाला गोदावरी, ललितपुर

पत्र संख्या: ०७५/७६
च.नं. १००

गोदावरी, ललितपुर
मिति २०७६/०१/०३

विषय: नमुनाहरु पहिचान सम्वन्धमा ।

श्री लेखनाथ खनाल
रसायन शास्त्र केन्द्रीय विभाग
त्रि.वि.वि. कीर्तिपुर।

प्रस्तुत विषयमा तहाँको आ.व. ०७५/०७६, च.नं. १०० मिति २०७५/०५/१५ को पत्र साथ वनस्पतिका नमुनाहरु प्राप्त भई व्यहोरा अवगत भयो। पत्र मार्फत ल्याइएका नमुनाहरुको पहिचान गरी प्राविधिक विशेषज्ञको प्रतिवेदन (पाना २) यसै पत्रसाथ संलग्न गरी पठाइएको व्यहोरा अनुरोध छ।

सुभाष खत्री
वरिष्ठ अनुसन्धान अधिकृत
(१६३६८१)

फोन नं.: ५१७४२७७, ५१७४०४७, फ्याक्स: ९७७-०१-५१७४३४९, पोष्ट बक्स नं.: ३७०८
Website: www.kath.gov.np

List of Identified plant species received from Mr. Lekha Nath Khanal, PhD Scholar, Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu with Ref. no. 100,2075/076 dated on 2075/05/15.

S. No.	Scientific name	Family	Local name (According to collectors)	Herbarium number
1	*Aster indamellus* Grierson	Asteraceae	Metok	L1
2	*Juniperus indica* Bertol.	Cupressaceae	Dhupi Lekali	L2
3	*Ephedra pachyclada* Boiss.	Ephedraceae	Tamchya Somlata	L3
4	*Arctium lappa* L.	Asteraceae	Jhisung, Chisung	L4
5	*Elaeagnus umbellata* Thunb.	Elaeagnaceae	Gunyenli	L5
6	*Clematis graveolens* Lindl.	Ranunculaceae	Bagh Junge	L6
7	*Artemisia roxburghiana* Wall. ex Besser	Asteraceae	Lekali pati	L7
8	*Artmisia stricta* Edgew.	Asteraceae	Seto Lekali pati	L8
9	*Taraxacum parvulum* Wall. ex DC.	Asteraceae	Dudhe Phool	L9
10	*Sida acuta* Burm. f.	Malvaceae	Balujhar	L10
11	*Crataeva unilocularis* Buch.-Ham.	Capparaceae	Sipligan	L11
12	*Ziziphus mauritiana* Lam.	Rhamnaceae	Bayer	L12
13	*Rubus ellipticus* Sm.	Rosaceae	Ainselu	L13
14	*Pyrus pashia* Buch.-Ham. ex D. Don	Rosaceae	Mayal	L14
15	*Dischidia bengalensis* Colebr.	Apocynaceae	Thirjo	L15
16	*Drynaria coronans* (Wall.ex Mett.) T. Moore	Polypodiaceae	Kamari	L16
17	*Mimosa rubicaulis* subsp. *himalayana* (Gamble) H. Ohashi	Fabaceae	Areli	L18
18	*Angiopteris helferiana* C. Presl.	Marattiaceae	Gaikhure	L19
19	*Trichosanthes wallichiana* Lour.	Cucurbitaceae	Indreni ko Laharo	L21
20	*Begonia megaptera* A. DC.	Begonaceae	Makargachi	L23

16.4.2019

Ganga Datt Bhatt

Research Officer

National Herbarium and Plant Laboratories (KATH), Godawari, Lalitpur, Nepal

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Grade sheets of the semester examinations

Tribhuvan University
Institute of Science and Technology
Dean's Office

SEMESTER EXAMINATION 2075

Name of Student: Lekha Nath Khanal
Level: Ph.D.
Department: Central Dept. of Chemistry

Exam Roll No.: 100019
Ph.D. Enrolment No.: 56/074
T.U. Regd. No.: 8103-88
Semester: I

Grade Sheet

Code No.	Course Title	Cr. Hrs.	Grade Point	Grade
PHS 911	Philosophy of Science	3	3.7	A-
RM 912	Research Methodology	3	3.7	A-
Sem 913	Seminar	3	3.7	A-

SGPA: 3.7

Verified By: Sandu

Date: Oct. 9, 2018

Institute of Science & Technology
Dean's Office
Kirtipur 2045

Asst. Dean

First semester grade-sheet

Tribhuvan University
Institute of Science and Technology
Dean's Office

SEMESTER EXAMINATION-2075

Name of Student: Lekh Nath Khanal
Exam Roll No.: 200018
Level: Ph.D.
Ph.D. Enrolment No.: 56/074
Department: Central Dept. of Chemistry
T.U. Regd. No.: 8103-88
Semester: 2

Grade Sheet

Code No.	Course Title	Cr. Hrs.	Grade Point	Grade
CHE 951	Advanced Research Methodology	3	4	A
CHE 953	Natural Products Chemistry	3	4	A
CHE 952	Seminar	3	4	A

SGPA: 4.00

Verified By: .....

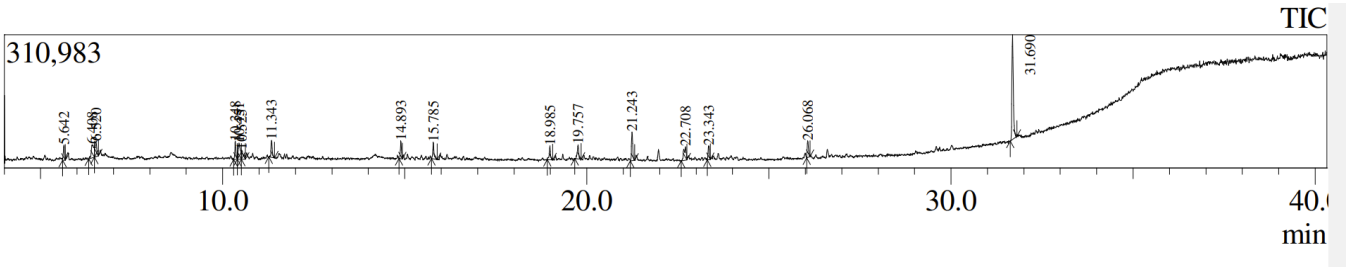
Date:- Sept. 15, 2019

Asst. Dean

Second semester grade-sheet

Chromatograms of GC-MS analysis

Chromatogram EO of *A. grandifolia*



Chromatogram of essential oil of *E. pachyclada*