BIOLOGICAL STUDIES OF SOME SELECTED MEDICINAL PLANTS FROM KATHMANDU VALLEY AND ISOLATION OF CHEMICAL COMPOUNDS

A DISSERTATION SUBMITTED FOR THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTERS DEGREE OF SCIENCE IN CHEMISTRY

Submitted by

Junu Kapali

Exam Symbol No: CHE 732/073

T.U. Regd. No: 5-2-33-89-2012

Submitted to

Central Department of Chemistry

Institute of Science and Technology

Tribhuvan University

Kirtipur

Kathmandu, Nepal

February, 2021

BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation entitled "Biological Studies of Some Selected Medicinal Plants from Kathmandu Valley and Isolation of Chemical Compounds" by Junu Kapali, under the supervision of Asst. Prof. Dr. Khaga Raj Sharma, Central Department of Chemistry, Tribhuvan University, Nepal, is hereby submitted for the partial fulfillment of the Master of Science (M.Sc.) Degree in Chemistry. This dissertation has been accepted for the award of a degree.

Supervisor
Asst. Prof. Dr. Khaga Raj Sharma
Central Department of Chemistry
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Internal Examiner
Prof. Dr. Niranjan Parajuli
Central Department of Chemistry
Tribhuvan University
Kirtipur, Kathmandu, Nepal

External Examiner
Prof. Dr. Daman Raj Gautam
Amrit Campus
Tribhuvan University
Lainchour, Kathmandu, Nepal

Head of the Department
Prof. Dr. Ram Chandra Basnyat
Central Department of Chemistry
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Date:………………

RECOMMENDATION LETTER

This is to certify that the dissertation work entitled "Biological Studies of Some Selected Medicinal Plants from Kathmandu Valley and Isolation of Chemical Compounds" has been carried out by Junu Kapali as a partial fulfillment of the Master of Science (M.Sc.) Degree in Chemistry under my supervision. To the best of my knowledge, this work has not been submitted to any degree in this institute.

Supervisor

Asst. Prof. Dr. Khaga Raj Sharma

Central Department of Chemistry

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date:………………

DECLARATION

I, Junu Kapali, hereby declare that the work presented is genuine work done originally by me and has not been published or submitted elsewhere for the requirement of a degree program. Any literature, data or works done by others, presented in this dissertation are cited, has been given due acknowledgement and listed in the reference section.

Junu Kapali

Central Department of Chemistry

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date:………………

DEDICATED TO MY PARENTS

Jyoti Kapali and Mina Kapali

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to my supervisor Asst. Prof. Dr. Khaga Raj Sharma of the Central Department of Chemistry at Tribhuvan University, for his continuous guidance, fruitful suggestion, practical help and encouragement throughout the period of research and dissertation writing. He was always been a supportive whenever I faced any troubles related with my work so that I am able to accomplish this task as per schedule.

I would like to acknowledge the opportunity provided by Head of the Central Department of Chemistry, Prof. Dr. Ram Chandra Basnyat, Tribhuvan University, Nepal. Moreover, I would like to thank all my respected teachers of CDC and administration staffs as well as laboratory staffs of the department for their continuous assistance throughout the dissertation work.

My thankful credit goes to Asst. Prof. Dr. Khaga Raj Sharma for FTIR analysis of respective fraction. I am also thankful to Mr. Karan Khadayat for being supportive whenever I faced any troubles at my lab work.

I am indebted to my friends Lalitkala Panday, Binod Gupta and Sarala Regmi for their continuous help and co-operation during my research work.

Last but not least, my warmest thank goes to my respected parents Jyoti Kapali and Mina Kapali, my brother Bishal Kapali for giving all the love, moral supports and encouragement.

Thank you all,

Junu Kapali

ABSTRACT

The aim of this study is to investigate the phytochemical and biological properties of some selected medicinal plants from Kathmandu valley and isolation of phytoconstituents from active plant extract. The methanolic extracts of *Ageratina Adenophora*, *Cupressus sempervirens* and *Lantana camara* aerial parts were analyzed for their phytochemical analysis, toxicity test, antioxidant activity, total phenolic and total flavonoid content, antidiabetic properties and isolation of chemical constituents from active plant fraction and FTIR analysis. The toxicity test was studied by brine shrimp lethality test. The antioxidant potential was evaluated by DPPH radical scavenging assay. The amount of total phenolic and flavonoid was estimated with the Folin-Ciocalteu reagent and aluminium chloride method, respectively. The α amylase enzyme inhibition activity was performed to evaluate the inhibition percentage for each extract. The phytochemical analysis showed the presence of various secondary metabolites like alkaloids, flavonoids, carotenoids, coumarins, glycosides, polyphenol, carbohydrate, saponin, tannins, terpenoids, quinones, and volatile oil & fats. The methanolic extracts of *Ageratina adenophora* was found to be cytotoxic against brine shrimp as shown by its LC_{50} value of 833.68 µg/mL. The methanolic extracts of *Lantana camara* (106.179 ± 11.390 µg/mL) showed the strongest DPPH radical scavenging activity as its IC_{50} values were close to standard ascorbic acid (17.456 ± 0.822 µg/mL). *Lantana camara* showed the highest phenolic (10.2 ± 0.343 mg GAE/g) and flavonoid content (1.872 ± 0.16 mg QE/g) respectively. In addition, *Ageratina adenophora* methanolic extracts were found to inhibit enzymatic activity of α-amylase under *in-vitro* starch digestion bioassay with the IC_{50} value of 1.843 ± 0.007 mg/mL. The FTIR measurement revealed the presence of C=O, O-H and C-H stretching bands at the functional group region.

Keywords: Phytochemical, antioxidant, phenolic, flavonoid, antidiabetic, α-amylase.

LIST OF ACRONYMS AND ABBREVIATIONS

TCM: Traditional Chinese Medicine

TKM: Traditional Korean Medicine

ROS: Reactive Oxygen Species

RNS: Reactive Nitrogen Species

RSS: Reactive Sulphur Species

BHT: Butylhydroxyl-toluene

BHA: Butylhydroxyanisole

TBHQ: tert-butylhydroquinone

PG: Propyl gallate

DPPH: 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazyl

HCA: Hydrocinnamic acid

HBA: Hydrobenzoic acid

DNJ: 1-deoxynojirimycin

LC_{50}: Lethality concentration for 50% mortality

HPLC: High Performance Liquid Chromatography

GC: Gas Chromatography

UPLC: Ultra Performance Liquid Chromatography

R_f: Retention factor

FTIR: Fourier Transform Infrared Spectroscopy

IR: Infrared radiation

HSCCC: High-Speed Countercurrent Chromatography

TPC: Total Phenolic Content

TFC: Total Flavonoid Content

GAE: Gallic acid equivalent

QE: Quercetin equivalent

IC_{50}: Inhibitory concentration for 50% concentration

STZ-NA: Streptozotocin-nicotinamide

HGO: Hepatic Glucose Output

HP: Hyperglycemic + Pyruvate

AF: Amentoflavone

BW: Bodyweight

TG: Triglycerides

TC: Total cholesterol

LDL: Low Density Lipoprotein

TLC: Thin Layer Chromatography

FCR: Folin-Ciocalteu reagent

NHPL: National Herbarium and Plant Laboratories

DMSO: Dimethylsulphoxide

PPA: Porcine pancreatic α -amylase

DCM: Dichloromethane

EtOAc: Ethylacetate

LIST OF TABLES

Table 1: Composition of artificial sea water 34

Table 2: Qualitative screening of phytochemicals present in different methanolic extracts of all plants 47

Table 3: Calculation of LC_{50} value of different plant extracts 48

Table 4: Percentage of radical scavenging with different concentration 50

Table 5: Comparison of IC_{50} values of different plants extracts with standard ascorbic acid 52

Table 6: Total phenolic Content (TPC) of different plant extracts 55

Table 7: Total flavonoid Content (TFC) of different plant extracts 57

Table 8: Percentage of α–amylase inhibition activity with different concentration 58

Table 9: Comparison of IC_{50} values of different plants extracts with standard acarbose 59

Table 10: Column chromatography and TLC report of different fractions 62

Table 11: Functional groups of active fraction 64

LIST OF FIGURES

Figure 1: Generation of reactive oxygen species (ROS) 4

Figure 2: Reaction mechanism of DPPH and antioxidant 6

Figure 3: Reaction mechanism of DPPH and ascorbic acid 6

Figure 4: Structure of phenolic acids 7

Figure 5: Chemical structure of amylopectin chain and amylose chain 10

Figure 6: Mechanism of action of α-amylase enzyme with starch substrate 12

Figure 7: Mechanism of action of α-amylase enzyme inhibition 12

Figure 8: Schematic diagram of methodology 32

Figure 9: The flow chart of fractionation and isolation of compounds 44

Figure 10: A plot of percentage radical scavenging activity with concentration of different plant extracts and ascorbic acid. 51

Figure 11: Free radical scavenging activity (IC_{50}) in different concentration of methanolic plant extracts 52

Figure 12: Variation of absorbance (nm) with concentration (µg/mL) for standard gallic acid 54

Figure 13: Total phenolic content of different methanolic plant extracts 55

Figure 14: Variation of absorbance (nm) with concentration (µg/mL) for standard quercetin 56

Figure 15: Total flavonoid content of different methanolic plant extracts 58

Figure 16: A plot of % inhibition with concentration (mg/mL) of different plant extracts and acarbose 59

Figure 17: α – amylase inhibition activity (IC_{50}) in different concentration of methanolic plant extracts 60

Figure 18: FTIR spectra of 2% EtOAc in hexane of *Lantana camara* 63

Figure 19: FTIR spectra of 10% EtOAc in hexane of *Lantana camara* 63

Figure 20: FTIR spectra of 15% EtOAc in hexane of *Lantana camara* 63

Figure 21: FTIR spectra of 30 % EtOAc in hexane of *Lantana camara* 64

TABLE OF CONTENTS

Page no.

BOARD OF EXAMINER **i**
RECOMMENDATION LETTER **ii**
DECLARATION **iii**
DEDICATION **iv**
ACKNOWLEDGEMENTS **v**
ABSTRACT **vi**
LIST OF ACRONYMS AND ABBREVIATIONS **viii**
LIST OF TABLES **xi**
LIST OF FIGURES **xii**
TABLE OF CONTENTS **xiv**

CHAPTER – 1

INTRODUCTION **1-17**

1.1 General Introduction 1
1.2 Antioxidants 3
1.2.1Reaction mechanism of DPPH and Ascorbic acid 6
1.3 Polyphenols 7
1.4 Flavanoids 8
1.5 Antidiabetic activity 9
1.5.1 α-amylase enzyme and its inhibition 10
1.5.2 Mode of action 12
1.6 Brine shrimp bioassay 13
1.7 Column chromatography 13
1.8 Fourier-transform infrared spectroscopy (FTIR) 14
1.9 Botanical Description of Plants 15
1.9.1 *Ageratina adenophora* 15
1.9.2 *Cupressus sempervirens* 15
1.9.3 *Lantana camara* 16
1.10 Objectives of study 17
1.10.1 General objectives 17

1.10.2 Specific objectives 17

CHAPTER-2

LITERATURE REVIEW 18-29

2.1 *Ageratina adenophora* 18
2.2 *Cupressus sempervirens* 21
2.3 *Lantana camara* 25

CHAPTER - 3

MATERIALS & METHODS 30-46

3.1 Chemicals 30
3.2 Equipments 30
3.3 Collection & Preparation of Plant Material 31
3.4 Extraction of the Dried Powdered Sample 31
3.5 Filtration of the Extract 31
3.6 Solvent Evaporation 31
3.7 Qualitative Phytochemical analysis 33
3.8 Biological activities 33
3.8.1 Brine shrimp bioassay 33
3.8.1.1 Materials and equipments 33
3.8.1.2 General Procedure 34
3.8.1.3 Preparation of artificial sea water 34
3.8.1.4 Culture and Harvesting of shrimps 34
3.8.1.5 Preparation of test extracts 34
3.8.1.6 Data analysis 35
3.9 Quantitative phytochemical analysis 36
3.9.1 Antioxidant Activity (Scavenging activity (DPPH) assay) 36
3.9.1.1 Preparation of DPPH solution (0.1mM) 37
3.9.1.2 Measurement of DPPH free radical scavenging activity 37
3.9.2 Total Phenolic Content (TPC) 37
3.9.2.1 Preparation of standard Gallic acid stock solution 37
3.9.2.2 Evaluation of Total Phenolic Content 38
3.9.2.3 Calculation of total phenolic content 38

3.9.2.4 Statistical analysis 38
3.9.3 Total Flavonoid Content 39
3.9.3.1 Preparation of Standard quercetin solution 39
3.9.3.2 Evaluation of Total Flavonoid Content 39
3.9.3.3 Calculation of total phenolic content 40
3.9.3.4 Statistical analysis 40
3.9.4 Antidiabetic activity 41
3.9.4.1 Preparation of reagent 41
3.9.4.2 Pancreatic a-amylase inhibition assays (Starch-Iodine colour assay) 42
3.9.5 Isolation of compounds 44
3.9.5.1 Extraction 44
3.9.5.2 Isolation of compounds by column chromatography 45
3.9.5.3 Analytical conditions for FTIR 45
3.9.6 Statistical Analysis 46

CHAPTER - 4

RESULTS AND DISCUSSION 47-64

4.1 Phytochemical screening 47
4.2 Biological Screening 48
4.2.1 Brine shrimp toxicity assay 48
4.3 Antioxidant Activity (DPPH free radical scavenging activity) 49
4.4 Determination of Total Phenolic Content (TPC) 53
4.4.1 Calculation of Total Phenolic Content (TFC) in different plant extracts 54
4.5 Determination of Total Flavonoid Content (TFC) 56
4.5.1 Calculation of Total Phenolic Content (TFC) in different plant extracts 57
4.6 Antidiabetic activity 58
4.7 Isolation of compounds 61
4.8 Chemical analysis of constituents of hexane fraction from FTIR analysis 63

CHAPTER – 5

CONCLUSION AND RECOMMENDATIONS 65-66

5.1 Conclusion 65

5.2 Recommendation 66

REFERENCE 67-77

APPENDIX-1 PHYTOCHEMICAL SCREENING 78-80

APPENDIX-2 PREPARATION OF REAGENTS 81

APPENDIX-3 82-84

APPENDIX-4 85

APPENDIX-5 86-89

CHAPTER - 1

INTRODUCTION

1.1 General Introduction

For millenia, medicinal plants have been used practically from the existence of human civilization on an empirical basis, without mechanistic knowledge on their pharmacological activities or active constituents.[1,2] Plants have been used as a traditional source for medicine. Traditional Chinese Medicine (TCM), Ayurveda, Kampo, Traditional Korean Medicine (TKM) and Unani employ natural products and have been practiced all over the world for hundred or even thousands of years.[3,4] The best-known example is the discovery of a potent anti-malarial drug, qinghaosu (artemisinin), which was extracted in the 1970s from Artemisia plants, used by Chinese herbalists for thousands of years as a remedy for many illnesses.[5]

It was only in the 18th century that James Lind, the Scottish naval surgeons, remembered as the first man in medical history, proved that drinking lemon juice could cure scurvy, Baron Anton Von Storck, who investigated poisonous herbs such as aconite and colchicum, and William Withering, who analyzed foxglove for the treatment of oedema, devised the basis for the rational pharmacological investigation of medicinal plants.[6] At the beginning of 19th century, the German apothecary assistant Friedrich Serturner, first, isolated the analgesic and sleep-inducing agent from tarry poppy seed juice *(Papaver somniferum)*.[7] As a result the era of modern drugs began and prompted the logical examination of other natural products.

Natural products, possess a unique and broad chemical diversity and have been evolved for optimal interaction with biological macromolecules, occupy only about 1% of all published chemical structures and the output of natural products has no counterpart in the available synthetic compounds or libraries; the statistical data, however indicate the advantage and potential of natural products for discovering new drugs or leads.[8,9] Natural products, containing inherently large-scale structural diversity than synthetic compound, have been the major resources of bioactive natural agents and will continually play as protagonists for discovering new drugs.[8] Currently, about 25–30% of all drugs available as

therapeutics are derived from natural products or are derivatives of a natural product.[10]

Various types of medicines, suchas anticancer, antihypertensive, antimigraine medication have benefited greatly from natural products. For instances, the Vinca alkaloids from *Catharanthus roseus*, the terpene paclitaxel from *Taxus baccata.*

Historically, most drugs were derived from natural products. Over 50% of all advanced clinical drugs are being either (semi) synthetic derivatives of compounds isolated from natural sources or synthetic mimetics of pharmacophores found in natural products.[11] Therefore, natural products play an important role in drug development programs. The medicinal plants have a long history of curative properties against various diseases. However, screening of plants for their activity is very essential and needs urgent attention in order to know the value of the higher plant. For being cheap, relatively safe and easily available, medicinal plants and herbs embody the foundation of traditional medicinal practice all over the world. The medicinal plants are center of research to find out novel lead compounds. The first commercial pure natural product introduced for therapeutic use is morphine and the first semi-synthetic pure drug aspirin, based on a natural product salicin is isolated from *Salix alba*. The compound galegine isolated from *Galega officinalis* was the lead compound for the development of metformin used in the treatment of type 2 diabetes. This led to the isolation of early drugs such as cocaine, codeine, digitoxin, quinine and pilocarpine. Drugs including paclitaxel from *Taxus brevifolia* for lung, ovarian and breast cancer, artemisinin from traditional Chinese plant *Artemisia annua* to combat multidrug resistant malaria, silymarin extracted from the seeds of *Silybum marianum* for the treatment of liver diseases are derived from natural products.

Nepal, harbors a distinct potential for traditional medicinal plants with its rich climatic and geographical variance, biodiversity and endogenous knowledge. Traditional medicinal plants are being used from ancient time in Nepal. 80 % of the Nepalese population are dependent on the traditional plant-based medicines for their primary health care needs.[12] Several kinds of research have

been conducted by National and International research community to their best in order to establish the scientific value and authenticity for the use of traditional medicinal plants. There is lack of proper governmental support, advance research equipments and sufficient funds for the researchers and research institutions to carry detail analysis on the medicinal plants of Nepal.[13]

1.2 Antioxidants

An antioxidant is a stable molecule that nullify free radical by accepting or donating electron(s) or an active hydrogen atom to eliminate the unpaired condition of the radical, thus reducing its capacity to damage. These antioxidants may directly react with the reactive species and terminate the chain reaction, while they may itself became a new free radical which are less active than those radicals they have neutralized. These newly formed radicals is again neutralized by other antioxidant or by other mechanism to terminate their radical status.[14]

Free radicals are a highly reactive chemical species with a single unpaired electron which are capable of undergoing reaction with the adjacent molecules suchas protein, lipids, carbohydrate and nucleic acids.[15] Free radical reactions could be one of the etiologic factors for many human diseases such as atherosclerosis, hemochromatosis, ischemic heart disease, diabetes, neurodegenerative diseases such as Alzheimer's disease, Parkinson, cardiovascular and hepatic disorders and disease conditions such as ageing process, inflammation and immune-suppression.[16] Free radicals are generated on exposure to UV light, ionizing radiation, heavy metal ions, smoking, chemicals and fried foods. In the biological system, free radicals are often derived from oxygen, nitrogen and sulphur molecule. These groups of molecules are reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS). ROS includes superoxide anion ($O_2^{\bullet-}$), perhydroxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($\cdot OH$), nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$) and other species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), hypobromous acid (HOBr) and peroxynitrite ($ONOO^-$). RNS are derived from nitric oxide through the

reaction with $O_2^{-\bullet}$ to form $ONOO^-$ and RSS are easily formed from thiols by reaction with ROS.[17]

During electron transport chain reaction, a small number of electrons "released" as a result molecular oxygen acquires an electron, thus, forming the oxygen free radical anion, superoxide anion, inside the living body. Superoxide anion O can capture further electron to form hydrogen peroxide. Hydrogen peroxide (HO) is toxic, injurious and can further react with "superoxide" anion, in the presence of ferrous ion or copper to form "hydroxyl" radical and "singlet oxygen". Whenever superoxide anion, O is formed in the tissues, it will lead to the formation of another free radical and hydrogen peroxide. This hydroxyl radical are extremely reactive and hence causes oxidative stress.[18]

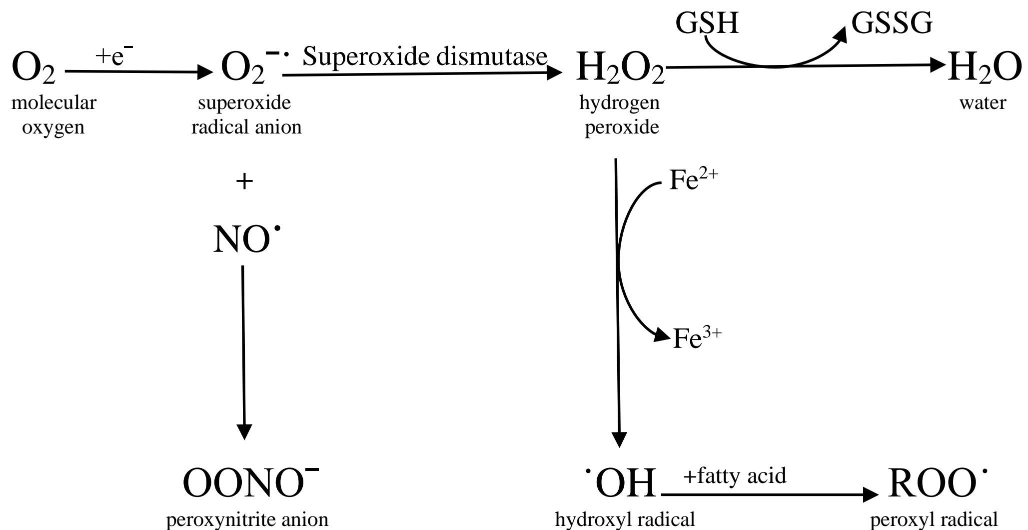


Figure 1: Generation of reactive oxygen species (ROS)[18]

It has been found that excessive ROS can have deleterious effects on many molecules including protein, lipids, RNA and DNA. ROS can also attack bases in nucleic acids, amino acids side chain in protein and double bonds in unsaturated fatty acids, thus leading to oxidative stress.[17] The term oxidative stress refers a shift towards a pro-oxidant environment in the balance between oxidant species formation and antioxidant defenses. Chemical compounds capable of producing potential toxic reactive oxygen species (ROS) are known as antioxidants. The oxidative stress significantly contributes to the pathogenesis of inflammatory diseases, cardio-vascular diseases, cancer, diabetes, alzheimer's, cataracts, autism and aging.[15]

Today, most of the food industry used synthetic antioxidants suchas butylhydroxyl-toluene (BHT), butylhydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ), propyl gallate (PG) in order to retard the lipid oxidation in foods. However, these synthetic antioxidants are not preferred for pharmacologic use due to the potential health hazards. Hence, insatiable interests have focused on identifying plant extracts to use as a dietary antioxidant supplements.[19]

The natural antioxidants have a wide variety of antioxidant compounds, suchas phenolic (phenol and polyphenols), flavonoids, carotenoids, steroids and thiol compounds that can help to prevent cellular damage from oxidative stress.[20] Eg. Green tea contains 50% flavonoids such as catechin and epicatechin that contribute to antioxidant activities. Polymeric tannins and monomeric flavonoids such as catechin and epicatechin in grape seed extracts could be responsible for their higher antioxidant activities.[19] Ginseng contains steroid-like compound, ginsenosides, which prevents free radical damage on the vascular endothelium.[21] Ginkgo reported strong antioxidant activities due to flavone glycosides that scavenge free radicals.[22]

For a compound to be an antioxidant, it must fulfill two condition.

1. When present at low concentration, compared to oxidizable substrate, it can significantly delay or prevent oxidation of the substrate.
2. The resulting radical formed on the polyphenol must be stable in order to prevent it from acting as chain propagating radical.[14]

Among various antioxidant assay we performed DPPH radical scavenging assay. DPPH is the most extensively used antioxidant assay for plant extract. DPPH is very stable, synthetic radical with an unpaired electron on nitrogen atom which is extensively delocalised over phenyl rings that are attached with the nitrogens of hydrazine moiety. It is a non-planar molecule with three benzene rings. It shows a dark-purple solution in ethanol, with a strong absorption at 517 nm. This purple colour in general, fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colourless/bleached product (i.e. 1,1-diphenyl-2-(2,4,6-

trinitrophenyl) hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band, which can be detected spectrophotometically.

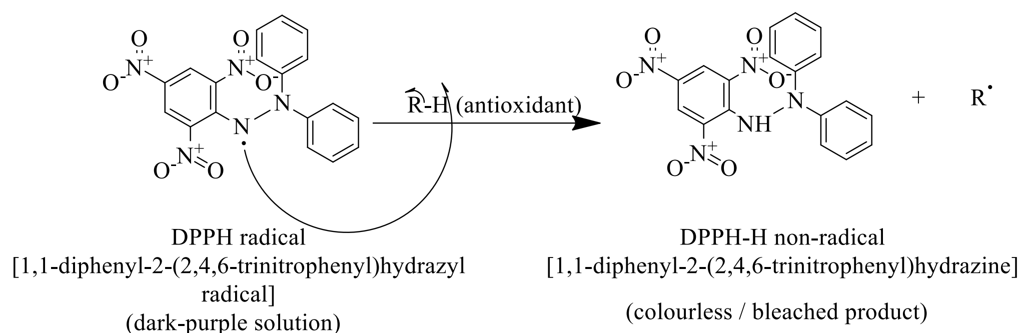


Figure 2: Reaction mechanism of DPPH and antioxidant

1.2.1 Reaction mechanism of DPPH and Ascorbic acid

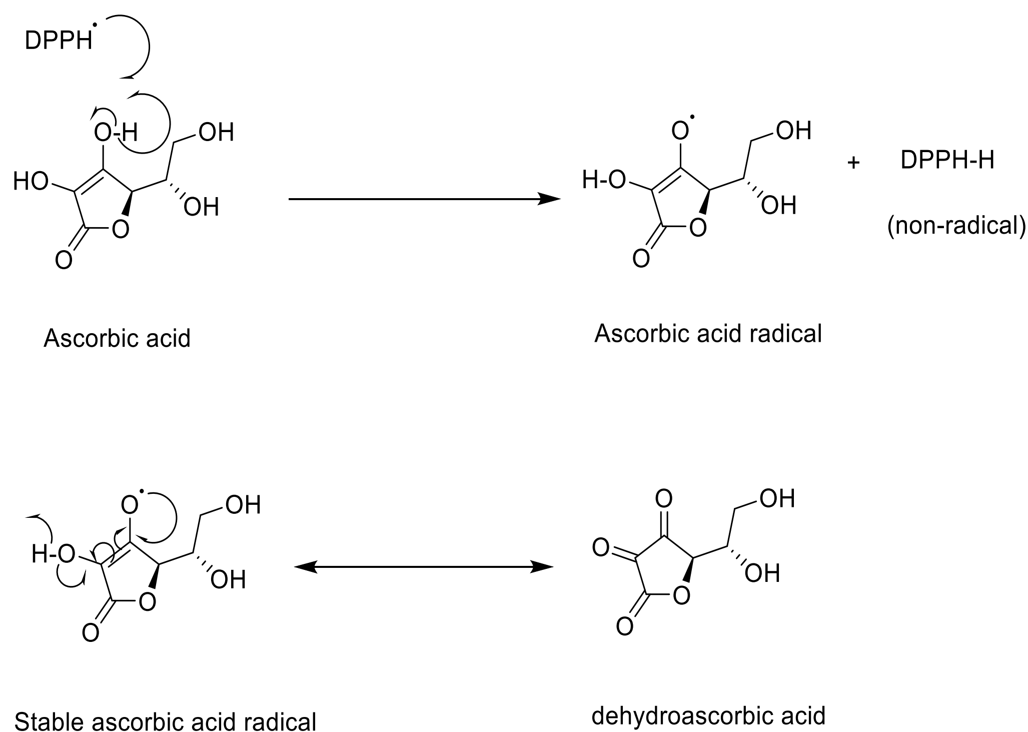


Figure 3: Reaction mechanism of DPPH and ascorbic acid

Two molecules of DPPH are reduced by one molecules of ascorbic acid. Thus molecules that are able to donate two or more than two hydrogen atom to DPPH radical and thus itself get oxidized act as an antioxidant.

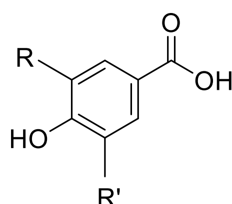
1.3 Polyphenols

Phenolics acid are one of the most widespread class of metabolites in nature, and their distribution is almost ubiquitous. They occurs in the form of esters, glycosides or amides, but rarely in free form. Phenolic compounds are synthesized in plants partly as a response to ecological and physiological pressure suchas pathogen and insect attack, UV radiation and wounding. They participate in the aromatic characteristics of plants and are very common. Variation in phenolic acids is in the number and location of hydroxyl groups on the aromatic ring.[23]

Phenolic compounds have an aromatic ring with one or more hydroxyl group and acts as antioxidants. Generally, two classes of phenolic compounds, eg. hydroxybenzoic acids and hydroxycinnamic acids, are found in plants. Phenolic compounds are secondary metabolities synthesized through shikimic acid and phenylpropanoid pathways.[24]

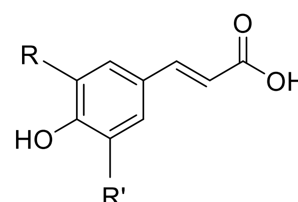
Structure of Phenolic acids

Benzoic acid derivatives



R = R' = H; *p*-hydroxybenzoic acid
R = OH, R' = H; protocatechuic acid
R = OCH3, R' = H; vanillic acid
R = R' = OH; gallic acid
R = R' = OCH3; syringic acid

Cinnamic acid derivatives



R = R' = H; *p*-coumaric acid
R = OH, R' = H; caffeic acid
R = OCH3, R' = H; ferulic acid
R = R' = OCH3; sinapic acid

Figure 4: Structure of phenolic acids

Phenolic acids have two parent structure - HCA & HBA. Hydrocinnamic acid, HCA derivatives includes ferulic, caffeic, p-coumaric and sinapic acid, while hydrobenzoic acid, HBA derivatives consists of gallic, vanillic, syringic and protocatechuic acids. These compounds play a crucial role in the cell wall during plant growth by protecting against stressed condition suchas infection, wounding and UV-radiation.[25]

Phenolic hydroxyl group are good hydrogen donors. H-donating antioxidants can react with ROS and RNS in a termination reaction, which breaks the cycle of generation of new radical. Following interaction with the initial reactive species, a radical form of the antioxidant is produced, having a much greater chemical stability than the initial radical. The interaction of the hydroxyl groups of phenolics with the π-electrons of the benzene ring gives the molecules special properties, most notably the ability to generate free radicals where the radical is stabilized by delocalization. The formation of these relatively long-lived radicals is able to modify radical-mediated oxidation process.[26]

The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate transition metal ions i.e. iron, copper, involved in the production of free radical.[27] However, phenolics can act as pro-oxidants by chelating metals in a manner that maintains or increases their catalytic activity or by reducing metals, thus increasing their ability to form free radicals.[28]

1.4 Flavonoids

Flavonoids are diverse polyphenolic compounds with a phenylbenzo-γ-pyrone structure which are widely distributed in the plant kingdom. They are synthesized by phenyl-propanoid pathway. Flavonoids are hydroxylated phenolic compounds.[29] They are abundantly found in natural sources like fruits, vegetables, seeds, nuts, flowers, tea, wine, honey and propolis and therefore form part of the normal diet of humans. Many reports claim the usefulness of flavonoids in medical conditions, including anti-inflammatory, oestrogenic, antimicrobial, antioxidant and chelating, vascular and antitumour activities.[20]

Chemically, flavonoid are 15-C-skeleton consisting of two phenolic ring A & B linked via a heterocyclic pyrane ring C. They are divided according to their biosynthetic origin into several classes such as flavones (eg. Flavone, apigenin, and luteolin), flavonols (eg. Quercetin, kaempferol, myricetin, and fisetin), etc.[30]

Flavonoids in food is responsible for colour, taste, prevention of fat oxidation, protection of vitamins and enzymes.[30] Flavonoids are strong antioxidants mainly due to their low redox potential and their capacity to donate several electron or hydrogen atoms.[20] Flavonoids break free radical chain reaction.

There is intense interest in flavonoids due to their antioxidant properties and their major role in the prevention of free radical-related diseases.

1.5 Antidiabetic activity

Diabetes mellitus is a heterogenous group of metabolic disorder characterized by hyperglycemia (elevated level of blood glucose) and insufficiency of secretion or action of insulin. Diabetes cause numerous complication affecting the vascular system, kidney, retina, lens, peripheral nerves, and skin.[31]

There are two broad etiopathogenetic categories of diabetes.

1.Type 1 diabetes (Juvenile onset diabetes): The cause is β-cell destruction usually leading to an absolute deficiency of insulin secretion results from a cellular mediated autoimmune destruction of the β-cells of the pancreas hence leading to absolute insulin deficiency. Most commonly occur in childhood or adolescence and also called insulin-dependent diabetes.

2.Type 2 diabetes (adult onset diabetes): The cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. This accounts 90-95% of those with diabetes, ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance. It is also known as non-insulin dependent diabetes.

Another form is gestational diabetes that develop during pregnancy. This condition develop during the 2 or 3 trimester of pregnancy. These women are at higher risk for developing type 2 diabetes within 5-10 years.[32]

The global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045.[33] As a complementary or alternative approach, herbal medicines with hypoglycemic activities are increasingly being used in the treatment of diabetes to confer less side effects and compatible with physiological system.[34] Only few active compounds have been approved for clinical use eg. metformin derived from *Galega officinalis*. Thus more research work are needed in the area of natural products with antidiabetic activity in the coming years.

1.5.1 α-amylase enzyme and its inhibition

Enzymes are biological catalysts which catalyzes specific biochemical reactions. Among these, α-amylase is one of them. α-amylase (α-1,4-glucan-4-glucanohydrolases) is a salivary or pancreatic enzyme that plays as important role in the digestion of starch and glycogen. α-amylase is a well-known endoamylase. The α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium.[35] Starch also known as amylum, is a polysaccharide of glucose made of two types of α-D-glucan chains, amylose and amylopectin. About 20% of dietary starch is amylose and the remainder is amylopectin. Amylose is a linear polymer of α-D-glucose unit linked by α-1,4 glycosidic bond. Amylopectin is a branched polymer of α-D-glucose units linked by α-1,4 and α-1,6 glycosidic bonds.[36]

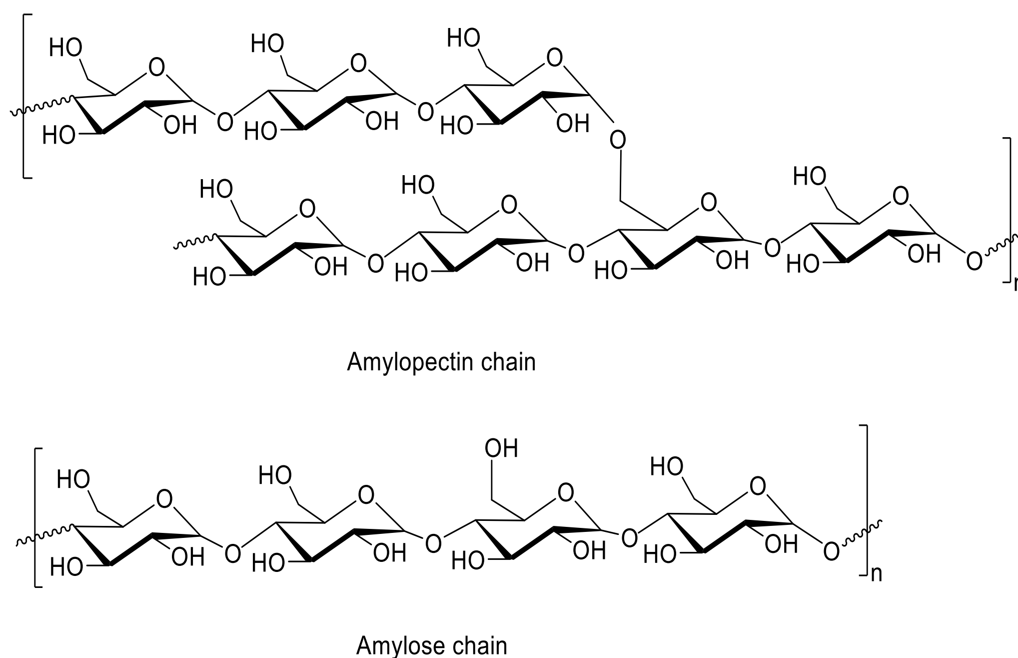


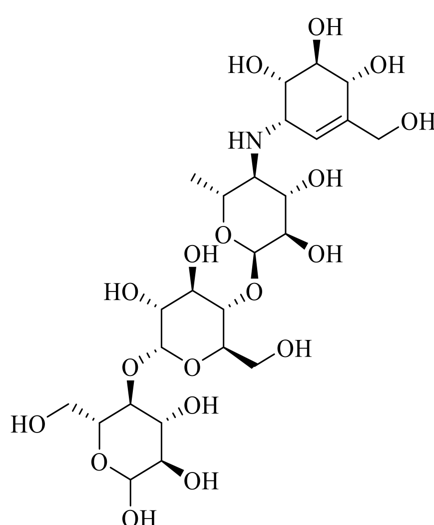
Figure 5: Chemical structure of amylopectin chain and amylose chain[36]

During metabolism, α-amylase (salivary and pancreatic) catalyses the initial hydrolysis of starch into smaller oligosaccharide through the cleavage of interior α-D-(1,4) glycosidic bonds. This resulting products of α-amylase digestion are called dextrins which is a mixture of maltose, maltotriose, and branched oligosaccharides of 6-8 glucose units that contain both α-1,4 and α-1,6 linkages. Neither terminal glucose residues nor α-1,6-linkages can be

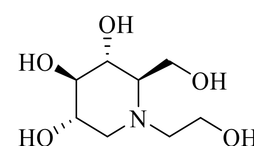
cleaved by α-amylase. Later on in the gut, intestinal brush border enzymes, maltase and isomaltase, finish the digestion of dextrins.[37]

In humans, four enzymes are involved in the complete digestion of starch into glucose. Salivary and pancreatic α-amylase are endohydrolases that cleaves the internal α-(1→4) bonds of starch into shorter linear and branched dextrin chain. The resultant mixture of dextrin is then further hydrolysed at the non-reducing ends into glucose by two small-intestinal brush-border exohydrolases: maltase glucoamylase and sucrose isomaltase.[35,37]

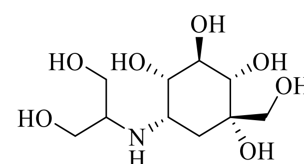
Suppression of the mammalian alpha amylase enzyme in the human digestive system would delay the degradation of starch and oligosaccharides to monosaccharides before they can be absorbed. This would decrease the absorption of glucose and consequently reduce postprandial blood glucose level. Acarbose, miglitol, voglibose and 1-deoxynojirimycin (DNJ) are commercially available glucose inhibitor widely used for the treatment of type 2 diabetes. Acarbose, a well known glucosidase inhibitors, is a pseudotetrasaccharide, consist of a polyhydroxylated aminocyclohexane derivative (valienamine or valienol) linked via its nitrogen atom to a 6-deoxyglucose, which is itself α-1,4-linked to a maltose moiety. It is a competitive inhibitor of α-amylase thus block the enzymatic reaction.[38]



Acarbose



Miglitol



Voglibose

These oral hypoglycemic agents produces myriad undesirable side effects. Thus, exploration of natural products as a therapeutic agents for the treatment

of diabetes is gaining more attention because they have less or no side effects and cost effective.

1.5.2 Mode of action

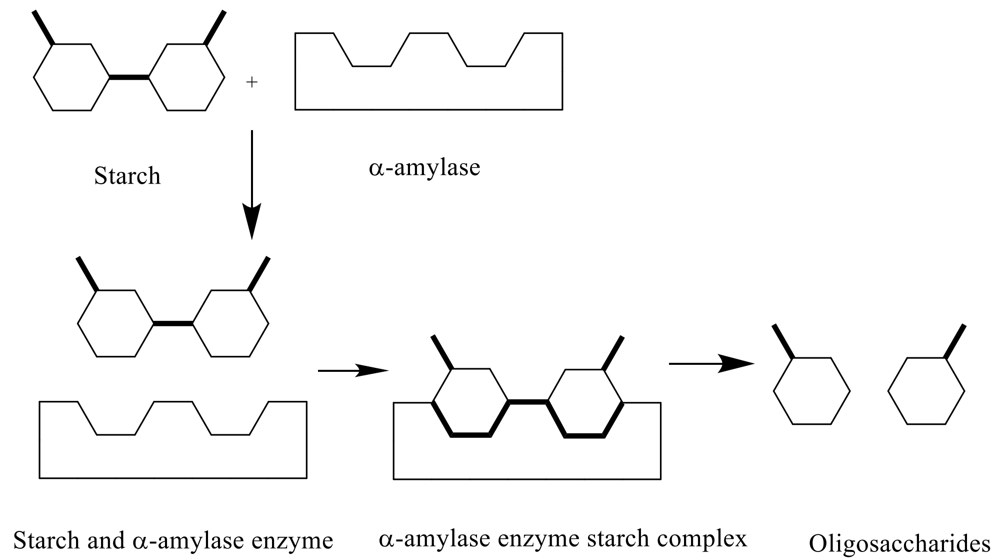


Figure 6: Mechanism of action of α-amylase enzyme with starch substrate

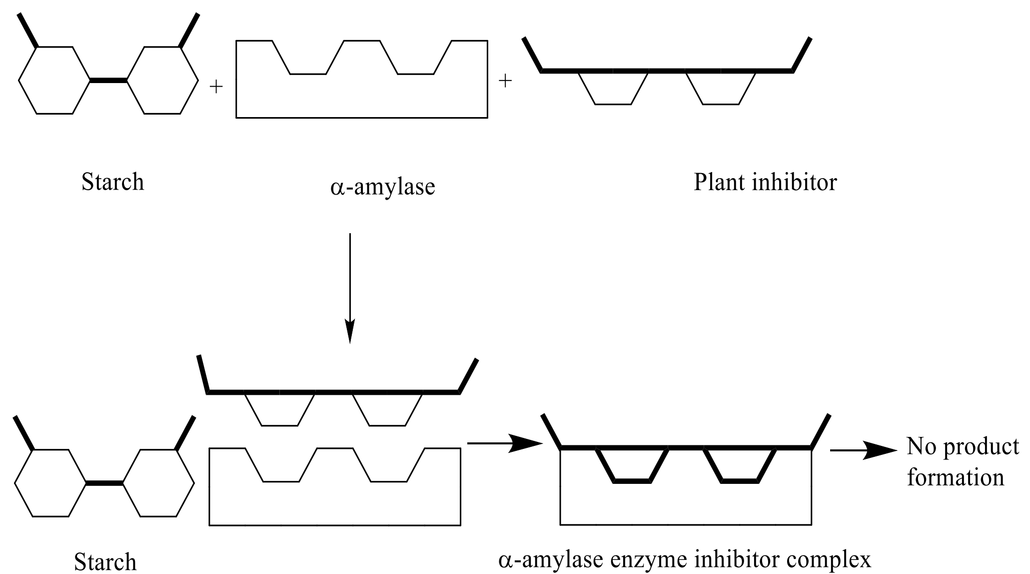


Figure 7: Mechanism of action of α-amylase enzyme inhibition

1.6 Brine shrimp bioassay

Brine shrimp lethality assay is an important tool for the preliminary cytotoxicity assay of plant extract and others based on the ability to kill a laboratory cultured larvae (nauplii). This assay was first proposed by Michael *et al.,* in 1956. Subsequently, it was further developed by others. This lethality assay has been successively employed as a bioassay guide for active cytotoxic and anti-tumor agents in 1982. This assay is proposed as a simple bioassay for natural product research. The newly hatched brine shrimp nauplii were exposed to different concentrations of plant extract for 24 hours. The number of motile nauplii was calculated for the effectiveness of the extract. It is a rapid (24 hours following introduction of shrimp), simple as no aseptic techniques are required, cost effective and requires small amount of test material. Bioactive compounds show toxicity towards brine shrimp larvae. Cytotoxicity was evaluated in terms of LC_{50} value (lethality concentration).

LC_{50} value is the measure of the cytotoxicity which is the concentration dosage required to kill the 50% of the exposed brine shrimps. LC_{50} and 95 % confidence intervals were determined from the 24 hour counts using the probit analysis method described by Finney. According to Meyer and others, LC_{50} value of 100 µg/mL or below was considered of having strong cytotoxic activity, LC_{50} values between 100 µg/mL and 500 µg/mL, and were categorized as having moderate cytotoxicity, LC_{50} values between 500 µg/mL and 1000 µg/mL, is considered to have weak cyto- toxic activity while LC_{50} values of greater than 1000 µg/mL is considered to be non-toxic. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number of nauplii being exposed in terms of percentage. This is to ensure that the death (mortality) of the nauplii is attributed to the bioactive compounds present in the plants extracts.[39]

1.7 Column chromatography

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with

the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster. Many modifications and improvements were made to column chromatography to derive the advanced chromatography techniques. The advanced forms of column chromatography are high performance liquid chromatography (HPLC), gas chromatography (GC), ultra performance liquid chromatography (UPLC), etc. Despite many advanced method of chromatography, column chromatography is widely used in science research and industry. This chromatography is a type of adsorption chromatography techniques. In this separation of components depends upon the extent of adsorption to stationary phase where the stationary phase is a polar solid material packed in a vertical column. When a mixture of mobile phase and samples to be separated are introduced from top to the column the individual components of mixture move with different rates. Those with lower affinity and adsorption to stationary phase move faster and eluted out first while those with greater adsorption affinity move or travel slower and get eluted out last. The solute molecule adsorb to the column in a reversible manner.[40,41]

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}}$$

1.8 Fourier-transform infrared spectroscopy (FTIR)

Fourier Transform Infrared (FTIR) Spectroscopy is one of the most widely used and important techniques to identify the chemical constituents and elucidate the chemical structures. The FTIR permits the analysis of all samples as small as a few microns in diameter. Chemical compounds have different chemical properties due to the presence of different functional groups so no two compounds produces the same exact IR spectrum. Thus, IR spectrum represent the fingerprint of a sample. The basic principle behind the technique is based on the vibrational motions of atom and chemical bond within molecules. When a beam of light containing the IR radiation band is passed through a sample, light

energy from the photons is absorbed by bonds and transformed into vibrational motions. Library searching in the IR region is a well established and powerful way of classifying and identifying compounds. The fundamental measurement obtained in FTIR is an infrared spectrum, which is a plot of measured infrared intensity versus wavelength (or frequency) of light. FT-IR spectroscopy is a rapid, non-destructive technique with minimum sample preparation necessary. Any sample in any state can be studied in short time hence it is sometimes referred to as the Felgett advantage.[42]

1.9 Botanical Description of Plants

1.9.1 *Ageratina adenophora*

Ageratina adenophora (Spreng.) R.M. King & H. Rob. [syn. *Eupatorium adenophorum Spreng*] commonly known as banmara, Crofton weed, Cat weed, Sticky snakeroot or Mexican devil is a many stemmed, perennial herbaceous shrub, 1-2 m high, reproduce by seed. The plants belongs to the family Asteraceae.[43] The plant is native to Mexico but has now spread to Hawaii, Philippines, China, Thailand, Australia, New Zealand, India, Nepal, southwestern USA, southern Europe, South Africa, Spain, California, Malaysia, Singapore, Indonesia, Papua New Guinea, Burma, Vietnam, Pakistan, Pacific Islands and the Canary Islands.[44]

In Nepal, juice of the plant is used as an antiseptic to treat cuts and wounds. Juice of root is useful for fever treatment. The leaf paste is applied to cure boils and is used to treat eyes insomnia. It is also used for green manure and bio-briquette.[45] In India, it is used as antiseptic and as a blood coagulant. A decoction of the plant has been recommended to treat jaundice and ulcer.[44] The plant is reported to possess diverse medicinal properties and finds use in Traditional Medicine Systems of India, Nigeria and other parts of the world as antimicrobial, antiseptic, blood coagulant, analgesic, antipyretic and phenobarbitone induced sleep enhancer, antiseptic, analgesic and antipyretic.[46]

1.9.2 *Cupressus sempervirens*

Cupressus sempervirens is a tree that grows up to 30 m tall. The leaves are 0.5 to 1 mm, dark green and obtuse. They are elliptical-oblong (rarely globose), green when young and shining yellowish-gray when ripe, with 8 to 14 short and

obtusely spiked scales. There are 8 to 20 seeds on each scale.[47] The plants belongs to the family Cupressaceae.[48] It was native to the Mediterranean basin. However, the plant was distributed in North Africa, Asia (Iran, Palestine, Jordan, Lebanon, Syria, Iraq, Turkey), Southern Europe (Greece and Italy) and Northern America.[49]

The parts of the plant used medicinally were the leaves and cones.[50] The drug was used externally for head colds, coughs and bronchitis.[47] A decoction of the cones and leaves of *Cupressus sempervirens* was used in a sitz bath three times a day for one week for haemorrhoids. The cones and leaves were used internally as an astringent. Externally, the extract of the cypress was incorporated in preparations (ointments and suppositories) and used to treat haemorrhoids, varicose veins and venous circulation disorders. The essential oil was used as antiseptic and an antispasmodic for stubborn coughs.[50] Cypress was also described as deodorant, and diuretic, to promote venous circulation to the kidneys and bladder area, and to improve bladder tone and as a co-adjuvant in therapy of urinary incontinence and enuresiss.[51]

1.9.3 *Lantana camara*

Lantana camara is locally known as banfanda. *Lantana camara* is a perennial aromatic shrub. It grows upto 2 to 3 meters and it can spread about 2.5 meters in wide. It belongs to family Verbenaceae. *Lantana camara* is a tropical origin plant and found mostly in the Nepal, south India, central and south America, Africa. *Lantana camara* is now spreaded to nearly 50 countries like America, Brazil, Mexico and etc. It has become very widespread in Australia, India and South Africa, infesting millions of hectares of land.[52]

Lantana camara has been reported to be used in traditional medicine system for the treatment of itches, cuts, ulcers, swellings, bilious fever, cataract, eczema and rheumatism. Different parts of the plants (leaves, flowers, roots etc.) are employed in the treatment of cold, headache, uterine haemorrhage, chicken pox, eye injuries, whooping cough, asthma, bronchitis and arterial hypertension. The fruits are effective in several conditions such as fistula, pustules, tumor and rheumatism. Leaves infusion used for bilious fever, vitiated condition of vata and kapha, eczema and eruptions. In the treatment of malaria, rheumatism and

skin rashes root of this plant much effective. *Lantana camara* oil is sometimes used against skin itches, as an antiseptic for wounds, and externally for leprosy and scabies.[53]

Thus, present study is focused on the chemical and biological activities of some selected plants collected from Kathmandu valley of Nepal. These plants were used by local people for different therapeutic purposes since many years. The study also concerns with the determination of important phytochemicals and connects them with medical uses which would help in assessment of new drugs and establish new drug for advance level of research.

1.10 Objectives of study

Varieties medicinal plants have been reported from different parts of Nepal for the treatment of different diseases. Despite of having great significant of medicinal plants, not much work has been reported on chemical and biological investigation on varieties of medicinal plants from Kathmandu valley in literature.

1.10.1 General objectives

Phytochemical and biological studies of some selected medicinal plants from Kathmandu valley and analysis of phytoconstituents from active plant extract.

1.10.2 Specific objectives

- To carry out the phytochemical analysis for the secondary metabolite of plant extracts collected from Kathmandu valley
- To perform the toxicity test of selected medicinal plant extracts
- To evaluate the antioxidant activity of selected plant extracts
- To determine the total phenolic and total flavonoid content of selected plant extracts
- To study the antidiabetic activity of plant extracts
- Isolation and purification of bioactive compounds from active plant extract by column chromatography
- FTIR analysis of active fraction obtained from column chromatography

CHAPTER-2

LITERATURE REVIEW

Literature review of the work done on the selected plant materials were composed from information published on some books, journal articles (Scholarly articles), website (Sci-hub, Google, Google scholar) and discussed on first come first basis.

2.1 *Ageratina adenophora*

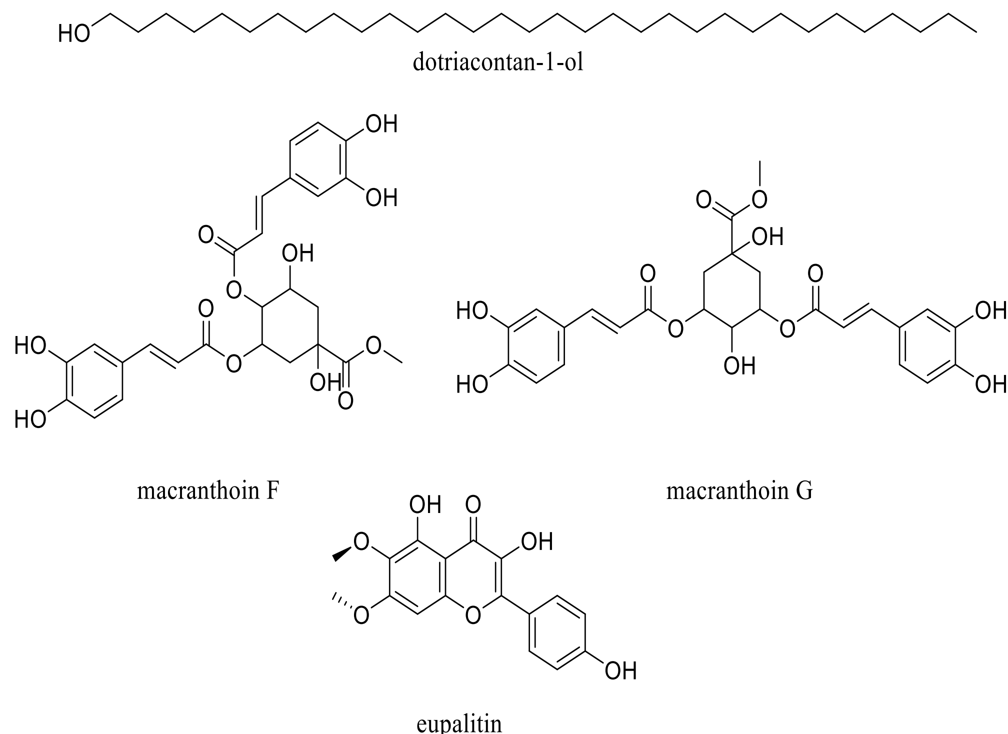
Structurally diverse chemicals including flavonoids, (mono-, sesqui-, di-, and tri-) terpenoids, pyrrolizidine alkaloids, phenylpropanoids, quinonoids, phytosterols and sesquiterpene lactones, polysaccharides, phenolic acids, coumarins, sterols and alkaloids essential oils, and some others have been reported from different parts of the *Ageratina adenophora.*[54]

Sesquiterpenes including chlorogenic acid (5-O-caffeoylquinic acid, 5-CQA), Neochlorogenic acid (3-O-caffeoylquinic acid, 3-CQA), cryptochlorogenic acid (4-O-caffeoylquinic acid, 4-CQA), 2-deoxo-2-(acetyloxy)-9-oxoageraphorone (DAOA), 9-oxo-agerophorone (OA), and 9-oxo-10, 11-dehydroagerophorone (ODA, Euptox A) were found in leaves of the *Ageratina adenophora.*[55]

Flavonoids (quercetagetin 7-beta-O-glucoside, 6-methoxykaempferol 7-methyl ether 3-beta-O-glucoside, quercetagetin 4^-methylether 7-beta-O-glucoside, 6-hydroxykaempferol-7-beta-O-glucoside and 6-methoxygenkwanin), coumarin (umbelliferone), a new phenyl glucoside (3-(2^-beta-O-pyranoglaucoside)-phenyl-2-trans-trans-propenoic acid) and dotriacontanol were isolated from the aerial parts of *Eupatorium adenophorum.*[56]

Semi-preparative high-speed countercurrent chromatography (HSCCC) successfully isolated five bioactive components from *Eupatorium adenophorum* Spreng crude extract. It includes caffeic acid, 40-methyl quercetagetin 7-O-(600-O-E-caffeoylglucopyranoside), quercetagetin 7-O-(600-O-acetyl-b-D-glucopyranoside), eupalitin 3-O-b-D-galactopyranoside, and eupalitin.[57]

A novel quinic acid derivative, 5-O-trans-o-coumaroylquinic acid methyl ester, together with chlorogenic acid methyl ester, macranthoin F and macranthoin G were isolated from the aerial parts of the *Ageratina adenophora* (Spreng.).[58]



Four new cadinane sesquiterpenes, including a dimeric cadinane derivative and a peroxide cadinane analogue, were isolated from the leaves of *Eupatorium adenophorum.* They are characterized as (+)-(5R, 7S, 9R, 10S)-2-oxocadinan-3, 6 (11)-dien-12, 7-olide, (+)-7,7′-bis [(5R, 7R, 9R, 10S)-2-oxocadinan-3, 6 (11)-dien-12, 7-olide], (+)-(5R, 7S, 9R, 10S)-7-hydroxy-7, 12-epidioxycadinan-3, 6 (11)-dien-2-one and (-)-(5R, 6R, 7S, 9R, 10S)-cadinan-3ene-6, 7-diol.[59]

Terpenes (2 monoterpenes and 9 sesquiterpenes) were isolated and identified from the aerial part of *Eupatorium adenophorum* Spreng. (or *Ageratina adenophora* (Spreng.) King and Robinson) plant. These include a new monoterpene, (-)-(1R*,2S,*4R*,5S*)-3,3-dimethyl-5 hydroxybicyclo[2,2,1] hept-2-yl methanol, two new cadinene sesquiterpenes, (-)-(5S*,6S*,7S*,9R*,10S*)7-hydroxy-5,7-epidioxycadinan-3-ene-2-one and (+)-(5S*,6R*,9R*,10S*)-5,6-dihydroxycadinan3-ene-2,7-dione, and eight known terpene compounds.[60]

Ten compounds including three previously undescribed benzofuran derivatives (7-hydroxy-dehydrotremetone, 7,10,11-trihydroxy dehydrotremetone, 10-oxo-7-hydroxy-nordehydrotremetone), a previously undescribed chromene derivative (5-b-glucosyl-7-demethoxy-encecalin) and a previously undescribed monoterpene glucoside (8-hydroxy-8-b-glucosyl-2-carene) were isolated and identified from the roots of *Ageratina adenophora.*[61]

The total phenolic content and total flavonoid content of chloroform, ethyl acetate, acetone, methanol and hydromethanol extracts were found in the range of 30.71±0.16 − 68.51±0.58 mg gallic acid equivalent/g and 18.93±1.76 − 32.25±1.15 mg quercetin equivalent/g respectively. Both TPC and TFC was highest in hydro-methanol extract followed by methanol extract.[62]

Antioxidant activity of the methanolic extracts (0.0005-0.05 mg/mL) of leaves of *Ageratina adenophora* were determined by spectrophotometric, using DPPH radical scavenging activity. *Ageratina adenophora* leaves neutralized 50% of free radicals at the concentration of 3.00 µg/mL.[63]

Quinic acid derivative i.e 5-O-trans-o-coumaroylquinic acid methyl ester, chlorogenic acid methyl ester, macranthoin F and macranthoin G, isolated from the aerial parts of the *Ageratina adenophora* (Spreng.) were tested for their potential antioxidant activity against DPPH (1,1-diphenyl-2-picryl hydrazyl) radical. Compounds chlorogenic acid methyl ester 2 and macranthoin G 4 showed scavenging activity against DPPH radical, with SC_{50} values 212.2 and 150.2 µM, respectively, but much weaker than the positive control resveratrol (SC_{50} 42.1 µM). SC_{50} value represents the concentration of a compound to scavenge 50% of DPPH radicals.[58]

The antioxidant potential of the inflorescence and root essential oils of *Ageratina adenophora* was investigated using DPPH radical-scavenging activities, compared with the standard synthetic antioxidant BHT. The IC_{50} (concentration to achieve 50% inhibition) of the inflorescence and root oils were 2.21 and 1.86 lg/mL, respectively, compared to the standard BHT (IC_{50} = 0.015 mg/mL).[64]

The hypoglycemic effects of aqueous and methanolic extracts prepared from aerial parts of *Ageratina petiolaris* were assessed in streptozotocin-

nicotinamide (STZ-NA) induced diabetic rats. The test extracts water (40 and 160 mg/kg) and methanol (67 and 268 mg/kg) reduced the increase in glucose blood glucose level at three hours after administration. L-chiro-inositol and chlorogenic acid shown to exhibit significant hypoglycemic activity but the exact mechanism by which it proceed is yet to be determined.[65]

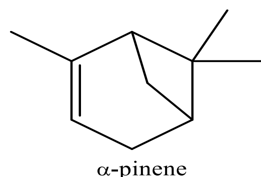
The hypoglycemic activity of the aqueous extract of *Ageratina petiolaris* aerial parts (160mg/kg, orally) was evaluated for the possible hepatic glucose output (HGO) inhibitory activity with a pyruvate tolerance test in 18-h fasted streptozotocin-nicotinamide (STZ-NA) Wistar rats after oral administration of the extracts. The plant extracts significantly reduced the hyperglycemic peak by approximately 20% at 30 min versus the hyperglycemic + pyruvate (HP) control group. This significant difference was maintained during the remainder of the test, showing an inhibitory effect on gluconeogenesis (decreased glucose output, preventing an increase in the blood glucose levels and sustaining this prevented increase after pyruvate administration).[66]

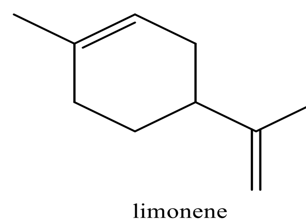
Thus far only few studies have been reported on the antidiabetic activities of *Eupatorium adenophorum*.

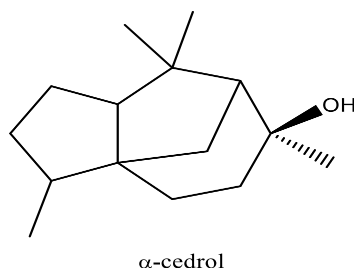
2.2 *Cupressus sempervirens*

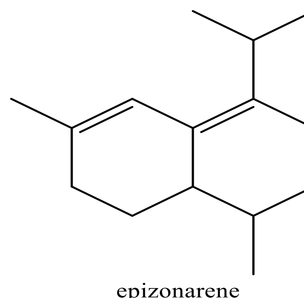
The preliminary phytochemical analysis showed that the plant contained alkaloids 0.7%, flavonoids 0.22%, tannin 0.31%, saponins 1.9% and phenols 0.067%.[67,68]

It appeared that the essential and volatile oils of the plant were differ according to the plant location and variety. Selim et al., isolated 20 compounds from the oil of Mediterranean cypress (*Cupressus sempervirens*), included: tricyclene, α-thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, δ-3- carene p-cymene, limonene, γ –terpinene, α-terpinolene, camphor, bronyl acetate, carvacrol, β-caryophyllene, α-humulene, germacrene-D, δ-cadinene and α-cedrol. However, the major components were included α-pinene which represented (48.6%), δ-3-carene (22.1%), limonene (4.6%) and α-terpinolene (4.5%).[69]









The essential oils isolated from Tunisian *Cupressus sempervirens* were ranged from 0.1 to 0.65% depending on the part of the plant analyzed. The greatest yields were in cones and leaves (0.65 and 0.43%, respectively) and the oil was lowest in the branches (0.1%). 52 compounds were identified accounting for 93.7, 94.82 and 95.8% of the total oil in leaves, cones and branches, respectively. The monoterpene fraction amounted 48.1 to 65.9%, sesquiterpenes accounted 27.3 to 45.01%, with low amount of diterpenes (less than 2.6%). In monoterpene fraction, hydrocarbon compounds were the major constituents, accounting 43.21 and 42.7% respectively in cones and leaves, and 60.4% in branches. The main monoterpene hydrocarbons were α-pinene 27.5% in leaves, 28.91% in cones and 35.8% in branches and δ-3-carene (5.8, 7.2 and 13.2%), respectively in cones, leaves and branches. In sesquiterpene fraction, sesquiterpene hydrocarbons were the major constituents 21.9% in leaves, 18.26% in cones and 14.9% in branches. The individual components in the leaves, cones and branches respectivily were: tricyclene, α-thujene; α-pinene; α-fenchene; sabinene; β-pinene; β-myrcene; α-phellandrene; δ-3-carene; 1.8.cineole; p-cymene; limonene; β-phellandrene; α-terpinolene; linalool; α-campholenal; camphre; borneol; δ-terpineol; myrtenal; terpen-4-ol; α-terpineol; iso-bornyl acetate; α-terpenyl acetate; longifolene; (Z)-caryophyllene; α-cedrene; α-humulene; ermacrene D; β-selinene; α-murrolene; epizonarene; β-bisabolene; cubebol; Cis-calmanene; δ-cadinene; α-copan-11-ol; α -calacorene; elemol; germacrene B; β-calacorene; caryophyllene oxide; α-cedrol; T-cadinol;

T-murrolol; manoyl oxide; abietatriene; abietadiene; nezukol; sempervirol; (Z)-tartarol.[67]

The essential oils obtained from fresh fruits and terminal branchlets with adherent leaves of *Cupressus sempervirens* L cv cereiformis growing in Iran, were analyzed by GC-MS. Thirteen components were identified in the essential oils. The main constituents of both fruits and leaves were α-pinene, Δ -3-Carene, α-terpenyl acetate and terpinolene. However, the volatile oil isolated from *Cupressus sempervirens* L cv cereiformis. fruits and leaves % respectively were: α-pinene 30.0 and 39.0, sabinene 2.0 and 3.0, β-pinene 2.6 and 2.2, myrcene 4.1 and 3.9, -3-carene 24.0 and 24.0, limonene 4.0 and 3.0, terpinolene 6.6 and 4.3, bronyl acetate trace and 1.7, α-terpenyl acetate 6.6 and 5.6, β-caryophyllene 1.2 and trace, α-humulene 1.3 and trace, germacrene D 4.0 and 1.7, while grouped compounds: (monoterpene hydrocarbons 73.3 79.4); (oxygencontaining monerpenes 6.6 and 7.3); (sesquiterpene hydrocarbons 10.5 and 1.7); (oxygen-containing sesquiterpenes 4.0 and trace).[70]

Glycosides from fresh cypres cones, *Cupressus sempervirens* were isolated by cold and hot ethyl acetate extraction. After enzymatic hydrolysis by means of β-glucosidase, 18 aglycones were released. The glycosidically bound volatile compounds amounted to 7-8 mg/kg. The main aglycones were 3-hydroxybenzoic acid methyl ester (15.5%) and thymoquinone (5-isopropyl-2-methyl-1,4-benzoquinone: 3.7-9.7%). Other important aglycones were perilla alcohol (3.6-8.2%), p-cymen-8-ol (5.3-6.4%), 2-phenylethanol (2.7-6.9%) and carvacrol (2.5-6.3%). There was no similarity between the glycosidically bound aglycones and the corresponding free compounds found in the essential oil.[71]

Diterpenes, 6-deoxytaxodione (11-hydroxy-7, 9(11), 13-abietatrien-12-one), taxodione, ferruginol, sugiol, trans-communic acid, 15-acetoxy imbricatolic acid and imbricatolic acid were isolated from *Cupressus sempervirens*.[72]

The total phenols content of *Cupressus sempervirens* fresh leaves was 4.35 (mg gallicacid/g extract) and the total flavonoids was 9.5 (mg quercetin/g extract).[73]

The fruits and seeds contained total free phenolic content of 1.96 mg/gGAE and 2.25 mg/gGAE, respectively. The saponin content determined with vanillin

reagent shows a good yield of 119.85 and 131.46 mg/gDE in ethyl acetate and butanolic extracts, respectively.[74]

The chloroform and methanol leaf extracts of *Cupressus sempervirens* were tested for antioxidant activity using the DPPH assay. Antiradical activity of the chloroform extract (50 µg/mL) was 6 %, while that of methanol extract (50 µg/mL) was 65 %.[75]

The antioxidant activities of *Cupressus sempervirens* fresh leaves by nitric oxide scavenging assay was 1.17 (mg quercetin /g extract), by reducing power assay was 2.85 (mg ascorbic acid/g extract), by metal chelating assay was 2.70 (mg EDTA /g extract) and by phosphomolbdenum antioxidant assay was 16.5 (mg Ascorbic acid/g extract).[73]

Antioxidant activity of the extracts of two varieties was determined using 2,2-diphenyl-1- picrylhydrazyl (DPPH) and N,N-dimethyl-p-phenylendiamine (DMPD) radical scavenging activity, metalchelation capacity along with ferric-(FRAP) and phosphor-molybdenum reducing antioxidant power (PRAP) tests. Antioxidant activity of the extracts was screened at 2000 µg/mL. In general, antioxidant activity of the extracts was observed to show a discrepancy according to the method used. For instance; the cone ethyl acetate extract of *Cupressus sempervirens var. horizantalis* displayed the highest DPPH radical scavenging activity (87.53±0.17%), while only six of the extracts had ability to scavenge DMPD radical varying from 6.06±0.23 to 30.34±0.69%. In the FRAP assay, the cone acetone extract of *Cupressus sempervirens var. horizantalis* exhibited the highest absorbance value, which was indicative of the highest antioxidant activity, although the extracts had generally low activity in the PRAP assay. The leaf methanol extract of *Cupressus sempervirens var. horizantalis* was the most active one. Concerning the results obtained from the metal-chelation assay, the cone and leaf methanol extracts of both varieties did not possess metal-chelation capacity. However, the leaf ethyl acetate extracts of *Cupressus sempervirens var. horizantalis* (75.86±0.33%) and *Cupressus sempervirens var. pyramidalis* (77.07±3.22%) showed the highest activity in this assay.[76]

The antioxidant activity of *Cupressus sempervirens* essential oil was evaluated by measuring the radicals-scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and by using the 2, 2'-azinobis(3- ethylbenzothiazoline-6-sulfonate) (ABTS) assay. Essential oil showed high antioxidant activity (7.7 µg/mL and 2.14 mM Trolox for DPPH and ABTS assays, respectively) when compared to BHT.[77]

The antioxidant activities of hydroethanolic extract of leaves of *Cupressus sempervirens* was studied *in-vitro* in comparison with ascorbic acid, and their correlation with *in-vivo* hepatoprotective activity in rat model of paracetamol-induced hepatotoxicity. *In-vitro* study revealed that the tested extracts contained abundant amount of phenolic and flavonoids compounds attributed to their effective antioxidant potential in different models. In *in-vivo* study, the pre-treatments with extract (250 mg/kg/day, po) or silymarin (100 mg/kg/day, po) for 4 weeks have good safety profile in normal rats and exhibited a marked hepatoprotection against single toxic dose of paracetamol (4 g/kg bw, po), significant preserving the normal liver function parameters, maintenance the hepatic redox status as evident from significant increase in antioxidant enzyme activities and reduced glutathione with inhibition of lipid peroxidation and protein oxidation, decreasing nitric oxide and tumor necrosis factor alpha, membrane stabilizing effects as confirmed from significant increase in the hepatic Na^+, K^+, ATPase activity and decrease in lysosomal enzyme activities which were changed in the untreated paracetamol-intoxicated rats.[78]

The ethyl acetate and butanolic extracts of *Cupressus sempervirens* (L) cones and seeds, collected from Laghouat (Algeria) was evaluated for the antidiabetic activity using α-amylase digestion enzyme. The results showed that phenolic and saponins extracts were found to inhibit enzymatic activity of α-amylase under *in-vitro* starch digestion bioassay and the values of the IC_{50} constants was determined for both seeds and cones extracts. The values ranged from 0.49 to 1.12 mg/mL. This paper is the first report on antidiabetic activity of saponins and phenolic extracts of cones and seeds from *Cupressus sempervirens (L)*.[74]

2.3 *Lantana camara*

Phytochemical analysis of the leaves of *Lantana camara* showed that the plant contained alkaloids, glycosides, steroids, saponins, flavonoids, coumarins, tannins, carbohydrates, hydroxy anthraquinones, anthraquinone glycosides , proteins, phytosteroids, fixed oils, fats, and triterpinoids.[79,80]

Quantitative phytochemical screening of *Lantana camara* showed that the leaves contain flavonoids (11.08±0.05 mg/g), tannins (9.0±0.03 mg/g), alkaloids (9.76±0.02 mg/g), saponin (6.07±0.06 mg/g), reducing sugar (4.86 ±0.05 mg/g) and carbohydrate (5.08± 0.03 mg/g). Micronutrients analysis showed vitamin A (0.50 mg/100g), vitamins C (6.5 mg/100g), vitamin E (1.6 mg/100g) and total phenolic compounds (2.36 Gallic Acid Equivalent [GAE]) [81]. Two new constituents, lantanoside and lantanone and the known compounds linaroside and camarinic acid were isolated from the aerial parts of *Lantana camara.*[82]

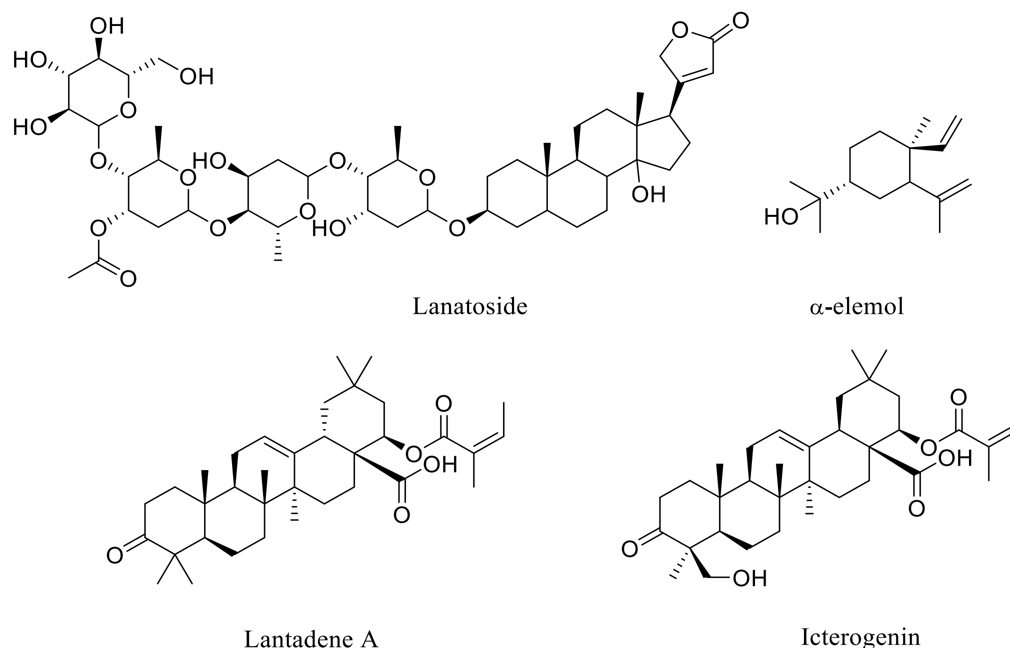
GC-MS profiling revealed the methanolic extract of *Lantana camara* contained 32 bioactive components. The identified major compounds included hexadecanoic acid (5.197%), phytol (4.528%), caryophyllene oxide (4.605%), and 9,12,15 octadecatrienoic acid, methyl ester, (Z,Z,Z)-(3.751%). Few other compounds identified in the extract included α-elemol, myristic acid, neophytadiene, furfuryl alcohol, propargyl alcohol, and acetic acid, fluoro-,ethylester.[83]

Pentacyclic triterpenoids (camaryolic acid, methylcamaralate, and camangeloyl acid), β-sitosterol 3-O-beta-D-glucopyranoside, octadecanoic acid, docosanoic acid, palmitic acid, oleanolic acid, lantanilic acid, camaric acid, lantadene A, lantadene B, icterogenin, and lantadene C were isolated from the aerial parts of *Lantana camara.*[84,85,86,87]

Triterpenoids (28-norolean-12,17-diene triterpene lantigdienone oxidized at C-11 and C-22 and camarinin and camangeloyl acid) were isolated from the aerial parts of *Lantana camara.*[88] Two new oleanane-type triterpenoids with an epoxy bridge between C-3 and C-25, named lantacamaric acids A and B, and seven known triterpenoids, two known iridoid glycosides, four known phenylethanoid

glycosides, one known flavonoid glycoside, and one known cyanogenic glycoside were isolated from the leaves and stems of *Lantana camara.*[89]

The polyphenol content of *Lantana camara* was 917.60 mg/100 g in the leaves and 328.56 mg/100 g in the stem, while flavonoids content was 3.29 mg/100 g in the leaves and 8.03 mg/100 g in the stem.[90]



In-vitro pharmacological activities revealed that phenolics, flavonoids, and different antioxidants effects of *Lantana camara* leaf extract varied with respect to different solvents extracts (ethyl acetate, methanol, acetone, and chloroform). The methanol solvent showed higher extractable compounds (14.4%) and contained the highest flavonoid (26.5 mgRE/g) and phenolic (92.8 mg GAE/g) content. DPPH radical scavenging assay showed IC_{50} value of 165, 200, 245, and 440 µg/mL for methanol, ethyl acetate, acetone, and chloroform extracts, respectively.[83]

Antioxidant activity of *Lantana camara* aerial parts methanolic extract, its fractions and three purified compounds (lantadene A, oleanolic acid, and lantanilic acid) were determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The methanolic extract showed 67% inhibition of DPPH free radical with EC_{50} value of 375 µg/mL, its three fractions were also active and exhibited 70%, 72%, and 65% inhibition respectively, with EC_{50} value of 375 µg/mL. Oleanolic acid showed 65% inhibition with EC_{50} 187µg/mL.[91]

The antioxidant activity, the total phenolic, and flavonoids contents of *Lantana camara* leaves were evaluated. The total phenolic content was (40.859±0.017) mg gallic acid/g in the leaves of *Lantana camara*, while the total flavonoids was (53.112±0.199) mg/g dry weight. Leaves extracts of the plant quenched DPPH in a dose dependent manner. Leaf extract of *Lantana camara* showed good radical scavenging activities (45–73%) at a concentration of 0.2–0.8 mg/mL in the reaction mixture. Leaves extracts exhibited a concentration-dependent reducing ability. It induced the maximum reducing power at 0.8 mg/mL.[92]

The methanolic extracts (2.5-80μg/mL) of the leaves of *Lantana camara* were investigated for their antioxidant activity, using DPPH radical scavenging model. The percentage inhibition of methanol extract of *Lantana camara* leaves extracts was concentration dependent with an effective concentration at fifty percent (EC_{50}) of 27.56±0.02 μg/mL compared to standard (ascorbic acid) with EC_{50} of 11.07±0.03μg/mL.[81]

The hypoglycemic activity of the methanolic extract of *Lantana camara linn* fruits (100 and 200 mg/kg BW, orally) was evaluated in normal and streptozotocin-induced diabetic rats. Methanolic extract of *Lantana camara* fruit 200 mg/kg produced significant reduction in fasting blood glucose levels in the normal and streptozotocin-induced diabetic rats. Methanol extract treated-diabetic rats showed improvement in parameter like body weight, HbA1c profile as well as histopathological studies showed regeneration of liver cells and so might be of value in diabetic treatment.[93]

The antihyperglycemic activity of the aqueous extract of the leaves of *Lantana camara* was evaluated, using both normoglycemic and alloxan induced hyperglycemic rats. The aqueous extract produced significant reduction of blood glucose concentration between 2-4 h of administration in alloxan-induced hyperglycemic rats at the tested doses (200 and 400 mg/kg). However, in normoglycemic rats, the extract at 400 mg/kg produced significant reduction of blood glucose between 2-4 h of administration.[94]

Oral administration of the methanol extract of the leaves of *Lantana camara* (200 and 400 mg/kg BW bodyweight) in alloxan-induced diabetic rats, showed significant ($p<0.01$) reduction in the blood glucose concentration in dose-

dependent manner. Treatment with 400 mg/kg of the methanol extract of *Lantana camara*, decreased blood glucose level to 121.94 mg/dL. Body weight significantly ($P<0.05$) increased to normal after treatment with extract. It was effective in oral glucose tolerance test as it decreased the elevated glucose after 1 h. The investigation of the biochemical parameters like triglycerides (TG), total cholesterol (TC), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density lipoprotein contend the methanol extract was effective against diabetes induced hyperlipidemia.[34]

CHAPTER - 3

MATERIALS & METHODS

3.1 Chemicals

All reagents and solvents used were of laboratory grade from E. Merck, T. Fisher Scientific and Qualigens Chemical Companies, India, purchased from the local vendors.

Isolation and separation of chemical constituents were carried out with the help of column chromatography using silica of 60-120 mesh. For thin layer chromatography "Silica gel-G" was used. The manufactures were Qualigen's Fine chemicals and Ranbaxy chemical respectively.

Pre-coated TLC aluminum plates with thickness of 0.2 mm were used to check the purity of the compounds. The plates were developed in different solvents and visualized under UV light, in iodine chamber and by charring with conc. H_2SO_4. Folin-ciocalteu reagent (FCR) for TPC was purchased from local vendor.

3.2 Equipments

Buchi RE111 Rotavapor was used for the evaporation of solvents. Absorbance for DPPH assay, TPC, TFC and antidiabetic activity were measured using 96 well plate reader. Grinder, Digital weighing machine (4 digit), Column chromatography (Fortuna W.G.C, *Optifit,* Germany), Micropipettes (Erba BIHOT), water bath (Physilab Scientific Industries, Ambalacantt, India) and others like filter paper (Whatmann), forceps, round bottom flask, conical flasks, funnels, glass rods, measuring cylinder, pipettes, test-tubes and glassware's were used during these work.

Other chemicals and reagents like DPPH, ascorbic acid, gallic acid, quercetin required for antioxidant, TPC and TFC were provided by Central Department of Chemistry and supervisor. Chemicals like aqueous Na_2CO_3, $NaNO_2$, NaOH, $AlCl_3$, methanol, etc. were available in laboratory.

3.3 Collection & Preparation of Plant Material

The plant material was collected in the month of April and June, 2019 from Kirtipur, during summer season. Then proper identification of plant was done from National Herbarium and Plant Laboratories (NHPL)/KATH, Godawari-5, Lalitpur, Nepal. At first the aerial parts of plant were thoroughly washed with tap water to remove dust, soil, bird's droppings, insect eggs etc. within them. The aerial parts of plant were dried under dry shade for at least two week. The dried parts were ground to coarse powder with the help of high capacity grinding machine. This process breaks the plant parts into smaller pieces thus exposing internal tissues and cells to solvents and facilitating their easy penetration into the cells to extract the constituents. Then the powdered sample was kept in clean closed glass containers till extraction. During grinding of sample, the grinder was thoroughly cleaned to avoid contamination with any remnant of previously ground material or other extraneous matters deposited on the grinder. The total weight of the dried powdered leaf was 250 gm which was measured using electronic balance.

3.4 Extraction of the Dried Powdered Sample

The fine powder of aerial parts of plants was dissolved in 250 mL methanol and it was thoroughly shaken to dissolve the powder into the solvent. Then it was kept in a closely covered glass jar for 36 hours with frequent agitation for more interaction between the powdered particles and the solvent. This process is termed as cold percolation. The cover of the jar was closed properly to resist the entrance of air in the jar.

3.5 Filtration of the Extract

After the extraction process the plant extracts was filtered with sterilized filter paper. The filtrate was collected in a beaker. The filtration process was repeated three times by using filter paper. Then the filtrate was taken into a volumetric flask and covered with aluminum foil paper for further use.

3.6 Solvent Evaporation

The filtrate was kept in rotary evaporator for evaporation of the solvent. The solution was also kept in the water bath at the temperature of $35^{O}C$ for solvent

evaporation. After running this procedure, a gummy concentrated extraction was obtained which was preserved in refrigerator at 4°C for further use.

The research/study design is given as follows:

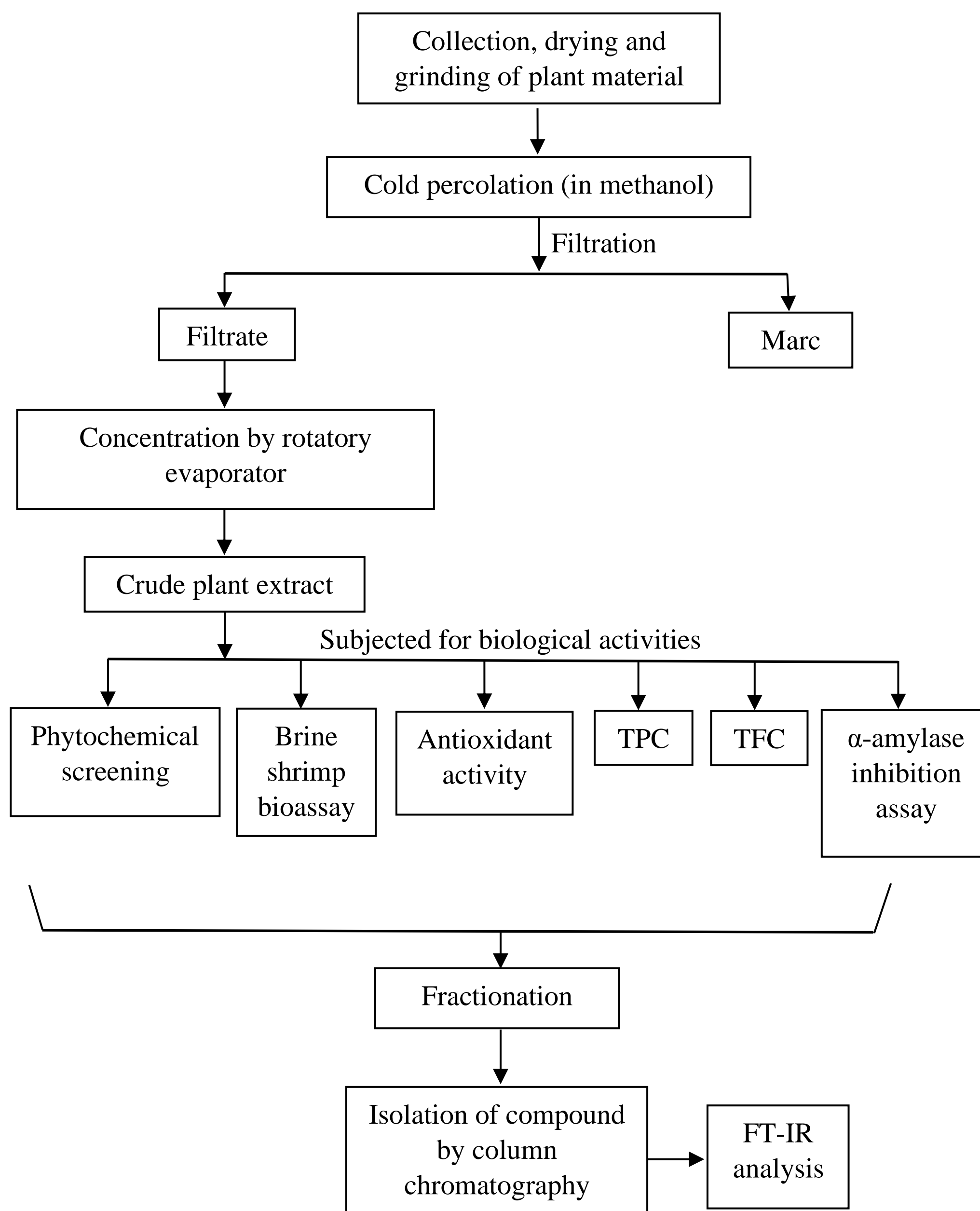


Figure 8: Schematic diagram of methodology

3.7 Qualitative Phytochemical analysis

Phytochemical analysis is the process of analyzing the presence of main groups of natural chemical constituents present in different plant extracts. The different phytochemical present in various extracts were identified by color reaction using different specific reagents. This result shows the presence of most of the phytochemicals in the polar extracts. The method employed for phytochemical analysis was based on standard protocol given by Ciulei I.[39] The procedure is given in detail in Appendix I and II.

3.8 Biological activities

The different chemical constituents present in the plants are responsible for its biological activity. Biological screening is a technique that involves the study of the potency of the crude plant extracts/fraction or isolated compounds at an arbitrarily fixed dose levels in a biological indicators using laboratory animals, isolated organ/tissues or using cell lines and prediction of its over the entire dosage range. Traditionally, people used natural products as medicine based on hit and trials method. Thus, biological screening is a way of measuring the potential of plant and plant products scientifically, thus allowing a decision to terminate or to proceed further for the medicinal use.

My present dissertation work involves the screening of methanolic extract of different selected plants for the brine shrimp toxicity activity.

3.8.1 Brine shrimp bioassay

The toxicity assay was performed by using brine shrimp assay following the standard protocol given by Meyer *et al.*[95]

3.8.1.1 Materials and equipments

1. Brine shrimp eggs (a few gram)
2. Beaker
3. Test tube (2x5 cm)
4. Artificial sea water
5. Disposable pipette
6. Magnifying glass
7. Test sample of plant extract

3.8.1.2 General Procedure

All the apparatus were sterilized before using for bioassay.

3.8.1.3 Preparation of artificial sea water

Freshly prepared artificial sea water needed for entire bioassay was prepared by dissolving the following chemical in distilled water.

Table 1: Composition of artificial sea water

S.N	Composition	Amount (g/L)
1.	NaCl	23.5
2.	Na_2SO_4	4
3.	KCl	0.68
4.	H_3BO_3	0.027
5.	$MgCl_2.2H_2O$	10.68
6.	$CaCl_2.2H_2O$	1.78
7.	$NaHCO_3$	0.197
8.	Na_2EDTA	0.0003

3.8.1.4 Culture and Harvesting of shrimps

Brine shrimps eggs were stored at -4°C before use. 10 mg of brine shrimps eggs were incubated on the beaker containing artificial sea water and illuminated with table lamp (80 watt) for 48 hours with the temperature adjusted at 23^{0}C. Nauplii show the body movement in response to light i.e. toward the source of light (positive phototaxis).

3.8.1.5 Preparation of test extracts

Stock solution was prepared by dissolving 20 mg of plant extract in 2 mL of methanol. Concentrations of 1000 µg/mL, 100 µg/mL, and 10 µg/mL were prepared by serial dilution from the stock solution. 2 mL of extracts solution from each concentrations (1000 µg/mL, 100 µg/mL, and 10 µg/mL) were transferred to nine different test tubes labelled as 1-9, three for each concentrations. Similarly, 2 mL of methanol were taken in three test tubes as a blank. After these, the test tubes were kept for 24 hours in order to evaporate

the whole solvent (methanol). After complete evaporation of the solvent, 5 mL of artificial sea water was added to each test tubes and the solution was gently shaken so that the dry compounds diffused adequately in the aqueous solution. Twenty matured shrimps were transferred to each test tubes. All the test tubes were maintained under illumination. The number of surviving nauplii were counted after 24 hours and the percentage of deaths at three dose levels and control were evaluated.

3.8.1.6 Data analysis

LC_{50} value is the lethal concentration required to kill 50% of the populations. It can be determined as follows:

If 'n' is the number of replicate (here three), 'x' is the log of constituents in mg/mL (log10, log100, and log1000 for three dose level respectively). 'y' is the prohibit for average survivor of all replicates.

We have,

$\alpha = \frac{1}{n}[\Sigma y - \beta \Sigma x]$……………………..(1)

$\beta = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$…………………………..….(2)

Where,

From probit regression,

$Y = \alpha + \beta X$…………………………....(3)

$X = \frac{(Y - \alpha)}{\beta}$ ………………………….....(4)

Where Y is constant having value 5 calculating LC_{50} values

Thus,

LC_{50} = antilog X…………………….(5)

Also,

Percentage mortality (% M) = $\frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}}$ x 100

In the present work, brine shrimp bioassay of different plants methanolic extracts were carried out and the lethal concentration value was calculated.

3.9 Quantitative phytochemical analysis

3.9.1 Antioxidant Activity (Scavenging activity (DPPH) assay)

Various methods are used to measure antioxidant activity of plants. The free radical scavenging activities of the extracts were determined in a rapid, simple and inexpensive method by using 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.[96] DPPH in oxidized form gives a deep violet color in methanol. An antioxidant compound donates the electron to DPPH thus causing its reduction and in reduced form its color changes from deep violet to yellow.

The percentage of the DPPH free radical scavenging activity was calculated by using the following equation:

$$\% \text{ Inhibition} = \left(\frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}}\right) \times 100$$

Where, A_{Control} = Absorbance of the DPPH

A_{Sample}= Absorbance of the DPPH + sample

The IC_{50} (50% inhibitory concentration) value is indicated as the effective concentration of the sample that is required to scavenge 50% of the DPPH free radicals. IC_{50} values were calculated using the inhibition curve by plotting extract concentration versus the corresponding scavenging effect.

3.9.1.1 Preparation of DPPH solution (0.1mM)

DPPH has the molecular weight of 394.32 g/mol. Thus, 3.9432 mg of the DPPH was carefully weighed and dissolved in methanol and the final volume was maintained to 100 mL and kept in dark place for further use.

3.9.1.2 Measurement of DPPH free radical scavenging activity

Firstly, 1 mg of the samples to be tested was dissolved in 1 mL 50% DMSO to get the stock solution of concentration of 1mg/mL (1000µg/mL). Different

concentrations of test samples of 0.01 mg/mL, 0.02 mg/mL, 0.04 mg/mL, 0.08 mg/mL, 0.16 mg/mL, 0.32 mg/mL were prepared from from stock solution. After that 100 µL of different plants extract at 500 µg/mL and 100 µL of DPPH reagent were loaded in 96 well plate in triplicate. Then incubated for 30 minutes in dark. After 30 minute, absorbance of all the samples was measured at 517 nm using micro-plate reader. Ascorbic acid of the concentration 0.01 mg/mL, 0.02 mg/mL, 0.04 mg/mL, 0.08 mg/mL, 0.16 mg/mL, 0.32 mg/mL and 0.5mg/mL was prepared as standard and its absorbance was also taken at 517 nm. For DPPH test, ascorbic acid of 20 µg/mL was used as positive control and 50% DMSO was used as negative control.

3.9.2 Total Phenolic Content (TPC)

The total phenolic content of all selected plant extracts were estimated using Folin-Ciocalteu reagent involving gallic acid standard. Folin-Ciocalteau (FC) is based on a chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The total phenolics content was determined according to Ainsworth *et al.,* & Lu *et al.,* with a slight modifications.[97,98]

3.9.2.1 Preparation of standard gallic acid stock solution

Gallic acid was prepared in ethanol since in methanol white precipitate were formed (can cause interference during measurement) while in ethanol no precipitate were observed. Gallic acid stock solution was prepared by dissolving 1 mg of gallic acid in 1 mL of ethanol. Various concentrations of gallic acid such as 10, 20, 30, 40, 50, 60, 70, 80 µg/mL were prepared from the stock solutions.[99]

3.9.2.2 Evaluation of Total Phenolic Content

The total assay mixture containing 20 µL of 10-80 µg/mL standard gallic acid, 100 µL Folin-Ciocalteu reagent followed by 80 µL 1 M Na_2CO_3 separately were incubated in dark for 15 min in dark and absorbance was measured at 765 nm. Similarly, the same procedure was performed for plant extracts. The results was

expressed as milligrams of gallic acid equivalent per gram of dry weight (mgGAE/g) of the extract using gallic acid standard curve.

3.9.2.3 Calculation of total phenolic content

TPC content compound concentration in extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of the extract which was calculated in all the extracts separately using the following formula:

$C = \frac{cV}{m}$…………(1)

Where,

C = Total phenolic content in milligrams per gram (mg/g) of dry plant material, in gallic acid equivalent (GAE)

c = Concentration of gallic acid established from the calibration curve in milligrams per milliliter (mg/mL)

V = Volume of the extract solution in milliliters (mL)

m = Weight of the extract in grams (g).

3.9.2.4 Statistical analysis

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

Y = mx + c……………….(2)

Where,

Y = Absorbance of extract

m = Slope from the calibration curve

x = Concentration of extract

c = Intercept

Using this regression equation concentration of extracts was calculated. Thus, with the calculated value of concentration of each extracts, the total polyphenolic content was calculated by the equation (2).

3.9.3 Total Flavonoid Content

Aluminium chloride complex forming assay was used to determine the total flavonoid content of the extracts. Quercetin was used as standard and flavonoid content was determined as quercetin equivalent.[100]

3.9.3.1 Preparation of standard quercetin solution

Stock solution of 1 mg/mL was prepared by dissolving 1 mg of quercetin in 10 mL of methanol or ethanol**.** Various concentrations of quercetin such as 10, 20, 40, 80, 160, 320 & 500 µg/mL were prepared from the stock solutions.

3.9.3.2 Evaluation of Total Flavonoid Content

The whole assay mixture containing 130 µL of 10-500 µg/mL standard quercetin, 60 µL ethanol, 5 µL of 10% $AlCl_3$ and 5 µL of 1M potassium acetate separately were incubated in dark for 30 min and absorbance was measured at 415 nm. Similarly, the same procedure was performed for plant extracts. The results was expressed as milligrams of quercetin equivalent per gram of dry weight (mgQE/g) of the extract using quercetin standard curve.

3.9.3.3 Calculation of total flavonoid content

The total flavonoid content in the extract was expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g) of the extract which was calculated in all the extracts separately using the following formula:

$$C = \frac{cV}{m} \text{............(1)}$$

Where,

C = Total flavonoid content in milligrams per gram (mg/g) of dry plant material, in quercetin equivalent (QE)

c = Concentration of quercetin established from the calibration curve in milligrams per milliliter (mg/mL)

V = Volume of the extract solution in milliliters (mL)

m = Weight of the extract in grams (g).

3.9.3.4 Statistical analysis

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

$Y = mx + c$……………….(2)

Where,

Y = Absorbance of extract

m = Slope from the calibration curve

x = Concentration of extract

c = Intercept

Using this regression equation concentration of extracts was calculated. Thus, with the calculated value of concentration of each extracts, the total flavonoid content was calculated by the equation (2).

3.9.4 Antidiabetic activity

Screening of plant material for α-amylase inhibitors was carried out in a 96 well microtitre plate according to Xiao *et al.,* based on the starch-iodine test with a slight modification.[101]

3.9.4.1 Preparation of working solutions

0.312 g of sodium dihydrogen phosphate (NaH_2PO_4) crystals were weighed out and dissolved in distilled water to make a final volume. Similarly, 0.356 g of disodium hydrogen phosphate (Na_2HPO_4) crystals were weighed, poured in 100 mL volumetric flask, dissolved and volume was made up to the mark by distilled water to make the final volume.

Then, 15 mL of 0.02 M NaH_2PO_4 solution and 15 mL of 0.02 M Na_2HPO_4 solution were mixed and 0.0105 g of NaCl was added. Finally, pH of the phosphate buffer solution was maintained at 6.9 by adding dilute HCl and dilute NaOH.

The concentration of HCl available in the laboratory was 11.14M. 4.48 mL of conc. HCl was pipetted out and 40 mL solution was prepared by adding distilled water in the volumetric flask.

0.1 g of soluble starch was weighed and taken in a beaker. About 3 mL of phosphate buffer was added and the whole content was warmed with constant stirring to dissolve the starch completely. Finally the volume of 10 mL was maintained by adding phosphate buffer.

The stock solution of all plant extracts was prepared by dissolving 500 mg in 1 mL of dimethylsulphoxide (DMSO). Different concentrations of test samples of 1.5625 mg/mL, 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL were prepared from stock solutions.

The enzyme solution was prepared by dissolving 0.2 mL of 9U α-amylase in 9 mL phosphate buffer of pH 6.9.

3.9.4.2 Pancreatic α -amylase inhibition assays (Starch-Iodine colour assay)

α-amylase activity can be evaluated *in-vitro* by hydrolysis of starch in presence of α-amylase enzyme. This process was enumerated by using iodine, which gives blue colour solution with starch. The reduced intensity of blue colour indicates the enzyme-induced hydrolysis of starch into the monosaccharides. If the substance/extract possesses α-amylase inhibitory activity, the intensity of blue colour will increase. In other words, the intensity of blue colour in test sample is directly proportional to α-amylase inhibitory activity. Thus, inhibition of α-amylase can lead to reduction in post prandial hyperglycemia in diabetic condition.

The total assay mixture composed of 20 µL 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride), 20 µL 4 units of (Porcine pancreatic α -amylase) PPA solution and 20 µL plant extracts at concentration from 1.5625-25 mg/mL (w/v) were incubated at 37°C for 10 min. Then 20 µL soluble starch (1%, w/v) was added to each reaction well and incubated at 37°C for 15 min. 1 M HCl (20 µL) was added to terminate the enzymatic reaction, followed by the addition of 100 µL of 5 mM iodine reagent. The colour change was noted and the absorbance was read at 620 nm on a 96 well microplate reader. The

control reaction representing 100% enzyme activity did not contain any plant extract. A dark-blue colour indicates the presence of starch; a yellow colour indicates the absence of starch while a brownish colour indicates partially degraded starch in the reaction mixture. The known PPA inhibitor, acarbose, was used a positive control at a concentration range of 1.5625-25 mg/mL. In the presence of inhibitors from the extracts the starch added to the enzyme assay mixture is not degraded and gives a dark blue colour complex whereas no colour complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase.

The IC_{50} values were determined from plots of percent inhibition versus log inhibitor concentration and calculated by logarithmic regression analysis from the mean inhibitory values. The IC_{50} values were defined as the concentration of the extract, containing the α-amylase inhibitor that inhibited 50% of the PPA activity. The other quantifiers were calculated as follows:

$$\text{\% Relative enzyme activity} = \left(\frac{\text{Enzyme activity of test}}{\text{Enzyme activity of control}}\right) \times 100$$

% Inhibition in the α-amylase activity = (100 - % Relative enzyme activity)

Where, Enzyme activity of test = Total starch – Remaining Starch

Enzyme activity of control = Starch only – (Starch + Enzyme)

3.9.5 Isolation of compounds

On the basis of antioxidant and antidiabetic activity, the aerial part of *Lantana camara* was selected as the potent extract for isolation of active phytoconstituents.

3.9.5.1 Extraction

For the isolation of the phytoconstituents, powdered aerial parts of *Lantana camara* (100 g) was extracted exhaustively with methanol by cold percolation method. The yield of the extract obtained was approximately 11.85 g. Then the methanol extract was fractionated by the solvent of increasing polarity successively. The methanol extract was treated with hexene, DCM and

ethylacetate in a separating funnel to obtain the respective fraction. The schematic diagram for fractionation is shown in figure.

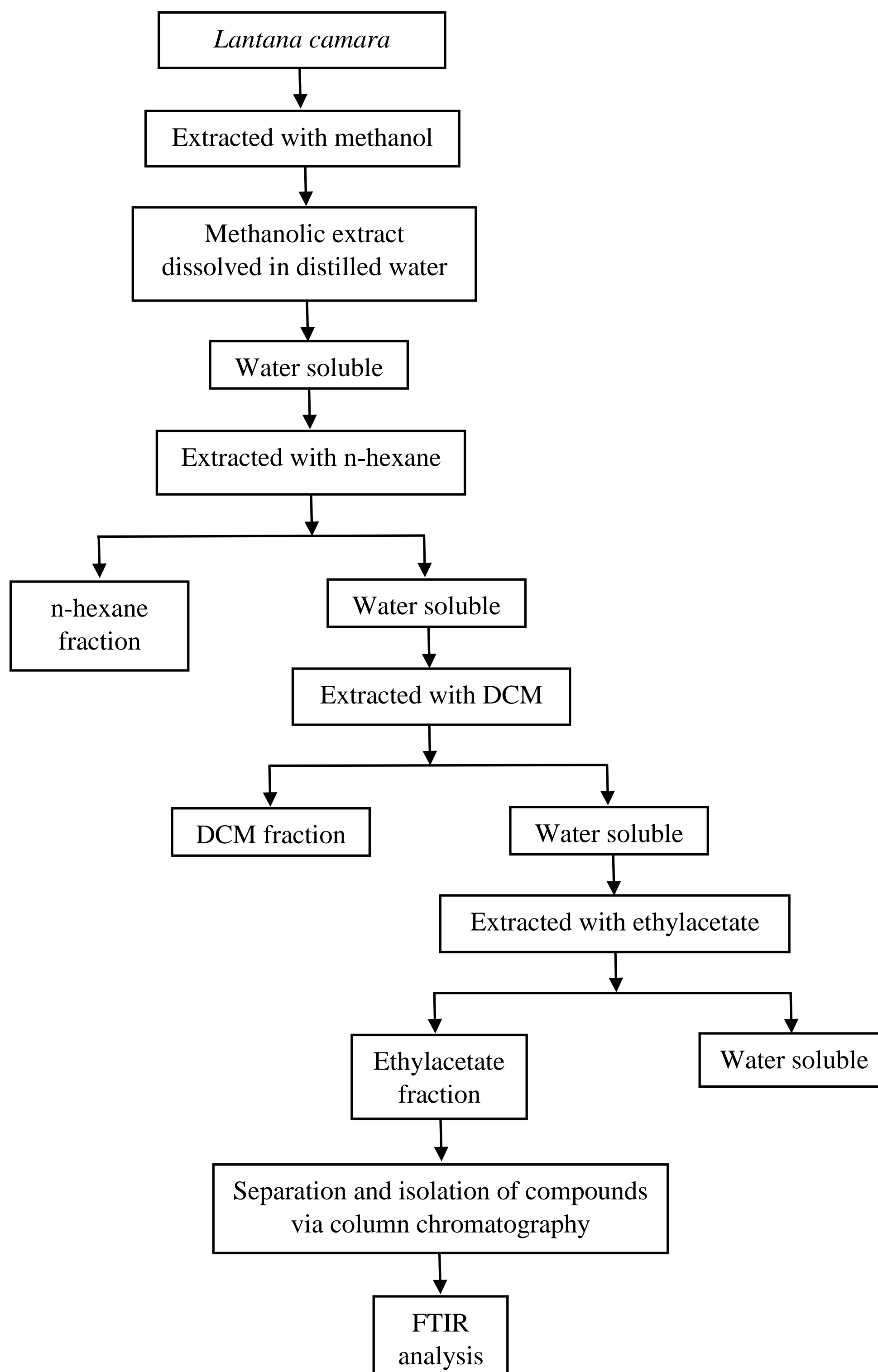


Figure 9: The flow chart of fractionation and isolation of compounds

3.9.5.2 Isolation of compounds from hexane fraction of aerial parts of *Lantana camara* by column chromatography

Hexane soluble fraction was subjected for column chromatography in order to isolate the pure chemical compounds. It was selected based on TLC report since good separation and large number of spots were observed in TLC. The hexane fraction (5 g) of methanol extract was first adsorbed on double (1:2) amount of silica gel and loaded on to a silica gel (100 g, E-Merck, 60-120 mesh) packed in a column having internal diameter 3 cm with the adsorbed height of 30 cm. The column was initially eluted with hexane and then with gradients of hexane-ethylacetate of increasing polarity and finally up to 60% ethylacetate in hexane. The fractionates were subjected for TLC. The number of spots in TLC was visualized under UV-visible light and iodine chamber.

3.9.5.3 Analytical conditions for FTIR

The FTIR instruments sends infrared radiation of about 400 to 4000 cm^{-1} through a sample, with some radiation absorbed and passed through. The absorbed radiation is then converted into rotational and/or vibrational energy by the sample molecules. The resulting signal at the detector presents as a spectrum, typically from 4000 cm^{-1} to 400 cm^{-1}, representing a molecular fingerprint of the sample. Each molecule or chemical structure will produce a unique spectral fingerprint, making FTIR analysis a great tool for chemical identification.

A crystalline solid that give a single spot on TLC analysis were subjected for FTIR. Sampling area of IR spectrometer accommodate a film of 0.01 mm or less in thickness. About 0.1 mg of sample were applied on the potassium bromide (KBr, spectroscopic grade) plate at 30 ^{0}C and were recorded with IR spectrometer. The potassium bromide plate was cleaned with ultra-pure organic solvents (Sigma-Aldrich,Germany). The instrument was continuously purged with argon for 40 min before and during measurements. Absorption spectra at a resolution of one data point per 1 cm^{-1} were obtained in the region between 4000 and 400 cm^{-1}. Scans were Fourier-transformed and averaged with the software.

3.9.6 Statistical Analysis

All the data measured in each experiment included 3 replications (n=3) and the results were represented as mean ± SD. The IC_{50} values were calculated using GraphPad Prism version 8.0.2 (263). The structure of the compound were drawn by using ChemDraw Professional version 16.0.1.1.

CHAPTER - 4

RESULTS AND DISCUSSION

4.1 Phytochemical screening

Phytochemical screening of the selected plants extracts was performed on the basis of the procedure put forwarded by Prof. I. Cuilei.[39] The result obtained from phytochemical screening for each methanolic plant extract is tabulated below.

Table 2: Qualitative screening of phytochemicals present in different methanolic extracts of all plants

S.N.	Group of compound	J_1	J_2	J_3
1.	Alkaloids	+	+	+
2.	Flavonoids	+	+	+
3.	Carotinoids	-	+	+
4.	Coumarins	+	+	+
5.	Glycosides	+	+	+
6.	Polyphenol	+	+	+
7.	Carbohydrate	-	+	+
8.	Saponin	-	-	+
9.	Tannins	-	+	-
10.	Terpenes/Sterols	+	+	+
11.	Quinones	+	+	-
12.	Volatile oil & fats	+	+	+

Where,

'+' represents presence and '-' represents absence

J_1 = *Ageratina adenophora* (Spreng.) R. King & H. Robinson

J_2 = *Cupressus sempervirens* L.

J_3 = *Lantana camara*

Results shows that glycosides and terpenoids were present in all the extract whereas few number of extract showed the presence of carbohydrate and saponins. Similarly, alkaloids and flavonoids were also present in all extracts. The absence of chemical compounds in some of the plant extract may be due to

the decomposition of compounds because of the heat during concentrating the extract by rota-evaporator.

The results presented in the table above are slightly different than the data present in the literature of some plants. The results of phytochemical screening for same samples may vary from the screening of same phytochemical constituent due to variation in altitude of plants, different environmental conditions, method and time of sample collection, extraction procedure and also due to lab setup and chemical grades.

4.2 Biological Screening

4.2.1 Brine shrimp toxicity assay

The biological activities of the different plant extracts were evaluated for their toxicity towards newly hatched brine shrimp larvae (*A. salina* Leach). In this study, the lethal concentration that kills 50% of exposed population of *A. salina* (LC_{50}) values of µg/mL obtained with the brine shrimp lethality assay for different concentration of plant extracts was determined and results obtained during these studies are shown in table no. 3.

Table 3: Calculation of LC_{50} value of different plant extract

Plant extract	*Ageratina adenophora*			*Cupressus sempervirens*			*Lantana camara*		
Z	10	100	1000	10	100	1000	10	100	1000
x	1	2	3	1	2	3	1	2	3
y	0	4.33	4.48	0	3.36	3.96	0	3.36	3.72
xy	0	8.66	13.44	0	6.72	11.88	0	6.72	11.16
x^2	1	4	9	1	4	9	1	4	9
$\sum x$	6			6			6		
$\sum y$	8.81			7.32			7.08		
$\sum xy$	22.1			18.6			17.88		
$\sum x^2$	14			14			14		
β	2.24			1.98			1.86		
α	-1.543			-1.52			-1.36		
X	2.921			3.293			3.419		
LC_{50}	833.68			1963.36			2624.22		

The degree of lethality was found to be proportional to the concentration of the extracts that is maximum mortalities of the brine shrimp larvae took place at the concentration of 1000 µg/mL and least mortalities at the concentration of 10 µg/mL. Those having LC_{50} values less than 1000 µg/mL are supposed to be pharmacologically active. The extract of *Ageratina adenophora* was found to be cytotoxic against brine shrimps as shown by its LC_{50} value of 833.68 µg/mL as its LC_{50} value was found to be lower than 1000 µg/mL. In addition, the extract of *Cupressus sempervirens* and *Lantana camara* showed positive results, indicating that the samples are biologically active and do not possess cytotoxicity. This variation in the results may be due to the altitude variations of collected plant or may be due to laboratory conditions.

For the bioactive compound of either natural or synthetic origin, this is a rapid and comprehensive test which utilizes a large number of organisms for statistical validation and requires no special equipment and relatively small amount of sample (2-20 mg or less) is necessary.[102] Although this method does not provide any adequate information regarding the mechanism of toxic action, it is a very useful method for the assessment of the toxic potential of various plant extracts. This method provides preliminary screening data that can be backed up by more specific bioassays.

4.3 Antioxidant activity (DPPH free radical scavenging activity)

In this study, the hydrogen atom or electron donation ability of each plant extract against DPPH free radical was measured from the bleaching of violet coloured DPPH solution at 517 nm. DPPH is scavenged by antioxidants through the donation of proton forming the reduced DPPH and the colour changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The degree of decolourization indicates the free radical scavenging potentials i.e. antioxidant potentials of the sample.

The DPPH radical assay was performed for each plant extracts by using ascorbic acid as standard according to the standard procedure. In this assay, the mixture of DPPH with different concentrations of extract solution and ascorbic acid were separately incubated at room temperature and absorbance was recorded at 517 nm by using micro-plate reader. The absorbance value decrease with the

increase in the concentration of antioxidant and antioxidant activity of the compound itself.

Table 4: Percentage of radical scavenging with different concentration

Plant extracts	% of scavenging	Concentration (µg/mL)						
		10	20	40	80	160	320	500
Ascorbic acid	% Sca. 1	61.9	69.35	74.71	80.46	88.51	92.72	95.78
	% Sca. 2	63.53	69.80	74.51	81.96	85.49	94.51	96.47
	% Sca. 3	57.53	66.41	76.06	81.08	86.49	93.44	95.37
Ageratina adenophora	% Sca. 1	1.92	10.73	18.77	22.99	27.59	44.83	52.87
	% Sca. 2	1.57	9.8	18.04	19.61	27.84	46.67	54.12
	% Sca. 3	2.32	8.11	16.99	21.24	27.24	48.65	53.67
Cupressus sempervirens	% Sca. 1	8.81	13.8	18	21.07	38.31	62.45	67.81
	% Sca. 2	10.2	12.89	14.12	21.57	36.89	64.71	67.84
	% Sca. 3	8.11	14.28	16.99	21.63	39	63.71	69.5
Lantana camara	% Sca. 1	8.43	22.22	27.97	39.85	61.3	65.9	74.33
	% Sca. 2	13.33	23.14	31.37	41.96	60.78	64.71	76.47
	% Sca. 3	13.13	23.94	28.19	36.29	59.46	66.41	75.67

In the present study, the % of scavenging effect on the DPPH radical was concomitantly increased with the increase in concentration of the methanolic extract from 0.01-0.5 mg/mL ie 10-500 µg/mL. The percentage inhibition of DPPH free radical of methanolic extract of *Lantana camara* were lower than *Cupressus sempervirens* and *Ageratina adenophora* and close to standard ascorbic acid.

The graphical representation of DPPH assay is given below.

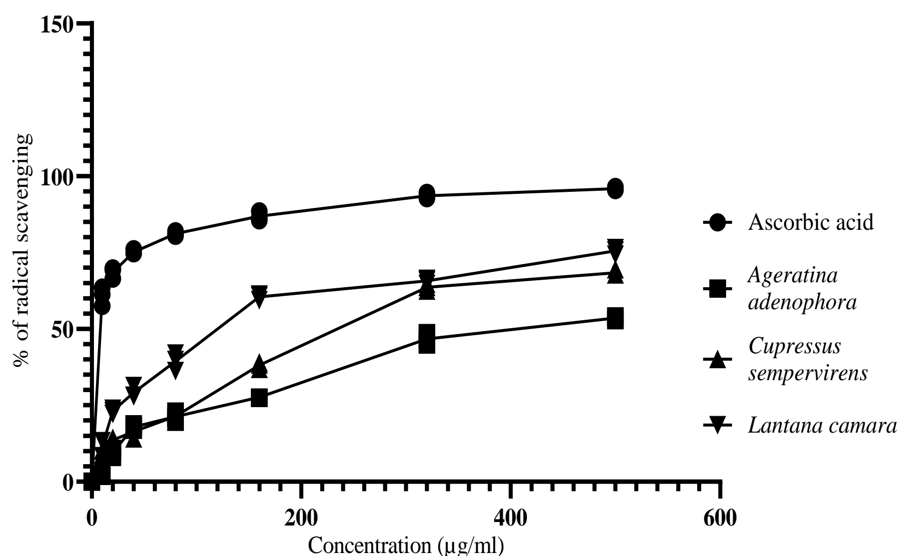


Figure 10: A plot of percentage radical scavenging activity with concentration of different plant extracts and ascorbic acid.

The linear regression of the percentage of radical scavenging versus concentration was used for the calculation of concentration of each plant extract required for 50 % inhibition of DPPH activity (IC_{50}). The antioxidant potential is in an inverse relation with IC_{50} value, lower value of IC_{50} indicates high antioxidant potential. The IC_{50} value of the plant extract along with the standard ascorbic acid is tabulated below.

Table 5: Comparison of IC_{50} values of different plants extracts with standard ascorbic acid

S.N.	Sample	IC_{50} (µg/mL)
1.	Standard ascorbic acid	17.456 ± 0.822
2.	*Ageratina adenophora*	422.200 ± 18.580
3.	*Cupressus sempervirens*	218.068 ± 4.405
4.	*Lantana camara*	106.179 ± 11.390

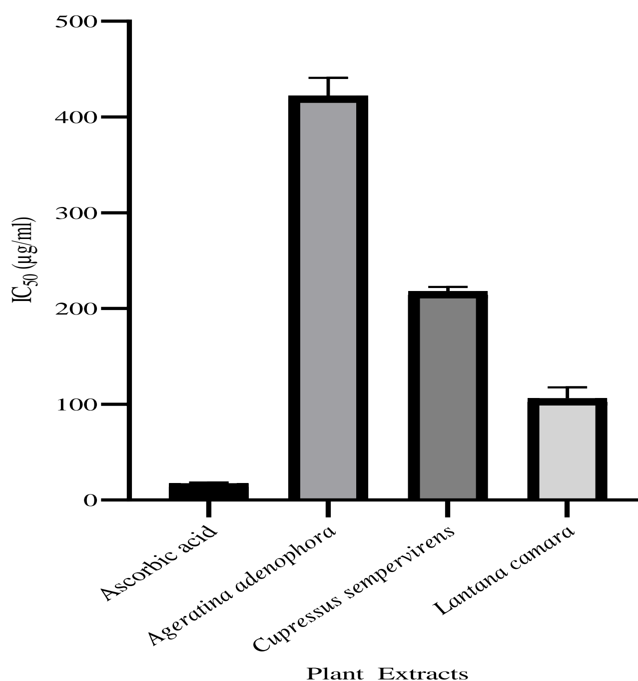


Figure 11: Free radical scavenging activity (IC_{50}) in different concentration of methanolic plant extracts

The table and the bar graph above showed that the plant extracts were potential antioxidants as their IC_{50} values were found to be close to the standard ascorbic acid taken. Among these, methanolic extracts of *Lantana camara* (106.179 ± 11.390 µg/mL) showed the strongest DPPH radical scavenging activity as their IC_{50} values were close to standard ascorbic acid (17.456 ± 0.822 µg/mL). Similarly, the IC_{50} values of methanolic extract of *Ageratina adenophora* and *Cupressus sempervirens* were found to be 422.200 ± 18.580 µg/mL and 218.068 ± 4.405 µg/mL.

The IC_{50} values of methanolic extract of *Lantana camara* (106.179 ± 11.390 µg/mL) was much lesser than the quantity (165 µg/mL) as reported by Swamy *et al.,* from the methanolic extract of *Lantana camara* collected from the tropical region of Malaysia.[83] This difference can be attributed to the influence of environmental factors and geographical location.

The formation of free radicals in the body may cause cell damage and induces various disorders. However, antioxidants from the natural source like plant can repair and neutralize these free radicals and thereby, these antioxidants can be very useful in preventing various disorders. The results reflect that the plant

studied above can act as a very good option in the field of medicine based on antioxidant property of natural products chemistry.

4.4 Determination of Total Phenolic Content (TPC)

The quantitative determination of total phenol was carried out using Folin-Ciocalteu reagent in terms of gallic acid equivalent. It involves the oxidation of phenols in alkaline solution by the yellow molybdotungstophosphoricheteropolyanion reagent and colorimetric measurements of the resultant molybdotungstophosphate blue. Polyphenols in the plant extracts react with specific redox reagent (FCR) to form a blue complex exhibits a broad light absorption depending on the qualitative and quantitative composition of phenol mixture, beside the pH of the solution that can be quantified by the UV-Visible spectrometry at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. The observation for absorbance for different representation, X-axis being plotted for concentration and Y-axis for absorbance. The numerical data is shown in Appendix 3.3.

The absorbance curve for standard gallic acid is shown in figure below:

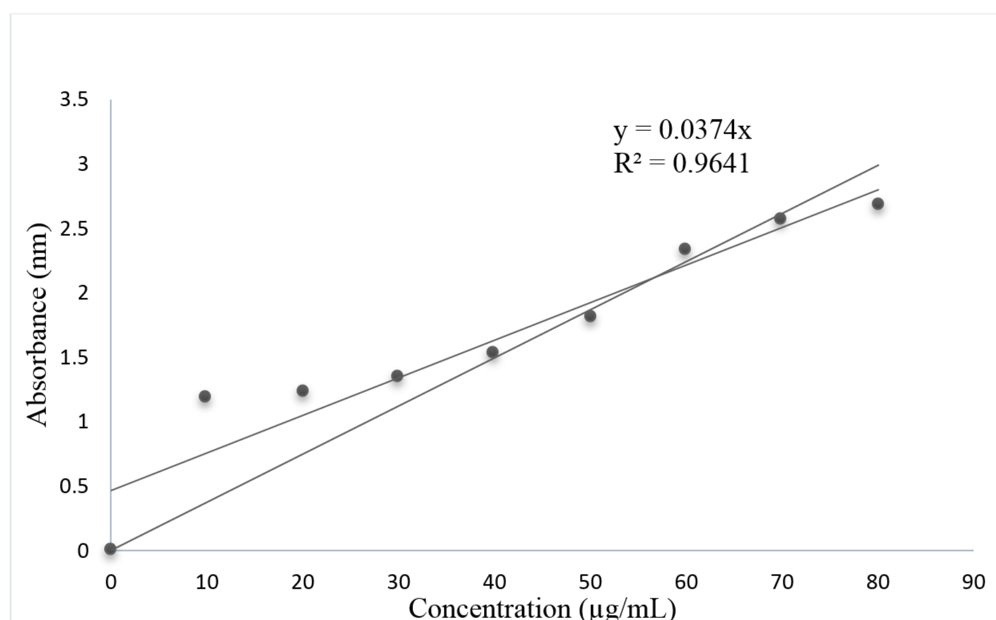


Figure 12: Variation of absorbance (nm) with concentration (µg/mL) for standard gallic acid

4.4.1 Calculation of Total Phenolic Content (TFC) in different plant extracts

The absorbance values of each extracts at different concentrations (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL, 80 µg/mL) were recorded at 765 nm by using micro-plate reader. The TPC in the plant extracts taken under study was calculated by using the regression equation $y = 0.0374x$, $R^2 = 0.9641$ of the curve obtained from the above graph followed by the formula cV/m and expressed as mg GAE per g of extract in dry weight. The TPC of different plant extracts (mg gallic acid equivalent per g dry extract) is tabulated below:

Table 6: Total Phenolic Content (TPC) of different plant extracts

S.N.	Name of plants	Absorbance			TPC (mg GAE/g)			Mean ± SD
		A1	A2	A3	C1	C2	C2	
1.	*Ageratina adenophora*	0.446	0.436	0.437	4.77	4.663	4.674	4.702± 0.059
2.	*Cupressus sempervirens*	0.388	0.406	0.415	4.15	4.342	4.438	4.31 ± 0.147
3.	*Lantana camara*	0.922	0.986	0.953	9.861	10.546	10.192	10.2 ± 0.343

The total phenolic content of all selected medicinal plant extracts showed varied data ranging from 4.31 ± 0.147 to 10.2 ± 0.343 mg GAE/g in *Cupressus sempervirens and Lantana camara* respectively. The total phenol content of rest of plant extract lied between these two extremes. However, extracts of *Cupressus sempervirens* exhibited little TPC. Swamy *et al.,* reported the highest phenolic content (92.8 ± 1.7 mg GAE/g) in the methanol leaf extract of *Lantana camara*.[83] Similarly, Ali *et al.,* reported the polyphenol content of *Lantana camara* 917.60 mg/100 g in the leaves and 328.56 mg/100 g in the stem.[90]

More conveniently total phenolic content in different plant extracts are represented in bar diagram.

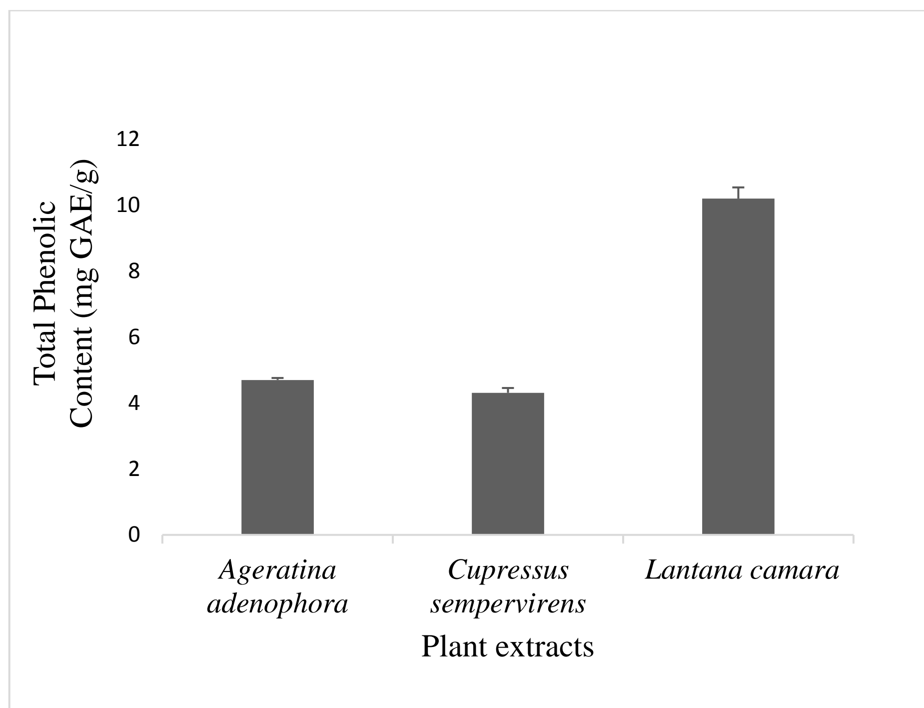


Figure 13: Total phenolic content of different methanolic plant extracts

Phenolic compounds have been known to possess antioxidant properties due to their free radical scavenging properties and act as a free radical terminators. It has been reported that extract containing large amount of polyphenol content possesses a greater antioxidant activity. These H-donating antioxidant react with ROS and RNS breaks the cycle of free radical generation in a termination reaction.[26]

4.5 Determination of Total Flavonoid Content (TFC)

The aluminium chloride colorimetric assay was used for the estimation of total flavonoid present in the methanolic extract of different plants according to the standard procedure given involving quercetin as standard. The flavonoids of the plant extracts in the presence of aluminium chloride forms an acid liable complexes, has an intense yellow fluorescence which can be observed under UV spectrophotometer at 415 nm.

The intensity of light absorption at that wavelength is proportional to the concentration of flavonoids. The numerical data of absorbance of different concentration of standard quercetin is shown in Appendix 3.4.

The absorbance curve for standard quercetin is shown in figure below:

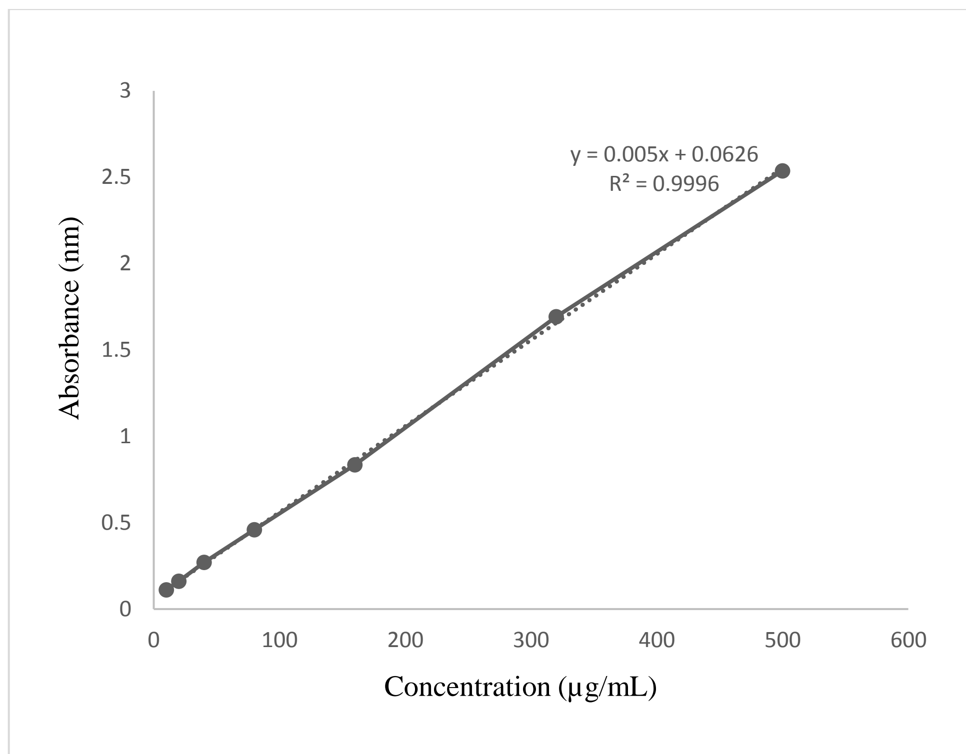


Figure 14: Variation of absorbance (nm) with concentration (µg/mL) for standard quercetin

4.5.1 Calculation of Total Phenolic Content (TFC) in different plant extracts

The absorbance values of each extracts at different concentrations (0.01 mg/mL, 0.02 mg/mL, 0.04 mg/mL, 0.08 mg/mL, 0.16 mg/mL, 0.32 mg/mL, 0.5 mg/mL) were recorded at 415 nm by using micro-plate reader. The TFC in the plants extracts taken under study was calculated by using regression equation y = 0.005x + 0.0626, R^2 = 0.9996 of the curve obtained from above graph followed by the formula cV/m and expressed as mg QE/g of extract in dry weight.

Those plant extracts which showed better antioxidant activities in this study also demonstrated higher total flavonoid content. Although quantitative determination of flavonoid compounds in plant extracts are hampered by their structural complexity, diversity, nature of analytical assay method, selection of standard and presence of interfering substance.

The TFC of different plant extracts (mg quercetin equivalent per g dry extract) are tabulated below:

Table 7: Total Flavonoid Content (TFC) of different plant extracts

S.N.	Name of plants	Absorbance			TFC (mg QE/g)			Mean ± SD
		A1	A2	A3	C1	C2	C2	
1.	*Ageratina adenophora*	0.069	0.072	0.071	0.512	0.752	0.672	0.645 ± 0.122
2.	*Cupressus sempervirens*	0.068	0.067	0.067	0.432	0.352	0.352	0.379 ± 0.046
3.	*Lantana camara*	0.084	0.088	0.086	1.712	2.032	1.872	1.872 ± 0.16

The total flavonoid content of different selected medicinal plants was found and the result revealed that the TFC varied from 0.379 ± 0.046 mg QE/g in *Cupressus sempervirens* to 1.872 ± 0.16 mg QE/g in *Lantana camara*. The total flavonoid content was lesser than (26.5 ± 0.5mgRE/g) were observed in the methanol leaf extract of *L. camara*.[83] The flavonoids content was 3.29 mg/100 g in the leaves and 8.03 mg/100 g in the stem as reported by Ali *et al.*[26] The total flavonoid content of different plant extracts is shown in following bar diagram.

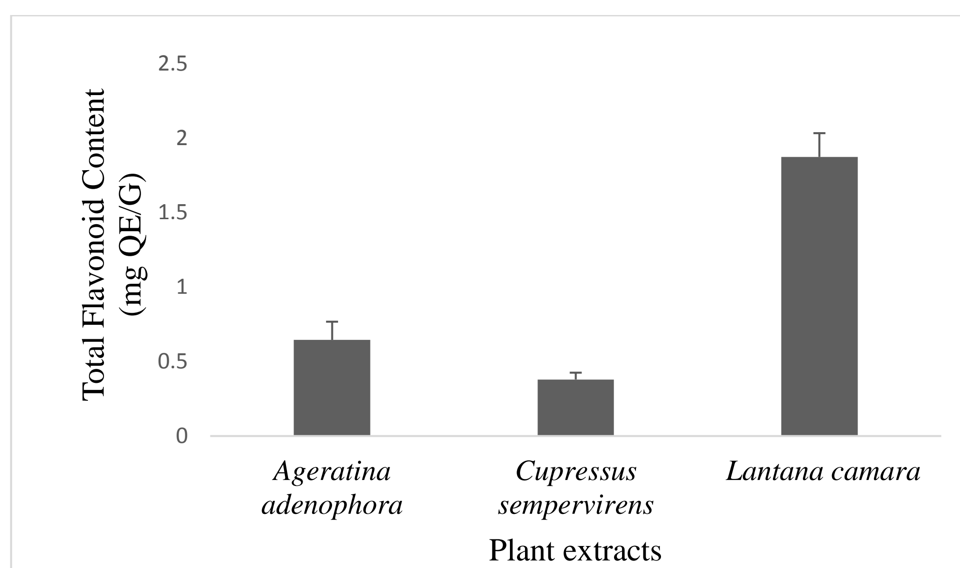


Figure 15: Total flavonoid content of different methanolic plant extracts

4.6 Antidiabetic activity

α – amylase inhibitory activity of plant extract was determined using quantitative starch-iodine method. The percentage of α – amylase inhibition activity is tabulated in table no. 8.

Table 8: Percentage of α – amylase inhibition activity with different concentration

Plant extracts	% of inhibition	Concentration (mg/mL)				
		1.5625	3.125	6.25	12.5	25
Acarbose	% Inh 1	83.974	86.874	89.926	90.511	94.912
	% Inh 2	84.955	87.24	89.936	91.066	96.945
	% Inh 3	84.692	86.73	88.997	90.296	95.746
Ageratina adenophora	% Inh 1	66.116	67.362	69.477	71.916	73.086
	% Inh 2	66.495	67.728	69.731	72.706	73.53
	% Inh 3	66.072	67.193	69.536	71.701	72.771
Cupressus sempervirens	% Inh 1	37.726	40.499	46.222	49.657	54.541
	% Inh 2	37.921	41.078	46.932	52.632	54.763
	% Inh 3	37.545	38.997	46.332	52.369	54.254
Lantana camara	% Inh 1	49.911	52.913	56.194	59.374	62.58
	% Inh 2	50.141	54.711	56.457	60.051	61.335
	% Inh 3	50.127	53.209	55.553	59.475	62.838

IC_{50} value is calculated using graph plotting % inhibition against concentration.

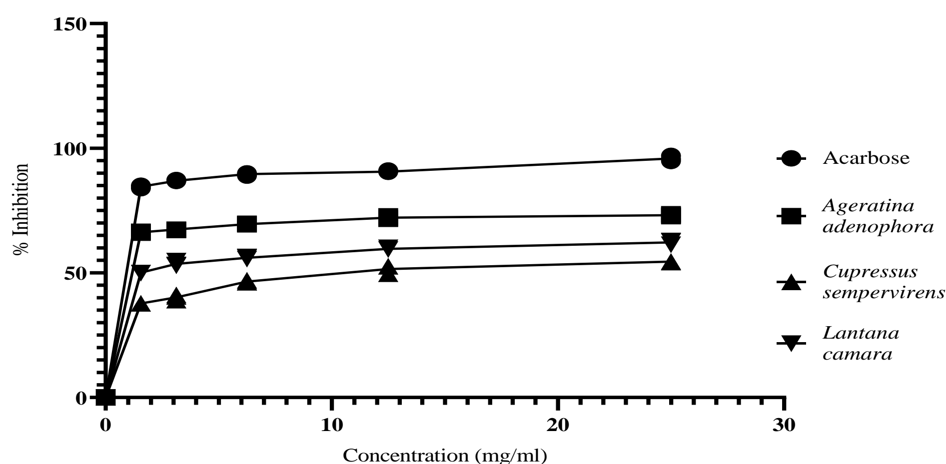


Figure 16: A plot of % inhibition with concentration (mg/ml) of different plant extracts and acarbose

Table 9: Comparison of IC_{50} values of different plants extracts with standard acarbose

S.N.	Sample	IC_{50} (mg/mL)
1.	Acarbose	1.251 ± 0.007
2.	*Ageratina adenophora*	1.843 ± 0.007
3.	*Cupressus sempervirens*	4.861 ± 0.209
4.	*Lantana camara*	2.750 ± 0.046

The IC_{50} value was calculated for all plant extract which is mentioned in tabulated form as well as in bar diagram with standard error bar.

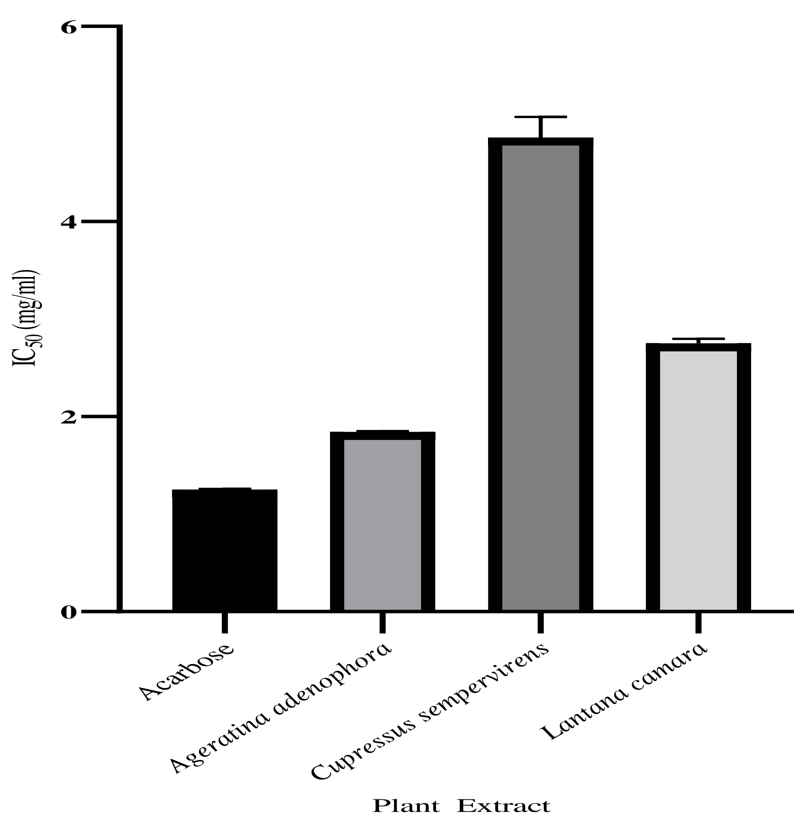


Figure 17: α – amylase inhibition activity (IC_{50}) in different concentration of methanolic plant extracts

Inhibitors of α – amylase delay the breaking down of carbohydrate in the small intestine and diminish the postprandial blood glucose excursion in a person suffering from diabetes. One of the strategies adopted to lower the levels of postprandial hyperglycemia involves the inhibition of carbohydrate digestive enzymes such as α – amylase in the gastrointestinal glucose absorption. This is

an attempt to search for alternative drug from medicinal plants with increased potency and lesser adverse effect than existing drugs.[103]

Synthetic drugs like acarbose, miglitol, metformin, etc. are used for the clinical treatment of type 2 diabetes. However, these drugs are reported to cause various side effects such as abdominal distention, flatulence and diarrhoea owing to the excessive inhibition of pancreatic α – amylase, which results in abdominal bacterial fermentation of undigested carbohydrates in the colon. Hence, at present there is an increasing interest among food scientists to identify natural sources of α – amylase and α – glucosidase inhibitors for the dietary management of type 2 diabetes.[38]

Though several studies showed the antidiabetic potential of these plants, no previous reports has been given on the mechanism by which it exerts this effect. In the present study methanolic extract of different plants were investigated for their potential to inhibit α – amylase activity. IC_{50} value of standard acarbose was found to be 1.251 ± 0.007 mg/mL. Among these plants, methanolic extracts of *Ageratina adenophora* showed highest α – amylase inhibitory activity with IC_{50} value of 1.843 ± 0.007 mg/mL. Similarly, methanolic extracts of *Lantana camara* and *Cupressus sempervirens* has the IC_{50} value of 2.750 ± 0.046 mg/mL and 4.861 ± 0.209 µg/mL respectively. Among the selected medicinal plants, *Ageratina adenophora* showed the highest percentage inhibition of alpha-amylase. Hepatic glucose output (HGO) inhibitory activity showed the decreased in glucose output on oral administration of *Ageratina adenophora* aerial parts (160mg/kg, orally) in the wistar rats.[66] Since, the plant showed significant inhibitory activity, further research based on isolation, purification and characterization which is responsible for inhibition can be carried out.

4.7 Isolation of compounds

On the basis of antioxidant and antidiabetic activity of the all methanolic plant extracts, *Lantana camara* extract was selected for the isolation of active constituents. After column chromatography TLC of each was performed. Fractions having similar TLC were mixed.

Results obtained after column chromatography is shown as below.

Table 10: Column chromatography and TLC report of different fractions

S.N.	Elution solvent system	Fraction number	Volume of eluent (mL)	Remarks	Color of spots
1	Hexane only	1-3	200	No spot	
2	2% EtOAc in hexane	4-6	200	Single spot	UV-active
3	5% EtOAc in hexane	7-9	200	Two spot	Green
4	10% EtOAc in hexane	10-12	200	Single spot	Orange
5	15 % EtOAc in hexane	13-15	200	Single spot	Light Yellow
6	20 % EtOAc in hexane	16-18	200	Multiple spot	Dark green
7	25 % EtOAc in hexane	19-21	200	Tailing	Green
8	30 % EtOAc in hexane	22-24	200	Single spot	Green
9	35 % EtOAc in hexane	25-27	200	Multiple spot	Green orange
10	40 % EtOAc in hexane	28-30	200	Tailing	Green
11	45 % EtOAc in hexane	31-33	200	Tailing	Green
12	50 % EtOAc in hexane	34-36	200	Multiple spot	Light Green
13	55 % EtOAc in hexane	37-39	200	Multiple spot	Orange green
14	60 % EtOAc in hexane	40-42	200	Tailing	Orange green

After column chromatography, TLC of each fraction was performed. Fractions having similar TLC were mixed. Fraction no. 4-6, 10-12, 13-15 and 22-24 was further analysed via FTIR.

4.8 FTIR analysis of hexane fraction

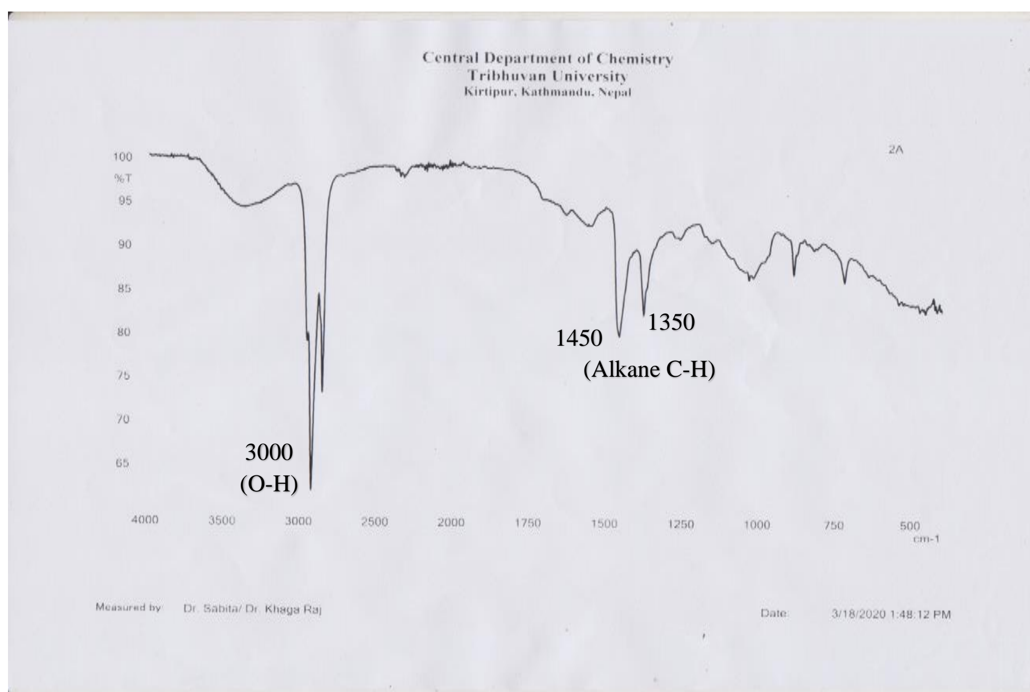


Figure 18: FTIR spectra of 2% EtOAc in hexane of *Lantana camara*

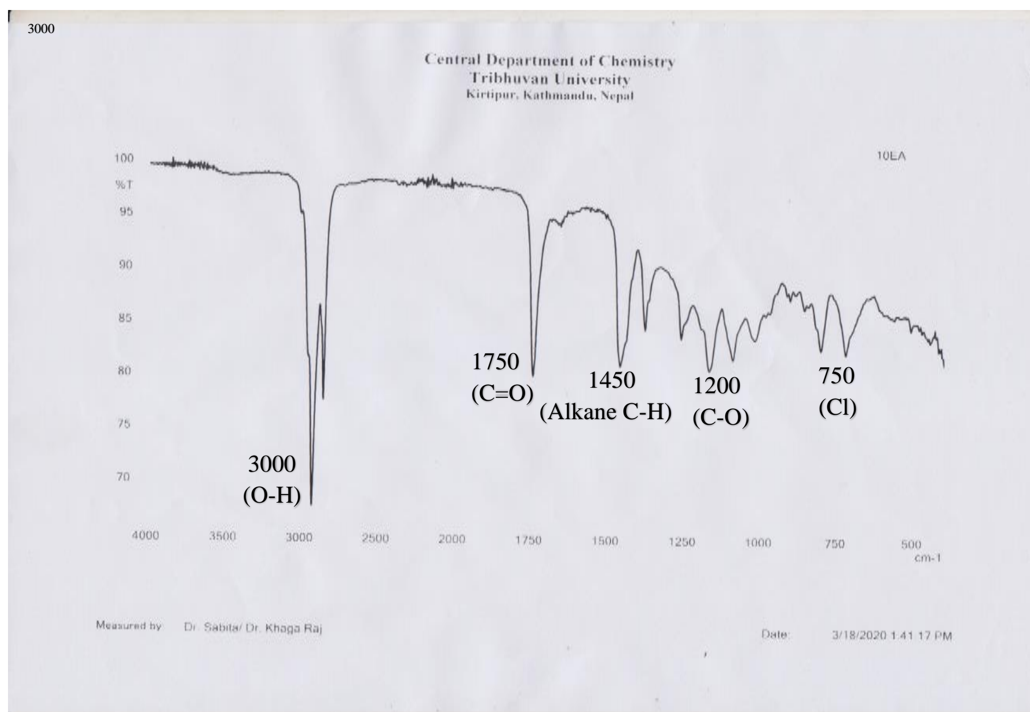


Figure 19: FTIR spectra of 10% EtOAc in hexane of *Lantana camara*

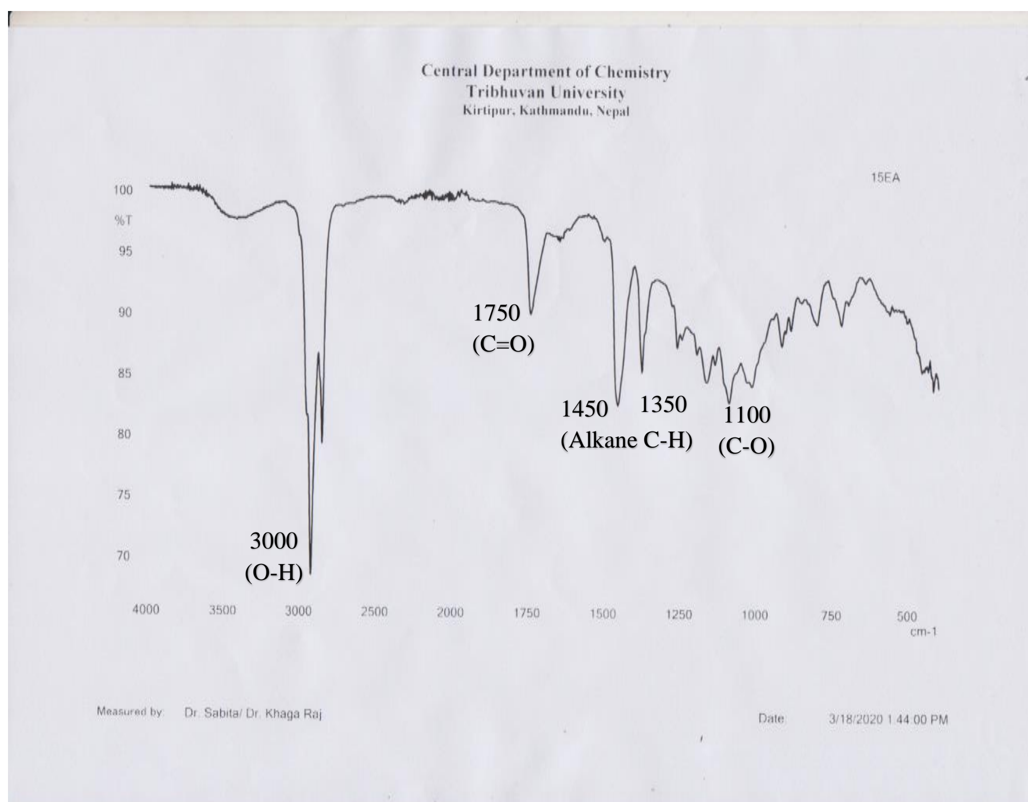


Figure 20: FTIR spectra of 15% EtOAc in hexane of *Lantana camara*

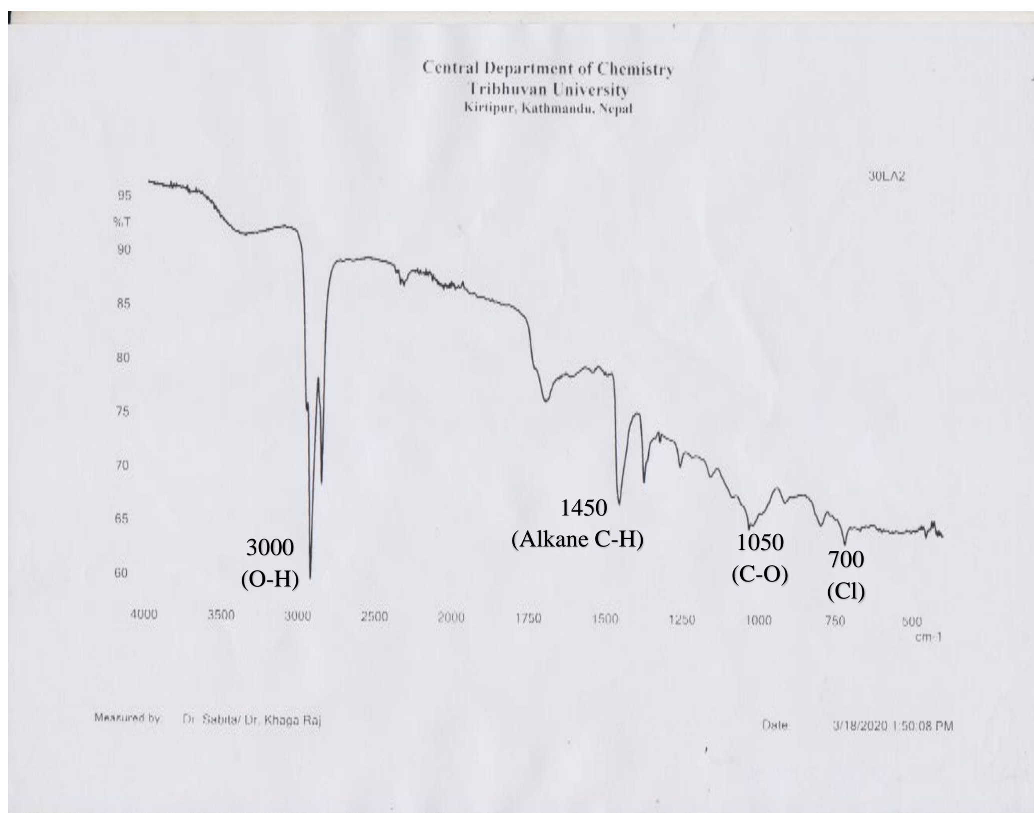


Figure 21: FTIR spectra of 30 % EtOAc in hexane of *Lantana camara*

Active fraction no. 4-6, 10-12, 13-15 and 22-24 was introduced into FTIR spectroscopy for the identification of possible functional group present in the sample. A sharp peak at 3000 cm^{-1} wave number indicate the O-H group of carboxylic acid. Similarly, a sharp peak at 1750 cm^{-1} indicate the carbonyl group (C=O) may be of aldehyde, ketone, carboxylic acid, ester, etc. A broad peak at 3400 cm^{-1} indicate the presence of aromatic C-H group present in the respective molecule.

Table 11: Functional groups of active fractions

Group or functionality	Assignment region (cm^{-1})
O-H (carboxylic acid)	3300-2500
Aromatic C-H bond	3400-3000
Aldehyde, ketone, carboxylic acid, ester	1750-1700
Alkanes	1470-1350
Alcohol, ether, ester, carboxylic acid, anhydrides	1300-1000
Chloride	785-540

The FTIR analysis indicated the presence of functional groups suchas O-H of carboxylic acid, carbonyl group (may be of aldehyde, ketone, carboxylic acid, ester), anhydride, aromatic C-H group, alkanes, etc as reported in the literature.[84,86,87,89]

CHAPTER – 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Phytochemical screening of methanolic extract of the plants showed the presence of alkaloids and flavonoids in all the extracts. Similarly, coumarins, glycosides, polyphenol, terpenoids and volatile oils were present in all the methanolic extracts. Carotinoids and carbohydrate were present in *Cupressus sempervirens* and *Lantana camara* respectively. Quinones were present in *Ageratina adenophora* and *Cupressus sempervirens*. Saponin was present only in *Lantana camara* and tannin only in *Cupressus sempervirens*.

The methanolic extract of *Lantana camara* was found to be less cytotoxic against brine shrimps as shown by its LC_{50} value of 2624.22 µg/mL. The methanolic extract of *Ageratina adenophora* was found to be cytotoxic against brine shrimps as shown by its LC_{50} value of 833.68 µg/mL as its LC_{50} value was found to be lower than 1000 µg/mL.

The methanolic extracts of *Lantana camara* (IC_{50} = 106.179 ± 11.390 µg/mL) showed the strongest DPPH radical scavenging activity as their IC_{50} values were close to standard ascorbic acid (IC_{50} = 17.456 ± 0.822 µg/mL) taken.

Methanolic extract of *Lantana camara* showed highest amount of total phenolic content (10.2 ± 0.343 mg GAE/g) followed by *Ageratina adenophora* (4.702 ± 0.059 mg GAE/g) which was determined spectrophotometrically.

Total flavonoid content of methanolic extract of *Lantana camara* (1.872 ± 0.16 mg QE/g) and *Ageratina adenophora* (0.645 ± 0.122 mg QE/g) was found to be highest. Methanolic extract of *Cupressus sempervirens* showed (0.379 ± 0.046 mg QE/g) lowest total flavonoid content.

During α – amylase inhibitory assay, the methanolic extract of *Ageratina adenophora* showed highest α – amylase inhibitory activity with IC_{50} value of 1.843 ± 0.007 mg/mL which is close to the acarbose with the IC_{50} value of 1.251 ± 0.007 mg/mL. Similarly, methanolic extracts of *Lantana camara* and

Cupressus sempervirens has the IC_{50} value of 2.750 ± 0.046 mg/mL and 4.861 ± 0.209 µg/mL respectively.

FTIR analysis revealed the presence of C=O group, O-H bond, aromatic C-H group in the respective fraction.

5.2 Recommendation

The present study strongly recommended the further work to isolate, purify, characterize, and standardize the bioactive constituents from the active extract of *Lantana camara*. *In-vitro* and *in-vivo* activity of these plant extracts could be performed which will be the part of drug discovery process.

Since, extracts of *Ageratina adenophora*, *Cupressus sempervirens* and *Lantana camara* are good sources of bioactive chemical constituents, it could be subjected to isolate the target compounds. These activities may find implication in the prevention of diabetic, cardiovascular complications and so on. Therefore, further investigations are required to justify the traditional medical applications of these plant.

REFERENCE

1. Kinghorn, A. D.; Pan, L.; Fletcher, J. N.; Chai, H. The relevance of higher plants in lead compound discovery programs. *Journal of natural products*. **2011,** *74* (6), 1539-1555.
2. Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of natural products*. **2012,** *75* (3), 311-335.
3. Fabricant, D. S.; Farnsworth, N. R. The value of plants used in traditional medicine for drug discovery. *Environmental health perspectives*. **2001,** *109* (1), 69-75.
4. Alves, R. R.; Rosa, I. M. Biodiversity, traditional medicine and public health: where do they meet?. *Journal of ethnobiology and ethnomedicine*. **2007,** *3* (1), 14.
5. Cui, L.; Su, X. Z. Discovery, mechanisms of action and combination therapy of artemisinin. *Expert Review of Anti-Infective Therapy*. **2009,** *7*, 999–1013.
6. Sneader, W. *Drug discovery: a history*. John Wiley & Sons. **2005,** *43*.
7. Serturner, F. W. Ueber das Morphium, eine neue salifahige Grundlage, und die Mekonsaure als Hauptbestandtheile des Opiums. *Annalen der Physik*. **1817,** *25*, 56-89.
8. Shen, J.; Xu, X.; Cheng, F.; Liu, H.; Luo, X.; Shen, J.; Jiang, H. Virtual screening on natural products for discovering active compounds and target information. *Current medicinal chemistry*. **2003,** *10* (21), 2327-2342.
9. Hong, J. Role of natural product diversity in chemical biology. *Current opinion in chemical biology*. **2011,** *15* (3), 350-354.
10. WHO. Traditional Medicine (2003). http://www.who. int/mediacentre/factsheets/2003/fs134/en/. Accessed 2 Jun 2017
11. Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the last 25 years. *Journal of natural products*. **2007,** *70* (3), 461-477.
12. Kunwar, R. M.; Nepal, B. K.; Kshhetri, H. B.; Rai, S. K.; Bussmann, R. W. Ethnomedicine in Himalaya: a case study from Dolpa, Humla, Jumla

and Mustang districts of Nepal. *Journal of ethnobiology and ethnomedicine*. **2006,** *2* (1), 27.

13. Pandey, B. R.; Shrestha, B. G. Status of Research on Medicinal Plants in Nepal: A Review. *International Journal of Medicine & Biomedical Sciences*. **2018,** *1*, 43-46.
14. Halliwell, B. Biochemistry of oxidative stress. *Biochemical society transactions.* **2007,** *35* (5), 1147-1150.
15. Rahman, K. Studies on free radicals, antioxidants, and co-factors. *Clinical interventions in aging*. **2007,** *2* (2), 219-236.
16. Kaur, K. Exploring the nootropic effect of *Juniperus recurva* extract: Possible involvement of acetylcholinesterase inhibition. *International Journal of Green Pharmacy (IJGP)*, **2019,** *13* (3).
17. Giles, G. I.; Jacob, C. Reactive sulfur species: an emerging concept in oxidative stress. *Biological chemistry*. **2002,** *383* (3-4), 375-388.
18. Chaterjea, M. N.; Shinde, R. Free radicals. Japee Brothers Medical Publishers. *New Delhi Indian.* **2007,** *156*, 629.
19. Rababah, T. M.; Hettiarachchy, N. S.; Horax, R. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. *Journal of Agricultural and Food Chemistry*. **2004,** *52* (16), 5183-5186.
20. Lotito, S. B.; Frei, B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon?. *Free Radical Biology and Medicine*. **2006,** *41* (12), 1727-1746.
21. Lu, J. M.; Yao, Q.; Chen, C. Ginseng compounds: an update on their molecular mechanisms and medical applications. *Current vascular pharmacology*. **2009,** *7* (3), 293-302.
22. DeFeudis, F. V.; Papadopoulos, V.; Drieu, K. *Ginkgo biloba* extracts and cancer: a research area in its infancy. *Fundamental & clinical pharmacology*. **2003,** *17* (4), 405-417.
23. Ralston, L.; Subramanian, S.; Matsuno, M.; Yu, O. Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using

soybean type I and type II chalcone isomerases. *Plant physiology*. **2005,** *137* (4), 1375-1388.

24. Saranraj, P.; Behera, S. S.; Ray, R. C. Traditional Foods from Tropical Root and Tuber Crops: Innovations and Challenges. In *Innovations in traditional foods*. Woodhead Publishing. **2019,** 159-191.
25. Naczka, M.; Shahidib, F. Extraction and analysis of phenolics in food. *Journal of Chromatography A*. **2004,** *1054*, 95–111.
26. Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture*. **2000,** *80* (7), 985-1012.
27. Yang, C. S.; Landau, J. M.; Huang, M. T.; Newmark, H. L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual review of nutrition*. **2001,** *21* (1), 381-406.
28. Croft, K. D. The Chemistry and Biological Effects of Flavonoids and Phenolic Acids. *Annals of the New York Academy of Sciences*. **1998,** *854* (1), 435-442.
29. Kumar, S.; Pandey, A. K. Chemistry and biological activities of flavonoids: an overview. *The scientific world journal*. **2013.**
30. Middleton, E. Effect of plant flavonoids on immune and inflammatory cell function. In *Flavonoids in the living system.* **1998,** 175-182.
31. Maritim, A. C.; Sanders, A.; Watkins Iii, J. B. Diabetes, oxidative stress, and antioxidants: a review. *Journal of biochemical and molecular toxicology*. **2003,** *17* (1), 24-38.
32. American Diabetes Association. Classification and diagnosis of diabetes: Standards of Medical Care in Diabetes—2020. *Diabetes care*. **2020,** *43* (1), S14-S31.
33. Saeedi, P.; Petersohn, I.; Salpea, P.; Malanda, B.; Karuranga, S.; Unwin, N.; Shaw, J. E. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas. *Diabetes research and clinical practice*. **2019,** *157*, 107843.
34. Ganesh, T.; Sen, S.; Thilagam, E.; Thamotharan, G.; Loganathan, T.; Chakraborty, R. Pharmacognostic and anti-hyperglycemic evaluation of

Lantana camara (L.) var. aculeate leaves in alloxan-induced hyperglycemic rats. *International Journal of Research in Pharmaceutical Sciences*. **2010,** *1* (3), 247-252.

35. Kandra, L. α-Amylases of medical and industrial importance. *Journal of Molecular Structure: THEOCHEM*. **2003,** *666*, 487-498.
36. Egharevba, H. Chemical properties of starch and its application in the food industry. *Chemical properties of starch. IntechOpen*. **2020.**
37. Whitcomb, D. C.; Lowe, M. E. Human pancreatic digestive enzymes. *Digestive diseases and sciences*. **2007,** *52* (1), 1-17.
38. Ren, L.; Cao, X.; Geng, P.; Bai, F.; Bai, G. Study of the inhibition of two human maltase-glucoamylases catalytic domains by different α-glucosidase inhibitors. *Carbohydrate research*. **2011,** *346* (17), 2688-2692.
39. Ciulei I. *Phytochemistry*, **2003,** *63*, 97-104.
40. Cuatrecasas, P.; Wilchek, M.; Anfinsen, C. B. Selective enzyme purification by affinity chromatography. *Proceedings of the National Academy of Sciences of the United States of America*. **1968,** *61* (2), 636.
41. Porath, J. From gel filtration to adsorptive size exclusion. *Journal of protein chemistry*. **1997,** *16* (5), 463-468.
42. Baranska, M.; Schulz, H.; Krüger, H.; Quilitzsch, R. Chemotaxonomy of aromatic plants of the genus Origanum via vibrational spectroscopy. *Analytical and bioanalytical chemistry*. **2005,** *381* (6), 1241-1247.
43. Parsons, W. T.; Cuthbertson, E. G. Noxious Weeds of Australia Inkata Press. *Melbourne/Sydney*. **1992.**
44. Sharma, O. P.; Dawra, R. K.; Kurade, N. P.; Sharma, P. D. A review of the toxicosis and biological properties of the genus *Eupatorium. Natural Toxins*. **1998,** *6* (1), 1-14.
45. Malla, B.; Chhetri, R. B. Indigenous knowledge on ethnobotanical plants of Kavrepalanchowk district. *Kathmandu University Journal of Science, Engineering and Technology*. **2009,** *5* (2), 96-109.
46. Lallianrawna, S.; Muthukumaran, R.; Ralte, V.; Gurusubramanian, G.; Kumar, N. S. Determination of total phenolic content, total flavonoid

content and total antioxidant capacity of *Ageratina adenophora* (Spreng.) King & H. *Rob. Science Vision*. **2013,** *13* (4), 149-156.

47. A guide to medicinal plants in north Africa. *Cupressus sempervirens*. IUCN Centre for Mediterranean Cooperation. **2005,** 106.
48. Databses, G. R. I. N. USDA, ARS, National Genetic Resources Program. Germplasm Resources Information Network-(GRIN)[Online Database], National Germplasm Resources Laboratory, Beltsville, Maryland. **2009.**
49. Chaudhary, H. J.; Shahid, W.; Bano, A.; Ullah, F.; Munis, F.; Fahad, S.; Ahmad, I. *In-vitro* analysis of *Cupressus sempervirens* L. plant extracts antibacterial activity. *Journal of Medicinal Plants Research*. **2012,** *6* (2), 273-276.
50. Rawat, P.; Khan, M. F.; Kumar, M.; Tamarkar, A. K.; Srivastava, A. K.; Arya, K. R.; Maurya, R. Constituents from fruits of *Cupressus sempervirens*. *Fitoterapia*. **2010,** *81* (3), 162-166.
51. Mahmood, Z.; Ahmed, I.; Saeed, M. U. Q.; Sheikh, M. A. Investigation of physico-chemical composition and antimicrobial activity of essential oil extracted from lignin-containing *Cupressus sempervirens*. *BioResources*. **2013,** *8* (2), 1625-1633.
52. Priyanka, N.; Joshi, P. K. A review of *Lantana camara* studies in India. *International Journal of Scientific and Research Publications*. **2013,** *3* (10), 1-11.
53. Remya, M.; Vashum, N.; Sivasankar, S. Bioactivity studies on *Lantana camara* linn. *International Journal of Pharma and Bio Sciences*. **2013,** *4* (1), 81-90.
54. Liu, P. Y.; Liu, D.; Li, W. H.; Zhao, T.; Sauriol, F.; Gu, Y. C.; Zhang, M. L. Chemical constituents of plants from the genus *Eupatorium* (1904–2014). *Chemistry & biodiversity*. **2015,** *12* (10), 1481-1515.
55. Liu, B.; Dong, B.; Yuan, X.; Kuang, Q.; Zhao, Q.; Yang, M.; Zhao, B. Enrichment and separation of chlorogenic acid from the extract of *Eupatorium adenophorum* Spreng by macroporous resin. *Journal of Chromatography B*. **2016,** *1008*, 58-64.

56. Rongtao, L.; Zhihui, D.; Jingkai, D. Chemical constituents from *Eupatorium adenophorum*. *Acta Botanica Yunnanica*. **1997,** *19* (2), 196-200.
57. Wei, Y.; Zhang, K.; Zhang, G.; Ito, Y. Isolation of five bioactive components from *Eupatorium adenophorum* Spreng using stepwise elution by high-speed countercurrent chromatography. *Journal of liquid chromatography & related technologies*. **2011,** *34* (20), 2505-2515.
58. Zhang, M.; Liu, W. X.; Zheng, M. F.; Xu, Q. L.; Wan, F. H.; Wang, J.; Tan, J. W. Bioactive quinic acid derivatives from *Ageratina adenophora*. *Molecules*. **2013,** *18* (11), 14096-14104.
59. He, L.; Hou, J.; Gan, M.; Shi, J.; Chantrapromma, S.; Fun, H. K.; Sung, H. H. Y. Cadinane sesquiterpenes from the leaves of *Eupatorium adenophorum. Journal of natural products*. **2008,** *71* (8), 1485-1488.
60. Zhao, X.; Zheng, G. W.; Niu, X. M.; Li, W. Q.; Wang, F. S.; Li, S. H. Terpenes from *Eupatorium adenophorum* and their allelopathic effects on Arabidopsis seeds germination. *Journal of Agricultural and Food Chemistry*. **2009,** *57* (2), 478-482.
61. Zheng, G.; Luo, S.; Li, S.; Hua, J.; Li, W.; Li, S. Specialized metabolites from *Ageratina adenophora* and their inhibitory activities against pathogenic fungi. *Phytochemistry*. **2018,** *148*, 57-62.
62. Tripathi, Y.; Saini, N. Total phenolic, total flavonoid content and antioxidant efficacy of leaves of *Eupatorium adenophorum. International Journal of Pharma and Bio Science*. **2019,** *10* (2), 157-166.
63. Ralte, V.; Sameul, L. *In vitro* antioxidant activity of *Ageratina adenophora* (King & Rob) and *Ipomoea cairica* (L) Sweet. *Science vision*. **2014,** *14* (3), 128-132.
64. Ahluwalia, V.; Sisodia, R.; Walia, S.; Sati, O. P.; Kumar, J.; Kundu, A. Chemical analysis of essential oils of *Eupatorium adenophorum* and their antimicrobial, antioxidant and phytotoxic properties. *Journal of pest science*. **2014,** *87* (2), 341-349.
65. Bustos-Brito, C.; Andrade-Cetto, A.; Giraldo-Aguirre, J. D.; Moreno-Vargas, A. D.; Quijano, L. Acute hypoglycemic effect and

phytochemical composition of *Ageratina petiolaris*. *Journal of ethnopharmacology*. **2016,** *185*, 341-346.

66. Mata-Torres, G.; Andrade-Cetto, A.; Espinoza-Hernández, F. A.; Cárdenas-Vázquez, R. Hepatic Glucose Output Inhibition by Mexican Plants Used in the Treatment of Type 2 Diabetes. *Frontiers in Pharmacology*. **2020,** *11*, 215.
67. Hassanzadeh Khayyat, M.; Emami, S. A.; Rahimizadeh, M.; Fazly-Bazzaz, B. S.; Assili, J. Chemical constituents of *Cupressus sempervirens* L. cv. Cereiformis Rehd. essential oils. *Iranian Journal of Pharmaceutical Sciences*. **2005,** *1* (1), 39-42.
68. Selim, S. A.; Adam, M. E.; Hassan, S. M.; Albalawi, A. R. Chemical composition, antimicrobial and antibiofilm activity of the essential oil and methanol extract of the Mediterranean cypress (*Cupressus sempervirens* L.). *BMC complementary and alternative medicine*. **2014,** *14* (1), 179-186.
69. Ismail, A.; Lamia, H.; Mohsen, H.; Samia, G.; Bassem, J. Chemical composition, bio-herbicidal and antifungal activities of essential oils isolated from Tunisian common cypress (*Cupressus sempervirens* L.). *Journal of Medicinal Plants Research*. **2013,** *7* (16), 1070-1080.
70. Miloš, M.; Mastelić, J.; Radonić, A. Free and glycosidically bound volatile compounds from cypress cones (*Cupressus sempervirens* L.). *Croatica chemica acta*. **1998,** *71* (1), 139-145.
71. Krishnaveni, M.; Amsavalli, L.; Chandrasekar, R.; Madhaiyan, P.; Durairaj, S. Antioxidant activity of plants at Govt. College of Engineering campus, Salem, Tamil nadu, India. *International Journal of Pharmaceutical Sciences Review and Research*. **2013,** *21* (1), 160-163.
72. Tumen, I.; Senol, F. S.; Orhan, I. E. Evaluation of possible in vitro neurobiological effects of two varieties of *Cupressus sempervirens* (Mediterranean cypress) through their antioxidant and enzyme inhibition actions. *Turkish Journal of Biochemistry/Turk Biyokimya Dergisi*. **2012,** *37* (1).

73. Shahid, W.; Durrani, R.; Iram, S.; Durrani, M.; Khan, F. A. Antibacterial activity *in vitro* of medicinal plants. *Sky Journal of Microbiology Research*. **2013,** *1* (2), 5-21.
74. Boussoussa, H.; Khacheba, I.; Berramdane, T.; Maamri, A.; Bendahgane, H.; Yousfi, M. *In vitro* antidiabetic effect of saponins and phenolic extracts from fruits and seeds of Algerian cypress tree: *Cupressus sempervirens* L. *Current Enzyme Inhibition*. **2018,** *14* (2), 92-96.
75. Asgary, S.; Naderi, G. A.; Shams Ardekani, M. R.; Sahebkar, A.; Airin, A.; Aslani, S.; Emami, S. A. Chemical analysis and biological activities of *Cupressus sempervirens var. horizontalis* essential oils. *Pharmaceutical biology*. **2013,** *51* (2), 137-144.
76. Fleming, T. PDR for herbal medicines: From Medical Economics Company. *New Jersy: USA*. **2000,** 253.
77. Toroglu, S. *In vitro* antimicrobial activity and antagonistic effect of essential oils from plant species. *Journal of Environmental Biology*. **2007,** *28* (3), 551-559.
78. Loizzo, M. R.; Tundis, R.; Menichini, F.; Saab, A. M.; Statti, G. A.; Menichini, F. Antiproliferative effects of essential oils and their major constituents in human renal adenocarcinoma and amelanotic melanoma cells. *Cell Proliferation*. **2008,** *41* (6), 1002-1012.
79. Ganatra, S.; Gurubaxani, S. Preliminary Phytochemical and TLC profiling of *Lantana camara* leaf extracts. *Journal of Chemical and Pharmaceutical Research*. **2016,** *8* (15), 614-617.
80. Pradhan, R. R.; Hati, D. K.; Samal, S. Pharmacognostical, phytochemical and antimicrobial studies on the leaves of *Lantana camara* Linn. *Der Pharmacia Lettre*. **2012,** *4* (6), 1648-1656.
81. Asadu, C. L.; Anosike, C. A.; Uzoegwu, P. N.; Abonyi, O.; Ezugwu, A. L.; Uroko, R. I. *In-vitro* antioxidant activity of methanol extract of *Lantana camara* leaves. *Global Veterinaria*. **2015,** *14* (4), 595-602.
82. Begum, S.; Wahab, A.; Siddiqui, B. S.; Qamar, F. Nematicidal constituents of the aerial parts of *Lantana camara*. *Journal of Natural Products*. **2000,** *63* (6), 765-767.

83. Swamy, M. K.; Sinniah, U. R.; Akhtar, M. *In-vitro* pharmacological activities and GC-MS analysis of different solvent extracts of *Lantana camara* leaves collected from tropical region of Malaysia. *Evidence-Based Complementary and Alternative Medicine*. **2015,** *9*.
84. Saraf, A.; Quereshi, S.; Sharma, K.; Khan, N. A. Antimicrobial activity of *Lantana camara* L. *Journal of Experimental Sciences*. **2011,** *2* (10), 50-54.
85. Shamsee, Z. R.; Al-Saffar, A. Z.; Al-Shanon, A. F.; Al-Obaidi, J. R. Cytotoxic and cell cycle arrest induction of pentacyclic triterpenoides separated from *Lantana camara* leaves against MCF-7 cell line in vitro. *Molecular biology reports*. **2019,** *46* (1), 381-390.
86. Sharma, O. P.; Dawra, R. K.; Makkar, H. P. S. Isolation and partial purification of *Lantana (Lantana camara* L.) toxins. *Toxicology Letters*. **1987,** *37* (2), 165-172.
87. Begum, S.; Zehra, S. Q.; Ayub, A.; Siddiqui, B. S. A new 28-noroleanane triterpenoid from the aerial parts of *Lantana camara* Linn. *Natural product research*. **2010,** *24* (13), 1227-1234.
88. Ono, M.; Hashimoto, A.; Miyajima, M.; Sakata, A.; Furusawa, C.; Shimode, M.; Yoshimitsu, H. Two new triterpenoids from the leaves and stems of *Lantana camara*. *Natural Product Research*. **2020,** 1-9.
89. Ali, A. A.; Elgimabi, M. E. Extraction and determination of antioxidants, polyphenols, flavonoids and antioxidants activity in some plants. *International Journal of Chemical Science.* **2015,** *13*, 1883-92.
90. Ayub, A.; Tauseef, S.; Ali, S. T. Antioxidant activity of the medicinal plant *Lantana camara* L. *FUUAST Journal of Biology*. **2017,** *7* (2), 227-230.
91. Naz, R.; Bano, A. Phytochemical screening, antioxidants and antimicrobial potential of *Lantana camara* in different solvents. *Asian Pacific Journal of Tropical Disease*. **2013,** *3* (6), 480-486.
92. Venkatachalam, T.; Kumar, V. K.; Selvi, P. K.; Maske, A. O.; Anbarasan, V.; Kumar, P. S. Antidiabetic activity of *Lantana camara* Linn fruits in normal and streptozotocin-induced diabetic rats. *Journal of Pharmacy Research*. **2011,** *4* (5), 1550-2.

93. Dash, G. K.; Suresh, P.; Ganapaty, S. Studies on hypoglycaemic and wound healing activities of *Lantana camara* Linn. *Journal of Natural Remedies*. **2001,** *1* (2), 105-110.
94. McLaughlin, J. L. Brine shrimp: a convenient general bioassay for active constituents. *Planta Medica*. **1982,** *45,* 31-32.
95. Demirkiran, O.; Sabudak, T.; Ozturk, M.; Topcu, G. Antioxidant and tyrosinase inhibitory activities of flavonoids from *Trifolium nigrescens* subsp. *petrisavi*. *Journal of agricultural and food chemistry*. **2013,** *61* (51), 12598-12603.
96. Ainsworth, E. A.; Gillespie, K. M. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. *Nature protocols*. **2007,** *2* (4), 875-877.
97. Lu, X.; Ross, C. F.; Powers, J. R.; Aston, D. E.; Rasco, B. A. Determination of total phenolic content and antioxidant activity of Garlic (*Allium sativum*) and Elephant Garlic (*Allium ampeloprasum*) by attenuated total reflectance–fourier transformed infrared spectroscopy. *Journal of agricultural and food chemistry*. **2011,** *59* (10), 5215-5221.
98. Javanmardi, J.; Stushnoff, C.; Locke, E.; Vivanco, J. M. Antioxidant activity and total phenolic content of Iranian Ocimum accessions. *Food chemistry*. **2003,** *83* (4), 547-550.
99. Chang, C. C.; Yang, M. H.; Wen, H. M.; Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of food and drug analysis*. **2002,** *10* (3).
100. Xiao, Z.; Storms, R.; Tsang, A. A quantitative starch? Iodine method for measuring alpha-amylase and glucoamylase activities. *Analytical biochemistry*. **2006,** *351* (1), 146-148.
101. Sarah, Q. S.; Anny, F. C.; Mir, M. Brine shrimp lethality assay. *Bangladesh Journal of Pharmacology*. **2017,** *12* (2), 186-189.
102. Kwon, Y. I.; Apostolidis, E.; Shetty, K. Evaluation of pepper (*Capsicum annuum*) for management of diabetes and hypertension. *Journal of Food Biochemistry*. **2007,** *31* (3), 370-385.

103. Qamar, F.; Begum, S.; Raza, S. M.; Wahab, A.; Siddiqui, B. S. Nematicidal natural products from the aerial parts of *Lantana camara* Linn. *Natural product research*. **2005,** *19* (6), 609-613.

APPENDIX-1

PHYTOCHEMICAL SCREENING

General Procedure

Extraction

About 100 g of air dried plant material was extracted successively with methanol by cold percolation method. Each extracts was concentrated in rotatory evaporator and was then subjected for phytochemical test following the given procedure as:

1. Test for reducing sugars (Fehling's Test)

The methanolic solution was mixed with distilled water first and the filtered. To each filtrate a few drops of Fehling's solution A and B (1:1) was added and warmed over a water bath for 30 minutes. The appearance of an orange red precipitate confirmed the presence of reducing compounds.

To this solution 5% (w/v) ferric chloride solution (3 drops) was added and observed. Formation of greenish blue color indicated the presence of polyphenols.

2. Test for basic alkaloids

The methanol extract (10 mL) was concentrated to yield a residue and dissolved in 3 mL of 2% (v/v) HCl. This solution was equally divided into two testtubes.

I. Meyer's Test: The first test solution was treated with three drops of Meyer's reagent. White precipitate indicated the presence of basic alkaloids.
II. Dragendorff's Test: The second test solution was treated with three drops of Dragendorff's reagent. White precipitate indicated the presence of basic alkaloids.

3. Test for glycosides

The methanol extract (8 mL) was concentrated to half the original volume and divided into two test tubes.

i. The first test solution (2 mL) was treated with 25% (v/v) ammonium hydroxide solution (2 mL) and shaken vigorously. A cherry red color indicated the presence of glycosides.

ii. Molisch's Test: The second test solution was treated with 5 ml Molisch's reagent and conc. H_2SO_4 was added drop wise from the side of the test tube without disturbing the solution. A violet ring at the junction of two liquid layers was observed which turned violet completely on shaking indicating the presence of glycosides or free sugars.

4. Test for quinones

To the methanolic extract (2 mL), freshly prepared ferrous sulphate solution (1 mL) and ammonium thiocyanate (few crystals) was added and treated with conc. H_2SO_4 drop by drop. The deep red color was persistent indicating the presence of quinones.

5. Test for saponins

The methanolic extract (4 mL) was concentrated and mixed with hot water (2 mL). The solution was shaken vigorously for 15 seconds. Formation of persistent foam indicated the presence of saponins.

6. Test for coumarins

Firstly, single pellets of KOH was taken and dissolved in 1 mL of ethanol. Then 1 mL of extract solution was added. The presence of precipitate indicates the presence of coumarins.

7. Test for flavonoids

5 mL of dilute ammonia solution was added to the aqueous filtered solution of each fraction followed by the addition of concentrated sulphuric acid. The appearances of yellow color indicated the presence of flavonoids. The yellow color disappeared after sometimes.

8. Test for terpenoids (Salkowski's Test)

Small amount of extract was dissolved in chloroform and equal volume of conc. H_2SO_4 was added. Reddish brown coloration at the interface of two layers indicated the presence of terpenoids.

9. Test for tannins

A quantity (2 g) of the extract (HE) was boiled with 5 ml of 45% ethanol for 5 min. The mixture was cooled and filtered. The filtrate was subjected to the following tests:

i. Lead acetate test: 1 ml of the filtrate was added to 3 drops of the lead acetate solution. A cream gelatinous precipitate indicates the presence of tannins.
ii. Ferric chloride test: A quantity (1 ml) of the filtrate was diluted with distilled water and added 2 drops of ferric chloride. A transient greenish to black color indicates the presence of tannins.

10. Test for polyphenols (Ferric chloride test)

Few drops of dilute ferric chloride solution was added to the methanolic solution of extracts. The formation of bluish black color indicates the presence of polyphenols.

11. Test for carotenoids (Carr - Price reaction)

Few drops of anhydrous solution of antimony trichloride in chloroform was added to 1 ml of extract. The formation of blue color indicates the presence of carotenoids.

APPENDIX-2

PREPARATION OF REAGENTS

1. Maeyer's reagent

Mercuric chloride (0.679 g) was weighed in a 50 mL volumetric flask and dissolved in a distilled water. To this solution, potassium iodide (2.5 g) was added. The scarlet red precipitate was dissolved by shaking and then diluted with distilled water upto the mark of volumetric flask.

2. Dragendorff's reagent

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

3. Molisch's reagent

α-napthol or thymol (5 g) was dissolved in methanol (50 mL).

4. Neutral ferric chloride solution

Ferric chloride (1 g) was dissolved in distilled water (100 mL). To this aqueous solution, sodium carbonate was added little by little with stirring until the slight turbidity persisted. The mixture was filtered and the colorless filtrate was used as neutral ferric chloride.

APPENDIX-3

Appendix 3.1: Brine shrimp assay

Sample	Concentration (ppm)	x = log (ppm)	Total number of nauplii	Death nauplii	Live nauplii	% Mortality	y = Probit
Lantana camara	10	1	20	0	20	0	0
	100	2	20	1	19	5	3.36
	1000	3	20	2	18	10	3.72
Ageratina adenophora	10	1	20	0	20	0	0
	100	2	20	5	15	25	4.33
	1000	3	20	6	14	30	4.48
Cupressus sempervirens	10	1	20	0	20	0	0
	100	2	20	1	19	5	3.36
	1000	3	20	3	17	15	3.96

Appendix 3.2: Absorbance and % inhibition of DPPH scavenging assay

Sample	Concentration (µg/mL)	Abs 1	Abs 2	Abs 3	% Inhi1	% Inhi 2	% Inhi 3
Ascorbic acid	10	0.101	0.093	0.11	61.3	63.53	57.53
	20	0.08	0.077	0.087	69.35	69.8	66.41
	40	0.066	0.065	0.062	74.71	74.51	76.06
	80	0.051	0.046	0.049	80.46	81.96	81.08
	160	0.03	0.037	0.035	88.51	85.49	86.49
	320	0.019	0.014	0.017	92.72	94.51	93.44
	500	0.011	0.009	0.012	95.78	96.47	95.37
Lantana camara	10	0.239	0.221	0.225	8.43	13.33	13.13
	20	0.203	0.196	0.197	22.22	23.14	23.94
	40	0.188	0.175	0.186	27.97	31.37	28.19
	80	0.157	0.148	0.165	39.85	41.96	36.29
	160	0.101	0.1	0.105	61.3	60.78	59.46
	320	0.089	0.09	0.087	65.9	64.71	66.41
	500	0.067	0.06	0.063	74.33	76.47	75.67
Ageratina adenophora	10	0.256	0.251	0.253	1.92	1.57	2.32
	20	0.233	0.23	0.238	10.73	9.8	8.11
	40	0.212	0.209	0.215	18.77	18.04	16.99
	80	0.201	0.205	0.204	22.99	19.61	21.24
	160	0.189	0.184	0.188	27.59	27.84	27.41
	320	0.144	0.136	0.133	44.83	46.67	48.65
	500	0.123	0.117	0.101	52.87	54.12	53.67
Cupressus sempervirens	10	0.238	0.229	0.238	8.81	10.2	8.11
	20	0.225	0.226	0.222	13.8	12.89	14.28
	40	0.214	0.219	0.215	18	14.12	16.99
	80	0.206	0.2	0.203	21.07	21.57	21.63
	160	0.161	0.161	0.159	38.31	36.89	39
	320	0.098	0.09	0.094	62.45	64.71	63.71
	500	0.084	0.082	0.079	67.81	67.84	69.5
	Control	0.261	0.255	0.259			

Appendix 3.3: Absorbance of gallic acid

Concentration (µg/mL)	Absorbance			
	A_1	A_2	A_3	Mean ± SD
10	0.987	1.292	1.259	1.17933±0.167
20	1.072	1.254	1.395	1.24033±0.162
30	1.328	1.337	1.381	1.34867±0.028
40	1.339	1.639	1.637	1.53833±0.172
50	1.782	1.762	1.872	1.80533±0.058
60	2.195	2.412	2.412	2.33967±0.125
70	2.349	2.593	2.733	2.55833±0.194
80	2.633	2.649	2.748	2.67667±0.062

Appendix 3.4: Absorbance of quercetin

Concentration (µg/mL)	Absorbance			
	A_1	A_2	A_3	Mean ± SD
10	0.109	0.12	0.103	0.110667±0.008
20	0.16	0.167	0.158	0.161667±0.004
40	0.249	0.273	0.288	0.27±0.019
80	0.432	0.446	0.498	0.458667±0.034
160	0.86	0.799	0.848	0.835667±0.032
320	1.585	1.682	1.809	1.692±0.112
500	2.614	2.305	2.694	2.537667±0.205

Appendix 3.5: Absorbance and % inhibition for enzyme assay

Sample	Concentration (mg/mL)	Abs 1	Abs 2	Abs 3
Acarbose	1.5625	3.354	3.36	3.381
	3.125	3.468	3.449	3.461
	6.25	3.588	3.554	3.55
	12.5	3.611	3.598	3.601
	25	3.784	3.827	3.815
Lantana camara	1.5625	2.015	2.004	2.024
	3.125	2.133	2.182	2.145
	6.25	2.262	2.25	2.237
	12.5	2.387	2.39	2.391
	25	2.513	2.44	2.523
Ageratina adenophora	1.5625	2.652	2.641	2.65
	3.125	2.701	2.689	2.694
	6.25	2.784	2.767	2.786
	12.5	2.88	2.883	2.871
	25	2.926	2.915	2.913

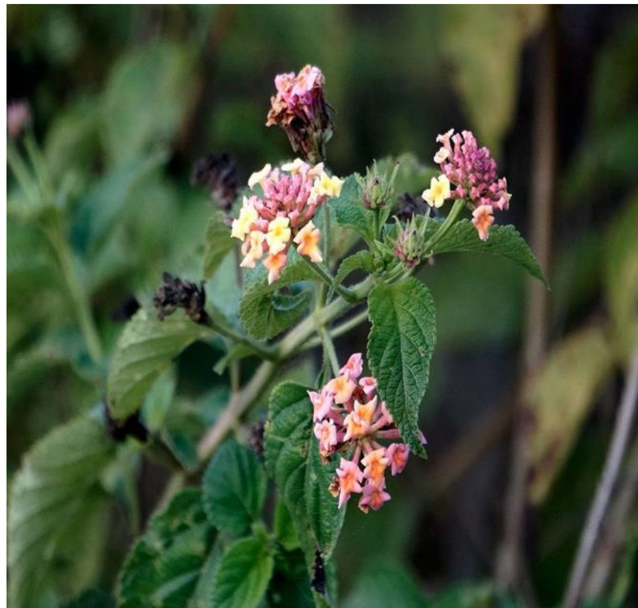
Cupressus sempervirens	1.5625	1.536	1.528	1.53
	3.125	1.645	1.651	1.587
	6.25	1.87	1.879	1.875
	12.5	2.005	2.101	2.112
	25	2.197	2.184	2.186
	Starch only	3.984	3.946	3.982
	Starch+Enzyme	0.053	0.051	0.056
	EA of control	3.931	3.895	3.926

APPENDIX-4

Photographs of selected plants

Ageratina adenophora

Cupressus sempervirens

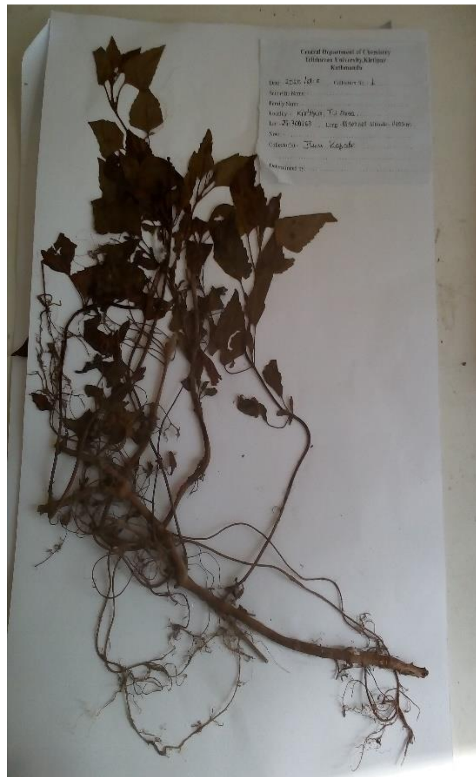


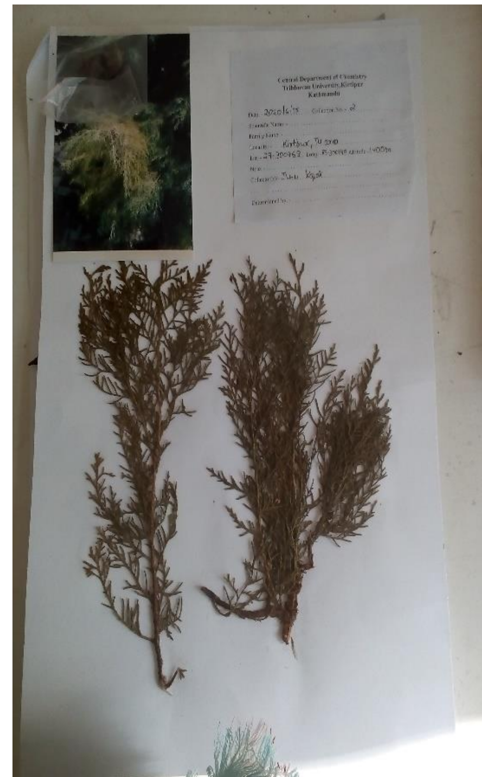
Lantana camara

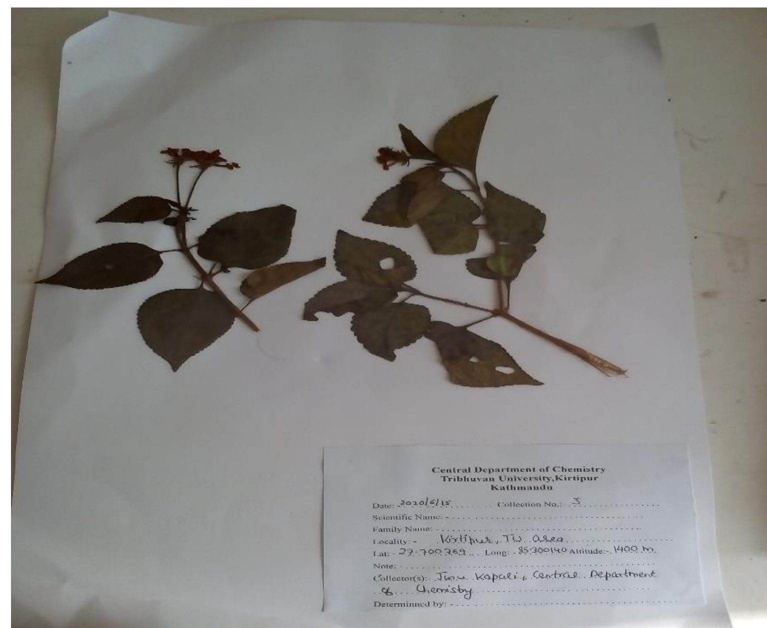
APPENDIX-5

Some photographs of laboratory work

Shade drying







Preparation of herbarium

Cold percolation

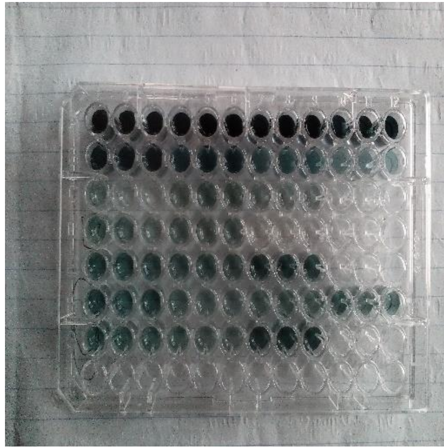
Rotary evaporator

Phytochemical screening

Stock solutions

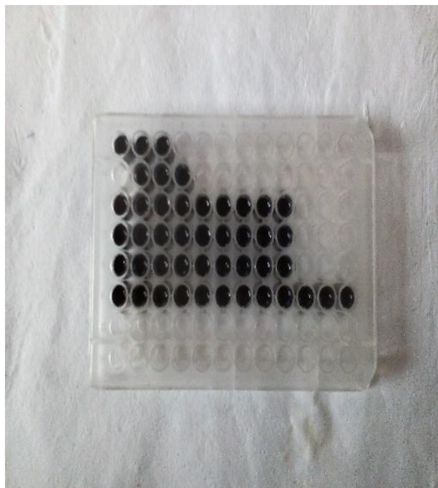
Brine shrimp bioassay

Antioxidant activity



Total phenolic content

Total flavonoid content



Antidiabetic activity

Fractionation

Column chromatography