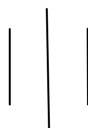
Phytochemical analysis of some medicinal plants indigenous to Nepal and study of antioxidant, antimicrobial and cytotoxicity effect of their extracts on various cell lines.

M. Sc. Thesis

2015



Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

A

Dissertation

For partial fulfillment of the requirement for the

Master of Science in Biotechnology

By

Krishna Thapa

Roll NO: BT 066/68

T.U.Regd. No: 5-2-37-580-2007

Phytochemical analysis of some medicinal plants indigenous to Nepal and study of antioxidant, antimicrobial and cytotoxicity effect of their extracts on various cell lines

M. Sc. Thesis

2015

Submitted to

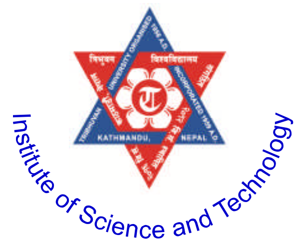
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Kirtipur, Kathmandu, Nepal

Submitted by
Krishna Thapa

Supervisors

Prof. Dr. Rajani Malla

Dr. Smita Shrestha



TRIBHUVAN UNIVERSITY
CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Ref. No.

Date : August 13, 2015

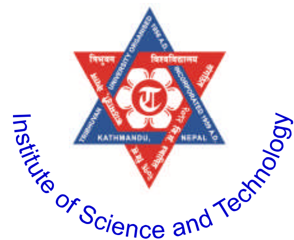
RECOMMENDATION

This is to certify that **Mr. Krishna Thapa** has successfully completed his dissertation work entitled **"Phytochemical analysis of some medicinal plants indigenous to Nepal and study of antioxidant, antimicrobial and cytotoxicity effect of their extracts on various cell lines"** under our supervision**.**

This thesis work was performed for the partial fulfillment for award of Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. We, hereby, recommend this thesis for final evaluation.

…………………………………………..
Dr. Rajani Malla
(Supervisor)
Head of the Department
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Nepal

…………………………………………..
Dr. Smita Shrestha
(Supervisor)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Nepal



TRIBHUVAN UNIVERSITY
CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Ref. No.

Date : August 13, 2015

CERTIFICATE OF EVALUATION

This is to certify that this thesis entitled **"Phytochemical analysis of some medicinal plants indigenous to Nepal and study of antioxidant, antimicrobial and cytotoxicity effect of their extracts on various cell lines"** presented to evaluation committee by **Mr. Krishna Thapa** is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

……………………………………………
Dr. Rajani Malla
(Supervisor)
Head of the Department
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

……………………………………………….
Dr. Rabindra Sapkota
(External Examiner)
Everest Biotech
Khumaltar, Lalitpur, Nepal

…………………………………………..
Dr. Smita Shrestha
(Supervisor)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

……………………………………………
Ms Jarina Joshi
(Internal Examiner)
Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

ACKNOWLEDGEMENT

First of all, I would like to express deepest gratitude to first supervisor; Assoc. Prof. Dr. Rajani Malla, Head, Central Department of Biotechnology, Tribhuvan University (TU), for her embracement and patience throughout the period of my staying at department, and for supporting me throughout the project. I am also extremely thankful to my second supervisor, Dr. Smita Shrestha, Central Department of Biotechnology, TU for providing all necessary guidance while pursuing this work. This thesis would not have materialized until their encouragement and good support.

I gratefully acknowledge the support of Dr. Deepak Pant, Faculty of Central Department of Botany and Dr. Suira Joshi, Ayurvedic herb specialist for identification of medicinal plants.

I would like to express my deep respect to Prof. Dr. Tribikram Bhattarai, former Head of Department; Prof. Dr. Mohan Kharel, Prof. Dr. Ganga Kharel, Prof. Dr. Krishna Das Manandhar, Dr. Sampoornanda Jha, Dr. Tilak R. Shrestha, Mrs Jarina Joshi and Mr. Balhari poudel, along with all teaching and non-teaching staffs of Central Department of Biotechnology. I owe my special thanks to Mohan Shrestha, Elen Pradhan, Gita Neupane and Binu Tamang Labrotary assistance for generous help whatever needed.

Sincere thank be given to Pradeep Shrestha for all of his necessary advice and selfless help during thesis work.

I wish to express my gratitude towards all of my friends, Supreet Khanal, Niroj Shakya, Nirman Nepal, Namita Bhattarai, Keshav GC, Preeti Regmi, Seeta Poudel, Bibek Vakta Mathema, Rajesh Lamichanne, Surendra Neupane, all of my classmates, my seniors and junior of this Department.

Similarly, I express my deepest gratitude to Dr. Bikash Pandey, General Manager at Everest Biotech for providing the cancer cell line and culture media and Department of plant resources, Thapathali, Kathmandu for providing the mice for research.

Last but not the least, I would like to express my sincere thankfulness to the family member's, colleagues and to all, who contributed directly and indirectly to the achievement of this work, those who stand behind the scene, thanks a lot.

Krishna Thapa

ACRONYMS

ATCC	American Type Culture Collection
CFU	Colony forming unit
DMSO	Dimethyl sulfoxide
DPPH	1, 1- DiPhenyl-2 PicryHydrazyl
DPR	Department of plant resources
EGF	Epidermal Growth Factors
ERK	Extracellular signal Regulated Kinase
FC	Folin–Ciocalteu phenol reagent
GR	Glutathione Reductase
GSK	Glaxo Smith Pharmaceuticals
GST	Glutathione S Transferase
HPLC	High Pressure Liquid Chromatography
IC_{50}	Inhibitory Concentration
IL	Inter leukin
Khz	Kilohertz
MHA	Muller Hinton Agar
MIC	Minimum inhibitory concentration
MIF	Migration Inhibitory Factor
MMP-2	Matrix MetalloProteinase-2
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole)
NA	Nutrient Agar
NAMC	Nepal Academy of Medical Council
NO	Nitric Oxide
OH^-	Hydroxyl Radical
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSA	Radical Scavenging Activity
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid reactive Substances
TNF-α	Tissue Necrosis Factor- α
WHO	World Health Organisation
YEPD	Yeast Extract Potato Dextrose

Acknowledgement i

Acronyms ii

List of Tables iii

List of figures iv

Abstract

CHAPTER : INTRODUCTION 1-7

1.1 Background 1

1.2 Plants as a source of secondary metabolites 1

1.3 Medicinal plants as a source of drugs 2

1.4 Economic benefits from medicinal plants 3

1.5 Scenario of Herbal medicine in Nepal 4

1.6 Research Plan 5

1.6.1 Hypothesis 5

1.7 Objectives 6

1.7.1 General Objectives 6

1.7.2 Specific Objectives 6

1.8 Rationale 6

1.9 Scope of the study 7

CHAPTER II: LITERATURE REVIEW 9-21

2.1 Rheum australe 9

2.1.1 Introduction 9

2.1.2 Habitat and Distribution 9

2.1.3 Botanical Description 9

2.1.4 Phytochemistry 10

2.1.5 Traditional and Ethnomedicinal value 10

2.1.6 Anti-Oxidant properties 10

2.1.7 Antimicrobial properties 11

2.1.8 Anticancer properties 12

2.1.9 Antitoxic properties 12

2.2 Tinospora cordifolia 13

2.2.1 Introduction 13

2.2.2 Habitat and Distribution 13

2.2.3 Botanical Description 13

2.2.4 Phytochemistry 14

2.2.5 Traditional and Ethnobotanical value 14

2.2.7 Antioxidant properties 15

2.2.6 Antimicrobial properties 15

2.2.8 Anti cancer properties 16

2.2.9 Anti toxic properties 16

2.3 Datura stramonium 16

2.3.1 Introduction 16

2.3.2 Habitat and distribution 17

2.3.3 Botanical description 17

2.3.4 Phytochemistry 18

2.3.5 Traditional and Ethnomedical value 18

2.3.6 Antioxidant properties 19

2.3.7 Antimicrobial properties 19

2.3.8 Anticancer properties 20

2.3.9 Antitoxic properties 20

Chapter III: MATERIALS AND METHOD **22-31**

3.1 Setting of Laboratory 22

3.2 Plant materials 22

3.2.1 Selection of plant materials 22

3.2.2 Collection and identification of the plant material 22
3.3 Chemicals and Equipment 23
3.4 Extraction 23
3.5 Phytochemical Screening 24
3.5.1 Detection of Alkaloids 24
3.5.1.1 Mayer Test 24
3.5.1.2 Wagner Test 24
3.5.2 Detection of Carbohydrates 24
3.5.2.1 Benedict's Test 24
3.5.2.2 Molisch's Test 24
3.5.3 Test for Proteins 24
3.5.3.1 Xanthoproteic Test 25
3.5.3.2 Biuret Test 25
3.5.4 Test for Phenols 25
3.5.4.1 Ferric Chloride Test 25
3.5.5 Test for Tannins 25
3.5.6 Resins Test 25
3.5.7 Test for Flavonoids 25
3.5.7.1 Shinoda Test 25
3.5.8 Test for Saponins 25
3.5.9 Test for Glycosides 25
3.5.9.1 Keller-Kilani Test 25
3.5.10 Test for Terpenoid 26
3.5.10.1 Libermann Burchard Test 26
3.5.10.2 Salkowski's Test 26
3.6 Determination of Total Flavonoid Content 26
3.7 Determination of Total Polyphenol Content 26
3.8 Determination of Antioxidant Activity 27
3.8.1 Measurement of DPPH Free Radical Scavenging Activity 27

3.9 Antimicrpbial Screening 28

3.9.1 Microorganism 28

3.9.2 Preparation of the standard culture Inoculums 28

3.9.3 Preparation of Extract 29

3.9.4 Antimicrobial Screening via Agar well Diffusion Technique 29

3.10 Biological Asssay 29

3.10.1 Animal 30

3.10.2 Peritoneal Macrophage isolation and culture 30

3.10.3 Cancer cell line 30

3.10.4 Cytotoxic Effect of extract on Hela cells and Peritoneal Macrophages 30

3.10.4.1 MTT Assay 30

3.11 Statistical Analysis: 31

CHAPTER IV: RESULTS **32-49**

4.1 Yield of Extracts 32

4.2 Phytochemical Screening 33

4.3 Determination of Total Flavonoid Content 37

4.4 Determination of Total Phenol Content 39

4.5 Estimation of Antioxidant Activity of the Plant Extract 40

4.5.1 DPPH free radical scavaanging activity 40

4.6 Antimicrobial Activity 42

4.6.1 Antibacterial Activity 42

4.6.2 Antifungal Activity 43

4.7 Cytotoxicity screening for Peritoneal Macrophages 44

4.7.1 Tinospora cordifolia 45

4.7.2 Reum austral 45

4.7.3 Datura stramonium 46

4.8 Cytotoxicity screening for Hela cells 46

4.8.1 Standard drug(5-Flurouracil) 47

4.8.2Tinospora cordifolia 47

4.8.3 Reum austral 48

4.8.4 Datura stramonium 48

4.8.5 IC_{50} value of Standard Drug(5-Flurouracil)and crude fraction of plant extract. 49

Chapter V: DISCUSSION **50-56**

5.1 Yield of Plant Extract 50

5.2 Phytochemical Screening 50

5.3 Total flavonoid Estimation 51

5.4 Total Polyphenol Estimation 52

5.5 Free Radical Scavenging Assay 52

5.6 Antimicrobial Activity 53

5.7 Cytotoxicity against Macrophages 54

5.8 Cytotoxicity against HeLa cell lines 54

Chapter VI: SUMMARY **57-58**

CHAPTER VII: CONCLUSION **59-60**

CHAPTERVIII: RECOMMENDATION **61**

References

Appendices

Photographs

LIST OF TABLES

Table 2 : Detailed description of each plant and their pharmacological properties.

Table 1.3 : Some pharmaceutical Drugs derived from ethnomedical lead.

Table 3.2.2 : Name, Collection site and Parts of plants used.

Table 4.1.1 : Percentage Yield and Physical characteristics of the crude extract of Methanol, chloroform and Hexane fraction.

Table 4.2.1 : Phytochemical screening of Methanol, Chloroform and Hexane fraction of Reum australe

Table 4.2.2 : Phytochemical screening of Methanol, Chloroform and Hexane fraction of Tinospora cordifolia

Table 4.2.3 : Phytochemical screening of Methanol, Chloroform and Hexane fraction of Datura stramonium

Table 4.6.1 : Antibacterial activity of different fraction of Plant extract.

List of figures

Fig 1.6.1 : Flow chart showing the experimental design during research work

Fig4.3.1 : Standard graph of the Quercetin

Fig4.3.2 : Total Flavonoid content in three fraction of each plant species.

Fig 4.4 : Standard graph of Gallic acid for the determination of Total Phenol content

Fig 4.4.2 : Estimation of Total Polyphenol Content in three fraction of each plant extract.

Fig 4.5.1 : Standard graph of %RSA of Ascorbic acid.

Fig4.5.2 : % Radical Scavenging Activity of three fraction of each plant extract.

Fig 4.5.3 : IC_{50} values of standard compound (Ascorbic acid) and fraction of Plant samples

Fig 4.7.1 : Cytotoxic effect of crude fraction of *Tinospora cordifolia* extract on peritoneal Macrophages

Fig 4.7.2 : Cytotoxic effect of crude fraction of *Reum austral* extract on Peritoneal Macrophages

Fig 4.7.3 : Cytotoxic effect of crude fraction of *Datura stramonium* extract o Peritoneal macrophages

Fig4.8.1 : %inhibition of standard drug(5-Flurouracil) on Hela cell lines

Fig 4.8.2 : Cytotoxic effect of crude fraction of *Tinospora cordifolia* on Hela cells

Fig 4.8.3 : Cytotoxic effect of crude fraction of *Reum australe* on Hela cells

Fig 4.8.4 : Cytotoxic effect of crude fraction of *Datura stramonium* on Hela cells

Fig 4.7.5 : IC_{50} of standard drug(5-Flurouracil) and crude fraction of Plant extract

ABSTRACT

Plants and plant-based products are the bases of many of the modern pharmaceuticals as well as traditional health care system we use today for various ailments especially the country like Nepal. Keeping therefore in view, the growing interest of the use of herbal drugs, the present study was undertaken with a view to evaluate the phytochemical and pharmacological activities of the stem of *Rheum australe* and *Tinospora cordifolia* and fruit part of *Datura stramonium*.

In this research plants were subjected to both chemical and biological assays. The results of phytochemical screening showed presence of all the necessary primary and secondary metabolites except tannins and resins. The total flavonoid content in crude methanolic extract of *T.cordifolia* was found to be highest i.e. 9.350±0.286 mg QE/g. Similarly the highest phenol content was also found about 39.2±2.173mg/g in methanolic extract of *T.cordifolia.* The free radical-scavenging activity of the extracts was tested through DPPH-method and the results were compared with Ascorbic acid. During our experiment, it was noted that the IC_{5o} value of the standard antioxidant Ascorbic acid was found to be 41.69±2.309 and the extracts exhibited a concentration-dependent antiradical activity by inhibiting DPPH- radical. Of the different extracts, crude methanol extract of *Tinospora cordifolia* exhibited the highest free radical scavenging activity of 87.5% followed by the methanolic extract of *Datura stramonium*(85.39%) and *Rheum austral*e(80.67%). While the hexane and the chloroform crude extract exhibited moderate level free radical scavenging activity. Crude Methanolic and chloroform extract of all the three plant demonstrated the significant activity on *Klebsiella pneumonia* and *Staphylococcus aureus* respectively as well as on *Saccharomyces cerevisae*. In our investigation, the extracts that were obtained demonstrated very nominal cytotoxic effect on the macrophage cells at a given concentration of 0-300µg/ml using MTT assay signifying that plant extract render no risk to the normal cell lines but provide platform to study cytotoxicity against Hela cell lines. The crude extract of *T.cordifolia* exhibited the pronounced effect on Hela cells in dose dependent manner especially the methanolic extract. Methanolic extract of *T. cordifolia* demonstrated the significant cytotoxicity (89.79±2.908) and lowest IC_{50} value(50.754±5.776). Lower the IC_{50} value higher the cytotoxicity effect. These findings suggest that medicinal plants taken under study show antibacterial and cytotoxic activity and show promising future for further researches.

Key words: Medicinal plants, Phytochemical screening, Antioxidant, DPPH, Flavonoid, Phenol, Cytotoxicity.

CHAPTER I: INTRODUCTION

1.1 Background

The relationship existing between plants and humans is as old as mankind, dating back to the origin of human civilization. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been derived from natural sources, many of these isolations were based on the uses of the agents in traditional medicine plants, making ethnobotany an interesting and important research field.(RATES, 2001).

The term ethnobotany was coined by John W. Harsberger (1896) Davis. EW (1995) and was considered as the art of collection of useful plants by a group of people and the description of the uses of plants. Over the last century, ethnobotany has evolved into a scientific discipline that focuses on the people-plant relationship in a multidisciplinary manner, incorporating not only collection and documentation of indigenous uses but also ecology, economy pharmacology, public health, and other disciplines. Ethnomedicine, a branch of ethnobotany, is a set of empirical local practices embedded in the indigenous knowledge of a social group often transmitted orally from generation to generation with intent to understand social, cultural, and economic factors influencing health problems and to overcome such problems. (Bhattarai, 1992). Herbal medicine also known botanical medicine or phytomedicine was prime healthcare system during the twentieth century when antibiotics or analgesics were not as yet discovered. However with the advent of allopathic system of medicine, herbal medicine gradually lost its popularity among people. Recently there has been a shift in universal trend from synthetic to herbal medicine, which can be said “Return to Nature”. Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments(Singh, 2007).

1.2 Plants as a source of secondary metabolites

Plants contain many active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids which are deposited in their specific parts such as leaves, flowers, bark, seeds, fruits, root, etc. The beneficial medicinal effects of plant materials typically result from the combination of these secondary products (Tonthubthimthong et al.,2001). These secondary products are known to possess antioxidant (Wong et al., 2009), antibacterial (Nair et al., 2005), antifungal (Khan and Wassilew, 1987), antidiabetic (Singh and Gupta, 2007, Kumar et al., 2008), anti-inflammatory, antiarthritic (Kumar et al., 2008), and radio-protective activity (Jagetia et al.,

2005). They are widely used in the human therapy, veterinary, agriculture, scientific research and countless other areas (Vasu .et al., 2006).

1.3 Medicinal plants as a source of drugs

The plants have been used for treatment of many ailments from time immemorial. Since the plants possess many more secondary metabolites, drugs from the plants can be easily derived with minimal negative effect, safe, less expensive and efficient such as anticancer drugs (Dewick,1996), antimicrobial drugs (Phillipson, Wright,1996).

According to World Health Organization (WHO), medicinal plants would be the best source to obtain variety of drugs. The WHO estimates that a minimum of 20,000 plants taxa has recorded medicinal uses all over the world. It is estimated that up to 70,000 plant species are used in folk medicine and a majority of these species are found in the Asia-Pacific region. Out of 255 drugs which are considered as basic and essential by the World Health Organization (WHO), 11% are obtained from plants. However medicinal properties of such plants have to be investigated and tested in order to authenticate its potentiality and safety. (Arunkumar, Muthuselvam, 2009). About 50 drugs have been discovered from ethnobotanical leads by translating folk knowledge into new pharmaceutical (Cox, 1994). The figures published vary in different reports and it must be much more by now. The names, sources of the plant and clinical uses of the selected drugs obtained from the plants are given in the following table.

Table 1.3: Some pharmaceutical Drugs derived from ethnomedical lead

Drugs	**Plants sources**	**Clicnical properties**
Atropine	*Atropa belladonna*	Anticholinergic
Berberine	*Berberis vulgaris*	Bacillary dysentery
Camptothecin	*Camptotheca acuminate*	Antitumor agent
Caffeine	*Camellia sinensis*	CNS stimulant
Cocaine	*Erythroxylum coca*	Local anaesthetic
Codeine, Morphine	*Papaver somniferum*	Analgesic, antitussive
Digitoxin	*Digitalis purpurea*	Cardiotonic
Emetine	*Cephaelis ipecacuanha*	Amoebicide, emetic
Ephedrine	*Ephedra sinica*	Sympathomimetic, antihistamine
Etoposide, Podophyllotoxin	*Podophyllum peltatum*	Antitumor agent
Galanthamine	*Lycoris squamigera*	Cholinesterase inhibitor
Hyoscyamine	*Hyoscyamus niger*	Anticholinergic
Menthol	*Mentha species*	Rubefacien
Quinine	*Cinchona ledgeriana*	Antimalarial, antipyretic

1.4 Economic benefits from medicinal plants

Medicinal plants offer alternative remedies with tremendous opportunities. They not only provide access and affordable medicine to poor people, they can also generate income, employment and foreign exchange for developing countries. Many traditional healing herbs and plant parts have been shown to have medicinal value, especially in the rural areas and that these can be used to prevent, alleviate or cure several human diseases. According to WHO (2008), 80% of the population in some Asian and African countries depend on traditional medicine for primary healthcare. In the last two decades, the developed and developing countries have witnessed a significant rise in the demand for herbal medicines with global sales estimated at US$ 60 billion WHO, 2003). Herbal treatment has become 6 lucrative in the international market generating US$ 14 billion for China in 2005 and US$ 160 million for Brazil in 2007. In addition, countries like Germany, Japan and Turkey have

contributed significantly to the economic boom in the phytomedical market (LAIRD *et al.*, 2003). The global market for the medicinal plants and herbal medicine is estimated to be worth US$800 billion a year (Rajasekharan and Ganesha,2002).

1.5 Scenario of Herbal medicine in Nepal

Nepal, a country with disproportionately rich cultural and ethnic diversity, is located between the latitudes 26° 22'-30° 27' N and longitudes 80° 40'-88' including endemic Himalayan flora and fauna. Its area, 147,181 km^2, represents just 0.1% of the global land surface. Remarkably, however, it claims over 2.04% of the world's flowering plant. Due to altitudinal and climatic variations, there are almost all types of climatic zones. There is a striking vertical zonation in natural vegetation and diversity in flora, with 118 ecosystems, comprising 75 vegetation types, and 35 forest types (MFSC, 2006).

It has been estimated that the Himalayan region harbors about 12,000 species of medicinal and aromatic plants, supporting the livelihood of about 600 million people living in the area (Pie Shengji, 2001). The flora of the Nepal Himalaya contains 10,167 plant species, of which over 7,000 are flowering plants and over 1,600 species are medicinal and aromatic herbs. The herbs, representing about 25% of the total country's vascular flora, are used under different traditional systems including the *Ayurveda*, Homeopathic, Home herbal (folklore) and *Amchi* (traditional Tibetan medicine) medicinal systems (Bhattarai, 1997). Thirty percent medicinal plant species of the country occur in the western part of the country and about 50% of the plants used as ethno-medicine in Nepal Himalaya have been documented. The management of medicinal and aromatic plants and knowledge of utilization of the resources therefore is of great importance and it can be promoted by considering and documenting the diversity of the medicinal plant resources and their indigenous knowledge of utilization. (Kunwar et al.,2006).

It is irony that people living in rural areas of Nepal are in deprived state. Still they have not access to modern facility of health system. They are bound to rely on age old practice, traditional healer leading to the death of large no of people. Thus, if we able to harness the medicinal plants and exploit their medicinal property, we not only enhance the health status of people but economic activities of country.

1.6 Research plan

1.6.1 Hypothesis

- Medicinal herbs have found to treat various ailments from biblical times. Thus they may possess active compound that can counter act various diseases.
- These active compounds can be evaluated and quantified by performing different bioassay.

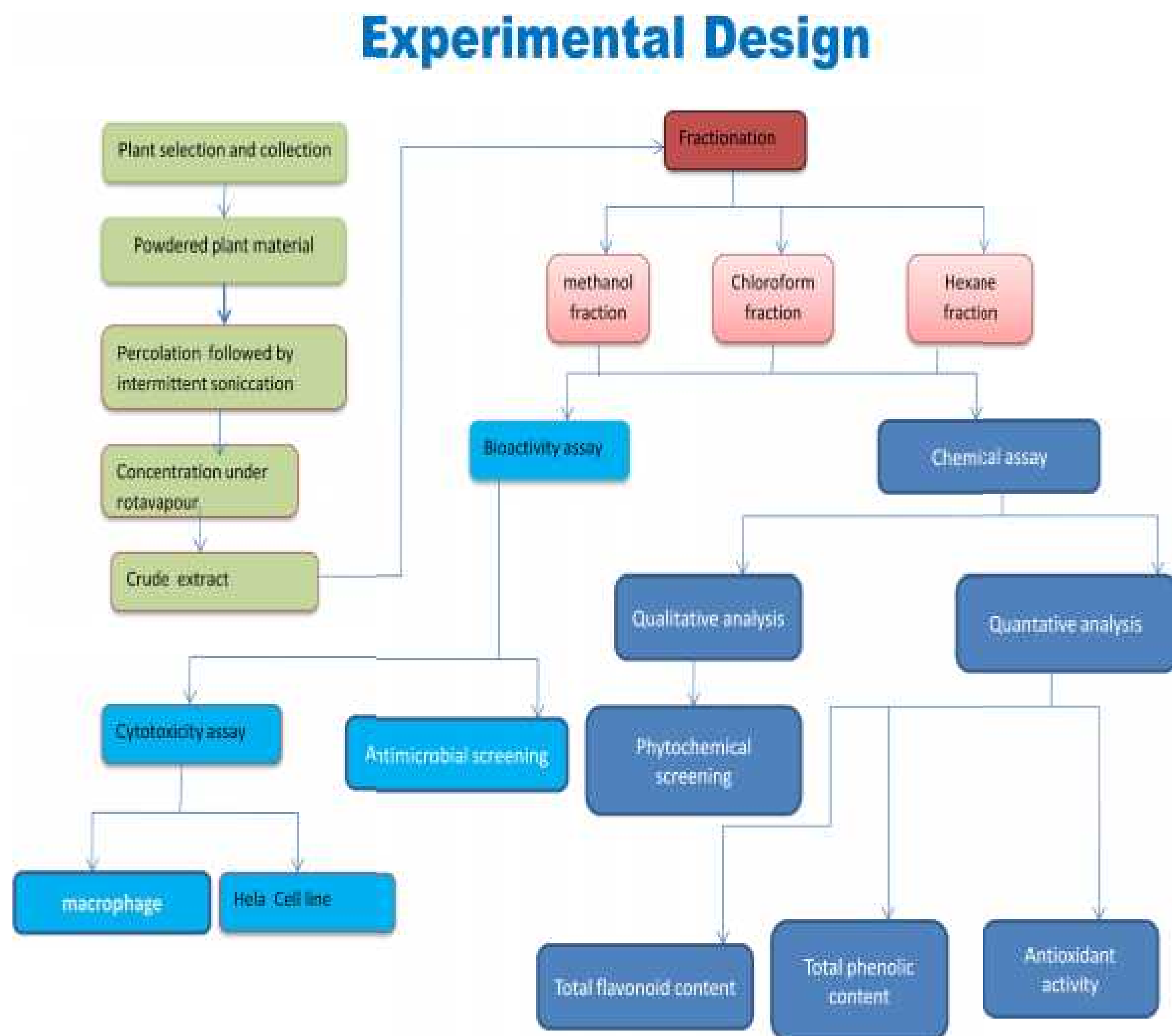


Fig 1.6.1: Flow chart showing the experimental design during research work.

1.7 Objectives

1.7.1 General objectives

The overall goal of this research is to check the presence of secondary metabolites in the chosen medicinal plants and observe cytotoxic effects of their various fractions on macrophages and cervical cancer cell lines (HeLa).

1.7.2 Specific objectives

1. Assement of phytochemical screening.
2. To quantify the flavonoid and phenolic content of various fraction of the chosen plants.
3. To evaluate the in vitro and in vivo antioxidant of various fraction of chosen medicinal plants.
4. To detect the antibacterial and antifungal activity of selected plant.
5. To observe and evaluate the cytotoxic effect on macrophages of selected medicinal plants
6. To assess the efficacy of selected medicinal plants on cervical cancer cell lines (HeLa).

1.8 Rationale

From time immemorial, humankind is utilizing plant source to alleviate or cure illnesses. Medicinal plants are the best sources for the antibacterial as well as antioxidant compounds. The extensive use of antibiotics against the microorganism has raised the global concern about drugs resistance against the pathogenic microorganism. Thus it is necessary to screen the possible medicinal plant that possesses the inhibitory action against the pathogenic microorganism. ROS with chemical species such as super oxide anions (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl free radicals (OH) which is known to induce damage to biomembranes, proteins, DNA, pathogenesis of human diseases such as cancer, aging, inflammatory response syndrome, respiratory diseases, liver diseases and atherosclerosis. Plants phenolics, in particular phenolic acid, tannins and flavonoids are known to be potent antioxidant and display a vast variety of pharmacological activities such as anti-inflammatory, anti-carcinogenics, antibacterial or antiviral and anticancer activities. (Diniset et al.,2002).

Cancer is one of the leading causes of death in the world. Although various chemical and radiotherapy treatment are available for the cancer but it does not alleviate or cure the disease. It rather reduces its proliferation. So the hunt for the novel anti cancer compound can lead to the discovery of innovative drugs that can completely treat the cancer.

Epidemiological studies have indicated the relationship between flavonoid intake and reduced risk of certain cancers (Lopez-Otin & Diamandis, 1998; Middleton et al.,2000).

1.9 Scope of the study

It is crystal clear that medicinal plants are known to possess the various secondary metabolites which are very fruitful in assessing the treatment of various ailments. Thus these herbal extract can be boon for the discovery of innovative drugs model. However its efficacy and specificity has to test.

CHAPTER II: LITERATURE REVIEW

Nature has been endowed with immense number of medicinal agents and an impressive number of modern drugs have been derived from natural sources, many of these isolations were based on the uses of the agents in traditional medicine (Cragg and Newman 2001). Herbal medicines are an essential and growing part of the international pharmacopeia. Knowledge of their medicinal properties is growing as a result of research and testing, which will make them an increasingly safe alternative or preferred option to allopathic medicine. In present scenario, people prefer the traditional medicine and plant based drugs. These revivals of interest in plant-derived drugs are mainly due to beneficial attributes such as cost effective, safety, efficacy than synthetic drugs having many more adverse consequences (Parekh and Chanda, 2007). There is a growing interest in correlating phytochemical constituents of a plant with its pharmacological activity. Such as antimicrobial, antidiabetic, anti-inflammatory, hepato-protective, anti-cancer etc. Scientists have even started correlating the botanical properties of plants with their pharmacological activity (Rawat et al. 1997). In future, more co-ordinated multidimensional research aimed at correlating botanical and phytochemical properties to specific pharmacological activities is expected (Dahanukar et al., 2000). Therefore, these scientific investigations may be utilized to develop drug for diseases as well as isolate the desired compounds responsible for the observed biological activity.

Table II: Detailed description of each plant and their pharmacological properties are given below.

Botanical name	Taxonomy				
	Division	Class	Order	Family	Vernacular name
R. australe	Magnoliophyta	___	Caryophyllales	Polygonaceae	Padamchal, Chulthi
T. cordifolia	Magnoliophyta	Magnoliopsida	Ranunculales	Menispermaceae	Gurjo,Guduchi,
D. stramonium	Magnoliophyta	Magnoliopsida	Solanales	Solanaceae	Dhaturo, Madak Dhattur,

2.1 Rheum australe

2.1.1 Introduction

Rheum australe D. Don (Synonym: *Rheum australe* Wall. ex Meisn.) is a himalayan plant known by various vernacular name. Aakchhyo, Atchowa, Chyurcha(Sherpa), Akase chuk, Padamchal, Chulthi amilo, Shankhatra (Nepali), Akcyowa, Rheuchini(Solukhumbu) Amalaparni, Amlavetasa, Pitamulika(Sanskrit) Amlaparni, Pitamuli, Gandhini Revatikaa. Revandachini (Ayurvedic) Indian Rhubarb, Himalayan Rhubarb, Red-Veined Pie Plant (English). It is an important medicinal plant, which finds an extensive use in Ayurvedic and Unani systems of medicine (Manandhar, 2002).

2.1.2 Habitat and distribution

The genus *Rheum* consists of approximately 60 perennial species distributed around the world and belongs to the family Polygonaceae (Li et al., 2003). There are seven species of *Rheum* in Nepal (Press et al., 2000). It is the Himalayan species, found wild at an altitude of 2000-3800m in Kashmir, Nepal, Sikkim, Bhutan and China. It is found in the alpine zone on rocky soil, moraines and crevices, between boulders and near streams in specific pockets. It is restricted to the temperate, sub-alpine, and alpine zones of the Himalayas. Well drained, porous, humus-rich soil is suitable for its cultivation. It prefers exposed or partially shaded habitat and can be cultivated at altitudes above 1800 m. (Press et al., 2000)

2.1.3 Botanical description

Rheum is a perennial plant that grows from thick short rhizomes. The plants have large leaves that are somewhat triangular shaped with long fleshy petioles. The flowers are small, greenish-white to rose-red, and grouped in large compound leafy inflorescences. The inflorescence is large, fastigiately branched and densely papilliferous. The flowers are pedicellate and dark purple. The perianth is spreading, 3–3.5 mm; the three outer parts of the perianth are smaller and oblong-elliptic. The filaments are subulate. The ovary is rhomboid-obovoid, and the stigma is oblate and muricate. The fruit is ovoid-ellipsoid, broadly ellipsoid or ovoid-oblong in shape, large (0.5–1.5 cm), long and purple, with wings more narrow than thick and notched at both ends. It flowers from June to August and fruits from July to September ((Li et al., 2003) Rheum is a perennial plant that grows from thick short rhizomes. The plants have large leaves that are somewhat triangular shaped with long fleshy petioles. The flowers are small, greenish-white to rose-red, and grouped in large compound leafy inflorescences. The inflorescence is large, fastigiately branched and densely papilliferous. The flowers are pedicellate and dark purple

2.1.4 Phytochemistry

The major phytoconstituents reported to have been isolated from the rhizomes are: free anthraquinones and their glycosides. stilbenes anthrones, chromones, oxantrone ethers esters, flavonoids carbohydrates, lignans, phenols and sterols. The frequently occurring active constituents of *Rheum australe* are anthraquinone derivatives. The anthraquinones, both with and without carboxyl groups are found in *Rheum australe*. Anthraquinones with carboxyl group include rhein, while those without carboxyl group include chrysophanol, aloe-emodin, emodin, physcion (emodin monomethylether), chrysophanein and emodin glycoside (Malik et al., 2010). Several complex compounds have also been isolated,including torachrysone-8-O-β_-d-glucopyranoside, sulphated emodin glucoside, acetylated chrysophanol glucoside ,6-methyl rhein ,6-methyl aloe-emodin, oxanthrone ether (revandchinone-4), oxanthrone esters (revandchinone-1 and revandchinone-2), and revandchinone-3. Other compounds, namely, naphthoquinones, rutin, rheinal, rhein 11-O-b-D-glucoside, torachrysone 8-O-b-D-glucoside, epicatechin, auronols (carpusin and maesopsin), the sulfated anthraquinone glycoside sulfemodin 8-O-b-D-glucoside (Krenn et al., 2003).

2.1.5 Traditional and ethnomedicinal value

The roots of *Rheum* species are traditionally used for blood purification and in the treatment of body pain, broken or fractured bones, chest pain, cold and cough, fever, headache, indigestion, intestinal worms, menstruation problems, sprain, stomach ache, constipation and swelling. The roots are also used to treat injured animals and in herbal baths. (Rokaya et al., 2010). Dyes for cosmetics, textiles (e.g. wool/ silk), wooden materials and food colorants are also extracted from Rheum roots. While spices and condiments are derived from the petioles. Juices from the shoot portion of the plant are taken for cold & cough chest pain, diarrhea, dysentery and swelling. Leaves and petiole are considered antihelminthic and appetizer. (Kunwar et al., 2006).

2.1.6 Anti oxidant effect

Oxidative stress is one of the major challenges responsible for the development and progression of certain life-threatening diseases and disorders like cancer, diabetes, hyperlipidaemia, neuronal degeneration and hepatotoxicity. Antioxidants from plant sources may be fruitful in their prevention and treatment. Since antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) possess certain threat due to their potent toxicity and DNA damage capacity, natural antioxidant from plant sources have received considerable attention because of their efficacy and safety in biological systems.

Rheum has been reported to possess protective effect in many inflammatory diseases and oxidative stress-related injuries. Methanolic and aqueous extracts of the roots of *R. australe* are reported to possess antioxidant potential (Rajkumar et al.,2010). The compounds like marsupsin and maesopsin obtained from the rhizome/root extracts of *R. australe* are found to possess antioxidant activity (Krenn et al., 2003). The most abundant stilbenoid piceatannol-4_-O-*β*-D-glucopyranoside (PICG) and its aglycon piceatannol (PICE) isolated from *R. emodi* rhizome displayed promising antioxidant activity in all the four assays i.e DPPH and superoxide anion radical scavenging, ferric reducing power, and inhibition of lipid peroxidation *in vitro..* The PICE also demonstrated the antioxidant activity at the cellular level on the model of hydrogen-peroxide-induced H9c2 rat cardiomyoblasts injury **(**Yuan-yuan Chai, 2012).

2.1.7 Antimicrobial effect

Many of the bacterial and fungal strains are found to be resistant against a wide variety of antibiotics. Thus it is necessary to study potential effect of medicinal plants on various microorganisms. Aloe-emodin, rhein and emodin obtained from commercial rheum possess significant antibacterial activity against four strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and also a strain of methicillin-sensitive *Staphylococcus aureus* (MSSA). Revandchinone-1 and 3 have shown only moderate antibacterial activity. Revandchinone-4 has been found to possess good antibacterial properties against some Gram-positive bacteria *(Bacillus subtilis, Bacillus sphaericus and Staphylococcus aureus)* using penicillin G as control, and Gram-negative bacteria *(Klebsiella aerogenes, Chromobacterium violaceum and Pseudomonas aeruginosa)* using streptomycin as control. Revandchinone-1, 3 and 4 also exhibit a moderate degree of antifungal activity against *Rhizopus oryzae* and *Aspergillus niger* using clotrimazole as control (Babu et al., 2003). Ethanolic extracts of Rheum australe rhizomes has shown to inhibit the growth of different filamentous fungi and bacteria such as *Shigella dysenteriae, Staphylococcus aureus, Salmonella typhimurium, Salmonella paratyphi, Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Fusarium chlamydosporum, Trichoderma viride, Rhizoctonia bataticola, Aspergillus niger* and *Alternaria alternate*(Aqil and Ahmad,2003).

2.1.8 Anticancer potential

A number of studies have shown that the main anthraquinones of *Rheum*, such as emodin, aloe-emodin, and rhein could inhibit the growth and the proliferation of various cancer cells. Emodin has found to suppress the active multiplication and proliferation of ovarian, breast, lung, liver, and prostate cancer cells. Emodin alone has the ability to induce the apoptosis by down-regulating the expression of macrophage migration inhibitory factor (MIF), matrix metalloproteinase-2 (MMP-2), and matrix metalloproteinase-9 (MMP-9) in ovarian cancer cells (Li, 2009). Moreover, emodin induced the apoptotic death in murine leukemia WEHI-3 cells and enhanced the phagocytosis in the leukemia animal model (Chang *et al.*, 2011). Emodin, is also capable of inhibiting cellular proliferation, inducing apoptosis, and preventing metastasis and these capabilities seem to act through tyrosine kinases, phosphoinositol 3-kinase (PI3K), protein kinase C (PKC), NF-kappa B (NF-κB), and mitogen-activated protein kinase (MAPK) signaling cascades (Huang,2006). The anticancer effect of aloe-emodin has been established in two human cancer cell lines, Hep G2 and Hep 3B. Aloe-emodin inhibited cell proliferation and induced apoptosis in both examined cell lines by different antiproliferative mechanisms (Kuo, Lin & Lin, 2003). Rhein, another major rhubarb anthraquinone, effectively inhibited the uptake of glucose in tumor cells, determining changes in membrane-associated functions, and led to cell death. Rhein lysinate has been reported to suppress the proliferation of ovarian cancer (SKOV-3), breast cancer cells (MCF-7M, SK-Br-3, and MDA-MB-231), phosphorylation of epidermal growth factor receptor and extracellular signal-regulated kinase (MEK and ERK) with or without epidermal growth factors (EGF) stimulation (Liu YJ, 2009). Methanolic and aqueous extracts of the Rheum australe rhizome has shown to induce concentration dependent cytotoxicity of human breast carcinoma (MDA-MB-435S) and liver carcinoma (Hep3B) cell lines due to the apoptosis of targeted cancerous cells (Rajkumar et al., 2011).

2.1.9 Antitoxic effect

The total tannin obtained from total Rheum extract showed the protective action of kidney function caused by chromium nephrotoxicity in rats. Total tannin also showed significant activity to scavange the hydroxyl radical which is considered to the dominant lesion product generated by hexavalent chromium. It has shown the ability to precipitate the metal ion. (Ling-na Zeng, 2013). α -Naphthylisothiocyanate is a toxicant that targets the bile ducts and causes an intrahepatic cholestasis that models human chronic cholangitic diseases. The intragastrical administration of rhein, aloe-emodin and physione to α-naphthylisothiocyanate-treated rats reduced significantly the serum level of both glutamate-pyruvate transaminase, glutamic oxaloacetic transaminase and the serum total

bilirubin, direct bilirubin, alkaline phosphatase, γ-glutamyltransferase and total bile acid which are the hepatic biochemical markers of cholestasis (Yan-Ling Zhao, 2009). The methanolic extract of rhizome of *Rheum australe* displayed mild yeast as well as mammalian intestinal a-glucosidase inhibitory activity. Besides Rhapontigenin, desoxyrhapontigenin, chrysophanol-8-O–β-D-glucopyranoside, torachrysone-8-O-β-D-glucopyranoside obtained from further fractionation of Rheum australe extract exhibited potent yeast a-glucosidase inhibition.(Babu,2004). Wang J *et al* (2009) assessed the value and toxic potential of rhubarb to treat the chronic renal failure (CRF), and the result indicated that there was an evidence of protective effect to CRF rats.

2.2 Tinospora cordifolia

2.2.1 Introduction

Tinospora cordifolia is one of the most important medicinal plants and occupy an important place in ayurveda system of medicine. It is known by different name such as gurjo (nepali), guduchi/amrita (Sanskrit), gulancha Tinospara(English), Shindilkodi (Tamil). Guduchi, its Sanskrit name, means one which protects the entire body. The term Amrita is attributed to its ability to impart youthfulness, vitality and longevity to the consumer (Sivarajan, Balachandean,1999). In Ayurvedic system of medicine it is designated as Rasayana drug recommended to enhance general body resistance, immune function, promote longivity and the body resistance against infection.

2.2.2 Habitat and distribution

T. cordifolia is a climbing shrub native to lower elevation in tropical areas and sub tropical regions up to an altitude of 300 meter. It prefers wide range of soil, acid to alkaline and it needs moderate level of soil moisture. The climber is found in scrub jungles, fences. It is indigenous to areas of India, Myanmar, Sri Lanka, Nepal China, Thailand, Philippines, Indonesia, Malaysia, Borneo, Vietnam, Bangladesh, North Africa, West Africa, and South Africa (Pendse, 1981; Singh, 2003)

2.2.3 Botanical description

T. cordifolia belongs to the family Menispermaceae which consists of about 70 genera and 450 species that are found in tropical lowland regions. They are generally climbing or twining, rarely shrubs. (Wealth of India 2003; Aima, 2003). It is a perennial deciduous twiner

with succulent stem. The bark is papery, creamy white to gray in appearence with large rosettelike lenticels. Leaves are simple, alternate or lobed, cordate, entire, 7-9 nerved; flowers are small cymose, yellow or greenish colour. Male and female flowers are formed on separate branches (Sinha,2004). The male flowers are small, yellow or green in colour, and occur in clusters in the axils of small subulate bracts. Sepals are 6, 3 outer very small, ovate-oblong, acute, the inner 3 larger, membranous, broadly elliptical, concave, yellow Petals are 6, equal, broadly spathulate, each loosely embracing a stamen, claw cuneate, reflexed to apex, pistillode. Female flowers usually solitary, similar to male, but sepals green, margins not reflexed, staminode short, linear. Carpels 1-3, widely separated on the short fleshy gynophores, dorsally convexed, and scarlet. The fruit are the size and shape of a large pea and turn from green to red when ripe in winter and mucilaginous (Kirtikar and Basu, 2005).

2.2.4 Phytochemistry

A diverse variety of compound have been isolated from *Tinospora cordifolia* plant and their structures were elucidated. They belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids,sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. The stem parts of the plant bear the alkaloid(Berberine, palmatine D, choline D, tinosporine, Magnoflorine, tetrahydropalmatine isocolumbin 18-norclerodane glycoside) (Padhya,1986), glycoside(Furanoid diterpene glycoside, Tinocordiside Syringin Syringin-apiosylglycoside, Tinocordifolioside, cordioside, cordifolioside A, cordifolioside B, palmatoside C31, palmatoside F31, cordiofoliside B2, cordifoliside) (Khan,1989) and sesquiterpenoid (Tinocordifolin) (Maurya and Hardass,1998). Various miscellaneous compound have also been reported in *T.cordifolia* such Nonacosan-15-one (a,4-dihydroxy-3-methoxy-benzyl)-4-(4-hydroxy-3-methoxy-benzyl)-tetrahydrofuran, Tinosponidine, cordifol, Cordifelone, Jatrorrhizine (Khaleque,1971).

2.2.5 Traditional and ethnobotanial uses

In traditional medicine, whole plant, root powdered, stem bark, decoction of root and stem, juice of the root, juice or paste of the leaves, and stem of the *T. cordifolia* are being used to treat various ailments such as Urinary diseases, syphilis, skin diseases, bronchitis (Treadway, 1998), vermifuge, jaundice, intestinal worms, sore eyes, syphilitic sores, antipyretic, antimalarial, chronic diarrhea, some form of obstinate chronic dysentery, intestinal problems diabetes, vaginal, urethral discharges,low fevers and enlarged spleen.(Kirtikar and Basu,1918). In ayurveda *Tinospora* has been described as gudchi (plant which protects from diseases). *T. cordifolia* has been described in ancient textbooks of Ayurveda including

Sushrut Samhita and Charak Samhita. Other synonyms used for Guduchi which refer to its various properties and uses, include Chhinnaruha/Chhinnodbhava (for the plant's capacity to grow from the cut side), Vatsadini (eaten by grazing animals),Pittaghni (bile destroying), Amruta (imparts immortality), Rasayana (capacity to improve quality of rasa, the primordial tissue which in turn strengthens all other tissues), Chakrangi/Chakra-akshana (for its wheel-like appearance), Jwaranashi/ Jwarari (potent antipyretic), Vayastha (prevents ageing), Amrutsambhava (ambrosia), Bhishakpriya (favourite of physicians), Saumya (not harmful) and Tikta (bitter taste).

2.2.6 Antioxidant property

T.cordifolia has been reported of its strong free radical scavenging properties against superoxide anion (O^{2-}) hydroxide radicals (OH^-), NO^- radicals and peroxynitrite anion ($ONOO^-$) (Rawal,2004). Methanolic extract of stem when administrated orally increased the erythrocytes, membrane lipid peroxidation and catalase activity (Stanely, Menon, 2003). *T.cordifolia* showed the ability to protect aflatoxin induced nephrotoxicity due to its ability to scavange the free radicals generated during aflatoxicosis. It is possible due to presence of alkaloid such choline, tinosporin, isocolumsbin, palmatine, palmatine, tetrahydropalmatine and magnoflorine. Oral administration of 2.5 and 5.0 g/body weight of aqueous extract of root of *T. cordifolia* resulted in a significant reduction in Thiobarbituric acid reactive substances(TBARS) and increased in reduced glutathione, catalase and superoxide dismutase in alloxan diabetic rats.(Stanely and Menon, 2001). Aqueous extract of *T.cordifolia* exhibited the protective role by preventing the radiation mediated 2-deoxyribose degradation by inhibiting the (Fe^{2+})-bipiridyl complex formation (Goel, 2002). The enhanced GSH level and enzyme activities involved in xenobiotic metabolism and maintaining anti-oxidant status of cells are suggestive of a chemo preventive efficacy of *T.cordifolia*.(Singh ,2006).

2.2.7 Antimicrobial properties

Extract of *Tinospora cordifolia* has shown significant activity against *Escherichia coil, Proteus vulgaris, Enterobacter faecalis, Salmonella typhi (Gram-negative), Staphylococcus aureus and Serratia marcesenses (Gram-positive).(*Jeyachandran, 2003) B*acillus cereus, Bacillus fusiformis, and Klebsiella pneumonia.* (Agnihotri, *2012).*

2.2.8 Anti cancer effect

The effect of hydroalcoholic extract of aerial roots of *Tinospora cordifolia* on swiss aqlbino mice revealed a significant increase in acid soluble sulfhydryl (-SH), cytochrome p(450) contents and enzyme activities of cytochrome p (450) reductase, cytochrome b5 reductase, GST, DT-diaphorase (DTD), SOD,catalase, GPX and GR activity in liver highlighting the chemopreventive role of T. cordifolia against carcinogenicity (Singh,2006). Intraperitonial administration of alcoholic extract of Tinospora has shown to slow down the growth of tumor growth and increase the life span of tumor bearing host. Thus demonstrating the anti tumor effect through destabilizing the membrane intregrity of Daltons lymphoma cells directly or indirectly (Singh, 2004). Similarly it has shown to up regulate the antitumor activity of tumor associated macrophage by enhancing the differentiation of tumor associated macrophages into the dendritic cells in response to granulocyte/macrophage colony stimulating factor, interleukin-4 and tumor necrosis factor. Dendritic cell in turn shows an enhanced tumor cytotoxity and production of tumoricidal soluble molecules like TNF, IL-1 and NO. (Singh,2005).

2.2.9 Anti toxic effects

T.cordifolia have found to demonstrate the protective activity by reducing the thiobarbituric acid reactive substances (TBARS) level and enhancing the anti oxidant enzymes like ascorbic acid, glutathione s transferase (GST) and glutathione reductase (GR) in kidney. Alkaloid compound such as chokine, tinosporin, isocolumbin, palmatine, tetrahydropalmatine and magnoflorine isolated from *Tinospora cordifolia* showed protection against aflatoxin-induced nephrotoxicity (Gupta,Sharma,2011). Stem and leaves extract have shown hepatoprotective effect in swiss albino male mice against lead nitrate induced toxicity and prevented the occurance of lead nitrate induced liver damage(Sharma, Panday, 2010).

2.3 Datura stramonium

2.3.1 Introduction

Datura stramonium is a widespread annual plant from the Solanaceae family. According the recent classifications, it has four varieties*, D. stramonium var. stramonium L. D. stramonium var. tatula L.,Torr., D. stramonium var. inermis Jacq. Timmerman* and *D. stramonium var. godronii Danert*, which had been considered for years by many botanists to be different species (Avery, 1959). It is one of the widely well known folklore medicinal herb. It is a wild

growing flowering plant. In Nepal, *D. stramonium* L. is commonly known as Dhaturo, Seto Dhaturo, Dhattur, and Madak. In Sanskrit language, it has several names such as Dhatturdhurtadhustur Unmatta, Kanakahwaya, Dewatakitawasturi Mahamohi Shivapriya, and Matulo Madanashchasya phale Matulaputraka. In Ayurvedic medicine, *D. stramonium* is described as a useful remedy for various human ailments including ulcers, wounds, inflammation, rheumatism and gout, sciatica, bruises and swellings, fever, asthma and bronchitis, toothache, etc (Kirtikar, Basu,1999). In the Hindu religion, the seed of *D. stramonium* is believed to be associated with the *God Shiva*, which can promote misuse of the plant on religious occasions, such as *Shivaratri* and *Swasthani Puja* (Gaire, 2008).

2.3.2 Habitat and distribution

Datura stramonium, the most common species within Solanaceae family, is native to Asia, but is also found in the United States, Canada, and the West Indies Central and South America, Europe Africa. It is widespread and cosmopolitan with higher abundance in temperate, tropical and subtropical regions. It is commonly found along riverbanks, roadsides and disturbed sites (Berkov, 2006). It is mainly distributed in the Himalayan region from Kashmir to Sikkim up to 2 700 m, in the hilly district of central and south India (Khare, 2007).

2.3.3 Botanical description

Datura stramonium L. (Family Solanaceae), also called Apple of Peru, Devil's Apple, Devil's Trumpet, Jamestown Weed, Mad-apple, Stinkweed or Thorn apple, is an erect, sub herbaceous annual up to 1.5 m high with dark green or purplish leaves which are usually paler below. The root is large, whitish in color, with a taproot system giving off many fibers. The branching stems are spreading and leafy, stout, erect, smooth, a pale yellowish green in color, branching repeatedly in a forked manner. Leaves are hairy, big, simple dentate, oval glabrous, apposite veins of leaves are pale black, stalked, 4-6 inch long, ovate and pale green. The upper surface is dark and grayish- green, generally smooth, the under surface paler, and when dry, minutely wrinkled. The flowers are ebracteate, ebracteolate, pedicellate, actinomorphic, bisexual, complete, regular, pentamerous, except fourth whorl and are hypogynou,with 5 stamens and superior ovary. The average length of flower is about 3 inches. The calyx is long, tubular and somewhat a swollen below and very sharply five angled surmounted by five sharp teeth. Corolla is funnel shaped. Each flower is

replaced by a hard fruit that is dry and spiny, and spheroid-ovoid in shape. These fruits are initially green, but become brown with maturity; they divide into four segments to release the seeds. The seeds are dull, irregular, and dark-colored; their surface may be pitted or slightly reticulated (Preissel and Preissel 2002; Das, 2012).

2.3.4 Phytochemistry

The major tropane alkaloids hyoscyamine and scopolamine and Sixty-four tropane alkaloids have been detected from *D. stramonium*. It is reported that the whole plant contains 0.26% alkaloids. The investigation on the production and distribution of hyoscyamine and scopolamine in *D.stromonium* in the different plant parts, at different stages of their life cycle showed the maximum contents were found in the stems and leaves of young plants, hyocyamine being always the predominant component. Alkaloids scopoline, 3-(hydroxyacetoxy) tropane, 3-hydroxy-6-(2-methylbutyryloxy) tropane, 3â-tigloyloxy-6-hydroxytropane, 3,7 dihydroxy-6-tigloyloxytropane, 3-tigloyloxy-6-propionyloxytropane, 3-phenylacetoxy-6,7-epoxytropane, 3-phenylacetoxy-6-hydroxytropane, aponorscopolamine, 3â,6â ditigloyloxytropane 7-hydroxyhyoscyamine are reported for the first time for this species(Strahil et al.,2006). Two new tropane alkaloids, 3-phenylacetoxy-6, 7-epoxynortropane and 7- hydroxyapoatropine were tentatively identified. Typical examples of minor alkaoids in *D. stramonium* are tigloidin, aposcopolamine, apoatropin, hyoscyamine N-oxide, 7-hydroxyhyoscyamine and scopolamine N-oxide (Das et al., 2012). Recently, Li *et al.*(2012) reported the different alkaloids from *D. stramonium* seeds such as N-trans-feruloyl tryptamine, hyoscyamilactol, scopoletin, umckalin, daturaolone, daturadiol, N-trans-ferulicacyltyramine, cleomiscosin A, fraxetin, 1-acetyl-7-hydroxbeta- carboline, and 7-hydroxy-beta-carboline-propionicacid.

2.3.5 Traditional and Ethnomedical properties

Plant derived drugs come into use in the modern medicine through the uses of plant material as indigenous cure in folklore or traditional systems of medicine. *Datura* has a very long history of being used as herbal medicine. In Western Nepal, leaves of *Datura* along with the leaves of *Cannabis sativa* and stem of *Neopicrorhiza scrofulariflora*, are pounded with water and applied to treat headaches. *Datura* seeds are crushed with grains of rice and taken orally to relieve indigestion. In parts of Central Nepal, fresh leaves are warmed and placed on a sprained body part repeatedly, before going to bed, for the alleged analgesic effect. Juice from the leaves is given with warm milk to expel intestinal worms, specifically tapeworm (Rajbhandari 2001). The seeds of the plant are medicinally the most active. The seeds of *Datura* are analgesic, anthelmintic and anti-inflammatory and as such, they are

used in the treatment of stomach and intestinal pain that results from worm infestation, toothache, and fever from inflammation. The juice of its fruit is applied to the scalp, to treat dandruff and falling hair. Externally, the plant is used as a poultice in treating fistulas, abscesses, wounds and severe neuralgia ointment for burns and rheumatism (Paolo, 2001). *Datura* is internally used in relieving the spasm of bronchitis in asthma, relax the smooth muscles of the bronchial tube, treatment of epilepsy madness and depression. It is also used in the treatment of Parkinsonism and Hemorrhoids. Its leaves, applied after roasting, are useful in relieving pain. Traces of scopolamine are also found in the plant, which is a potent cholinergic-blocking hallucinogen that has been used to calm schizoid patients. Its leaves, containing hyoscyamine and atropine, can be used as an immensely powerful mind-altering drug. The growing plant works as an insect repellant, which protects neighboring plants from insects (Das et. al 2012).

2.3.6 Antioxidant property

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants. A free radical, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are dangerous substances produced in the body along with toxins and wastes responsible for damage to cell structures, nucleic acids, lipids and proteins. The aqueous extracts of leaf, stem bark and roots of *D. metel* showed phytochemical and antioxidant activities. The aqueous extract of the plant displayed antioxidant activity of between 49.30-23.82% and can consider the plant as a natural source of antioxidants (Akharaiyi, 2011).

2.3.7 Antimicrobial effects

Ethanol extract of *Datura stramonium* displayed the excellent inhibitory action against *Klebsiella pneumonia* followed by *Staphylococcus aureus* whereas minimal inhibitory activity against Salmonella typhi*.* The methanol extracts of aerial parts of *D. stramonium* exhibited wide range of the bactericidal activity against Gram-positive bacteria in a dose-dependent manner. However *Escherichia coli* and *Pseudomonas aeruginosa* showed more or less resistance against methanolic extract. The aqueous extract showed activity on only *staphylococcus aureus*. *D. stramonium* was also equally potent as vibriocidal against various strains of *Vibreo cholera* and *Vibreo parahaemolyticus* (Eftekhar, 2005). Acetone extracts of *D.stramonium* have been reported to have antifungal activity against several fungi including *Penicillium expansum*, *Aspergillus niger*, *Aspergillus parasiticus*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Trichoderma harzianum*, *Phytophthora nicotiana*, *Pythium ultimum* and *Rhizoctonia solani (*Mdee et al ,2009).

2.3.8 Anticancer effects

Three flavonoidal aglycones viz; F1 (Chrysin), F2 (Kampferol) and novel F3(3,7-dimethylether quercetin), isolated from acetone wash of fresh leaves of *Datura stramomium* L, were found to be significantly cytotoxic against three tumor cell lines (liver, cervix & breast). Compound chrysin was noticed only potent against liver cell line. On the other hand highly oxygenated compounds Kampferol) and and 3, 7- dimethylether quercetin recorded higher activities against both cervix and breast tumor cell line than compound chrysin (Meselhy, 2012). Seed Extract of datura plant when used at different concentrations of 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75 and 5 mg/ml on mammary adenocarcinoma (AMN3), brain cancer, and normal rat embryonic fibroblast (Ref3) cell lines, it was noted that the extract displayed the significant cytotoxic effect by decreasing the viability of AMN3 (42.91%) and brain cell lines (32.79%). However, it produced little effect on viability of normal cell line Ref3 (Merza, 2010**).** It was demonstrated in the experiment, when 1mg/mL of *Datura* aqueous extract was treated on Breast (MDA-MB231), head, neck (FaDu), and lung (A549) cancer cell lines for 24 and 48 hours, there was significant cytotoxicity on MDA-MB231(40%) and FaDu cells(65%) for 24 hours exposure resulting in a appreciable decrease in cell survival. However, A549 cells were found to be resistant to cell killing induced by exposure to the extract for 24 hours. Similar activity was exhibited when treated for 48 hours i.e MDA-MB231 (61%),FaDu (63%), and A549 (22%). (Ahmad et al. 2009).

2.3.9 Antitoxic effects

The seed powder of *Datura stramonium* when tested for its hypoglycemic activity in normal and alloxan-induced diabetic rats, it was found that there was significant reduction in blood glucose at the 8 h at the graded doses of 25, 50 and 75mg/kg. The effect was found to be dose dependent with all treatments at the doses administered (Murthy, 2004). The ethanolic extract of *D. stramonium* leaf displayed the excellent anti-inflammatory activity against carrageenan induced paw edema in rats. It was noted that Maximum activity was observed i.e 39.43% inhibition of the edema, when the extract was administered in doses of 200mg/kg at 3-hour intervals. The inhibitory effect of the extracts on carrageenan-induced edema involved the release of histamine and serotonin that partly inhibit mast cell mediator (Sonika, 2010). *Datura stramonium* seed is a fruitful anodyne or antidote for counterattacking the central cholinergic symptoms of organophosphate (OP) poisoning because of the presence of several atropine and other anticholinergic compounds. Bania *et al*., (2004) determined the beneficial effect of *Datura* seed extracts following a severe OP

poisoning. He demonstrated that *stramonium* seeds when heated in water to make 2 mg/ml atropine solution and administered to male rats as a single intraperitoneal injection 5 min before the subcutaneous injection of 25 mg/kg of dichlorvos, there was significantly increased in survival rate in a rat model with severe OP poisoning. D. stramonium contains a variety of alkaloids, including atropine and scopolamine, having anticholinergic and broncho dilating activity. Atropine and scoplamine dilate bronchial smooth muscle and ease asthmatic attacks by blocking the muscarine receptors (M2 receptors) on airway smooth muscle and submucosal gland cells. However when exposure to the asthamatic pregnant women lead to the death of foetus due to the desensitization of nicotinic receptors by the continuous release of the acetylcholine (Pretorius, Marx, 2006).

CHAPTER III: MATERIAL AND METHOD

3.1 Setting of Laboratory

This thesis work was conducted on the laboratory of the Central Department of Biotechnology, Tribhuvan University..

3.2 Plant materials

3.2.1 Selection of plant materials

Different plants were checked and were selected based on availability, their application as medicinal plants as reported in various literature and suggestion from Dr. Suira Joshi (Ayurvedic herb specialist, Ayur polyclinic, Lalitpur, NAMC NO.330).

3.2.2 Collection and identification of the plant material

Different parts of plants were collected from the different parts of Nepal as indicated. All the plants sample collected were fully grown, healthy and free from contamination by different fungal and bacterial diseases. These all the sample collected were identified by Dr.Suira Joshi and Dr. Deepak Raj Panta.

Table 3.2.2 Name, Collection site and parts of plants used

S.N	Scientific Name	Family	Vernacular Name	Collection Site	Parts Used
1	*Rheum australe*	Polygonaceae	Padamchal	Rasuwa	stem
2	*Tinospora cordifolia*	Menispermaceae	Gurjo	Panaute	stem
3	*Datura stramonium*	Solanaceae	Daturo	Panaute	fruit

3.3 Chemicals and Equipments

All chemicals and organic solvents used during research were of analytical grade and were purchased from Merck Co. Pvt. Ltd, Hi-Media Pvt. Ltd, Glaxo Smith Pharmaceuticals (GSK), and Qualigens. Ultra sonicator used for crude extraction was purchased from LOBA Life™. The rotary evaporator used for concentration of the extracts was from Hanshin Scientific Co., South Korea. The Spectrophotometer used for anti-oxidant study was Genesys manufactured by Thermo Scientific™, USA. The ELISA reader used for cell cytotoxicity assay was MultiSkan EX from Thermo Scientific™, USA. The CO_2 incubator used for animal cell culture was from SHELDON LABS, USA. The Confocal inverted microscope used for animal cell culture was purchased from OLYMPUS, USA. The T-25 culture flask, 96 well cell culture plate, 100mm culture plate, and mice housing cage was obtained from Tarson, USA. The 0.2 µM syringe filter was purchased from Tarson, USA. All culture media used were from Hi-Media Lab. Pvt. Ltd.

3.4 Extraction

First of all plants parts obtained were shade dried in enclosed places at the room temperature. The shaded dried parts of the plant were crushed into fine powder with the help of electric crusher. Then they were subjected to the extraction technique: percolation followed by intermittent ultrasonication. During the percolation process, the known weights of the plant parts were subjected into the three solvent(methanol,chloroform and hexane) at the ratio of 1:10 and they were allowed to stand for 2 days in conical flasks. On the third day, percolated plant materials were introduced in the intermittent sonication i.e 30 khz at 60° C for 30 minutes of 2 hour cycle. The sonicated products were slowly filtered into the reagent bottle using the filter paper. The solvent/filtrate thus obtained were subjected into rotatory vaccum evaporator for the evaporation at the reduced pressure. The resulting extract were transferred into the clean, dry and weighted vial and allowed to dry at the room temperature. Thus finally obtained plant extract were the crude extract of the respective plant materials. The percentage yields of the extract were calculated using following formula:

$$\text{Percentage yield (\%)} = \frac{\text{Dry wt.of crude extract}}{\text{Dry wt.of plant material}}$$

3.5 Phytochemical screening

The preliminary qualitative phytochemical analysis was carried out using plant extract of three different fraction (i.e. methanol, chloroform and hexane) to investigate the presence the important primary and secondary metabolites. The protocol that was followed on the basis of book "phytochemical Methods (Harborne, J.B. 1973, Trease et al., 1989) with some modification.

3.5.1 Detection of alkaloids

Each of the three crude extract of each plant was individually dissolved in the few ml of dilute hydrochloric acid and filtered and subjected for two different tests.

3.5.1.1 Mayers test (Evans and Trease, 2009): 2 to 3 drops of Mayers reagent was added with filtrate obtained. Formation of white/cream ppt indicates the presence of alkaloid.

3.5.1.2 Wagners test: 1ml of HCL was mixed with the few drops of filtrate obtained and red/brown ppt indicates the presence of alkaloid.

3.5.2 Detection of carbohydrates

Each crude plant extract (100mg) was dissolved in distilled water (10ml) and filtered.

3.5.2.1 Benedict's test: 0.2ml of Crude extract was mixed with 2ml of Benedict's reagent and boiled. A reddish brown precipitate formed indicates the presence of the carbohydrates.

3.5.2.2 Molisch's test: 1 ml of Crude extract was mixed with 2 or 3 drops of Molisch's reagent and the mixture was shaken properly. After that, 2ml of concentrated H_2SO_4 was poured carefully along the side of the test tube. Appearance of a violet ring at the interface indicates the presence of carbohydrate.

3.5.3 Test for proteins

Each fraction of crude plant extract (100mg) was dissolved in distilled water (10ml) and filtered.

3.5.3.1 Xanthoproteic test: 1ml of filtered crude extract solution was mixed with 2 drops of conc. nitric acid and appearance of white or pink color represents the presence of protein.

3.5.3.2 Bi-uret test: 2ml of extract was treated with 2 drops of 2%copper sulphate, 1ml of 95%ethanol and potassium hydroxide. Pink color in alcoholic layer indicates the presence of protein.

3.5.4 Test for phenols

3.5.4.1 Ferric chloride test (Braemers test): Each crude extract was mixed with 2% solution of $FeCl_3$ (1:1). A blue-green or black coloration indicates the presence of phenols.

3.5.5 Test for tannins: 1ml of crude extract was mixed with 5ml of 1% gelatin and NaCl. Presence of white precipitate represents tannin.

3.5.6 Resins test: It was done by acetone water test in which crude extracts were treated with 5 ml acetone. Then equal volume of water was added and shaken and formation of turbidity indicates the presence of resins.

.

3.5.7 Test for flavonoids

3.5.7.1 Shinoda test: Crude extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added drop wise. Appearance of Pink scarlet or red orange color after few minutes indicates the presence of flavonoid.

3.5.8 Test for saponins

Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam is taken as an indication for the presence of saponin.

3.5.9 Test for glycosides

3.5.9.1 Keller-kilani test (cardiac glycosides): Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2ml of concentrated H_2SO_4. A brown ring at the interface indicates the presence of cardiac glycosides.

3.5.10 Test for terpenoid

3.5.10.1 Libermann Burchard test: 1ml of crude extract was treated with 1ml of $CHCl_3$ and resulting solution was treated with few drops of acetic anhydride. Finally few drops of concentrated H_2SO_4 were added from the side of the tube and red/pink color formation indicates the presence of terpenoid.

3.5.10.2 Salkowski's test: 2ml of chloroform and 3ml of conc. Sulphuric acid was added to 5ml of crude extract solution. Formation of yellow ring at the interface that turns reddish brown in few minute indicates the presence of terpenoid.

3.6 Determination of Total Flavonoid Content. (Chang et al., 2002)

The total flavonoid content in the plant extract was estimated using the Aluminium chloride ($AlCl_3$) colorimetric method with slight modifications. First of all 10mg/ml stock of plant extract was prepared in absolute methanol. Then 0.25 ml of extract (10 mg/ml) was separately taken in clean test tubes. To each of the test tube, 0.75 ml of ethanol was added followed by 0.05 ml of the 10% aluminum chloride, 0.05 ml of the 1 M potassium acetate (CH_3COOK) and 1.4 ml of the distilled water. The reaction mixture was shaken and allowed to stand for about 30 minutes in room temperature. The absorbance of the mixture was measured at 415 nm using the UV – visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5).The standard calibration curve was obtained with the help of quercetin (Sigma) standard solutions in ethanol with the concentration ranging from the 10-100µg /ml and for the blank all the chemicals added were same except plant sample instead methanol. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass (mg QE/g). For each experiment the three replications were used for the accuracy and reproducibility of results.

3.7 Determination of Total Polyphenol Content (Ainsworth and Gillespie, 2007)

The total phenol content of the three plant species having different fraction (i.e methanol, chloroform and hexane) were determined using the Folin–Ciocalteu phenol reagent with slight modification. First of all 2.5mg/ml stock of crude plant extract were prepared. During the process, 0.1 ml of each extract (2.5 mg/ml) was mixed with the 1 ml of Folin–Ciocalteu

phenol reagent i.e. 1:10 dilution with the d/w (Merck Specialities pvt Ltd, India) followed by the addition of 0.8ml of aqueous 1 M Na_2CO_3 solution in a clean test tube. The resulting solution was shaken well and allowed to incubate for about 15 minute in room temperature and the absorbance of the reactants was measured at 765 nm using the UV- visible spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). For the blank absolute methanol was used instead of test solution and Gallic acid (Moly Chem, Mumbai, India) was used as the standard. Based on this standard calibration graph, the concentrations of total phenolic content of the individual plants samples were quantified. The total polyphenol content was expressed in terms of the milligrams of the Gallic acid equivalent per gram of the dry mass (mg GAE/g). For each extract, three replicates were performed for the reproducibility of results.

3.8 Determination of antioxidant activity

The antioxidant of the given plant sample were calculated by DPPH free radical scavenging activity:

3.8.1 Measurement of DPPH free radical scavenging activity

The stable 1, 1- diphenyl-2 picryhydrazyl (DPPH) was used to analyse the antioxidant activity of the crude extract of various fraction. It is based on free radical scavenging activity following the protocol of Singh *et al*., 2002 with slight modification. During the process different concentration of plant extract (10-100µg/ml) and ascorbic acid (10-100µg /ml) were prepared in methanol on the clean and clear test tubes. Then 1ml of sample volume was taken in the test tube followed by the equal volume of 0.2mM DPPH solution. The reaction mixture were shaken well and incubated in dark room for about 30 minutes. DPPH solution was taken as control and methanol as a blank or baseline solution for spectrophotometer (Thermo Fisher Scientific, Genesystem-10-5). The absorbance was measured on spectrophotometer at 517 nm.

Now the radical scavenging activity of the sample in percentage was calculated using the following formula.

$$\% \, Radical\ scavenging\ activity = [\frac{Control\ abs - sample\ abs}{Control} abs)]\ x100\%$$

Standard graph was plotted taking the concentration on the X-axis and percentage scavenging activity on the Y-axis. . Based on this graph, IC_{50} value of each sample was calculated.

The IC_{50} value of given sample was calculated by the given formula below:

$$IC50 = EXP\ (LN\ (conc. > 50\%) - ((pi > 50\% - 50)/\ (pi > 50\% - pi < 50\%) * LN\ (conc. > 50\%/conc. < 50\%)))$$

3.9 Antimicrobial Screening

The antimicrobial test was performed by modified agar diffusion method(perez.c,1990). During this process, fixed volume of the plant extract solution was placed in the equal sized well bored on the solidified agar plates along with positive and negative control. On the basis of diameter of the clear halo zone of the inhibition around the well, the efficacy of the plant extract against microorganism was evaluated. This is only qualitative parameter to determine antimicrobial efficacy.

3.9.1 Microorganism

The individual pure culture of bacteria: *Klebsiella pneumonia, Salmonella typhimurium, Staphylococcus aureus, and* yeast: *Saccharomyces cerevisae,Candida albicans, Pichia stipititis* were used for antimicrobial assay.

3.9.2 Preparation of the standard culture Inoculum

First of all pure culture of bacteria were streaked on the different nutrient agar plates and incubated on the incubator at 37°C for about 24 hours. From the agar plated single isolated pure colony was obtained. The entire isolated colony was checked by performing different biochemical test. Now each distant colony was aseptically transferred to the Luria Bertani (LB broth) for the suspension culture with the help of the sterilized inoculating loop. The inoculated bottles were kept on the shaking incubator at 37°C and 120 rpm for overnight. The cell suspension in culture was maintained at 1-1.8 $\times 10^8$ cfu/ml or the turbidity of the bacterial suspension was adjusted at the 0.5 McFarland standards. Whereas for the yeast culture, the organisms were first streaked in the potatoes dextrose agar and single colony was isolated. Now the isolated colony was inoculated in the YEPD broth and maintained at 25°C for 72 hours for antifungal screening.

3.9.3 Preparation of extract

Each of the fractions of plant extract (i.e. methanol, chloroform and hexane) was prepared at a concentration of 50mg/ml. Plant extract solution was prepared using the DMSO as solvent and stored in refrigerator until use.

3.9.4 Antimicrobial screening via Agar well diffusion technique (Perez, 1990)

Sterile petriplate were taken and MHA (bacteria) and YEPD agar (fungi) was prepared. Into the sterile petriplate, media was poured maintaining the thickness about 4mm and allowed to solidify. Now the plates were subjected to overnight incubation to observe any kind of cross contamination. The standard suspension of culture maintained at 1-1.8 $\times 10^8$ cfu/ml was inoculated into the sterile plate with the help of sterile cotton swab. The cotton swab was uniformly rubbed into media at an angle of 45° in order to maintain the uniform growth of microorganisms. The inoculated MHA plates were allowed to diffuse for 15 minutes in biosafety cabinet. With the help of 6mm sterile borer, 5 wells were prepared in each MHA plates. Each of the three fractions of plant extract (i.e. methanol, chloroform and hexane) were prepared in the DMSO at a concentration of 50mg/ml and placed in the first three wells at volume of 40µl with the help of sterile micropipette. The DMSO was taken as a negative control and Chloramphenicol as a positive control at a concentration of 50mcg/disc for bacteria and Gentamycin at a concentration of 10mcg/disc for fungi. The inoculated plates were incubated 24 and 72 hours in incubator at 37° and 25° for bacteria and yeast respectively. After designated period of incubation, zone of inhibition around the wells were observed and their diameter was noted for further analysis.

3.10 Biological assays

The three different fraction of each plant extract (i.e. methanol, chloroform and hexane) were evaluated for their biological assay. During the procedure, each plant extract were treated with Human cervical cancer cell line (Hela) and murine peritoneal macrophages to analyze their cytotoxicity effect. The viability of Hela cell lines and macrophages were determined on the basis of standard MTT assay with minor alteration. (Roomi et al.,2006; Nair and Varalakshmi,2011).

3.10.1 Animal

BALB/c mice (2-3 months) were obtained from Department of plant resources, Thapathali, Kathmandu. They were housed under the standard environmental condition with five per cages and fed with standard pellets and tap water. The animals quarters were maintained at 21-24°C with 12-hrs light –darkness cycle. These experimental works were performed under the guidelines of Institutional animal care and Use committee.

3.10.2 Peritoneal macrophage isolation and culture

Peritoneal macrophages were isolated from BALB/c mice as previously described (Miles et al., 2000; Al-Bayaty et al., 2010) with minor modification. Briefly, BALB/c mice of around 2-3 months were injected intraperitoneally with 3 ml sterile 3% starch solution dissolved in PBS. After 3 days, the mice were euthanized aseptically and the peritoneal exudates were isolated by cervical dislocation and peritoneal lavage by freshly prepared PBS. The isolated cells were centrifuged and the erythrocytes were lysed using erythrocyte lysis buffer. The macrophages thus obtained were washed once with cold PBS, resuspended and cultured in RPMI-1640 medium supplemented with 10% heat inactivated FBS, 1% penicillin–streptomycin. By Trypan blue exclusion, the cell counting was revealed >95% viability. The numbers of viable macrophages were counted using the haemocytometer chamber.

3.10.3 Cancer cell line

Standard human cervical cancer cell line (Hela ATCC CCL-2) was provided by the Everest Biotech™, khumaltar, Lalitpur, Nepal and was in optimal growth condition having the confluency of 90%.

3.10.4 Cytotoxic Effect of extract on Hela cells and Peritoneal macrophages.

3.10.4.1 MTT assay

The cytotoxicity of the extracts was tested against Hela cells and macrophages on the basis previously described protocol with minor alteration.(Escribano et al.,1999) During the process, Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 100µg/ml streptomycin and 100 µ/ml penicillin at 37°C with 5% CO_2. For

experiments, cells were plated in 96-well plates (10^5cells/well for adherent cells or 0.3×10^6 cells/well for suspended cells in 100µl of medium). After 24 h, the extracts at concentration of 0-300µg/ml dissolved in DMSO (1%) were added to each well and incubated for 24 hours at 37°C, 5% CO_2 (Sheldon Labs^{TM}). Control groups received the same amount of DMSO. Anticancer drug (5-fluro-uracil, 5-50µg/ml) was used as positive control and DMSO as negative control and DMSO only as blank. At the end of 24 hour incubation, the medium in each well was replaced by fresh medium (100µL) containing 0.5mg/mL of MTT. Growth of Hela cells and macrophages was quantified by the ability of living cells to reduce the yellow dye 3-(4, 5-dimethyl- 2- thiazolyl) 2, 5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product. After 4 hours of incubation, the formazan product of MTT reduction was dissolved in 100µl DMSO, and absorbance was measured using ELISA spectrophotometric reader at wavelength of 540nm.

3.11 Statistical analysis:

The statistical analysis was performed by using the Software's Microsoft Office Excel, 2007 and Graph Pad prism 5. All the experiments were performed in triplicates and the data are reported as mean±SD. Linear regression analysis was used to calculate total phenol content, flavonoid content and DPPH radical scavenging value.

CHAPTER IV: RESULTS

4.1 Yield of extracts

The extract of three fraction of three plants species were obtained using extraction technique known as ultrasonication followed by rotavapor evaporation. The extracts thus obtained were found to have differences in physical properties as well as their yield. The highest yield of extract was found in methanol fraction of *Tinospora cordifolia* and lowest in hexane fraction of *Datura stramonium*. The yield percentage and characteristics of extract has been tabulated below.

Table 4.1.1 Percentage Yield and physical characteristics of the crude hexane, chloroform, and methanol extract

Plant extract	**Dry weight taken (gm)**	**Wt. of extracts (gm)**	**Percentage yield (%)**	**Parts Used**	**Characteristics of extracts**	
					Color	**Consistency**
Methanol Fraction						
Rheum australe	32	2.45	7.65	**Stem**	**Black**	**Powder**
Tinospora cordifolia	32	3.17	9.9	**Stem**	**Dark brown**	**Greasy (sticky)**
Datura stramonium	32	2.31	7.21	**Fruit**	**Brown**	**Sticky**
Chloroform fraction						
Rheum australe	40	0.98	2.45	**Stem**	**Yellow**	**Powder**
Tinospora cordifolia	40	1.15	2.87	**Stem**	**Dark brown**	**Greasy**
Datura stramonium	40	0.95	2.37	**Fruit**	**Dark green**	**Oily(greasy)**
Hexane fraction						
Rheum australe	45	0.74	1.64	**Stem**	**Faint yellow**	**Powder**
Tinospora cordifolia	45	0.97	2.15	**Stem**	**Green**	**Greasy**
Datura stramonium	45	0.69	1.53	**Fruit**	**Light green**	**Oily(greasy)**

4.2 Phytochemical screening

The phytochemical investigations of three fractions of each medicinal plant have shown the presence of primary and secondary metabolites. The different primary and secondary metabolites present were carbohydrates, proteins, alkaloid, flavonoid, phenol, saponins, terpenoids, glycosides and many other phytochemicals. However, the content of various secondary metabolites varied in different fraction of plant extract while some were absent in the plant extract such as tannin, gelatin, fat and oils etc. The secondary metabolites are biologically active compound and found to exhibit wide range of biological and pharmacological function. They are highly used in the anti-inflammatory, anti-diabetic, anti-cholesterol, antimicrobial, anti-cancer etc. A detailed outcome of the phytochemical investigation of three fraction of *Rheum australe, Tinospora cordifolia* and Datura *stramonium* are elucidated below.

Table 4.2.1 Phytochemical screening of methanol, chloroform and hexane fraction of *Rheum australe*

S. N	Pytochemical test	Reagent used	Samples	Intensity	Observation	Result
1.	Carbohydrates test	Molish reagent	Rheum meoh	+++	Formation of violet ring	positive
			Rheum chl	+++	Formation of violet ring	positive
			Rheum hex	++	Formation of violet ring	positive
		Benedicts reagent	Rheum meoh	+	Formation of violet ring	positive
			Rheum chl	+	Formation of violet ring	positive
			Rheum hex	+	Formation of violet ring	positive
2.	Protein tests/ amino acid test	Xanthoproteic test	Rheum meoh	+++	Formation of rose pink colour	positive
			Rheum chl	+	Formation of rose pink colour	positive
			Rheum hex	+	Formation of rose pink colour	positive
		Biurets test	Rheum meoh	++	Formation of pink/violet	positive
			Rheum chl	-	Formation of pink/violet	negative
			Rheum hex	-	Formation of pink/violet	negative
3.	Fats and oil test	Filter paper press test	Rheum meoh	-	Formation of oily stain	negative
			Rheum chl	-	Formation of oily stain	negative
			Rheum hex	-	Formation of oily stain	negative
4.	Alkaloids test	Mayers test	Rheum meoh	+++	Formation of yellow cream ppt	positive
			Rheum chl	++	Formation of yellow cream ppt.	positive
			Rheum hex	++	Formation of yellow cream ppt	positive
		Wagners test	Rheum meoh	++	Formation of reddish brown ppt	positive
			Rheum chl	++	Formation of reddish brown ppt.	positive
			Rheum hex	++	Formation of reddish brown ppt	positive
5.	Flavonoid tests	Lead acetate test	Rheum meoh	+++	Formation of yellow ppt	positive
			Rheum chl	++	Formation of yellow ppt	positive
			Rheum hex	++	Formation of yellow ppt	positive
		Shinoda test	Rheum meoh	+++	Formation of red/orange color	positive
			Rheum chl	++	Formation of red/orange color	positive
			Rheum hex	++	Formation of red/orange color	positive
6.	Phenol tests	Ferric chloride test	Rheum meoh	+++	Formation of bluish black ppt	positive
			Rheum chl	++	Formation of bluish black ppt.	positive
			Rheum hex	++	Formation of bluish black ppt	positive
7.	Tannins / gelatin tests	Gelatin tests	Rheum meoh	-	Formation of white ppt.	negative
			Rheum chl	-	Formation of white ppt.	negative
			Rheum hex	-	Formation of white ppt.	Negative
8.	Resins test	Acetone water test	Rheum meoh	-	Appearance of turbidity	Negative
			Rheum chl	-	Appearance of turbidity	Negative
			Rheum hex	-	Appearance of turbidity	Negative
9.	Saponins test	Foam tests	Rheum meoh	++	Produce foam	Positive
			Rheum chl	+	Produce foam	Positive
			Rheum hex	-	Produce foam	Negative
10.	Glycosides test	Keller-killani tests	Rheum meoh	+	Formation of green/ blue color	Positive
			Rheum chl	+	Formation of green/ blue color	Positive
			Rheum hex	+	Formation of green/ blue color	Positive
11.	Pytosterol/ terpenoid test	Liberman Buchard test	Rheum meoh	+	Formation of Dark green	Positive
			Rheum chl	+	Formation of Dark green	Positive
			Rheum hex	+	Formation of Dark green	Positive
		Salkowski test	Rheum meoh	+	Formation of yellow ring	Positive
			Rheum chl	+	Formation of yellow ring	Positive
			Rheum hex	+	Formation of yellow ring	Positive

+++strong signal; ++intermediate signal; +weak signal; - no signal

Table 4.2.2 Phytochemical screening of methanol, chloroform and hexane fraction of *Tinospora cordifolia*.

S.N	Pytochemical test	Reagent used	Samples	Intensity	Observation	Result
1.	Carbohydrates test	Molish reagent	Tino meoh	+++	Formation of violet ring	Positive
			Tino chl	+++	Formation of violet ring	Positive
			Tino hex	++	Formation of violet ring	Positive
		Benedicts reagent	Tino meoh	+++	Formation of violet ring	Positive
			Tino chl	++	Formation of violet ring	Positive
			Tino hex	+	Formation of violet ring	Positive
2.	Protein tests/ amino acid test	Xanthoproteic test	Tino meoh	++	Formation of rose pink colour	Positive
			Tino chl	+	Formation of rose pink colour	Positive
			Tino hex	+	Formation of rose pink colour	Positive
		Biurets test	Tino meoh	++	Formation of pink/violet	Positive
			Tino chl	+	Formation of pink/violet	Positive
			Tino hex	+	Formation of pink/violet	Positive
3.	Fats and oil test	Filter paper press test	Tino meoh	-	Formation of oily stain	Negative
			Tino chl	-	Formation of oily stain	Negative
			Tino hex	-	Formation of oily stain	Negative
4.	Alkaloids test	Mayers test	Tino meoh	+++	Formation of yellow cream ppt	Positive
			Tino chl	++	Formation of yellow cream ppt.	Positive
			Tino hex	+	Formation of yellow cream ppt	Positive
		Wagners test	Tino meoh	++	Formation of reddish brown ppt	Positive
			Tino chl	+	Formation of reddish brown ppt.	Positive
			Tino hex	+	Formation of reddish brown ppt	Positive
5.	Flavonoid tests	Lead acetate test	Tino meoh	+++	Formation of yellow ppt	Positive
			Tino chl	++	Formation of yellow ppt	Positive
			Tino hex	++	Formation of yellow ppt	Positive
		Shinoda test	Tino meoh	+++	Formation of red/orange color	Positive
			Tino chl	++	Formation of red/orange color	Positive
			Tino hex	++	Formation of red/orange color	Positive
6.	Phenol tests	Ferric chloride test	Tino meoh	+++	Formation of bluish black ppt	Positive
			Tino chl	++	Formation of bluish black ppt.	Positive
			Tino hex	++	Formation of bluish black ppt	Positive
7.	Tannins / Gelatin tests	Gelatin tests	Tino meoh	-	Formation of white ppt.	Negative
			Tino chl	-	Formation of white ppt.	Negative
			Tino hex	-	Formation of white ppt.	Negative
8.	Resins test	Acetone water test	Tino meoh	-	Appearance of turbidity	Negative
			Tino chl	-	Appearance of turbidity	Negative
			Tino hex	-	Appearance of turbidity	Negative
9.	Saponins test	Foam tests	Tino meoh	+++	Produce foam	Positive
			Tino chl	++	Produce foam	Positive
			Tino hex	+	Produce foam	Positive
10.	Glycosides test	Keller-killani tests	Tino meoh	++	Formation of green/ blue color	Positive
			Tino chl	+	Formation of green/ blue color	Positive
			Tino hex	+	Formation of green/ blue color	Positive
11.	Pytosterol/ terpenoid test	Liberman Buchard test	Tino meoh	+	Formation of Dark green	Positive
			Tino chl	-	Formation of Dark green	Negative
			Tino hex	-	Formation of Dark green	Negative
		Salkowski test	Tino meoh	++	Formation of yellow ring	Positive
			Tino chl	-	Formation of yellow ring	Negative
			Tino hex	-	Formation of yellow ring	Negative

+++strong signal; ++intermediate signal; +weak signal; - no signal

Table4.2.3 Phytochemical screening of methanol, chloroform and hexane fraction of *Datura stramonium*

S.N	Pytochemical test	Reagent used	Samples	Intensity	Observation	Result
1.	Carbohydrates test	Molish reagent	Dat meoh	+++	Formation of violet ring	Positive
			Dat chl	+++	Formation of violet ring	positive
			Dat hex	+++	Formation of violet ring	positive
		Benedicts reagent	Dat meoh	+++	Formation of violet ring	positive
			Dat chl	++	Formation of violet ring	positive
			Dat hex	++	Formation of violet ring	positive
2.	Protein tests/ amino acid test	Xanthoproteic test	Dat meoh	+++	Formation of rose pink colour	positive
			Dat chl	++	Formation of rose pink colour	positive
			Dat hex	+	Formation of rose pink colour	positive
		Biurets test	Dat meoh	+++	Formation of pink/violet	positive
			Dat chl	++	Formation of pink/violet	positive
			Dat hex	+	Formation of pink/violet	positive
3.	Fats and oil test	Filter paper press test	Dat meoh	-	Formation of oily stain	negative
			Dat chl	-	Formation of oily stain	negative
			Dat hex	-	Formation of oily stain	negative
4.	Alkaloids test	Mayers test	Dat meoh	+++	Formation of yellow cream ppt	positive
			Dat chl	+++	Formation of yellow cream ppt.	positive
			Dat hex	+++	Formation of yellow cream ppt	positive
		Wagners test	Dat meoh	+++	Formation of reddish brown ppt	positive
			Dat chl	+++	Formation of reddish brown ppt.	positive
			Dat hex	+++	Formation of reddish brown ppt	positive
5.	Flavonoid tests	Lead acetate test	Dat meoh	+++	Formation of yellow ppt	positive
			Dat chl	++	Formation of yellow ppt	positive
			Dat hex	++	Formation of yellow ppt	positive
		Shinoda test	Dat meoh	+++	Formation of red/orange color	positive
			Dat chl	++	Formation of red/orange color	positive
			Dat hex	++	Formation of red/orange color	positive
6.	Phenol tests	Ferric chloride test	Dat meoh	+	Formation of bluish black ppt	positive
			Dat chl	+	Formation of bluish black ppt.	positive
			Dat hex	+	Formation of bluish black ppt	positive
7.	Tannins / gelatin tests	Gelatin tests	Dat meoh	-	Formation of white ppt.	negative
			Dat chl	-	Formation of white ppt.	negative
			Dat hex	-	Formation of white ppt.	negative
8.	Resins test	Acetone water test	Dat meoh	-	Appearance of turbidity	negative
			Dat chl	-	Appearance of turbidity	negative
			Dat hex	-	Appearance of turbidity	negative
9.	Saponins test	Foam tests	Dat meoh	+++	Produce foam	positive
			Dat chl	+	Produce foam	positive
			Dat hex	+	Produce foam	positive
10.	Glycosides test	Keller-killani tests	Dat meoh	-	Formation of green/ blue color	negative
			Dat chl	-	Formation of green/ blue color	negative
			Dat hex	-	Formation of green/ blue color	negative
11.	Pytosterol/ terpenoid test	Liberman Buchard test	Dat meoh	-	Formation of Dark green	negative
			Dat chl	-	Formation of Dark green	negative
			Dat hex	-	Formation of Dark green	negative
		Salkowski test	Dat meoh	++	Formation of yellow ring	positive
			Dat chl	+	Formation of yellow ring	positive
			Dat hex	+	Formation of yellow ring	positive

+++strong signal; ++intermediate signal; +weak signal; - no signal

4.3 Determination of Total Flavonoid content

Standard graph of Quercetin was obtained taking its concentration ranging from the 10 µg/ml – 100 µg/ml with an equation of y = 0.0081X R^2=0.975. Based on this equation, total amount of the flavonoid present in three fraction of each plant species was determined. The results are expressed in mg QE /g ± SD.

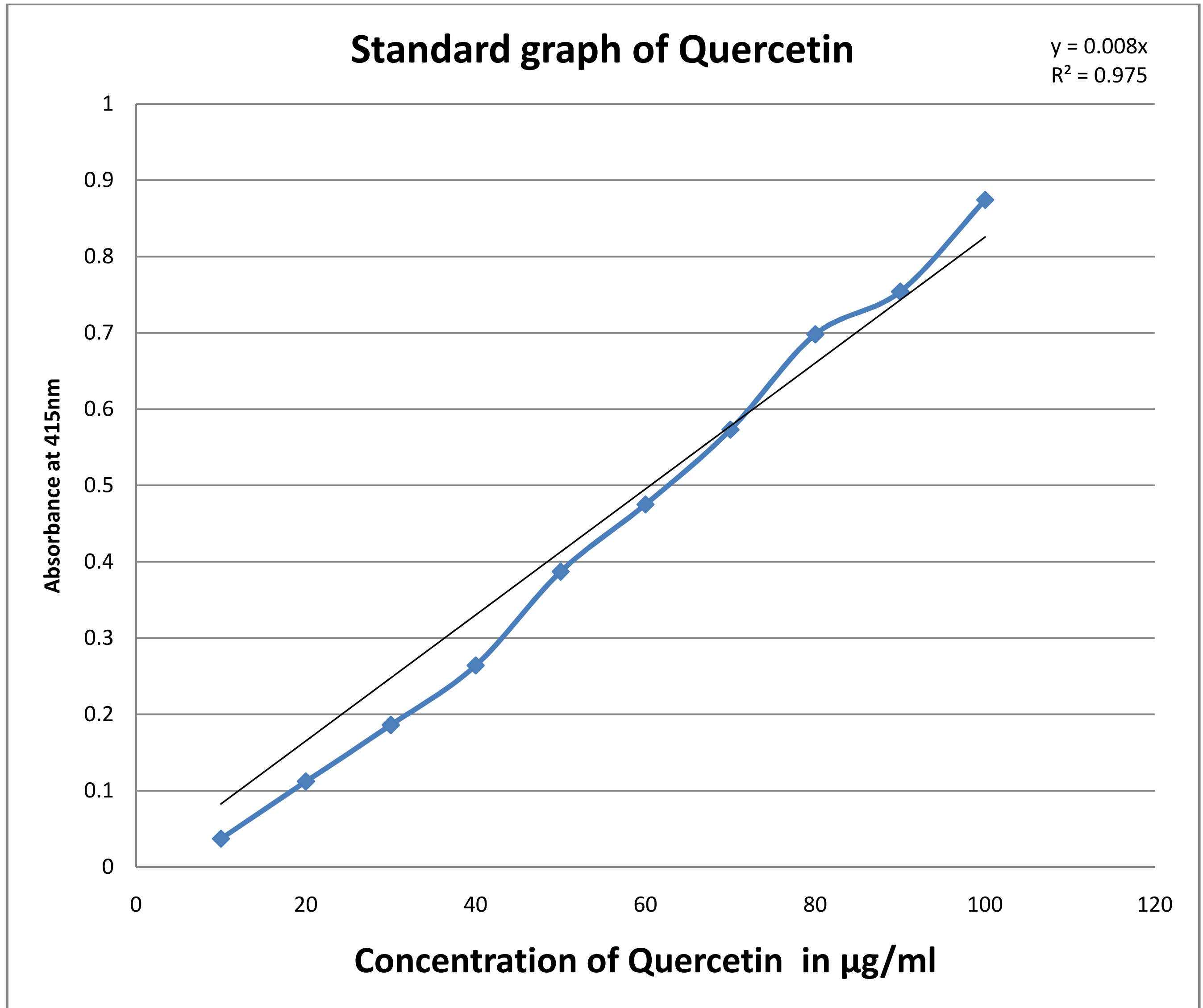


Figure 4.3.1 Standard graph of the Quercetin

The highest amount of flavonoid content was found in the methanol fraction whereas lowest amount in the hexane fraction. Methanol fraction of Tinospora cordifolia was found to contain 24.038 ±0.704 mgQE/g and hexane fraction was found to contain 9.679±0.693 mg QE/g. The flavonoid content of other fraction of each plant species remained in between these values.

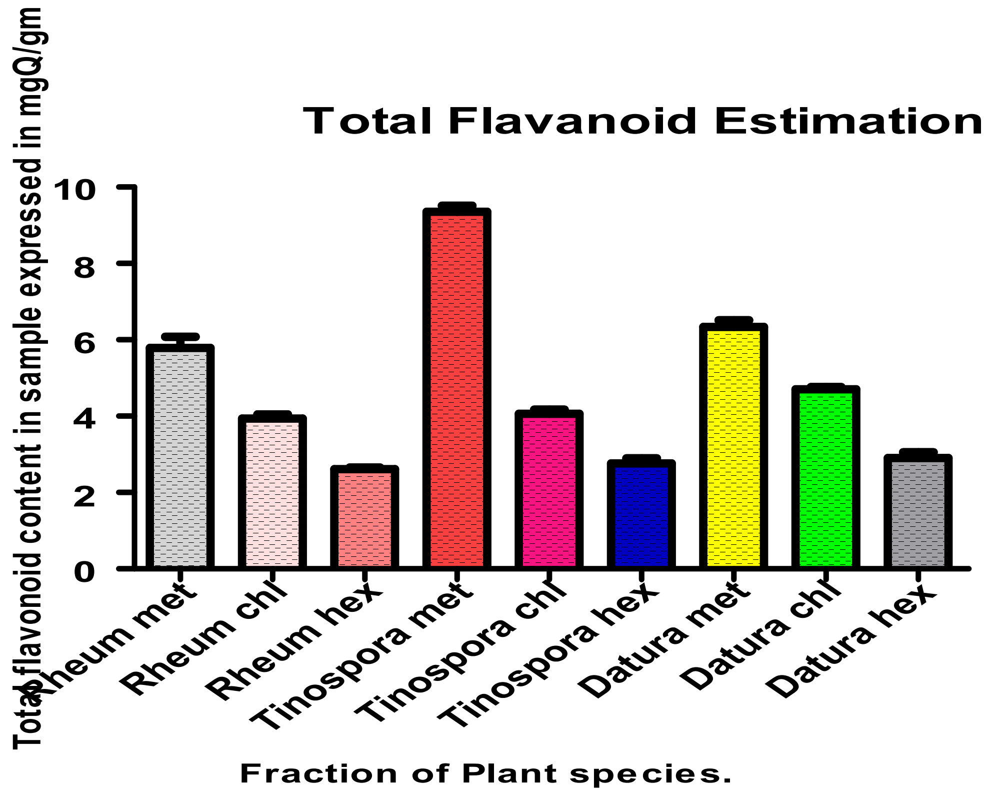


Figure 4.3.2 Total flavonoid content in three fraction of each plant species.

The error bars represent the standard deviation from mean value (mean±SD).

4.4 Determination of Total Phenol content

A calibration curve Y = 0.006x, R^2 = 0.993 was obtained by using the standard solution of gallic acid ranging from the concentration of 10µg/ml to 100µg/ml as shown on figure 4.4A . Based on this equation, the concentration of the total phenol content present in three fraction of each plant species was evaluated and the results are expressed as mg GAE/g ± SD.

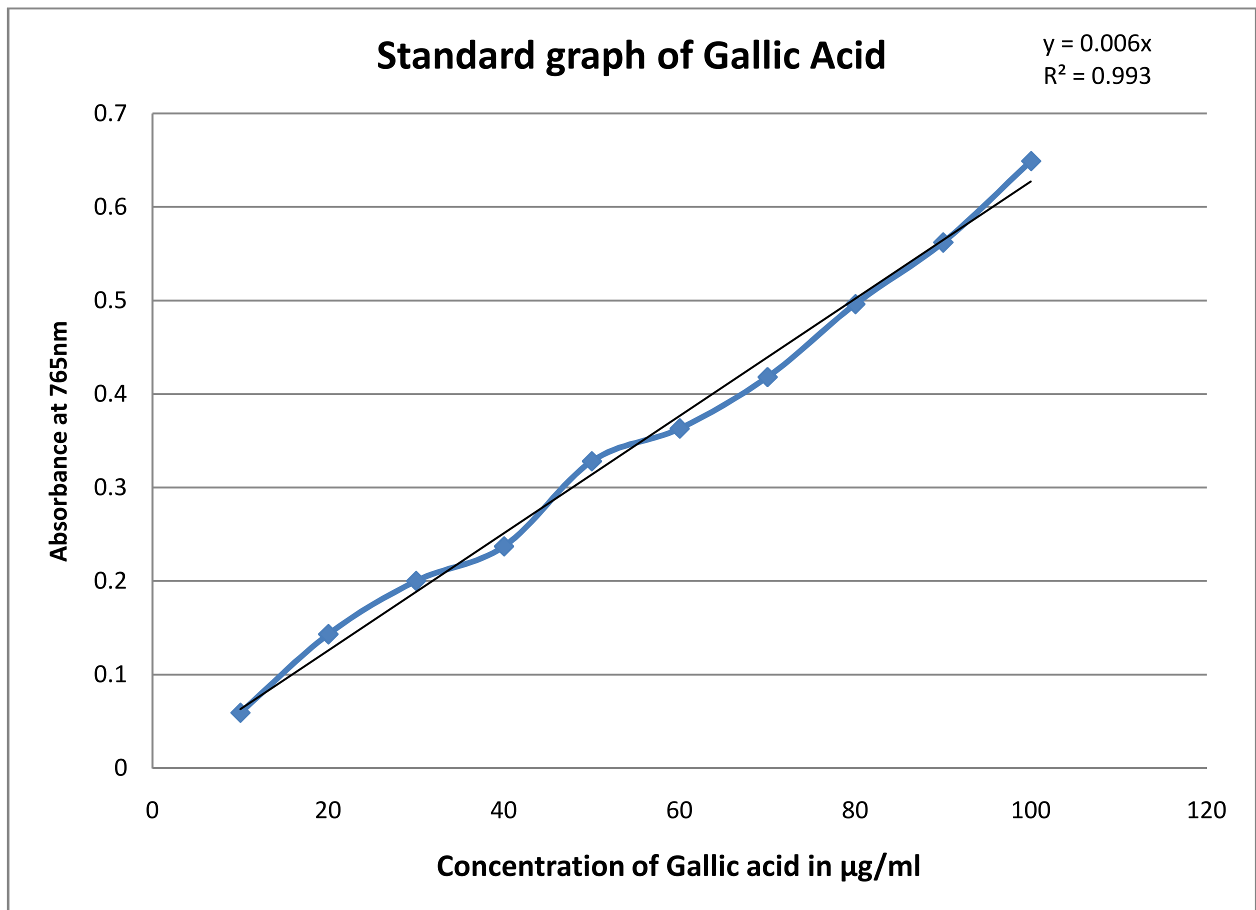


Figure4.4: Standard graph of Gallic acid for the determination of Total Phenol content

Highest concentration of phenol was found in methanol fraction of each plant species. *Tinospora cordifolia* was found to contain highest amount phenol content of 60.488±5.37 mg GAE/g dry wt. the chloroform extract of *Datura stramonium* showed the lowest content of polyphenol with the value of 18.08±2.43mg GAE/g dry w. the phenol content of three fraction of

each plant extract was found in between these values. The graphical representation of total polyphenol estimation has given below.

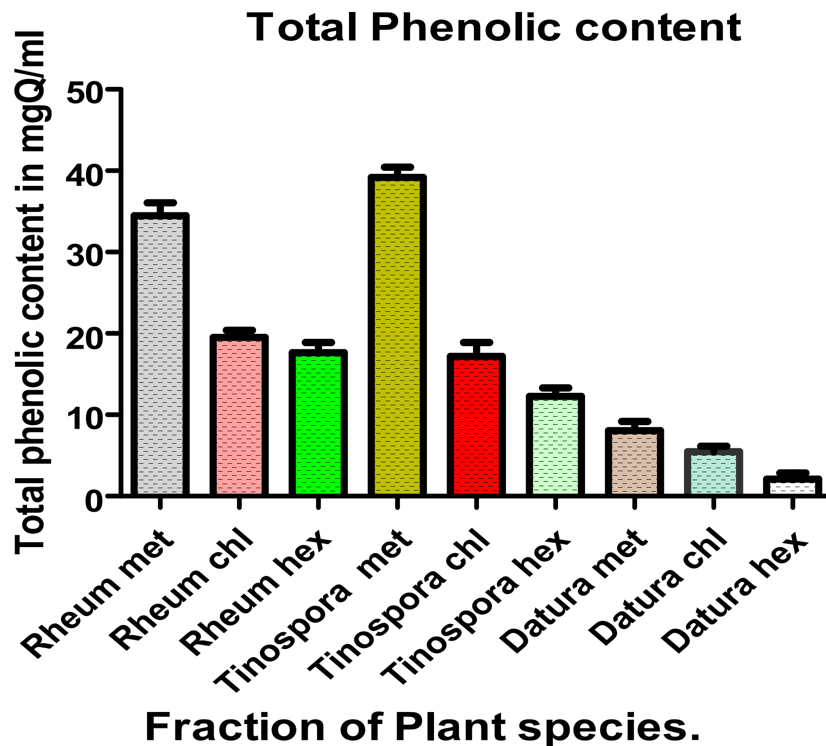


Figure 4.4.2: Estimation of Total Polyphenol Content in three fraction of each plant extract.

The error bars represent the standard deviation from mean value (mean±SD)

4.5 Estimation of Antioxidant activity of the plant extract

4.5.1 DPPH free radical scavenging activity

Antioxidant activity of three fractions each plant extract was determined using the solution of DPPH (0.2mM) and taking Ascorbic acid as the pure antioxidant reference compound. IC_{50}value was calculated for each sample taking the concentration vs. % radical scavenging activity following the formula used by Maes *et al.*, 2010.The IC_{50} value for Ascorbic acid was found to be 41.696 ±2.308. In the similar way IC_{50} value for the plan species was found minimum for *Tinospora cordifolia* of methanol fraction (50.542±3.285) and maximum for

chloroform fraction of *Datura stramonium* (110.746±2.0589). The species that has lower IC_{50} are considered as the best antioxidants and vice versa. The IC_{50} value of other fraction of each plant extract is found to be in between these values. The graph representing the % scavenging and IC50 value are given below.

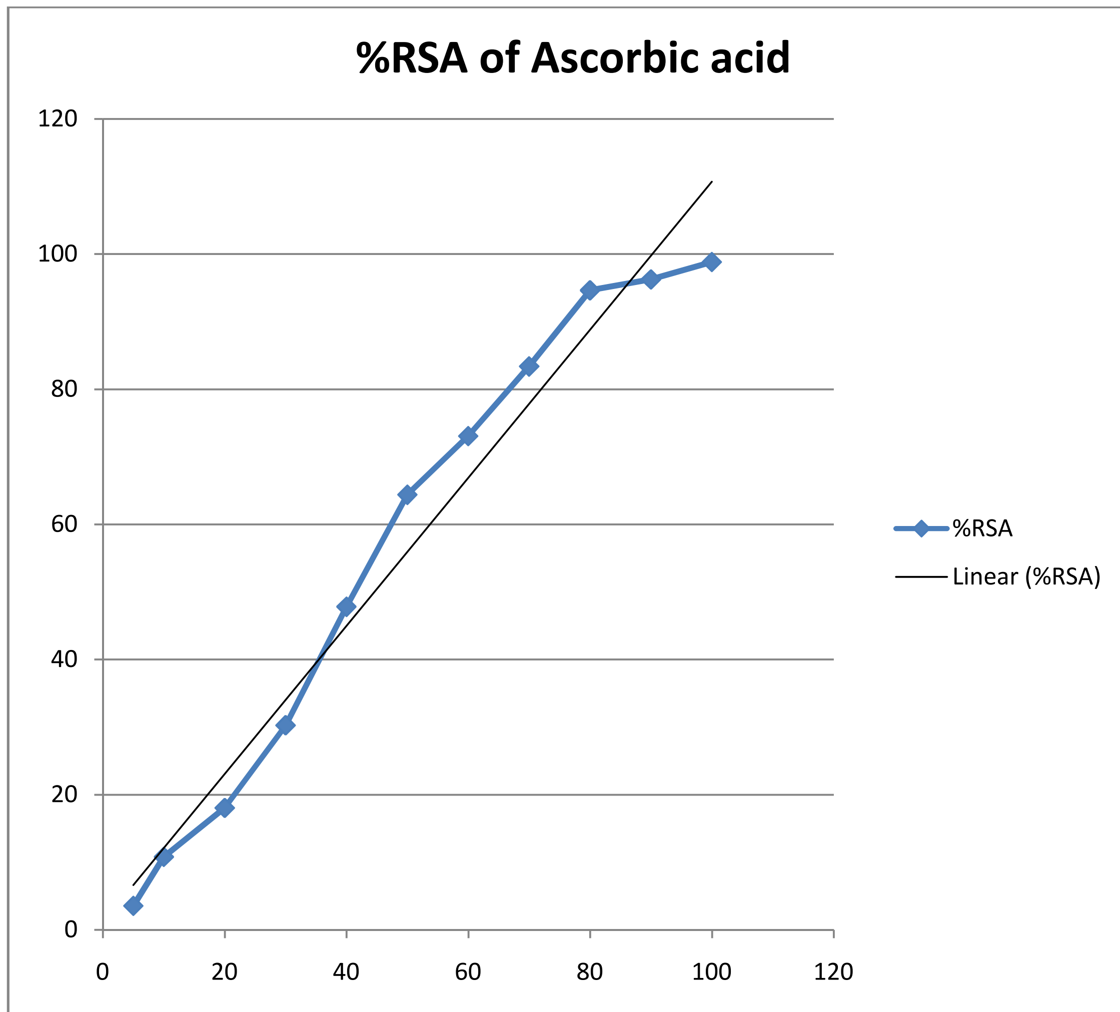


Figure 4.5.1: standard graph of %RSA of Ascorbic acid.

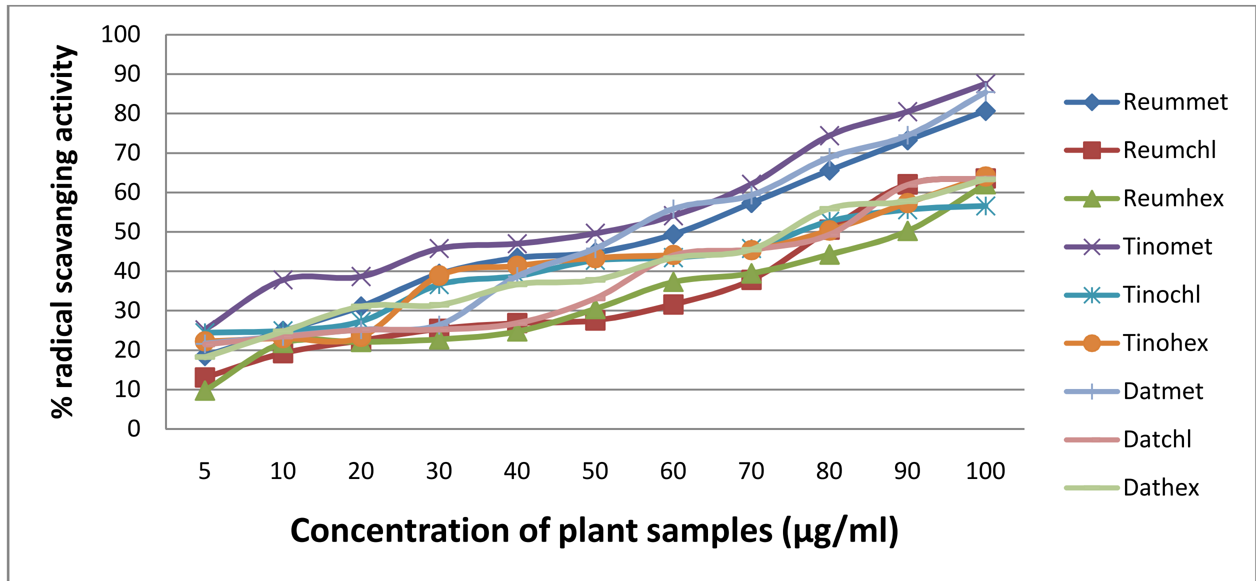


Figure 4.5.2 %Radical Scavenging Activity of three fraction of each plant extract.

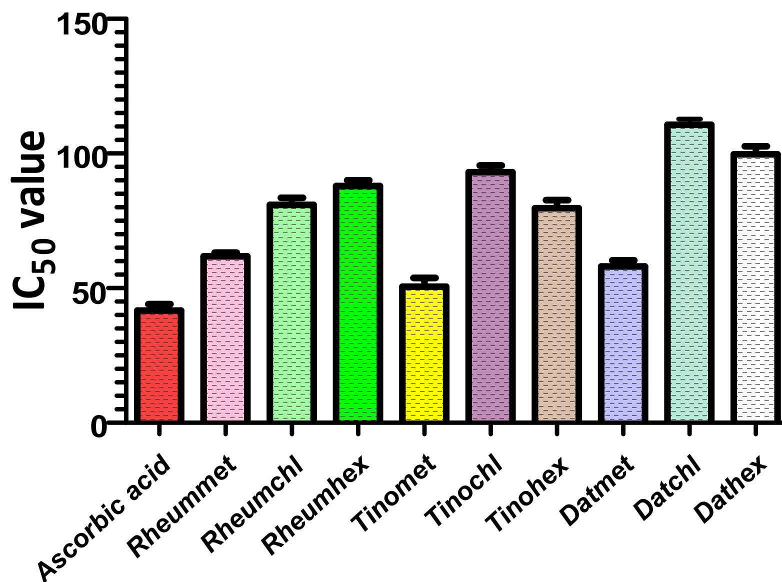


Figure 4.5.3 IC_{50} values of standard compound (Ascorbic acid) and fraction of plant samples.

4.6 Antimicrobial activity

4.6.1 Antibacterial activity

The antibacterial activity of three fraction of each plant extracts was tested against ATCC cultures of *Staphylococcus aureus, Salmonella typhimurium, Klebsiella pneumonia.* Antibiotic Chloramphenicol was taken as a positive control and DMSO (the solvent of the plant extract) was taken as a negative control.The results were expressed with Zone of inhibition on mm.

Tables 4.6.1: Antibacterial activity of different fraction of plant extract

Zone of inhibition(mm) with well diameter (6mm)			
Bacterial strain ATCC no.	***Salmonella typhimurium* 14028**	***Klebsiella pneumonia* 27853**	***Staphylococcus aureus* 25525**
Plant Extract			
Rheum australe			
Rheum-Met	18	22	18
Rheum-Chl	16	14	16
Rheum-Hex	18	14	16
Tinospora cordifolia			
Tino-Met	16	16	12
Tino-Chl	16	18	16
Tino-Hex	16	16	10
Datura stramonium			
Dat-Met	14	18	14
Dat-Chl	12	12	18
Dat-hex	12	12	12
Chloramphenicol (50mcg/disc)	30	18	36
DMSO(control)	6(no inhibition)	6(no inhibition)	6(no inhibition)

4.6.2 Antifungal activity

Similarly antifungal activity was carried out against *Candida albicans, Saccharomyces cerevisae* and *Pichia* using the three fraction of each plant extract. Gentamycin was taken as positive control and DMSO as negative control. The overall activity has been tabulated below.

Tables 4.6: Antifungal activity of different fraction of plant extract.

Zone of inhibition(mm) with well diameter (6mm)			
Name of fungi	***Saccharomyces cerevisae***	***Pichia stipititis***	***Candida albicans***
Plant Extract			
Rheum australe			
Rheum-Met	10	10	12
Rheum-Chl	14	12	10
Rheum-Hex	12	10	10
Tinospora cordifolia			
Tino-Met	12	12	14
Tino-Chl	10	10	12
Tino-Hex	10	10	8
Datura stramonium			
Dat-Met	14	12	14
Dat-Chl	12	12	10
Dat-hex	10	10	10
Gentamycin (50mcg/disc)	6(no inhibition)	12	10
DMSO(control)	6(no inhibition)	6(no inhibition)	6(no inhibition)

4.7 Cytotoxicity screening for peritoneal macrophages

Screening for the cytotoxic effect of the crude fraction of plant extract on peritoneal macrpophages was conducted using MTT assay. None of extract demonstrated any significant inhibitory activity on peritoneal macrophages for the given experimental concentration.(i.e.0-300µg/ml) whereas DMSO exhibited the null effect on the peritoneal macrophages.

4.7.1 *Tinospora cordifolia*

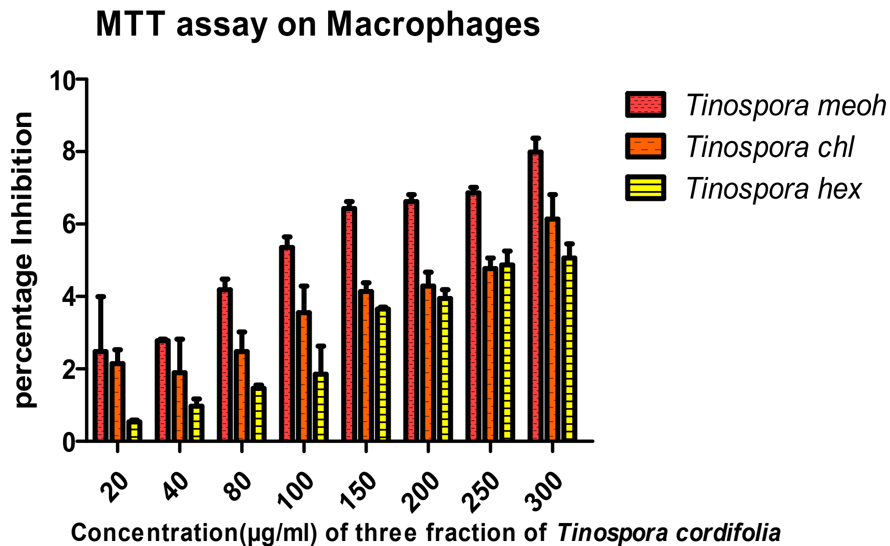


Figure 4.7.1 Cytotoxic effect of crude fraction of *Tinospora cordifolia* extract on peritoneal macrophages.

The error bars are represented as mean±SD

4.7.2 Rheum australe

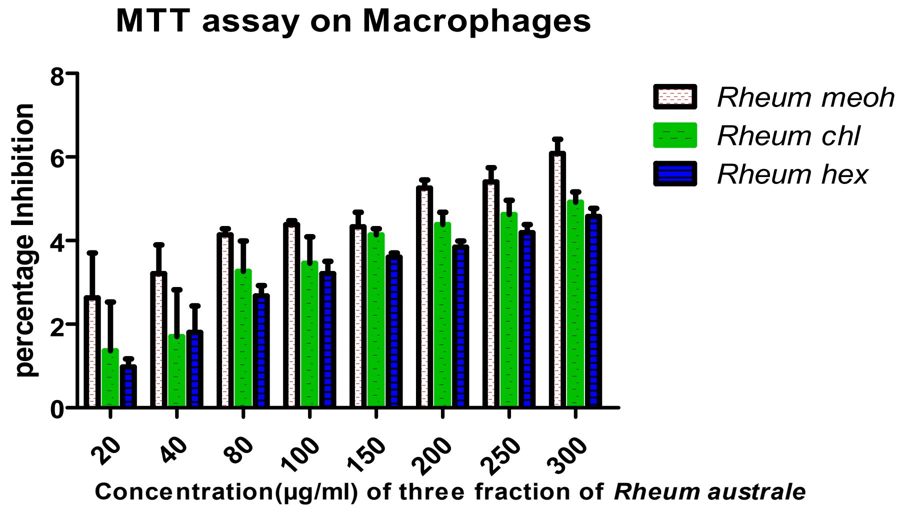


Figure 4.7.2: Cytotoxic effect of crude fraction of *Rheum australe* extract on peritoneal macrophages.

The error bars are represented as mean±SD

4.7.3 *Datura stramonium*

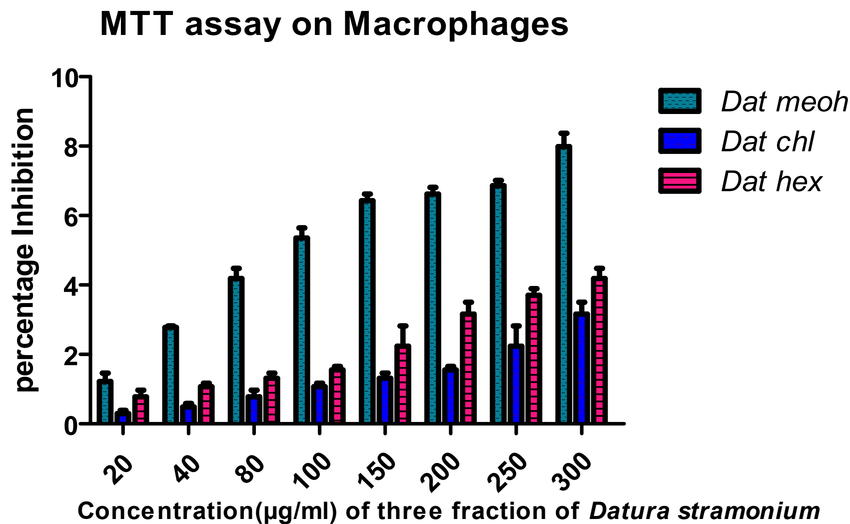


Figure 4.7.3: Cytotoxic effect of crude fraction of *Datura stramonium* extract on peritoneal macrophages.

The error bars are represented as mean±SD

4.8 Cytotoxicity screening for Hela cells

Human cervical cancer cell lines "Hela cells" was used to assess the cytotoxic effect of three fraction of crude plant extract as a anti cancer effect by performing the MTTassay. The standard drug(5-flurouracil) was taken as a positive control and it exhibited the strong dose dependent inhibition on hela cell lines.(Zhang et al.). The DMSO was taken as negative control and it did not show any inhibitory effect on the Hela cell line. The significant cytotoxicity effect was demonstrated by the crude extract of methanolic fraction of *Tinospora cordifolia* and least by the chloroform fraction of *Datura stramonium*. Moreover, the IC_{50} value of drug was recorded as 24.605±5.688 and IC_{50} value for the plant species was found minimum for *Tinospora cordifolia* of methanol fraction(50.754±5.776) and maximum for chloroform fraction of *Datura stramonium* (178.105±4.763).The least the IC_{50} value, more potent the anti cancer activity. The IC_{50} value of other fraction of each plant extract is found to be in between these values. The graph representing the % inhibition and IC_{50} value of drug and crude fraction of plant extract are given below.

4.8.1 Standard drug (5-Flurouracil)

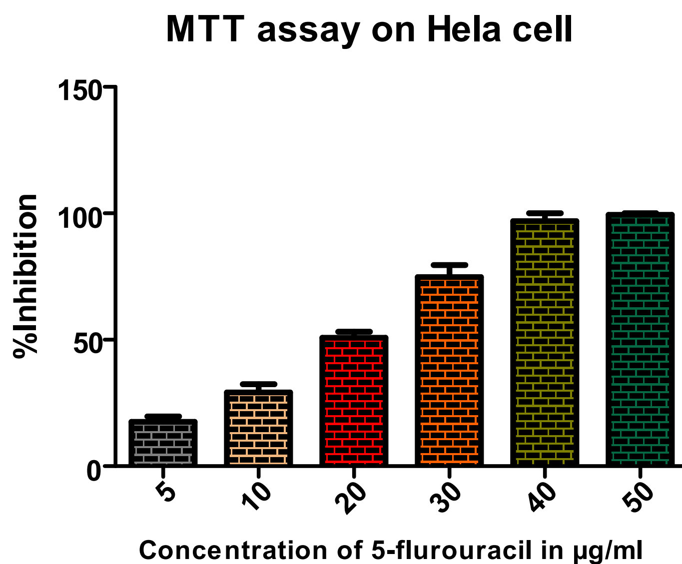


Figure 4.8.1 %inhibition of standard drug(5-Flurouracil) on Hela cell lines

The error bars are represented as mean±SD

4.8.2 *Tinospora cordifolia*

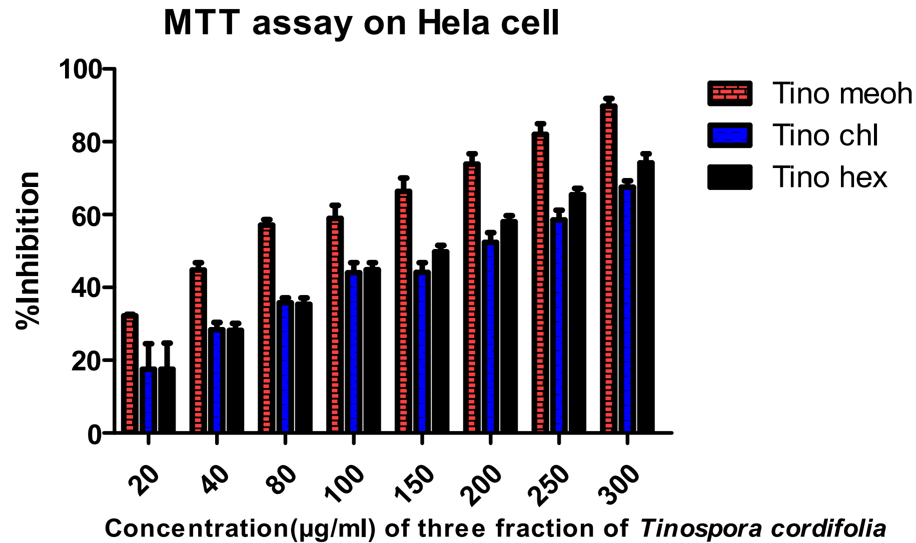


Figure 4.8.2: Cytotoxic effect of crude fraction of *Tinospora cordifolia* on Hela cells

The error bars are represented as mean±SD

4.8.3 *Rheum australe*

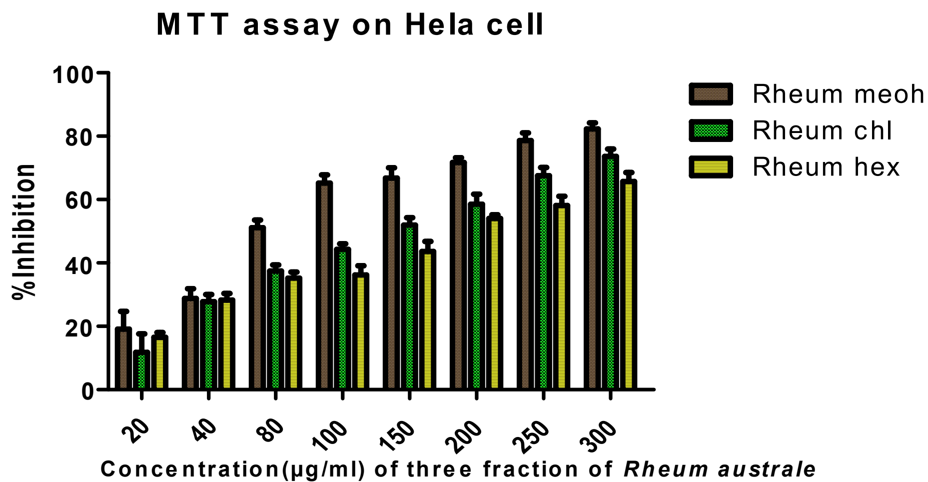


Figure 4.8.3: Cytotoxic effect of crude fraction of *Rheum australe* on Hela cells

The error bars are represented as mean±SD

4.8.4 *Datura stramonium*

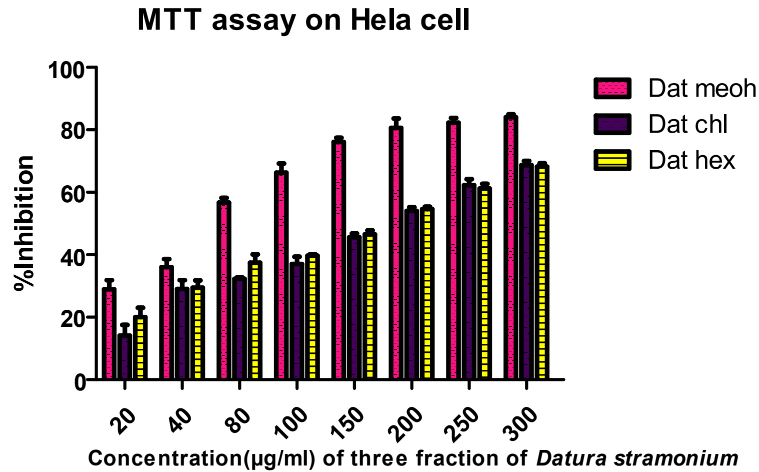


Figure 4.8.4: Cytotoxic effect of crude fraction of *Datura stramonium* on Hela cells

The error bars are represented as mean±SD

4.8.5 IC_{50} value of standard drug (5-Flurouracil) and crude fraction of extracts

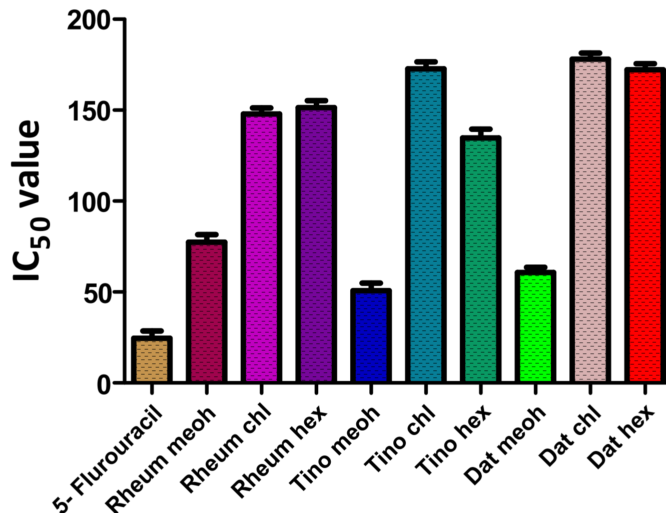


Fig 4.8.5: IC_{50} of standard drug (5-Flurouracil) and fraction of plant extract.

The error bars represent the standard deviation from the experimental mean values

Chapter V: DISCUSSION

Medicinal have been used for the treatment of various ailments and are also written in oldest repository like *Rigveda*. High value medicinal plants have served as a major source of drugs for centuries, and about half of the pharmaceuticals in use today are derived from medicinal plants while the use of indigenous drugs from plant origin form major part of complementary and alternative medicines in the form of herbal drugs and Ayurvedic medicines. Many such high value medicinal plants have been equally used in other forms of alternative medicines such as Chinese, Tibetan and Homoeopathic medicine (Lin et *al*., 2005). Nepal too is rich in biodiversity and possesses a variety of medicinal and aromatic plants in different geo-climatic region. Himalayan plants have been said to have very high chemical diversity and thus offers a potential source for the discovery of new and useful natural products for use in medicines.

5.1 Yield of plant extract.

The yield of plant extract depends on the parts of the plant material used; different solvent used as well as the chemical composition and content of the plant phyto-chemicals that are present within the plant species. A significant variation has been obtained on the yield of the extract. Out of three solvent i.e methanol, chloroform and hexane used, the highest yield was obtained in methanol followed by the chloroform and hexane. It could be the high polarity of methanol which has the ability to dissolve large number of primary as well as secondary metabolites.

5.2 Phytochemical screening

Qualitative phytochemical screening is an essential step towards discovery of new drugs as it provides the information regarding the presence of a particular primary or secondary metabolites in the plant extract of clinical significance. The presence of any significant bioactive natural product indicates the necessity of separation of the compound from the mixture of compounds through suitable chromatographic techniques. Standard methods were used for preliminary phytochemical screening of methanol, chloroform and hexane extracts of R. australe to know the nature of phytoconstituents present in it. The results showed presence of all the necessary primary and secondary metabolites except tannins and resins. This finding has evident from the research work of (Shwokat et al., 2012). Similarly extracts prepared in different solvents were screened for the presence or absence of phytochemicals *T. cordifolia.* Tannins was absent, while, alkaloids, flavonoids, cardiac

glycosides, carbohydrates, proteins and steroids were present in all the extracts. Saponins were present only in methanolic extracts. The results corroborates with the results of Tanwar *et al*., (2012). Sivakumar and Dhana Rajan, (2011) also reported the presence of wide range of phytochemicals in different solvent extracts of *T. cordifolia* stem. While phytochemical screening for major constituents was undertaken using standard qualitative method for *D.stramonium*. The result clearly revealed that the fruits of plant contains all the phytochemicals screened for with the exception of tannins, and resins in all the three extracts in accordance with Aderotimi and Samuel, (2006). However there may be differences in the phytochemicals screened compare with others. This might be due to geographical locations of the plant as well as method of extraction procedure.etc.

5.3 Total flavonoid estimation.

Flavonoids are a group of polyphenolic phytochemicals that include flavones, isoflavones, (iso) flavanones, flavonols, catechins, anthocyanidins and chalcones.(Sanderson et al, 2004). Flavonoids are known to have widely diverse beneficial biological effects, such as anti-inflammatory (Middleton, 1998), antioxidant (Pietta, 2000), antiviral (Jassim and Naji, 2003), and anticancer effects (Adlercreutz, 2002).Certain flavonoids, such as the isoflavone genistein, are estrogenic whereas others such as chrysin, can interfere with steroid synthesis and metabolism (Zand et al., 2000).

The total flavonoid content of *R. austral* was in the range of 2.608±0.085mgQE/g to 5.780±.501mgQE/g with the highest in crude methanolic extract. However the finding of my result was different with respect to research activity of Aslam et al. (2012). It might be due to variation in the solvent, standard compound, parts and geographical location of plants collected etc. While the total flavonoid content in *T. cordifolia* varied from 2.766± 0.220 mg QE/g to9.350±0.286mgQE/g. weight as suggested by the research work of N. Praveen et al. (2012). The highest of them was found in the methanol extract. The total flavonoid content of the crude extract of *D. stramonium* varied from 2.900±0.262mgQE/g to 6.337±0.297mg QE/g with highest content in methanolic extract (6.3375±0.297mgQE/g). However the result was contrast to previous finding of P. Suresh Kumar et al., (2008). Whereas research work of Aderotimi and Samuel (2006) reported the absence of flavonoid. It might be due to the different parts and varied geographical location of the plants.

5.4 Total Polyphenol Estimation

Phenol is an another salient class of secondary metabolites having the potent and strong antioxidant property like suppression of free radical producing enzymes, strong ROS scavenging efficiency etc (Atmani et al., 2009. It has been found that wide variety of phenolic compound has shown the antiproliferative activity against cancer cell by suppressing the metabolic enzymes responsible for stimulation of potential carcinogens (Newman et al., 2002).The phenolic content in crude extract of *R.australe* varied in the range of 17.644±2.16 to 34.488±2.734 mg GAE/g in accordance with Aslam et al. (2012) with highest in methanolic extract because of its ability to dissolve polyphenol component. In *T. cordifolia*, the highest phenol content was found in methanolic extract about 39.2±2.173 mg GAE/g. However the result is different from the work of Sivakumar V (2010). They reported the lower content of phenol in stem of *T. cordifolia*. While the total phenolic content of the crude extract *D. stramonium* varied from 2.11 ±1.3 to 8.06 ±1.953 mg GAE/g with highest in methanolic extract in accordance to the research of Sharma and Sharma, (2013).

5.5 Free Radical scavenging assay

Free radicals are well known reactive molecules mainly derived from univalent reduction of oxygen. Free radicals are highly reactive and present challenges to the cellular morphology and functional integrity a decreased in membrane fluidity, loss of enzymes, receptor activity and damaged to membrane proteins leading to cell inactivation and disease conditions. Scavenging and diminishing the formation of oxygen-derived species are not 100% efficient. Thus micro nutrients or antioxidants taken as supplements are particularly important in diminishing the cumulative oxidative damages. (Vani et al., 1997).

The free radical-scavenging activities of the extracts were tested through DPPH- method (Singh *et al*., 2002) and the results were compared with Ascorbic acid. DPPH is usually used as a substrate to evaluate anti oxidative activity of antioxidants. The method is based on the reduction of methanolic DPPH- solution in the presence of a hydrogen donating antioxidant to the yellow-coloured diphenylpicrylhydrazine. Antioxidant activity DPPH inhibition of the plant extract is expressed as % inhibition of stable radical or inhibition concentration fifty (IC_{50}) in reference to standard compound. The plant extract with lowest IC_{50} value is considered having better antioxidant properties. During our experiment, it was noted that the IC_{5o} value of the standard antioxidant Ascorbic acid was found to be 41.69±2.309. While the methanolic extract of all the plant samples selected have the lowest IC_{50} value exhibiting the better antioxidant property. There has been a well documented positive correlation

between polyphenolic content of the extracts and its antioxidant activity (Huang and Mau, 2006). In this study, the extracts exhibited a concentration - dependent antioxidant activity by inhibiting DPPH- radical. Of the different extracts, methanol extract of *Tinospora cordifolia* exhibited the highest free radical scavenging activity of 87.5% followed by the methanolic extract of *Datura stramonium*(85.39%) and *Rheum austral*(80.67%).While the crude hexane and the chloroform extracts of all the three plants exhibited the moderate level free radical scavenging activity. It might be attributes by the presence lower amount of flavonoid and phenol compound whereas methanolic extract better dissolves the phenols and flavonoids resulting the highest antioxidant activity.
However ascorbic acid showed higher degree of free radical scavenging activity than that of the extracts at low concentration points.

5.6 Antimicrobial activity

It is irony that Pharmacological sciences have begotten tremendous number of novel antibiotics but microorganisms have demonstrated increased level of resistance against these drugs. It might be because of the expression of resistance genes against antibiotics as well as its hapazardous use. For example *P. aerigunosa* and *S. aureus* (Nordmann et al., 2009). However many more medicinal herbs can be used for treating microbial pathagons such as cholera, diarrhea, dysentery, typhoid and bacterial enteritis (Cowan 1999). Considering the vast potentiality of plants as the source of antimicrobial drugs with reference to the antibacterial and antifungal, a systematic investigation was undertaken to screen the local flora for antibacterial and antifungal activity from *Tinospora cordifolia*, *Rheum austral* and *Datura stramonium*. These all of the plant extract exhibited the significant activity against the microorganisms chosen during the research. Methanolic extracts of *Rheum austral* demonstrated significant inhibitory activity against yeast and bacteria such as: *Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa* as reported (Aqil and Ahmad, 2003). Similarly methanolic and chloroform fraction of *Tinospora cordifolia* exhibited antimicrobial activity against both gram positive and gram negative bacteria as well as fungi in accordance with the work of Jeyachandran (2003); Nagaprashanthi(2012). Methanolic and chloroform fraction have significant activity on *Klebsiella pneumonia* and *Staphylococcus aureus* respectively as well as on saccharomyces cerevisae *as suggested by the* Eftekhar, (2005). This study has provided some biochemical basis of the medical use of the extracts in the treatment of infections, as a potential source of useful drugs and phytochemicals.

5.7 Cytotoxicity against Macrophages

Macrophages are the normal immune cells which are very sensitive and get activated when foreign particles entered into the body. They are often involved in many different protective biological processes such as defense against microbial tissue, remodeling during embryogenesis, wound healing, removal of damaged or senescent cells subsequent to injury or infection, recognize and killing of tumor cells. It has been found that immuno-modulatory functions of macrophages have been demonstrated through the release of reactive oxygen species including NO, TNF-α and various cytokines like IL-10, IL-12, IL-4 etc (Hamilton, 1992). Similarly many cancer vaccines have also been shown to elicit potent immuno cellular responses by activation and production of IL-12 and TNF-a (Dalgleish, 2000). Thus, macrophages form suitable model to study the cytotoxic effects of various fraction of chosen plant extracts. In our investigation, the crude extracts that were obtained demonstrated very nominal or little cytotoxic effect on the macrophage cells at a given concentration of 0-300µg/ml using MTT assay. The results were further confirmed by using visual microscopy where the cells were observed for their morphological changes upon extract treatment. This result illustrates that plant extract render no risk to the macrophage but provide platform to study cytotoxicity against various cell lines. However various crude plant extract namely *R. australe, T.cordifolia,* and *D.stramonium* have played significant part in activating macrophages to enhance immune response by producing the large no NO, TNF-α, and IL-12 leading to anti-tumour, wound healing and antibacterial effects via Th-1 and Th-2 cytokine regulation in vivo. (Kounsar et al., 2011; Upadhyaya et al.,2011).

5.8 Cytotoxicity against HeLa cell lines

Oxidative stress and damages are the major threat and herculean task for human health-related researchers. There are many more important free radicals responsible for oxidative damage commonly termed as reactive oxygen species (ROS) (Sies H 1997). These reactive oxygen species are involved in increased mitochondrial malfunction, decrease in membrane fluidity, loss of enzyme receptor activity, damage to membrane proteins and genomic instability thereby leading to the carcinogenesis (Pelicano et al., 2004). Carcinogenesis is a process of deregulation of live and dead cell ratio, where the cells undergo mutation to form tumor and finally lead to the state of cancer. The principal methods of cancer treatment include chemotherapy radiotherapy and surgery.

Chemotherapy is a systemic treatment, to which the whole body is exposed. Among the most successful chemotherapeutic agent are Cisplatin, Mitomycin and Docetaxel. However all of these agents enhance serious side effects or long term complication (Fan W et al., 1998).

Plants have a long history of use in the treatment of cancer and consist of ample source large no of secondary metabolites such as phenolic acid, phenolic, diterpenes, flavonoid, tannins, glycosides, saponins etc which can used as reservoir of drug components with anticancer and antitumor properties.

An ideal chemopreventive agent is expected to inhibit, delay or reverse the process of carcinogenesis through its antioxidant potential and/or its cytotoxicity and/or apoptosis-inducing property. In present scenario, human cervical cancer cell lines 'HeLa' provides a suitable platform to study cytotoxicity of the three crude extract of each plant samples namely *Rheum austral, Tinospora cordifolia* and *Datura stramonium* against the cancer cells utilizing MTT assay, well-established and sophisticated technique in cancer biology. (Roomi et al., 2006; Nair and Varalakshmi 2011). Such investigation of medicinally important plant against the cancer cell lines help to flourish the anticancer property and pave the way to develop the plant extract as effective chemopreventive agent or drug model. In our experimental work, methanolic fraction of each plant extract demonstrated the effective inhibitory activity against the HeLa cell lines. It may be attributed by it high antioxidant property augmented with triterpenoids, phenolics, flavonoides and other phytochemical alone or in combination.

The cytotoxicity of methanol, chloroform and hexane fraction of crude Rheum austral extract was characterized by a steady increase in the percentage cytotoxicity with respect to extract dosage. However, the methanolic fraction exhibited the highest percentage of inhibition with the lowest IC_{50} value. (fig 4.8.1). The significant inhibitory activity against cancer cell lines might be cumulative effect of different component present in plant extract such as emodin, aloe emodin. Emodin is capable of inhibiting cellular proliferation, induction of apoptosis, and prevention of metastasis. These capabilities are reported to act through tyrosine kinases, phosphoinositol 3- kinase (PI3K), protein kinase C (PKC), NF-kappa B (NF-kB), and mitogen-activated protein kinase (MAPK) signaling cascades. Aloe-emodin anti-proliferative property has been demonstrated through the p53 and its downstream p21 pathway. (Qing Huang et al., 2006)

Moreover the finding is further supported by the work of Naveen et al., (2012) and images taken using light microscopy during the experiment of MTT assay. Similarly the crude extract of *T. cordifolia* exhibited the pronounced effect on Hela cell in dose dependent manner especially the methanolic extract. Methanolic extract of *T. cordifolia* demonstrated the significant cytotoxicity (89.79±2.908) and lowest IC_{50} value. Lower the IC_{50} value higher the cytotoxicity effect. Whereas other fraction exhibited moderate level of inhibition at the same concentration. The experimental work corroborates the research activity of Jagetiaa et al., (1998). They demonstrated that *T. cordifolia* killed the HeLa cells very effectively in vitro when exposed at 0-100g/ml of extract of methanol. Moreover, the work is further supported by the research work of Rao S K, Rao PS, (2010). It has been reported that all parts of *D. stramonium* are poisonous if ingested by human or livestock. However, it could be used for medicinal purposes (King 1984, Mann 1992). The current investigation of various fraction of *D.stramonium* appeared that methanolic fraction exert the significant antiproliferative effect whereas the chloroform and hexane fraction demonstrated the moderate antiproliferative effect. The anticancer effect of *Datura* extract was supported by previous study in which compound isolated from the *D. stramonium* exhibited significant cytotoxic activities against liver, cervix and breast cell lines (Meselhy,2012) as well as the research activity of Ala'a H et al.(2010) and Iman M. Ahmad et al.(2009).

.

Chapter VI: Summary

This thesis describes the phytochemical investigation, quantification, antioxidant and cytotoxic property of methanol, chloroform and hexane fraction of *Tinospora cordifolia*, *Rheum australe* and *Datura stramonium*.

In present study, stem of *T.cordifolia* and *R. australe* and fruit of *D. stramonium* were collected from various parts of Nepal. The plant materials were authenticated by Taxonomist Dr. Deepak Raj Pantha (Central Department of Botany, Kirtipur) and Dr. Suira Joshi (Ayurvedic herb specialist, Ayur polyclinic, Lalitpur, NAMC NO.330).

After authentication, the plant materials were subjected to drying at room temperature in the shade until they were free from the moisture and subjected to size reduction to get coarse powder and then the uniform powder was subjected to physic-chemical evaluation with parameters.

32 grams plant materials were subjected to ultrasonication with methanol, Chloroform and hexane solvent followed by rotavapor evaporation. The residues obtained were subjected to preliminary phytochemical investigation to know the presence of various constituents in selected plant materials.

The result of preliminary phytochemical investigation has indicated the presence of carbohydrates, proteins, alkaloids, flavonoids, polyphenol, glycosides steroids, terpenoids, tannins and saponins in selected plants. During the quantification of polyphenols and flavonoids, the flavonoid content was found to be less significant amount with respect to the polyphenol contents. Among the three plants evaluated, the *Tinospora cordifolia* was found to contain highest content followed by the other plants. Moreover the methanolic content was found to content higher polyphenol and flavonoid yield. However, overall yield was satisfactory with respect to other research paper.

Evaluation of in-vitro antioxidant studies were carried out by using DPPH as a source of free radical and Ascorbic acid as a standard. The change in coloration of the resulting solution from violet to yellow is regarded as the presence of scavenging activity of the test extract. The radical scavenging activity of all the fraction of plant extract was found to be quite appreciable. Among the various fraction, methanolic extract render the pronounced antioxidant activity and *T.cordifolia* exhibited the highest percentage of radical scavenging activity although *R. australe* and *D.stramonium* demonstrated the equivalent degree of radical scavenging activity with comparable IC_{50} value with standard Ascorbic acid.

Moreover, the results of polyphenol and flavonoid corroborate the antioxidant property demonstrated by the selected medicinal plants.

Antimicrobial activity of methanol, chloroform and hexane extract were evaluatated in vitro against three bacterial species namely *K. pneumonia*, *S. typhimurium* and *S. aureus* and three fungal species namely *S. cerevisae, C. albicans* and *Pichia stipititis*. All extracts studied in this work showed antimicrobial activity against the test microorganisms. A lot new finding has been disclosed during the experimental work. Methanolic fraction of R. austral demonstrated the better inhibition of the *K. pneumonia* with regard to the standard drug I.e Chloramphenicol. Methanolic fraction of all the plant extract exhibited the significant inhibition activity against resistant *S. aureus*. However, activity of plant extract against the fungi was not satisfactory but *S.cerevisae* resistant against the Gentamycin was least sensitive against the Methanolic fraction of all plant extract.

As expected all the fractions of plant extract were least or null cytotoxic to the peritoneal macrophages. They demonstrated no harm to the natural immune cell. However all the fraction of plant extract were capable of producing the pronounced effect on the HeLa cell, especially the methanol fraction. The methanol fraction of *T. cordifolia, R australe* and *D.stramonium* displayed the significant cytotoxicity against the cancer cell lines whereas the chloroform and hexane exhibited the moderate level of cytotoxicity but their significance cannot be neglected. The overall activity might be contributed by the antioxidant property of the plant. This consummate study gives insight on medicinal property of the plant chosen namely *T. cordifolia, R australe* and *D.stramonium* and hence, guides to utilize the medicinal property of plant in various biological and pharmacological applications.

CHAPTER VII: CONCLUSION

Use of different medicinal plants to treat different diseases has been used since prehistoric times in traditional health practice systems like Aayurveda and Chinese medicine. Medicinal plants play vital role on the Nepalese livelihood and use of medicinal plant is frequent in several regions, especially in the rural areas of Nepal where the health facilities are not accessible. Though modern medical science is making its way to the grassroots, a vast majority of the rural people of Nepal are still dependent on traditional healers and medicinal plants for their primary health care. Although, Nepal is a natural storehouse for medicinal plants and several tons of plant raw materials have been exported to Asian countries (including India) and Europe and America, the contribution of this sector to national economy is still nominal and there is still dearth of knowledge regarding the collection and its application in modern medicine. Moreover, there are hapazardous collections of the medicinal plant for their immediate need without analyzing its future impact. As a consequence, medicinal plants are declining day by day and some are at the point of extinction due to their random collection. Thus it is necessary to facilitate rural people regarding the proper collection, importance in modern medicine, value in the international market and well documented books regarding the every aspect of medicinal plants. To fulfill every aspect, rigorous study and exploration are required. This thesis work is single leap to disclose the selected indigenous medicinal plant of Nepal.

The medicinal plants namely *R.austrle, T.cordifolia* and *D. stramonium* were collected on basis of expert advice, locally availability, medicinal properties etc. Phytochemical analysis, quantification, antioxidant efficacy, antibacterial and cytotoxic activity were performed. From our study it was concluded that various phytochemical, including phenols and flavonoids were present in selected parts of the plants. Antioxidant activity of all three extracts was investigated using DPPH radical scavenging method. Antioxidant efficacy of methanolic fraction of all the extract was found to be highest in comparison to other fraction. Strong antioxidant property of plant indicate possible use of plant in atherosclerosis, coronary heart diseases, Parkinson’s diseases, diabetes, neurodegenerative (Alzheimer’s disease), inflammatory response syndrome, respiratory diseases, liver diseases ,aging and cancer. Antibacterial activity test showed some antibacterial potency of the extracts even though it was very lower as compared to the standard antibiotics which serve as a clear indication of the potential of these extracts for further chemical and pharmacological studies. However, antifungal activity of the extract was not satisfactory as expected but there was new finding regarding the resistant exhibited by the *S.cervisae*

against the Gentamycin. So it alarms the increasing drugs resistant among the various microorganisms. As expected, the peritoneal macrophages were viable against the various fraction of plant extract. The plant extract render no threat or non toxic to the normal immune macrophage cells. However, the various fraction of plant extract significantly demonstrated the toxic property against the cancer cell lines chosen (i.e. HeLa) in dose dependent manner. This indicates that the activity is selective against the cancer cell lines and that the isolation of the particular compounds responsible for this activity may prove to be invaluable. Thus the toxic property of plant can be exploited to devise the chemotherapeutic agent against the cancer and tumor treatment. This overall research gives abysmal information of the selected indigenous medicinal plant of Nepal and their prospect in pharmacological science and scientific research and validation.

CHAPTERVIII: RECOMMENDATION

From this preliminary research I would like to make the following recommendations.

- Plants produce a wide range of secondary metabolites, which may prove to be invaluable in development of drugs, flavors, fragrances, dyes, anti-oxidants, insecticides, antimicrobial and anticancer etc. It is thus important to locate and determine the role of these secondary metabolites in plants and unravel their biosynthesis.

- Research on the non-volatile compounds warrant further study. Further analysis must be conducted using different detectors and chromatographic techniques such as liquid chromatography, mass spectroscopy, HPLC, to provide a greater insight of the photochemical composition of this species.

- Metabolomics investigates the end products of cellular functions. The use of metabolomics in plant studies will enable the characterization and differentiation of genotypes and phenotypes based on the levels of metabolites, and also aid in the rapid screening of multiple extracts giving an exceptionally broad overview of the chemistry. This may also provide a means of improving the production of certain metabolites in plants through genetic engineering.

- Nepal has favorable climate and geographical variability that support growth and cultivation of such high valued plants. Thus, there is an urgent need for improved management and commercialization of medicinal plants and still there is a large untapped reservoir waiting to be investigated.

Plants and their parts selected for research

Stem parts of *Reum australe*

Stem parts of *Tinospora cordifolia*

Fruit parts of *Datura stramonium*

Phytochemical screening

Antioxidant activity by DPPH method

Antimicrobial screening

1. Methanol
2. Chloroform
3. Hexane
4. DMSO

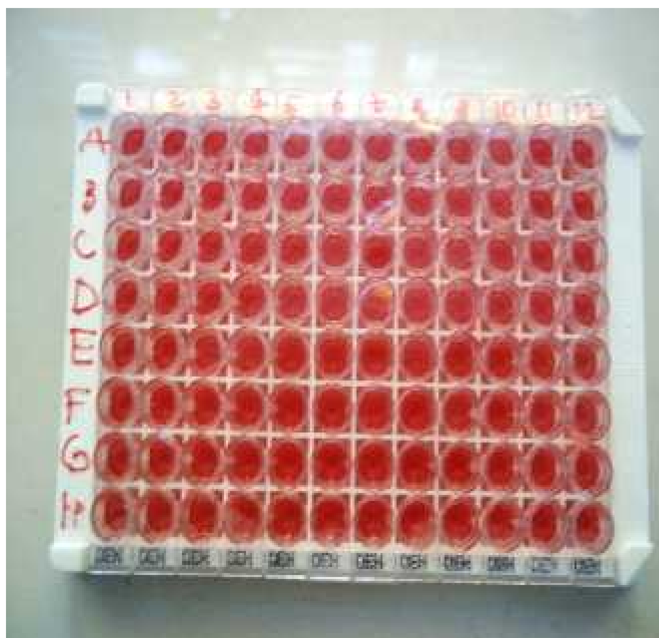
Activity of three fraction of *D. stramonium* on *K.pneumonia*

1. Methanol
2. Chloroform
3. Hexane
4. DMSO

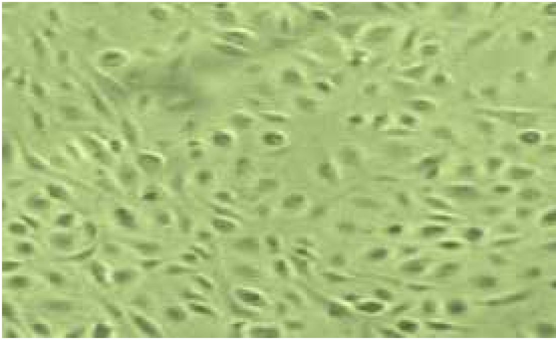
Activity of three fraction of *D.stramonium* on *S. cerevisae*

Injection of starch solution in peritonial cavity

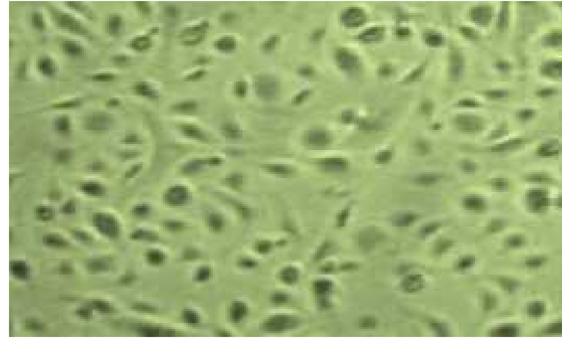
Observation of HeLa cell growth



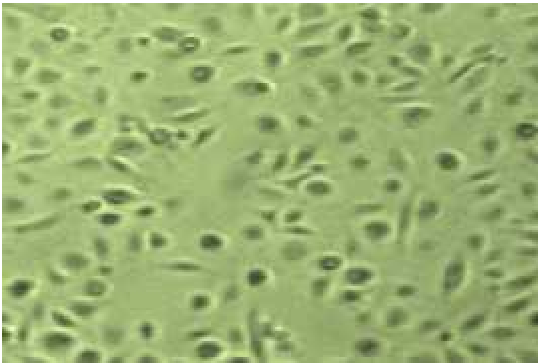
Cell culture plating for Cytotoxicity



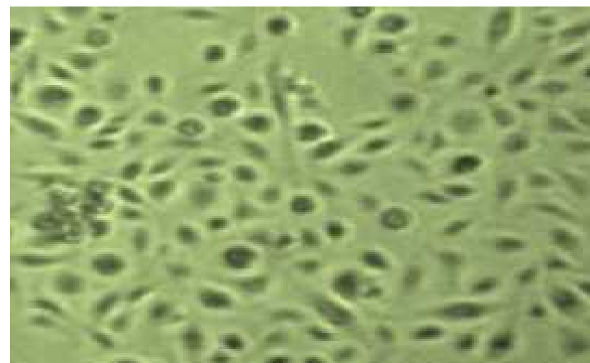
A. Control (100X)



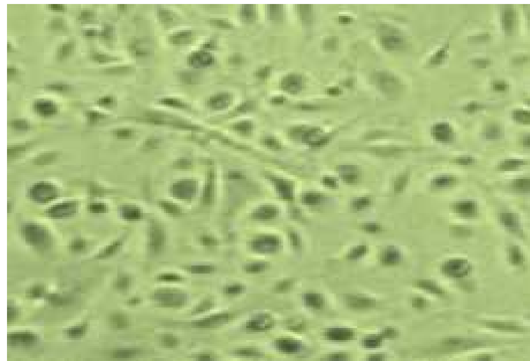
B. Concentration at 20µg/ml(100X)



C. Concentration at 100µg/ml(100X)

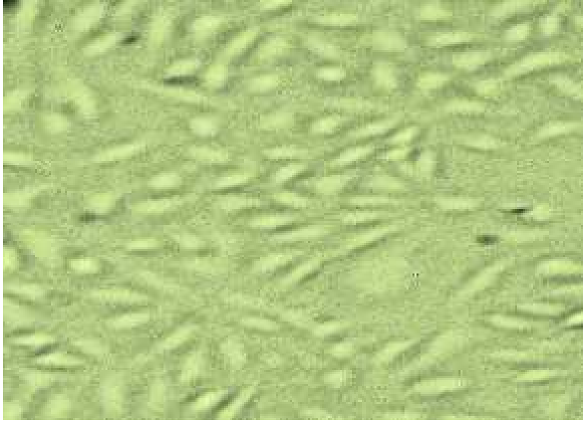


D. Concentration at 200µg/ml(100X)

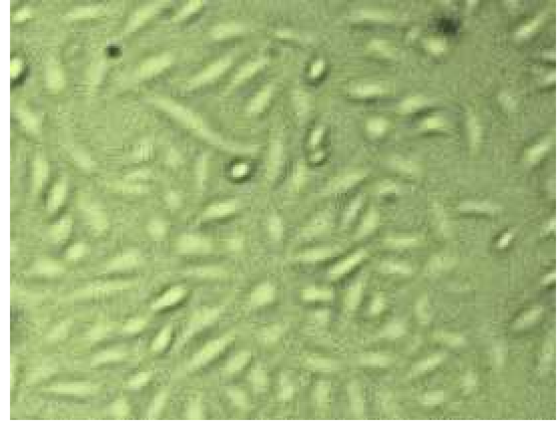


E. Concentration at 300µg/ml(100X)

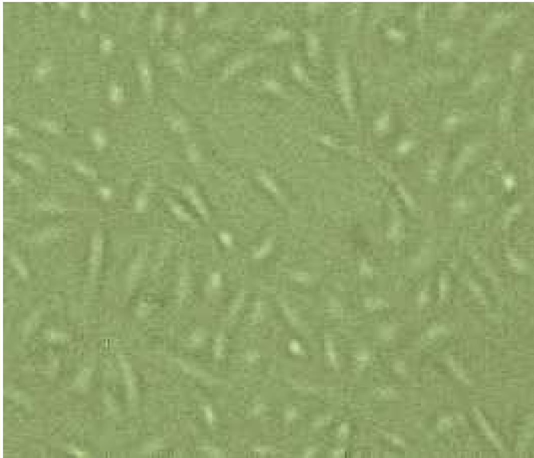
Cytotoxic effect of methanol fraction of Tinospora cordifolia on Peritonial macrophages.



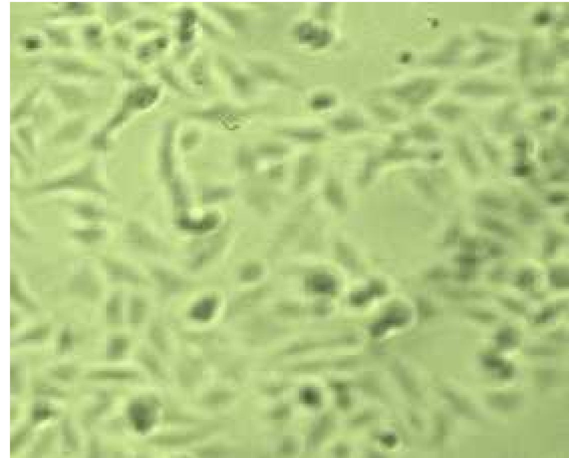
A. Control (100X)



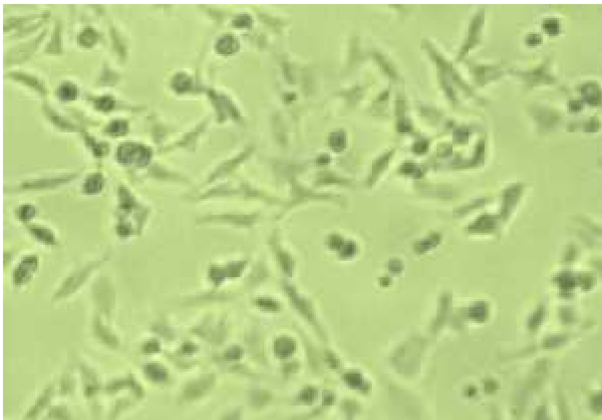
B. concentration at 20µg/ml (100X)



C. Concentration at 100µg/ml (100X)



D. Concentration at 200µg/ml(100X)



E. Concentration at 300µg/ml(100X)

Cytotoxic effect of methanolic extract of Tinospora cordifolia on HeLa cells.

References

Adams O. D, & Hamilton T. A. (1992). Molecular basis of macrophages activation and its origins New York: Oxford University Press.Pp. 75–114.

Aderotimi Banso and Samuel Adeyomo (2006). Phytochemical screening and antimicrobial assessment of Abutilon mauritianum, Bacopa monnifera and Datura stramonium BIOKEMISTRI 18(1):39-44.

Adlercreutz H, (2002). Phyto-oestrogens and cancer. Lancet Oncology 3: 364–373.

Agarwal S. K., Singh S. S., Verma S., Kumar S., (1999) Ind J Chem., , 38B, 749−751.
Aima RK. (2003) "Pictorial Guide to Plants". Natraj Publishers First Edition Dehradun; pp. 454–5.

Ahmad, I., Z. Mehmood and F. Mohammad,(1998). Screening of some Indian medicinal plants for their antimicrobial properties. J. Ethnopharmacol., 62: 183-193.

Akharaiyi F C (2011) Antibacterial, Phytochemical and Antioxidant activities of Datura metel International Journal of PharmTech Research CODEN (USA): Vol.3, No.1, pp 478-483.

Ala'a H Merza, Khalil Z Al- Jeboori, Nahi Y Yaseen.(2010) Cytotoxic Effect of Datura stramonium Extract on Cancer Cell lines. Iraqi Journal of Veterinary Medicine Vol. 34, No. 1.

Al-Bayaty F, Abdulla M, Mohamed I and Hussein S (2010). Effects of Malaysian medicinal plants on macrophages function in vitro study. J Med Plant Res. 4(14): 1459-1463.

Anjana Agnihotri, Kapil kumar, Archana Srivastav and Seema Bhaduria (2012.) Antibacreial potential of Tinospora cordifoli aagainst resistant human pathogen bacteria Vol 4, Suppl 5.

Aqil F, Ahmad I, (2003). Broad-spectrum antibacterial and antifungal properties of certain traditionally used Indian medicinal plants. World Journal of Microbiology and Biotechnology 19, 653–657.

Arunkumar, S., Muthuselvam (2009). Analysis of phytochemical constituents and antimicrobial activities of aloevera L. against clinical pathogens. World J. Agril. Sc., 5(5): 572 576.

Atmani D, Chaher N, Berboucha M, Ayouni K, Lounis H, Boudaoud H, Debbache N, Atmani D (2009). Antioxidant capacity and phenol content of selected Algerian medicinal plants. Food Chem.112: 303- 309.

Avery A, Satina S, Rietsema (1959) J. Chron Bot; 20:1.

B Krishna Murthy, S Nammi, M K Kota, R V Krishna Rao, N Koteswara Rao, A Annapurna.(2004) Evaluation of hypoglycemic and antihyperglycemic effects of Datura metel (Linn.) seeds in normal and alloxan-induced diabetic rats. Journal of Ethnopharmacology: 91: 95–98.

Babu K. S., Srinivas P. V., Praveen B, Kishore K. H., Murthy U. S., & Rao J. M. (2003). Antimicrobial constituents from the rhizomes of Rheum emodi. Phytochemistry, 62, 203–207.

Bania TC, Chu J, Bailes D, O'Neil (2004) M. Jimson weed extract as a protective agent in severe organophosphate toxicity. Acad Emerg Med.; 11(4): 335-338.

Berkov S, Zayed R, Doncheva T. (2006). Alkaloid patterns in somevarieties of Datura stramonium. Fitoterapia.; 77(3):179-182.

Bhattarai NK (1992): Folk herbal remedies of Sindhupalchok district, central Nepal. Fitoterapia, 63(2):145-155.

Bhattarai, N.K. (1997). Biodiversity-people interface in Nepal. NWFP 11. Medicinal plants for forest conservation and health care. FAO & UN Rome, Italy.

Borgia M, Sepe N, Borgia R, Ori-Bellometti M.(1981). Pharmacological activity of an herbal extract: controlled clinical study. Curr Ther Res; 29: 525-536.

Castleman M.(1991) The Healing Herbs: The Ultimate guide to the curative powers of nature's medicine. Emmaus PA: Rodale Press: 305-307.

Chang LW, Yen W J, Huang, SC and Duh PD (2002) Antioxidant activity of Sesame Coat. Food Chemistry 78: 347-354

Chang YC, Lai TY, Yu CS, Chen HY, Yang JS, Chueh FS, Lu CC, Chiang JH, Huang WW, Ma CY, Chung JG, (2011). Emodin induces apoptotic death in murine myelomonocytic leukemia WEHI-3 cells in vitro and enhances phagocytosis in leukemia mice in vivo. Evid Based Complement Alternat Med 2011: 523-535.

Chaudhary R, Jahan S, Goyal P K(2008). Chemopreventive potential of an Indian medicinal plant (tinospora cordifolia) on skin carcinogenesis in mice. J en viron pathol toxicol oncol; 27:233-43.

Comer, M and E Debus. (1996). A partnership: Biotechnology, biopharmaceuticals and biodiversity. Pp. 488-499.

Cowan, M.M. (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 564-582.Cox, PA. (1994). The ethnobotanical approach to drug discovery: Strengths and limitations. Pp. 25-41

Cragg GM, Newman DJ (2001) Medicinals for the Millennia. Annals of the New York Academy of Sciences 953: 3-25.

Criagg, G.M., David, J.N. (2001). Natural product drug discovery in the next millennium. J. Pharm. Biol., 39: 8-17.

D.r. Kumar Naveen , Shikha Swati , V. Cijo George, P. K. Suresh and Kumar Ashok(2012) anticamcer and anti metastatic activities of Rheum emodi rhizome chloroform extract. Asian Journal of Pharmaceutical and Clinical Research; Vol 5, Issue 3, ISSN - 0974-2441

Dahanukar SA, Kulkarni RA, Rege NN (2000) Pharmacology of medicinal plants and natural products. Indian Journal of Pharmacology 32: S81-S118.

Dalgleish A G. (2000). Cancer vaccines. British Journal of Cancer, 82, 1619–1624.

Das S, Kumar P, Basu SP. (2012). Review article on phytoconstituents and therapeutic potentials of Datura stramonium linn. J Drug Del Therapy; 2(3): 4-7.

Das S, Kumar P, Basu SP. (2012). Review article on phytoconstituents and therapeutic potentials of Datura stramonium Linn. J Drug Deliv Ther.; 2(3): 4-7.

Davis EW: (1995)Ethnobotany: an old practice, a new discipline. In Ethnobotany: Evolution of Discipline Edited by: Schultes RE, Reis SV. Dioscorides Press, Oregon; 40-51.

D.R. Naveen Kumar, Swati Shikha, V. Cijo George, P. K. Suresh And R. Ashok Kumar (2012). Asian Journal of Pharmaceutical and Clinical Research Vol 5.

Dewick, P.M. (1996). Tumor inhibition from plants: Tease and Evans.

Duke J A. (1997) Green Pharmacy. Emmaus PA: Rodale Books,: 507

Edoga, H.O., Okwu, D.E., Mbaebie, B.O.(2005). Phytochemicals constituents of some Nigerian medicinal plants. Afr. J. Biotechnol., 4(7): 685-688.

Eftekhar F, Yousefzadi M, Tafakori V (2005). Antimicrobial activity of Datura innoxia and Datura stramonium. Fitoterapia.; 76(1): 118-120.

Elizabeth A Ainsworth & Kelly M Gillespie (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nature Protocols 2, 875 - 877

Escribano J, Piqueras A, Medina J, Rubio A, Alvarez-Orti M, Fernandez J A(1999). Production of a cytotoxic proteoglycan using callus culture of saffron corms (Crocus sativus L.) J Biotechnol. 73(1):53-59.

Fan W, Johnson KR, and Miller MC,(1998). In vitro evaluation of combination Chemotherapy against human tumor cells. Oncology Report, 5(5): 1035-1042.

Gaire BP. (2008) Monograph on Datura stramonium. Kaski, Nepal: Pokhara University Press: 1 114.

Goel HC, Prem Kumar I, Rana SV. (2002). Free radical scavenging and metal chelation by tinospora cordifolia, a possible role in radioprotection. Indion j Exp Biol; 40:727-34.

Gupta R (2011). Ameliorate effect of Tinospora cordifolia root extract on histopathological and biochemical changes induced by aflatoxin-b (1) in mice kidney.Toxicol Int;18:94-99.

Harborne J.B. (1973). Phytochemicals Methods. Chapman and Hall Ltd., London, pp. 49-188.

Huang Q, Shen H. M, Shui G, Wenk M. R, & Ong C. N. (2006). Emodin inhibits tumor cell adhesion through disruption of the membrane lipid Raft-associated integrin signaling pathway. Cancer Research, 66, 5807–5815.

Iman M Ahmad, Maher Y Abdalla, Noor H Mustafa , Esam Y Qnais , Fuad A Abdulla. (2009) Datura Aqueous Leaf Extract Enhances Cytotoxicity via Metabolic Oxidative Stress on Different Human Cancer Cells. Jordan Journal of Biological Sciences. Volume 2, Number1, ISSN 1995-6673 Pages 9 – 14.

Iman M. Ahmad, Maher Y Abdalla, Noor H Mustafa, Esam Y Qnais , Fuad A Abdulla (2009) Datura Aqueous Leaf Extract Enhances Cytotoxicity via Metabolic Oxidative Stress on Different Human Cancer Cells Volume 2, Number 1, Pages 9 – 14.

Jagetia GC, Venkatesh P, Baliga MS. (2005). Influence of seed extracts of S. cumini on mice exposed to different doses of γ radiation. J. Radiat. Res. 46: 59-65.

Jagetiaa G C, Nayaka V and Vidyasagar M S, (1998)Evaluation of the antineoplastic activity of gudchi (Tinospora cordifolia) in culturesd HeLa cells, Cancer Lett, 127(1-2) 71.

Jassim S.A, Naji M.A,(2003). Novel antiviral agents: a medicinal plant perspective. Journal of Applied Microbiology 95: 412–427.

Jeyachandran R, Xavier TF, Anand SP (2003). Antibacterial activity of stem extracts of tinospora cordifolia (willd) hook. F & thomson. 40-43.

K M Meselhy. (2012) Cytotoxic and insect -repellent activities of surface flavonoids from Datura stramonium L. Grown in Egypt. Life Sci J; 9(4):3154-3158.

K. Suresh Babu, Ashok K. Tiwari, Pullela V. Srinivas, Amtul Z. Ali, B. China Rajuaand J. Madhusudana Rao (2004). Yeast and mammalian a-glucosidase inhibitory constituents fromHimalayan rhubarb Rheum emodi Wall.ex Meisson. Bioorganic & Medicinal Chemistry Letters 14 (2004) 3841–3845.

Khaleque A, Miah MAW, Huq MS and Abdul BK. (1971). Pak J Sci Ind Res.; 14, 481.

Khan MA, Gray AL and Waterman PG. (1989). Tinosporaside, an 18- norclerodane glucoside from T.cordifolia. Phytochemistry; 28: 273- 275

Khan M, Wassilew, S.W. (1987). Natural pesticides from the neem tree and other tropical plants. (Eds) Schmutterer, H. and Asher, K. R. S., Germany: Digitalverlag GmbH, pp.645-650.

Khare CP (2007). Indian medicinal plants. Delhi: Rajkamal Electric Press.: 203.

King FB (1984). Plants, People, and Paleoecology. Illinois State Museum Scientific Papers, Vol. 20.

Kirtikar KR and Basu BD. (1918) Indian Medicinal Plants, Lalit Mohan Basu, Allahabad; vol. I, pp. 75–80.

Kirtikar KR and Basu BD. (2005) Indian Medicinal Plants. Second Edition, Edited and enlarged by Blatter E, Caius JF and Mhaskar KS. International Book Distributers, Dehra Dun, Vol 1, pp. 77-81.

Kirtikar KR, Basu BD. (1999) Indian medicinal plants. 2nd ed.Volume III. Dehradun: International Book Distributors: 1783-1787.

Kounsar F, Rather M A, Ganai B. A & Zargar M A. (2011). Immuno-enhancing effects of the herbal extract from himalayan rhubarb Rheum emodi wall. Ex Meissn. Food Chemistry, 126, 967–971

Krenn L, Presser A, Pradhan R, Bahr B, Paper D. H, Mayer K. K, et al. (2003). Sulfemodin 8-O-b-D-glucoside, a new sulfated anthraquinone glycoside, and antioxidant phenolic compounds from Rheum emodi. Journal of Natural Products, 66, 1107–1109

Krenn L, Pradhan R, Presser A, Reznicek G, & Kopp B. (2004). Anthrone C Glucosides from Rheum emodi. Chemical and Pharmaceutical Bulletin, 52, 391–393.

Krenn L., Presser A., Pradhan R., Bahr B., Paper D. H., Mayer K. K., Kopp B., (2003). J Nat Prod. 66, 1107−1109.

Krenn L.,Pradhan R., Presser A., Reznicek G., Kopp B., (2004) Chem Pharm Bull. 52, 391−393.

Kumar A, Krishan MRV, Aravindan P, Jayachandran T, Deecaraman M, Ilavarasan R, Padmanabhan A. (2008). Anti diabetic activity of Syzygium cumini seed and its isolate compounds against streptozotocin induced diabetic rats. J. Med. Plants. Res. 2: 246-249.

Kumar A, Krishan MRV, Aravindan P, Jayachandran T, Deecaraman M, Ilavarasan R, Kumar RM, Padmanabhn A. (2008). Anti inflammatory activity of Syzygium cumini seed. Afr. J. Biotechnol. 7: 941-943.

Kumar KE, Mastan SK, Reddy KR, Reddy GA, Raghunandan N, Chaitanya G. (2008). Anti arthritic property of methanolic extract of Syzygium cumini seed. Int. J. Integr. Biol. 4: 55-60.

Kunwar R.M, Nepal B.K, Kshhetri H.B, Rai S.K, Bussmann R.W,(2006). Ethnomedicine in Himalaya: a case study from Dolpa, Humla, Jumla and Mustang districts of Nepal. Journal of Ethnobiology and Ethnomedicine 2, 27.

Kunwar,R.M. (2006). Non-timber forest products of Nepal: A sustainable management approach. International Tropical Timber Organization, Japan and Center for Biological Conservation, Nepal. 423.

Kuo P, L Lin, T C, & Lin C. (2002). The antiproliferative activity of aloe-emodin is through p53-dependent and p21-dependent apoptotic pathway in human hepatoma cell lines. Life Sciences, 71, 1879–1892.

Kuo PL, Lin TC, Lin CC, (2003). The antiproliferative activity of aloe-emodin is through p53-dependent and p21-dependent apoptotic pathway in human hepatoma cell lines. Life Sci 71(16):1872-1892.

Laird, S., Johnstone, S., Wynberg, R., Lisinge, E. and Lohan, D.(2003). Biodiversity Access and Benefit-Sharing Policies for Protected Areas: An Introduction. United Nations University Institute of Advanced Studies (UNU/IAS), Tokyo, Japan.

Li A, Bao B, Grabovskaya-Borodina A.E, Hong S.P, McNeill J, Mosyakin S.L, Ohba H, Park C.W, (2003).Flora of China. Vol.5 (Ulmaceae through Basellaceae). Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis, pp. 341–350.

Li J, (2009). Emodin Reverses Paclitaxel Resistance and Inhibits Invasive Activity of Paclitaxel Resistant Ovarian Cancer Cells. Shandong Universtity: Jinan.

Li J, Lin B, Wang G, Gao H, Qin M. Chemical constituents of Datura stramonium seeds. Zhongguo Zhong Yao Za Zhi. 2012; 37(3): 319-322.

Lin YC, Lee ACC, Kemper KJ and Berde CB (2005) Use of complementary and alternative medicine in pediatric pain management service: a survey. Pain Med 6: 452-458.

Ling-na Zenga, Zhi-jie Maa, Yan-ling Zhaoa, Lin-dong Zhang, Rui-sheng Li e, Jia-bo Wanga, Ping Zhanga, Dan Yana, Qi Li, Bing-qian Jianga, Shi-biao Pua, Yang Lua, Xiao-he Xiaod .(2013). The protective and toxic effects of rhubarb tannins and anthraquinones in treating hexavalent chromium-injured rats: The Yin/Yang actions of rhubarb Journal of Hazardous Materials) 1– 9; 246– 247.

Liu B., Yang J., Wang S., Huaxi Yaoxue Zazhi, (2007). 22, 33−35.

Liu YJ, Zhen YS, Shang BY, Zhen YS,(2009). Rhein lysinate suppresses the growth of tumor cells and increases the anti-tumor activity of taxol in mice. Am J Chin Med 37(5): 923-931.

Lopez-Otin, C. & Diamandis, E. P. (1998). Breast cancer and prostate cancer: an analysis of common epidemiological, genetic, and biochemical features. Endocrine Reviews, 19, 365−396.. Agarwal S. K., Singh S. S., Verma S., Kumar S., .(2000) J Ethnopharmacol. 72, 43−46.

Malik S, Sharma N, Sharma U. K, Singh N. P, Bhushan S, Sharma M, et al. (2010). Qualitative and quantitative analysis of anthraquinone derivatives in rhizomes of tissue culture-raised Rheum emodi Wall. plants. Journal of Plant Physiology, 167, 749–756.

Manandhar N.P, (2002). Plants and People of Nepal. Timber Press Inc., Portland, OR. MoFSC/DF, 2008/2009. Hamro Baan (Our Forest). Department of Forest, Ministry of Forest and Soil Conservation, Nepal (in Nepali).

Mann, J.(1978). Secondary Metabolism. Oxford University press, London, pp. 154.

Maurya R and Hardass.(1998).“Tinocordifolin, a sesquiterpene from T.cordifolia.” Phytochemistry; 49: 1343-6.

Mdee LK, Masoko P, Eloff JN. (2009). The activity of extracts of seven common invasive plant species on fungal phytopathogens. S Afr J Bot.; 75(2): 375-379.

Mendonca-Filho RR (2006) Boiactive phytocompounds: New Approaches in the phytosciences. Modern Phytomedicine, Wiley-VCH Verlag Gmbh and Co. KGaA: 1-24

MFSC (2006). Third National Report to the Convention on Biological Diversity. Ministry of Forests and Soil Conservation, HMNG-Nepal.

Middleton E Jr, Kandaswami C, Theoharides TC (2000). Effect of plant flavonoids on on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol rev. 2000 Dec;52(4):673-751.

Miles E.A, Wallace F.A, Calder P C, (2000). Dietary fish oil reduces intercellular adhesion molecule 1 and scavenger receptor expression on murine macrophages. Atherosclerosis 152, 43–50.

Mohd Aslam, Rameshwar Dayal, Kalim Javed, Nazeem Fahamiya, Mohd Mujeeb, Asif Husain (2012). Pharmacognostical and Phytochemical Evaluation of Rheum emodi Wall Current Pharma Research ISSN: 2230-7842 CPR 2(2), 471-479.

Mojab F, Kamalinejad, M, Ghaderi, N., Vanidipour, H.R. (2003). Phytochemicals screening of some species of Iranian plants. Iran. J. Pharm. Res., 3: 77-82.

Nagaprashanthi.Ch, Rafi khan.P, Gopi chand.K, Aleemuddin MA, Rajiya Begum.G (2012). In vitro Antimicrobial Activity of Tinospora cordifolia and its Phytochemical screening International Journal of PharmTech Research CODEN (USA) Vol.4, No.3, 1004-1008.

Nair R, Kalariya T and Chanda S (2005). Antibacterial activity of some selected Indian medicinal flora. Turk J Biol, 29: 41-47.

Nair S and Varalakshmi KN (2011). Anticancer, cytotoxic potential of Moringa oleifera extracts on HeLa cell line. J Nat Pharm. 2(3): 138-142.

Newman DJ, Gragg GM, Holbeck S, Sausville EA (2002). Natural products as leads to cell cycle pathway targets in cancer chemotherapy. Cur. Cancer Drug Targets. 2: 279-308.

Nordmann P., Cuzon G., Naas T. (2009). The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis 9: 228–236.

P Suresh Kumar, S Sucheta, V Sudarshana Deepa, P Selvamani and S. Latha(2008) Antioxidant activity in some selected Indian medicinalplants African Journal of Biotechnology Vol. 7 (12), pp. 1826-1828.

Padhya MA. (1986). “Biosynthesis of Isoquinoline alkaloid berberine in tissue cultures of T. cordifolia” Indian drugs; 24:47-48.

Pallavi Sharma1 and Ram Avatar Sharma(2013). Comparative Antimicrobial Activity and Phytochemical Analysis of Datura stramonium L. Plant Extracts and Callus In vitroEuropean Journal of Medicinal Plants 3(2): 281-287.

Paolo MG. (2001). Traditional antihelmintic, antiparasitic and repellent uses of plants in Central Italy. J Ethnopharm; 68(1-3): 183-192.

Parekh, J., Chanda, S. (2007). Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. Afr. J. Biomed. Res., 10: 175-181.

Parekh, J., Chanda, S. (2008). Phytochemicals screening of some plants from western region of India. Plant Arch., 8: 657- 662.

Peirce A. (1999). The American Pharmaceutical Association practical guide to natural medicines. New York: William Morrow and Company Inc.pp 12

Pelicano H, Carney D, Huanga P (2004). ROS stress in cancer cells and therapeutic implications. Drug Resistance Updates.; 7: 97–110.

Pendse VK, Mahavir MM, Khanna KC and Somani SK.(1981) Anti-inflammatory and related activity of Tinospora cordifolia (Neem giloe). Indian drugs; 19: 14-71.

Perez C PM, Bazevque P (1990) An antibiotic assay by the agar well diffusion method. Acta Biologet Med Exp. 15: 113-115.

Phillipson, J.D., Wright, C.W.(1996). Plants With Antiprotozoal Activity : Tease and Evans,

Pharmacognosy, 14th edn., WB Saunders Company, London, pp. 612.

Pie Shengji j (2001). Ethnoecological approaches of traditional medicine studies: Some experiences from Asia. Pharmacoceutical Biology 39:74-79.

Pietta P.G, (2000). Flavonoids as antioxidants. Journal of Natural Products 63: 1035–1042.

Preissel U, Preissel HG. (2002) Brugmansia and Datura: Angel's Trumpets and Thorn Apples. New York: Firefly Books: 106-129.

Press J.R, Shrestha K.K, Sutton D.A, (2000). Annotated Checklist of the Flowering Plants of Nepal. The Natural History Museum, London.

Pretorius E, Marx J. (2006) Datura stramonium in asthma treatment and possible effects on prenatal development. Environ Toxicol Pharmacol.; 21(3): 331-337.

Qing Huang, Guodong Lu, Han-Ming Shen, Maxey C.M. Chung, Choon Nam Ong (2006). Anti-Cancer Properties of Anthraquinones from Rhubarb. in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/med.20094

R Jeyachandran,T Francis Xavier and S P Anand (2003). Antibacterial activity of stem extracts of Tinospora cordifolia (Willd) Hook. f & Thomson Vol : XXIII(1) July, August, September;40-3

Raaman N(2006). Phytochemical techniques. New India Publishing Agency pp19 -24.

Rajbhandari KR.(2001). Ethnobotany of Nepal. Kathmandu Kishor Offset Press Private Limited.: 142-143.

Rajkumar V, Guha G, Kumar A.S., (2011). Apoptosis Induction in MDA-MB-435S, Hep3B and PC-3 cell lines by Rheum emodi Rhizome Extracts. Asian Pacific Journal of Cancer Prevention 12, 1197–1200.

Rajkumar V., Guha G., Kumar R.A. (2010). Antioxidant and anti-cancer potentials of Rheum emodi rhizome extracts. Evidence-based Complementary and Alternative Medicine, doi: 10. 1093

Rao S K, Rao PS. (2010). Alteration in the radiosensitivity of HeLa cells by dichloromethane extract of guduchi(Tinospora cordifolia). Integer Cancer Ther; 9:378-84.

Rao SK, Rao PS, Rao BN. (2008). Preliminary investigation of the radiosensitizing activity of guduchi (tinospora cordifolia) in tumoring bearing mice. Phytother Res; 22:1482-9.

RATES, S.M.K., (2001). Plants as source of drugs. Toxicon, 39, 603-613.

Rawat AK, Mehrotra S, Tripathi SC, Shome U (1997) Hepatoprotective activity of Boerhavia diffusa L. roots - a popular Indian ethnomedicine. Journal of Ethnopharmacology 56: 61-66

RP Singh, KN Chidambara Murthy, GK Jayaprakasha(2002). Studies on antioxidant activity on pomegranate (Punica granatam) peel and seed extract using in vitro models. Journal of agricultural and food chemistry.Vol:50; 81-86.

Rokaya M.B., Münzbergová Z., Timsina B. (2010). Ethnobotanical study of medicinal plants from the Humla district of western Nepal. J. Ethnopharmacol. 130, 485–504.

Roomi MW, Ivanov V, Kalinovsky T , Niedzwiecki A and Rath M (2006). Suppression of human cervical cancer cell lines Hela and Do Tc2 4510 by a mixture of lysine, proline,ascorbic acid and green tea extract. Int J Gynecol Cancer. 16(3):1241-1247.

Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A and Rath M (2006). Suppression of human cervical cancer cell lines Hela and DoTc2 4510 by a mixture of lysine, proline, ascorbic acid, and green tea extract. Int J Gynecol Cancer. 16(3): 1241-1247.

Sanderson T.J, Hordijk J, Denison M.S, Springsteel M.F, Nantz M.H, Van den Berg M,(2004). Induction and ınhibition of aromatase (cyp19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxıcologıcal Scıences 82: 70–79.

Sharma V, Panday D(2010) beneficial effect of Tinospora cordifolia on blood profiles in male mice exposed to leads toxicol Int; 17:8-11.

Sharma V, Panday D.(2010). Protective role of Tinospora cordifolia against lead- induced hepatoxicity. Toxicol Int; 17: 8-11.

Showakat Ahmad Awani, Mir Ashfaq, K. W. Shah and Dharmendra Singh(2012) Phytochemical screening of methanolic extract of Podophyllum hexndrum Royal and Rheum emodi Wall .J. Curr. Chem. Pharm. Sc.: 2(2), 125-128 ISSN 2277-2871.

Sies H. (1997) Oxidative stress: Oxidants and Antioxidants. Experitmental Physiol.; 82: 291-5.

Singh A (2007) Herbal medicine–dream unresolved. Pharmacognosy Reviews. 2: 375-376.

Singh J, Sinha K, Sharma A, Mishra NP and Khanuja SP. (2003) Traditional uses of Tinospora cordifolia (Guduchi) J Med Aromat plant Sci; 25: 748-51.

Singh N, Singh SM, Shrivastava P (2004). Immunomodulatory and antitumor action of medicinal plant Tinospora cordifolia are mediated through activation of tumor associated macrophages..Immunopharmacol Immunotoxicol; 26(1):146-62.

Singh N, Gupta M. (2007). Effect of ethanolic extract of Syzygium cumuni seed powder on pancreatic islets of alloxen diebetic rats. Indian J. Exp. Biol. 45: 861-867.

Singh N, Singh SM, Shrivastava P. (2005). Effect of tinospora cordifolia on the antitumor activity of tumor–associated macrophage derived dendritic cells. Immunopharmacol immunitoxicol; 27(1):1-14.

Singh RP, Banerjee S, Kumar PV, Raveesha KA, Rao AR. (2006). Tinospora cordifolia induces enzymes of carcinogen/ drug metabolism and antioxidant system and inhibits lipid peroxidation in mice.phytomedicine; 13:74-84.

Singh RP, Bannerjee S, Kumar PV, Raveesha KA, Rao AR. (2006) Tinospora cordifolia induces enzymes of carcinogen/drug metabolism and antioxidant system, and inhibits lipid peroxidation in mice.Phytomedicine;13:74-84

Singh S. S., Pandey S. C., Singh R., Agarwal S. K. (2005), Ind J Chem44B. 1494−1496.

Sinha K, Mishr NP, Singh J, Khanuja SPS. (2004). Tinospora cordifolia, a reservoir plant for therapeutic application. Indian Journal of Traditional Knowledge,; 3(3): 257-70.

Sivarajan VV, Balachandean I(1999). Ayurvedic Drugs and their plants sources. New Delhi: Oxford and IBH publishing Co. Pvt. Ltd;

Sonika G, Manubala R, Deepak J. (2010) Comparative studies on anti-inflammatory activity of Coriandrum sativum, Datura stramonium and Azadirachta indica. Asian J Exp Biol Sci.; 1(1): 151-154.

Stanely Mainzen, Prince P, Menon VP (2003.) Hypoglycemic and hypolipidimic action of alcoholic extract of tinospora cordifolia roots in chemical induced diabetic in rats. Phytother Res; 17:410-3.

Stanely Mainzen, Prince P, Menon VP, (2001). Antioxidant action of Tinospora cordifolia root extract in alloxan diabetic rats. Phytother Res; 15(3):213-8

Strahil Berkov, Rawia Zayed , Tsvetelina Doncheva (2006). Alkaloid patterns in some varieties of Datura stramonium. Fitoterapia. 77: 179–182.

Suresh B. K., Srinivas P. V., Praveen B., Hara K. K., Suryanarayana M. U., Madhusudana R. (2003) J. Phytochemistry. 62, 203−207.

Tanwar S, Jain J, Verma S and Solanki D (2012). Standardization and phytochemical evaluation of Tinospora cordifolia (willd.) Miers. (Menispermaceae). International Journal of Pharmacy and Pharmaceutical Sciences.1 (4): 219-223.

T.C. Dinis, C.L. Santosa and L.M. Alemida. (2002). The apoprotein is the preferential target for deoxynitirte induced LDL damage protection by phenolic acids. Free radical res. 36 (2): 531-43.

Thippeswamy G, salimanth BP. (2007) Induction of caspase-3 activated DNase mediated apoptosis by hexane fraction of tinospora cordifolia in EAT cells. Environ Toxicol Pharmacol;23 212-20.

Thomas A. Hamilton, Jennifer A. Major and Guy M. Chisolmt. The Effects of Oxidized Low Density Lipoproteins on Inducible Mouse Macrophage Gene Expression Are Gene and Stimulus Dependent Departments of Immunology and Cell Biology, Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195.

Tonthubthimthong P, Chuaprasert S, Douglas P, Luewisutthichat W. (2001). Supercritical CO_2 extraction of nimbin from neem seeds an experimental study. J. Food Eng. 47: 289-293.

Treadway S. (1998) Exploring the universe of ayurvedic botanicals to manage bacterial infections. Clinical Nutrition Insights; 6 (17): 1-3.

Trease G E, Evans W.C. (1989). Pharmacognosy, 11th ed., Bailliere Tindall, London, pp. 45-50.

UNDP, UNEP, World Bank and WRI. 2000. World Resources 2000-2001. Washington DC, World Resources Institute.

Upadhyaya R, P R Sharma V, Anita KV. (2011) Assessment of th multifaceted immunomodulatory potential of the aqueous extract of Tinospora cordifolia. Res J Chem Sci; 1:71-9.

Vasu, K., Goud, J.V., Suryam, A., Singara, Chary, M.A. (2009). Biomolecular and phytochemical analyses of three aquatic angiosperms. Afr. J. Microbiol. Res., 3(8):418-421.

V. Sivakumar, M.S. Dhana Rajan And M.S. Riyazullah (2010). Preliminary Phytochemical Screening and Evaluation Of Free Radical Scavenging Activity Of Tinospora Cordifoli. An international Journal of Pharmacy and Pharmaceutical Sciences vol 2, Issue 4.

V Sivakumar and Dhana Rajan MS (2011). Standardization & Characterization of Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thoms. Plant stem extract in different solvent fractions. Asian Journal of Biochemical and Pharmaceutical Research Issue: 4 (Vol. 1).

Wang J, Zhao Y, Li H, Zhao H, Zhang P, Jin C, (2009). Assessment of the renal protection and hepatotoxicity of rhubarb extract in rats. J Ethnopharmacol 124(1): 18-25.

Wang X, Lous Z, Mikage M, Namba T. (1988). Pharmacognostical studies on the Chinese crude drug da-huang rhubarb II. Botanical origin of three unofficial da-huang. Shoyakugaku Zasshi; 42: 302-309.

Wealth of India: A dictionary of Indian Raw Materials and Industrial Products. First Edition Vol X. New Delhi: CSIR; 2003. Anonymous; pp. 251–2.

WHO, (2003). WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants. WHO - World Health Organization, Geneva, Switzerland.

WHO (2008). Traditional medicine: fact sheet No 134. WHO-World Health Organization, Geneva, Switzerland.

Wong SK, Lim YY, Chan EW. (2009). Antioxidant properties of Hibiscus species variation, altitudinal change costal influence and floral colour change. J. Tro. For. Sci. 21: 307-315.

Nair R, Kalariya T, Chanda S. (2005). Antibacterial activity of some selected Indian medicinal flora. Turk. J. Biol., 29: 41-47.

Yan-Ling Zhao, Jia-Bo Wang, Guang-De Zhou, Li-Mei Shan and Xiao-He Xiao (2009) Investigations of Free Anthraquinones from Rhubarb Against α-Naphthylisothiocyanate-induced Cholestatic Liver Injury in Rats. Basic & Clinical Pharmacology & Toxicology, 104, 463–469.

Yuan-yuan Chai, FangWang, Yan-li Li, Ke Liu, and Hui Xu (2012) Antioxidant Activities of Stilbenoids fromRheum emodi WallEvidence-Based Complementary and Alternative Medicine Volume 2012, Article ID 603678, pp 7.

Zand R.S, Jenkins D.J, Diamandis E.P, (2000). Steroid hormone activity of flavonoids and related compounds. Breast Cancer Research Treatment 62: 35–49.

APPENDIX

1.Reagents used for Phytochemical Screening

i. Mayer's Reagent: (Raaman, 2006): Freshly prepared by dissolving a mixture of $HgCl_2$ (1.36 g) and of KI (5.00 g) in water (100.0 ml).

ii. Dragendorff reagent: (Raaman, 2006) Solution A: Dissolve 0.5 g bismuth nitrate in 20 ml of 20% acetic acid. Solution B: 5 ml of a 40% KI solution in water. Just before use, mix 20 ml solution A, 5 ml solution B and 70 ml water

iii.Benedict Reagent: (Raaman, 2006): Solution A: Dissolve 8.65g of CuSO4.5H2O in 75 ml of H2O. Solution B: Dissolve 86.5 g of sodium citrate and 50 g of anhydrous Na2CO3 in 400 ml water. Mix Benedicts solution A slowly into solution B and make up the volume to 500 ml

2. Preparation of 1 M Na_2CO_3 -100 ml:

10.599 gram of the Na_2CO_3 (Merk Specialities Pvt. Ltd, Mumbai, India) was carefully weighed and then dissolved in distilled water and the volume was adjusted to 100 ml at the end.

3. Preparation of Glacial acetic acid (20%) - 200 ml:

40 ml of the commercially supplied glacial acetic acid (Thermo Fisher Scientific, India) was taken and mixed with ethanol. Finally the volume was adjusted to 200 ml by the addition of ethanol.

4. Preparation of Aluminium Chloride (10%) -100 ml:

10 gram of the commercially supplied aluminium chloride (Merk Specialities Pvt. Ltd, Mumbai, India) was weighed and dissolved in water. Finally the volume was maintained to 100 ml.

5. Preparation of 1M potassium acetate (CH_3COOK) – 100 ml:

Weigh 9.814 gram of the potassium acetate Merk Specialities Pvt. Ltd, Mumbai, India) and dissolve on water. Finally maintain the volume to 100 ml by the addition of water.

6. Preparation of 0.2mM DPPH solution - 100 ml:

1, 1- diphenyl-2 picryhydrazyl (DPPH) has the molecular weight of 394.32 gm/mol. Thus, 100 ml of 0.2mM solution of DPPH is prepared by weighing the 7.886 mg of the DPPH carefully and dissolving it on ethanol and finally maintaining the volume to 100 ml by addition of ethanol.

7. Preparation of the Folin – Ciocalteu phenol reagent (1: 10)

6 ml of the commercially supplied Folin – Ciocalteu phenol reagent (Merk Specialities Pvt. Ltd, Mumbai, India) was taken and mix it with 54 ml of the distilled water to prepare the 60 ml of 1: 10 dillution of Folin – Ciocalteu phenol reagent.

8. Composition of Nutrient agar media:

The composition of nutrient agar media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

S.N	Components	Gram/L
1	Peptic digest of animal tissue	5.0
2	Beef extract	1.5
3	Yeast extract	1.5
4	Sodium chloride	5.0
5	Agar	15.0
6	P^H	7.4 ± 0.2

9. Composition of Luria Bertani broth, (LB) Miller media:

The composition of Luria Bertani broth, (LB) Miller media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) are as follow.

S.N	Components	gram/L
1	Casein enzyme hydrolysate	10
2	Yeast extract	5.0
3	Sodium chloride	10.0
4	Final PH	7.5± 0.2

10. Preparation of 0.5 McFarland standards- 100 ml

The components that were used for the preparation of the 0.5 McFarland standard is as follows

S.N	Components	Amount in ml
1	Sulfuric acid, 0.18 M	99.5
2	Barium chloride, 0.048 M	0.5

11. Composition of Mueller Hinton Agar (MHA)

The composition of Mueller Hinton agar (MHA) media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

S.N	Components	gram/L
1	Beef infusion form	300
2	Casein hydrolysate	17.5
3	Starch	1.56
4	Agar	17
5	Final PH	7.3 ± 0.2

12. Composition of Cell Culture PBS: (Dulbecco and Vogt, 1954)

S.N	Chemical	Concentration (mM)
1	NaCl	137.93
2	KCl	6.67
3	Na_2HPO_4	8.06
4	H_3BO_3	1.47
5	Final P^H (at 25oC)	7.40 ±0.2