CHEMICAL AND PHARMACOLOGICAL EVALUATION OF ETHNOMEDICINAL PLANTS OF SIKKIM

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This Thesis is

dedicated to all of my

beloved students

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CERTIFICATE

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PREFACE

Natural products are viable sources and resources for drug discovery and development. The continual emergence of new natural product with novel chemical structures, various biological activities along with the potential for chemical modification and synthesis bode well for the utility of natural products as lead molecules in drug discovery.

Like the combinatorial chemistry, nature itself presents the most diverse and complete source of leads. According to the new approach that unites the strengths of combinatorial chemistry and natural product identification in a process, referred to as combinatorial biosynthesis, in which a natural product is identified and the genetic basis of it is elucidated and modified to produce different biologically active products of therapeutic potency. Combinatorial biosynthesis utilizes enzymes from various natural product source biosynthetic pathways to create novel chemical structures.

Herbs and herbal medicine have been an integral part of our health care for centuries and will continue to develop and expand in our future health care system. Sikkim is a small state, has the highest biodiversity and harbors approx. 4000 vegetal species that encompass enormous therapeutic potential. In this context, these study protocols, aimed at testing for safety and pharmacological efficacy of the plants used in traditional system of medicine may be the cornerstone of modern and rational drug development.

The work embodied in this thesis is a humble and sincere effort to explore and screen three ethnomedicine *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn. used by traditional healers of Sikkim in their health care. The finding of this investigation revealed that the crude extracts of these plants are very useful as anti-inflammatory, antipyretic, hepatoprotective, antoxidant, antimicrobial and hypoglycemic agents. The constituents of these plants were isolated and purified, analyzed the chemical structures of the pure compounds as well as their activities were evaluated.

The thesis has been segregated into 10 well-defined chapters and finally a summary and conclusion has been drawn along with the summary of the whole work. At the end of each chapter the necessary references have been enlisted. The investigated work deals with a scientific approach to explore the pharmacological activities of the compounds isolated from *Urtica parviflora*, *Callicarpa arborea* and *Morinda citrifolia*, three ethnomedicinal plants from the Sikkim Himalayan region. As the ethnomedicinal uses of these plants have been validated scientifically, these can be utilized against various diseases in a cost effective manner and also as a basis for further scientific study.

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30-9-2008

(Prasanna Kumar Kar)

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CHAPTER 1

INTRODUCTION

1.0 Introduction

1.1 Sikkim: The geography and population

Sikkim is a beautiful tiny Himalayan state situated on the flanks of Eastern Himalayas at the latitude of 27°00'46" to 28° 07' 48" and 88°00'58" to 89°55'20' longitude north. The state is bounded by Tibet in the North and North-East, Bhutan in the South- East, Nepal in the West and West Bengal in the South. The Pangolakha Range of mountains separates Bhutan, Singalila range separates Nepal and Chola range separates Tibet from Sikkim. The state has a very rugged topography defined with towering mountains and deep valleys. Even though the total geographical area is only 7096 sq. kms, its altitude varies from 244 mt. at Melli to 8,598 mt. at Mt. Kanchandzonga forming the mountaineering climate. The other important peaks in the state such as Kumbhakana (7,711 mt.), Penden (6706 mt.), Narsing (5,825 mt.), Kabru Dome (6,545) are also found in the Kanchandzonga range. Glaciers descend from these mountaineering peaks to form the source for important rivers like Teesta and Rangit in Sikkim (Anonymous 1, 2007; Pant, 2003).

Due to its rugged topography, land locked condition and altitudinal variations the state is facing backwardness compared to the other states in India but the richness of flora, fauna, water falls, hot springs, Orchids, Rhododendron, snow capped mountains, adventure trekking, world renowned Monasteries, river rafting, customs and culture offer a great potential for tourism development (Rai *et.al.,* 2000). The total population of Sikkim as per the Census of Sikkim is 540,851 in the year 2001. The overall density of population in the state is 76 persons per sq km, which is one of the lowest in India. The total number of literates (7 years and above) in the state is 234,135 of whom 137,745 are male and 96,390 are female. The Schedule Caste population according to census 2001 was 5.03 % and the Schedule Tribe population 20.62%. The main language of Sikkim is Nepali. Besides Nepali Bhutia, Limbu, Hindi, English are also the running languages.

1.2 Tribal Communities

India is one of the twelve mega diversity countries in the world and has 17,000 flowering plants. Among the 25 hotspots in the world, the Eastern Himalayas and the Western Ghats are the two hotspots of India. The country possesses a total of 427 tribal communities. The largest proportion of tribes is found in Mizoram (95%), followed by Lakshadweep (93%), Nagaland (88%), Meghalaya (86%) and Arunachal Pradesh (64%) (Kala, 2005).

Tribal peoples throughout the world including India, have developed their own cultures, customs, religious rites, taboos, legends and myths, folk tales, medicine and food habits. They are the repository of accumulated experience and of the knowledge of indigenous vegetation which can be utilized in tribal development. These days greater emphasis is being laid on this traditional knowledge and on the 'bioprospecting' of natural products as a new source of food and medicine (Ganesan *et al.*, 2007).

In early times mankind developed, through observation and experience; knowledge of the properties of plants as a source of food and medicines. Although food and medical facilities are more readily available to most of the people in our times, still in several underdeveloped and less accessible areas of the country, food deficiency and lack of medical facilities are prevalent. Plant parts like fruits, tubers, flowers, leaves, etc., are consumed as principal or supplementary food and employed as medicines (Singh, 2001).

1.2.1 Tribal medicine

Tribal medicinal practices first came to be of interest mainly to the anthropologists and ethnographers from the western countries. The subject still continues to draw their interests' world over. Their interests in the matter have mainly to project the indigenous knowledge base of the tribal people regarding their health and survival. But more often than not the tribal practices have also been highly publicized as exotic cultural elements mixed with voodoo and witchcraft. The role of the shaman, magic, divination and cure has thus been overhyped. Of late, of course the social and psychological support value of tribal etiology is being widely projected (Roy Burman, 2003).

The interest in the tribal medicinal practices as evident in Sikkim has also followed more or less the same course as in other tribal areas. Initially the western colonial administrator/ ethnographers documented in details about the medicinal practices of the Lepcha and Bhutias, the two main autochthonous tribes of the states, in their monographs that covered almost all the facets of tribal life. While Waddell dealt more with the Tibetan-Mahayana Buddhist influence on the Sikkimese people, Gorer stressed more on the influence of the earlier Bon religion and the ancient pre-Bon animistic religion on the lives of the Lepchas. Similar descriptive accounts are also available in the later studies by some of the local people, like Foning or even in the western stints-as dealt by Barbara Aziz (Aziz, 1996). Taking this into consideration the present study does not aim at repeating such descriptive accounts of the ritualistic dimension of medicinal practices of the local tribal. The anthropologists summarize the physical reasons attributed to diseases by the tribals are: 1. weather 2. Food 3. Accidents

and natural calamities. However they suggest that treatment of diseases among the tribes may not resemble the modern scientific method as prevalent in western medicine, but it would be wrong to assure that it has no science. It rather moves in a way which covers not only a scientific principle, but also involves the society at large to over come the catastrophe. In the recent years tribal medicine is being supported by many so as to able to combine it with modern medicine. Many are also stressing that Tribal medicine should be incorporated with the primary health scheme.

1.3 Flora and Fauna

The state is listed among the world's ten most critical centers for biodiversity and endemism. Even though the state has only 0.2% of the countries geographical area it houses over 4500 species of flowering plants, 550 species of Orchids, 362 species of Ferns and its allies, 11 species of oaks, 9 species of Tree Ferns, 30 species of Primulas and 20 species of Bamboos. The faunal wealth of Sikkim comprises of 144 species of mammals, 600 species of birds, 400 species of butterflies and moths and 33 species of reptiles. Several species of medicinal plants and herbs are found throughout the state (Poudyal, 2006).

1.4 The medicinal plants

In the tropical zone the followings are some of the important medicinal plants: *Terminalia chebula* (Harra), *Terminalia bellerica* (Barra), *Emblica officinalis* (Amla) are less frequently available. *Costus speciosus* (Bet Lauri), *Dioscorea bulbifera* (Githa tarul), *Piper retrofaction* (Chaba), *Piper longum* (Pipla), *Calotropis gigantia* (Ank), *Gloriosa superba* (Langare tarul). The natural habitats of *Gloriosa* are over exploited. *Ocimum Sanctum* (Tulasi), *Adathoda vasica* (Asuru), *Melia azadirachta* (Bakaina), *Azadirachta indica* (Neem), *Aegle marmelos* (Belpata) very rare in occurrence, *Vitex nigundo* (Sewali), *Woodfordia fruticosa* (Dhunyeri), *Bacopa monnieri* (Brahmi), *Alstonia scholaris* (Chatiwan), *Bassia butyraceae* (Chiwri), *Garuga pinnata* (Dabdabe), *Oroxylum indicum* (Totala), *Tinospora cordifolia* (Gurjo), *Aloe barbadensis* (Ghew kumari), *Holarrhena* (Kiramar), *Smilax* sp (Kukurdaine), *Lawsonia inermis* (Mehendi), *Boerhavia diffusa* (Punarnava), *Moringa oleifera* (Sajana).

Orchids: Orchids mostly epiphytic are dominant in the area, the following are some of the important ones - *Vanda cristata, Dendrobium aphyllum, D. nobile, D. eriaeflorum, D.cumulatum, Cymbidium pendulum, Eria pania, E. paniculata, E. bambusifolia, E. stricta, E. graminifolia,, Cryptochilus sanguines, Parpax elwesii, Bulbophylum hirtus, B. bisectum, B. affine, B. scabratum, B. thomsonii, B. cauliflorum, C. fimbriata, C. cristata, Liparis cordifolia, Malaxis acuminata, M. latifolia, Pholidota recurva.*

Oil bearing seed plants: *Pyrularia edulis* (Amphi), *Gynocardia odorata* (Gantee), *Viburnum mullah* (Ghora Khari), *Bassia butyraceae* (Chiwri), *Juglans regia* (Okhar) are important ones. *Thysanolaena maxima* (Amliso) is recently recognized as economically important grass having multipurpose uses such as broom grass for its inflorescence, fodder grass, soil conserving plant and also as firewood.

1.5 Herbal medicine

Medicinal herbs have been used for thousands of years. Through lore, documentation, and experimentation herbal medicine has withstood the scientific trends of the 20th century. There has been a re-emergence of interest in herbal alternatives for medical treatment. There are, however, challenges to effective treatment using herbs. We lack standardization criteria, knowledge, and understanding of herbal applications and trust and knowledge from the traditional medicine sector. These challenges can be overcome with realization of respect, understanding, and acceptance from the traditional medical sector and the alternative herbal medicine sector (Maggie, 2004).

Herbal medicine and herbal use dates back 60,000 years. In addition, ritualistic and mythological use combined with medicinal use has resulted in misinformation and countless misinterpretations of the use of herbs. The challenge before us is to separate knowledge from lore and misinformation from factual data to create herbal alternatives in our technologically oriented world.

The challenge is for traditional Western medicine and herbal medicine to work collaboratively to improve the individual's health care quality. Traditional Western practitioners utilize technology and research findings to assist diagnosis, treatment, and prevention. Herbal medicine uses a gentle, less intrusive approach to health care. The emphasis on prevention is a predominate factor in herbal health care. Perhaps most important, herbal medicine supports and strengthens the body's complex physical, emotional, and spiritual system to heal the body as a whole.

1.5.1 A brief history of herbology

Although evidence has been found that dates the use of herbs to the Neanderthal era, written descriptions of curative herbs, minerals, and animals date back only to the 1st century A.D. with Dioscorides's De Materia Medica (Hobbs, 1995). With over 950 entries, complete with description, illustration, medical properties, application, and contraindications, this work is, perhaps, one of the most important pieces of herbal information even today. The Greek physician Hippocrates, considered the father of modern medicine, utilized natural remedies

such as vinegar, honey, and herbs for preventative medicine. Many of his concepts are still used in herbal practices as an integral part of health care regime.

The Chinese played an important role in the development of herbal medicine. Even today, many herbalists use or specialize in Chinese herbs for treatment of physical and emotional ailments. Text dated to the 3rd century B.C. and written on silk discusses exercise and diet. One manuscript included in this text, Prescriptions for Fifty-Two Ailments describes more than 250 substances derived from herbs, wood, grains, legumes, fruits, vegetables, and animal parts. By the 1st century A.D. the Chinese Classic of the Materia Medica was compiled as a descriptive interpretation of herbs and their medicinal effects (Balick et al., 2002).

Ayurvedic herbal medicine originated in India probably about 2000 years ago and emphasizes herbal uses, breathing exercises, yoga, and dietary considerations. Ayurveda focuses on a person's overall health imbalance as a means of diagnosis and treatment, rather than on a single symptom. Treatment begins with an evaluation of the patient's doshas (attributes, heredity makeup) that consist of vata (air), pitta (fire), or kapha (water) and herbal and diet recommendations are prescribed.

During the 15th and 16th centuries, European influence on the medicinal use of herbs is well documented. Herbs gained mystical superstitious importance with accounts of their use in magical powers and ritualistic use by witches. During this time thousands of women and men were executed in an effort to rid the world of impending evil. However, also during this era, the rise of the neighborhood apothecary became a familiar sight. Herb gardens were located near the apothecary, where the proprietor could easily harvest herbs to make tinctures and ointments. Physicians regularly patronized these establishments to obtain what they needed to effectively treat their patients.

Nicholas Culpeper wrote The Complete Herbal in 1651 and linked the use of herbs, medicine and astrology together. Connecting these three areas of study as one science was a popular focus during 16th and 17th century Europe. Culpeper's work was highly regarded for its thoroughness in cataloging plants and herbal usages. He also was known for his translation of London Pharmacopoeia from Latin to English, which was considered, for a time, to be the physicians' Bible. The translated work became even more popular and useful because it was made available to commoners and was instrumental in sharing herbal and home remedies to the people.

Herbal usage and study began to take a more scientific approach during the 17th and 18th centuries. Carl von Linne introduced the world to the concepts of genus and species as

descriptive accounts of plants, thereby establishing a universal system for describing and categorizing plants. His work Species Plantarum is evidence of the new descriptive system that is still so important in herbal usage today. As explorers discovered the Americas, they also discovered Native American Indian medicine. Although at first thought of as uncivilized people, after a disastrous winter, American Indians became an important information resource to the settlers. Goods were traded for knowledge, and early immigrants were taught which herbs to use to build endurance and treat injuries and simple physical ailments. This knowledge was passed from generation to generation, and until the 1940s herbal treatment was still an important element of health care.

Along with Native American Indian herbal knowledge, Shakers also became a resource, not only for knowledge but for commercial herbal medicinal products. Between 1787 and 1892, Shaker medicine grew from wild crafted herbs to cultivated herbs; from intercommunity healing to pharmaceutical suppliers (Miller, 1998).

As medicine and technology progressed in the 20th century, our culture began to treat herbal medicine with disdain. Reliance on synthetic application and symptomatic treatment has become the focus of health care today. The medical community has, in general, lost touch with our herbal heritage. There is too little knowledge and lack of documented research in the United States for traditional Western medicine to effectively integrate herbal medicine into their treatment.

Today, the U.S. Food and Drug Administration (FDA) prohibit the marketing of herbal products as anything but food supplements. Manufacturers are prohibited from making suggested herbal treatments for specific ailments on labels. It is important to note that many prescribed and over the-counter medications still contain herbal components. Compounds such as glycosides, found in the leaves of wild cherry trees are used in cough remedies and medical tonics. Digitalis, a cardiac stimulant, contains powdered foxglove (*Digitalis purpurea*) and is another example of a glycoside component used in traditional medicine. Yet another example of a glycoside, salicin, found in the bark of the white willow (*Salix* sp.) is one of the components in aspirin. Alkaloids are another compound used in traditional medicine. Used in small doses, plant alkaloids can be found in quinine, codeine, morphine, and nicotine. Plant tannins are valued for their astringent qualities and used to make skin and blood vessels constrict. Sulfur from garlic (*Allium sativum*), glycosides from mustard (*Sinapis* sp.), and alkaloids in water lilies (*Nymphaea* sp.) are used in the production of antibiotics.

In 1998, Time magazine reported that 60 million Americans use herbal supplements, spending over $12 billion dollars on natural herbal products. The World Health Organization estimates that healing herbs are the primary medical treatment for two thirds of today's population.

1.5.2 Herbs for prevention and treatment

Research of herbal applications to health care has been done primarily abroad in Europe, India, and Asia. Some of the best and most extensive studies have taken place in Europe where the effectiveness of herbal supplements (termed Phytomedicines in Europe) is well documented. Phytomedicines are produced as tablets or capsules under stringent guidelines and incorporated into conventional medical practice. These products are sold as health care herbal supplements and have been successfully integrated into traditional Western medical applications. These same products can be found in the United States in the food supplement section of many grocery store, pharmacy, and health stores.

Herbs and herbal medicine have been an integral part of our health care for centuries and will continue to develop and expand in our future health care system. Individuals are seeking alternatives to traditional Western medicine at a surprising rate, and all of us in health care related fields must begin to expand our views to encompass all modalities. Wellness, as a whole, is physical, emotional, and spiritual, and it is the responsibility of all of us to ensure that each individual receives complete health care. To successfully integrate herbology with traditional Western medicine, a call for thorough clinical research of herbal applications is necessary to provide educated answers and quality care to patients. Traditional Western and alternative/ herbal medicine can complement each other with understanding and appreciation for the science and art of health.

By definition, the word natural is an adjective referring to something that is present in or produced by nature and not artificial or man-made. When the word natural is used in verbiage or written, many times it is assumed that the definition is something good or pure. However, many effective poisons are natural products (Schoental, 1965). The term natural products today is quite commonly understood to refer to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine. Nature has provided many things for humankind over the years, including the tools for the first attempts at therapeutic intervention. Natural products are generally either of prebiotic origin or originate from microbes, plants, or animal sources (Nakanishi 1, 1999; Nakanishi 2, 1999). As chemicals, natural products include such classes of compounds as terpenoids, polypeptides, amino acids, peptides, proteins, carbohydrates, lipids, nucleic acid bases, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and so forth. Natural products are not just accidents or products of convenience of nature.

More than likely they are a natural expression of the increase in complexity of organisms (Jarvis, 2000). Interest in natural sources to provide treatments for pain, palliatives, or curatives for a variety of maladies or recreational use reaches back to the earliest points of history. Drug development over the years has relied only on a small number of molecular prototypes to produce new medicines (Harvey, 2001). Indeed, only approximately 250 discrete chemical structure prototypes have been used up to 1995, but most of these chemical platforms have been derived from natural sources. While recombinant proteins and peptides are gaining market share, low molecular-weight compounds still remain the predominant pharmacologic choice for therapeutic intervention.

1.5.3 Herbal Medicine in therapeutics

An herb is a plant or plant part used for its scent, flavor or therapeutic properties. Herbal medicine products are dietary supplements that people take to improve their health. Many herbs have been used for a long time for claimed health benefits. They are sold as tablets, capsules, powders, teas, extracts and fresh or dried plants. However, some can cause health problems, some are not effective and some may interact with other drugs we are taking. Research into the use of plant-derived natural products alone in just the field of medicine covers a broad spectrum of activities (Dahanukar *et al.*, 2000, Havsteen, 1983, Wrigley *et al.*, 1997, Yao *et al.*, 1998, Yu *et al.*, 2000). Examples of such biological activity profiles would include, but are not limited to nootropics, psychoactive agents, dependence attenuators, anticonvulsants, sedatives, analgesics, anti-inflammatory agents, antipyretics, neurotransmission modulators, autonomic activity modulators, autacoid activity modulators, anticoagulants, hyoplipidemics, antihypertensive agents, cardioprotectants, positive ionotropes, antitussives, There are many medicinal plants on which significant research leads have been obtained with respect to their pharmaceutical potential for which processing and agrotechnology need to be established (**Table 1.1**) and drugs for which no synthetic one is currently available are given in **Table 1.2** (Kumar *et al*, 1997).

Table 1.1 Medicinal plants on which significant research leads with their pharmaceutical potential have been obtained (Kumar *et al*, 1997).

Andrographis paniculata	*Coleus forskohlii*	*Picrorhiza kurroa*
Artemisia annum	*Commiphora wightii*	*Sida rhombifolia*
Boswellia serrata	*Curcuma longa*	*Taxus baccata*
Centella asiatica	*Phyllanthus amarus*	*Withania somnifera*

Table 1.2 Medicinal plant drugs for which no synthetic one is currently available (Kumar *et al.*, 1997).

Drug	Plant Source	Use
Vinblastine	*Catharanthus roseus*	Anticancer
Vinblastine	*Catharanthus roseus*	Anticancer
Ajmalacine	*Catharanthus roseus*	Anticancer, hypotensive
Rescinnamine	*Rauvolfia serpentina*	Tranquilizer
Reserpine	*Rauvolfia serpentina*	Tranquilizer
Quinine	*Cinchona sp.*	Antimalarial, dysentery
Pilocarpine	*Pilocarpus jaborandi*	Antiglucoma
Cocaine	*Erythroxylum coca*	Topical anaesthetic
Morphine	*Papaver somniferum*	Analgesic
Codeine	*Papaver somniferum*	Anticough
Atropine	*Atropa belladonna*	Spasmolytic, cold
Atropine	*Hyoscyamus niger*	Spasmolytic, cold
Cardiac glycosides	*Digitalis sp.*	Congestive heart failure
Artemisinin	*Artemesia annua*	Antimalarial
Taxol	*Taxus baccata*	Cancer, antitumour
Taxol	*Taxus brevifolia*	Cancer
Berberine	*Berberis*	Leishmaniasis
Pristimerin	*Celastrus paniculata*	Antimalarial
Quassinoids	*Ailanthus*	Antiprotozoal
Plumbagin	*Plumbago indica*	Antibacterial, antifungal
Diospyrin	*Diospyros Montana*	Antifungal
Gossypol	*Gossypium sp.*	Antispermatogenic
Allicin	*Allium sativum*	Antifungal, amoebiasis
Ricin	*Ricinus communis*	Amoebiasis
Emetine	*Cephaelis ipecacuanha*	Amoebiasis
Glycyrrhizin	*Glycyrrhizia glabra*	Antiulcer
Nimbidin	*Azadirachta indica*	Antiulcer
Catechin	*Acacia catechu*	Antiulcer
Sophoradin	*Sophora subprostrata*	Antiulcer
Magnolol	*Magnolia bark*	Peptic ulcer
Forskolin	*Coleus forskohlii*	Hypotensive, cardiotonic
Digitoxin, Digoxin	*Digitalis, Thevetia*	Cardio tonic
Thevenerin	*Thevetia*	Cardio tonic
Nerrifolin	*Thevetia*	Cardio tonic
Podophyllin	*Podophyllum emodi*	Anticancer
Indicine N-oxide	*Heliotropium indicum*	Anticancer
Elipticine	*Ochrosia*	Anticancer
Homoharringtonine	*Cephalotaxus*	Anticancer
Camptothecine	*Camptotheca acuminata*	Anticancer

1.6 Natural product research and development

The World Health Organization estimates that approximately 80 percent of the world's population relies primarily on traditional medicines as sources for their primary health care (Farnsworth *et al.*, 1985). Over 100 chemical substances that are considered to be important drugs, which are either currently in use or have been widely used in one or more countries in the world, have been derived from 100 different plants as described in Table 1.3. Approximately 75 percent of these substances were discovered as a direct result of chemical studies focused on the isolation of active substances from plants used in traditional medicine (Cragg 1 *et al.*, 2001; Cragg 2 *et al.*, 2001).The number of medicinal herbs used in China in 1979 has been estimated to be numbered at 5267 (Nakanishi 1,1999; Nakanishi 2, 1999). More current statistics based on prescription data from 1993 in the United States show that over 50 percent of the most prescribed drugs had a natural product either as the drug or as the starting point in the synthesis or design of the actual end chemical substance (Newman, 2000). Thirty-nine percent of the 520 new drugs approved during the period 1983 through 1994 were either natural products or derivatives of natural products (Harvey, 2001). Indeed, if one looks at new drugs from an indication perspective over the same period of time, over 60 percent of antibacterial and antineoplastic were again either natural products themselves or based on structures of natural products. Of the 20 top-selling drugs on the market in the year 2000, 7 of these were either derived from natural products or developed from leads generated from natural products. This select group of drugs generates over 20 billion U.S. dollars of revenue on an annual basis (Grabley, *et al.*, 2003; Harvey, 2001).

Drug development over the years has relied only on a small number of molecular prototypes to produce new medicines (Harvey, 2001). Indeed, only approximately 250 discrete chemical structure prototypes have been used up to 1995, but most of these chemical platforms have been derived from natural sources. While recombinant proteins and peptides are gaining market share, low molecular-weight compounds still remain the predominant pharmacologic choice for therapeutic intervention (Grabley *et al.*, 2003).Just a small sampling of the many available examples of the commercialization of modern drugs from natural products along with their year of introduction, indication, and company are: Orlistat, 1999, obesity, Roche; Miglitol, 1996, antidiabetic (Type II), Bayer; Topotecan, 1996, antineoplastic, SmithKline Beecham; Docetaxel, 1995, antineoplastic, Rhône-Poulenc Rorer; Tacrolimus, 1993, immunosuppressant, Fujisawa; Paclitaxel, 1993, antineoplastic, Bristol-Myers Squibb. The overwhelming concern today in the pharmaceutical industry is to improve the ability to find new drugs and to accelerate the speed with which new drugs are discovered and developed. This will only be successfully accomplished if the procedures for drug target elucidation and lead compound identification and optimization are themselves optimized. Analysis of the human genome will

provide access to a myriad number of potential targets that will need to be evaluated (Grabley *et al.*, 2003; Harvey, 2001). The process of high-throughput screening enables the testing of increased numbers of targets and samples to the extent that approximately 100,000 assay points per day are able to be generated. However, the ability to accelerate the identification of pertinent lead compounds will only be achieved with the implementation of new ideas to generate varieties of structurally diverse test samples (Grabley *et al.*, 2003; Harvey, 2001; Harvey, 1999). Experience has persistently and repeatedly demonstrated that nature has evolved over thousands of years a diverse chemical library of compounds that are not accessible by commonly recognized and frequently used synthetic approaches.

Natural products have revealed the ways to new therapeutic approaches, contributed to the understanding of numerous biochemical pathways and have established their worth as valuable tools in biological chemistry and molecular and cellular biology. Just a few examples of some natural products that are currently being evaluated as potential drugs are given in **Table 1.3**.

Table 1.3 Natural drugs having high pharmaceutical potential (Grabley *et al.*, 2003).

Natural Product	Source	Target	Indication	Status
Manoalide	Marine sponge	Phospholipage-A2 Ca^{2+} release	Anti-inflammatory	Clincial trials
Dolastatin 10	Sea hare	Microtubules	Antineoplastic	Nonclinical
Staurosporine	Streptomyces	Protein kinase C	Antineoplastic	Clinical trials
Epothilone	Myxobacterium	Microtubules	Antineoplastic	Research
Calanolide A, B	Tree	DNA polymerase action on reverse transcriptase	AIDS	Clinical trials
Huperzine A	Moss	Cholinesterase	Alzheimer's disease	Clinical trials

The costs of drug discovery and drug development continue to increase at astronomical rates, yet despite these expenditures, there is a decrease in the number of new medicines introduced into the world market. Despite the successes that have been achieved over the years with natural products, the interest in natural products as a platform for drug discovery has waxed and waned in popularity with various pharmaceutical companies. Natural drugs having numerous biological activity profiles are in use such as nootropics, psychoactive agents, dependence attenuators, anticonvulsants, sedatives, analgesics, anti-inflammatory agents, antipyretics, neurotransmission modulators, autonomic activity modulators, lavones activity

modulators, anticoagulants, hyoplipidemics, antihypertensive agents, cardioprotectants, positive ionotropes, antitussives, antiasthmatics, pulmonary function enhancers, antiallergens, hypoglycemic agents, antifertility agents, fertility-enhancing agents, wound healing agents, dermal healing agents, bone healing agents, compounds useful in the prevention of urinary calculi as well as their dissolution, gastrointestinal motility modulators, gastric ulcer protectants, immunomodulators, hepato-protective agents, myelo-protective agents, pancreato-protective agents, oculo-protective agents, membrane stabilizers, hemato-protective agents, antioxidants, agents protective against oxidative stress, antineoplastics, antimicrobials, antifungal agents, antiprotozoal agents, antihelminthics, and nutraceuticals (Dahanukar *et al.*, 2000).

Many frontiers remain within the field of natural products that can provide opportunities to improve our quality of life. Fungal disease has historically been a difficult clinical entity with which to effectively deal. Fungal diseases can include more than just a mycosis and can also include allergic reactions to fungal proteins and toxic reactions to fungal toxins. Mycoses as a group include diseases that are significantly more serious and life-threatening than nail infestations, athlete's foot, or "jock-itch." Indeed, increasing numbers of overly healthy individuals are becoming victims of the complications of fungal infestation. The reasons for this are that increasing numbers of people are receiving immunomodulatory treatment for an organ transplant or some underlying chronic systemic pathology, antineoplastic chemotherapy for cancer, or have been the recipients of proper or improper use of powerful antibiotics. Additionally there are a number of individuals within society that are infected with the human immunodeficiency virus (HIV). The available drugs to treat mycoses have been limited (Barrett, 2002). Furthermore, in this armamentarium, there are problems with dose-limiting nephrotoxicity, the rapid development of resistance, drug–drug interactions of concern, and a fungistatic mechanism of action. Thus there is an urgent need for the development of more efficacious antifungal agents with fewer limitations and less side effects. Ideally such compounds should possess good distribution characteristics, a novel mechanism of action, and a broad-spectrum fungicidal activity. The discovery and isolation of an echinocandin-type lipopeptide (FR901379) and lipopeptidolactone (FR901469) from microbes has been a significant achievement. These compounds are water soluble and inhibit the synthesis of 1, 3-b-glycan, a key component of the fungal cell wall. Furthermore, since the cell wall is a feature particular to fungi and is not present in eukaryotic cells, such inhibitors certainly have the potential to demonstrate selective toxicity against the fungi and not against the animal or human host. The ultimate modifications of the lipopeptide and lipopeptidolactone referenced above led to the discovery of micafungin (FK463), which is currently in phase III clinical trials. This work along with the relatively recent approval of caspofungin (Merck) as a therapeutic

agent for the treatment of disseminated aspergillosis are significant achievements in that they demonstrate that a melding of the proper research to identify and develop appropriate targets with the chemical and biological diversity found in natural products can be very rewarding. Much ado has been made over recent years about endocrine disruptors and their effects on humans (Crews *et al.*, 2000). It needs to be recognized that endocrine disruptors are not just synthetic chemicals but can also be natural products. The use of natural product endocrine disruptors may provide significant insight into our understanding of the mechanisms by which the evolution of the genome can protect transactivation of the sex hormone receptors and aid in the development of drugs, which can protect the embryo during its development from hormone disruptive effects. Diabetes is a multi-systemic affliction, having impact on nearly every body organ. As a disease, it kills more individuals on a per annum basis than AIDS and breast cancer combined (Shapiro *et al.*, 2002). The impact on the quality of life of an individual suffering with diabetes is profound. A number of natural products currently exist that demonstrate hypoglycemic activity. Indeed, depending upon the source that one might use, there are approximately 800 to 1200 plants that exhibit hypoglycemic activity. While research and development efforts in this particular area thus far are largely restricted to traditional medicine uses, future research may well identify a potent antidiabetic agent. The incidences of neuropsychiatric disorders are steadily increasing as our population increases in size and age. Such disorders include, but are not limited to, seizure disorders, schizophrenia, dementia, mania, aggression, memory loss, psychoses, age-related cognitive decline, depression, anxiety states, mood disorders, substance abuse, and substance dependence. There is a large body of data available that suggests the use of many natural products as potential treatments for these conditions and other neuropsychiatric disorders (Chung *et al.*, 1995; Lake, 2000; Bindseil *et al.*, 2001). Indeed, a number of plant extracts have been associated with the treatment of various categories of mental symptoms and various types of receptor selectivity (Chung *et al.*, 1995). A very controversial potential psychotherapeutic agent is *Gingko biloba* (Fugh-Berman *et al.*, 1999). A lack of understanding of mechanism of action, misidentification of materials, contamination of materials, intrinsic toxicity, and absence of standardization all contribute to this controversy. Further fractionation, isolation, and characterization of active components of these and other plants will undoubtedly lead to the discovery of novel neuropsychiatric agents as well as the debunking of other alleged therapies. There are numerous blood-based diseases that afflict humans. These would include, but are not limited to, anemia, blood group incompatibility, blood protein disorders, bone marrow diseases, hemoglobinopathies, hemorrhagic diatheses, leukemia, disorders of leukocyte dysfunction, platelet disorders, and erythrocyte aggregation disorders.

A number of natural products have been reported in the literature to be of value in the treatment of Epstein-Barr virus infection, leukemia, thrombosis and coagulopathies, malaria, anemia, and bone marrow diseases (Miles *et al.*,1998). Extracts from the fungus *Trichothecium roseum*, the sea cucumber *Cucumaria japonica*, the legume *Amorpha fruitcosa*, the tree *Magnolia officinalis*, and others may be useful in the therapeutic management of Epstein-Barr virus infection. Extracts from the basidiomycetes *Mycena pura* and *Nidula candida* may be useful in the treatment of leukemia. Compounds isolated from *Streptomyces platensis* may be useful in the treatment of thrombocytopenia. Compounds obtained from the marine sponge *Aplysina archeri* have been reported to inhibit the growth of the feline leukemia virus. Scalarane-type bishomo-sesterterpenes isolated from the marine sponge *Phyllospongia foliascens* have been reported to exhibit cytotoxic, antithrombocytic, and vasodilation activities. It should be noted that a number of natural products are based on the coumarin nucleus and as such may exhibit antithrombotic and antipatelet activities. A number of blood-sucking animals have small, low-molecular-weight proteins in their saliva that interfere with the clotting of blood and therefore might be of value as potential anticoagulants. Streptomyces, hygroscopicus and ascomyceticus manufactures a macrolide that has been reported to have immunosuppressant activity and may prove to be beneficial in preventing transplant rejection in humans. It is entirely possible that these compounds and others offer sufficient structural diversity, range of biological activities and differing mechanisms of action that new, safer, and more efficacious drugs to treat blood-based disorders could well burgeon from this library. A wide variety of natural products are claimed to possess immunosuppressant activity, but it is often difficult to dissect this activity away from associated cytotoxicity (Mann, 2001).

Since the first heart transplant in the late 1960s, medicine has progressed to the point where most organ transplants have become relatively routine procedures. The survival of individuals with transplants is owed in large part to the discovery of the fungal metabolite cyclosporine A in 1970 and its widespread use starting in 1978. Indeed, cyclosporine A has achieved such success that it is currently being evaluated for value in the treatment of Crohn's disease, systemic lupus erythematosus, and rheumatoid arthritis. Research efforts abound in the area of natural products and immunosuppression. A methyl analog of oligomycin F isolated from *Streptomyces ostreogriseus* has been reported to quite effectively suppress B cell activation and T-cell activation in the presence of mitogens at concentrations comparable to that of cyclosporine A. Concanamycin F first isolated from *Streptomyces diastatochromogenes* in 1992 has been found to possess a wide array of biological activities including immunosuppressive and antiviral activities. The experimental immunosuppressant (+)-discodermolide isolated from the marine sponge *Discodermia lavones* exhibits relatively nonspecific immunosuppression, causing the cell cycle to arrest during G_2 and M phases. This compound's current primary

interest is as a potential antineoplastic agent since it stabilizes microtubules and prevents depolymerization, effectively causing cell cyclic arrest during the metaphase to anaphase transition. This same mode of activity is shared with Taxol (Paclitaxel), the epothilones, eleutherobin, and the sarcodictyins. The didemnins, cyclic peptides, were first isolated from the marine tunicate *Trididemnum solidum* and exhibit immunosuppressive activity through a generalized cytotoxicity mediated by inhibition of progression through the G_1 phase of the cell cycle by an unknown mechanism. The trichopolyns I to V from the fungus *Trichoderma polysporum* are lipopeptides that suppress the proliferation of lymphocytes in the murine allogeneic mixed lymphocyte response assay. Triptolide from the plant *Tripterygium winfordii* demonstrates immunosuppressant activity through the inhibition of IL-2 receptor expression and signal transduction.

The novel heteroaromatic compound lymphostin, obtained from Streptomyces KY11783 has demonstrated immunosuppressant activity through its potent inhibition of the lymphocyte kinase. Over the last decade, research activities on immunosuppressants of natural product origin have focused on the mechanisms of inhibition of T-cell activation and proliferation. This approach has been fruitful, leading to the generation of significant information about signaling pathways between T cells, greater detail about the roles of T cells in immune function, and the discovery of Tacrolimus (Prograf) from the soil fungus *Streptomyces tsukubaensis*. As immunological research progresses, increasingly more potential targets will be elucidated for immunomodulatory therapeutic intervention. Natural products will undoubtedly provide a sound platform for the delivery of natural-product-based therapeutic agent candidates. Natural-products-based anticancer drug discovery continues to be an active area of research throughout the world (Da Rocha *et al.*, 2001; Mann, 2002; Mehta *et al.*, 2002; Schwartsmann *et al.*, 2002). While cancer incidences and the frequencies of types of cancer may vary from country to country, the most common sites for the development of neoplasia are generally considered to be the breast, colon, rectum, prostate, cervix, uterus, esophagus, stomach, pancreas, liver, lung, urinary bladder, kidney, ovary, oral cavity, and blood (leukemia and non-Hodgkin lymphoma) (Schwartsmann *et al.*,2002).

Currently, the chemotherapeutic management of these tumors involves a variety of different plant-based chemicals that are either currently in use or in clinical trials and include such drug classes as the *Vinca* alkaloids, lignans, taxanes, stilbenes, flavones, cephalotaxanes, camptothecins, and taxanes. Despite the wide range of organ structure, type, and function, great similarities exist between the organs with regard to the pathogenesis of cancer. As more and more details of the molecular biology of cancer are revealed, more targets will present themselves for possible therapeutic chemical intervention in the growth and development of neoplasms. A final note with regard to this approach is that it is important to appreciate that

the distinction between chemopreventive agent and chemotherapeutic agent can become quite blurred. A recurrent theme in neoplasia is the alteration of cell cycle control. One therapeutic approach to the treatment of neoplasia is the development of a treatment that would return to normal the altered cell cycle (Sausville *et al.*, 2000). The introduction of active agents derived from natural sources into the anticancer weaponry has already significantly changed the futures of many individuals afflicted with cancer of many different types. Continued research into natural sources will continue to deliver newer and more promising chemicals and chemical classes of anticancer agents with novel mechanisms of action that will improve survival rates to even higher degrees. For the period 1983 to 1994, seven out of 10 synthetic agents approved by the Food and Drug Administration (FDA) for use as antivirals were based on a natural product. These drugs are famciclovir, stavudine, zidovudine, zalcitabine, ganciclovir, sorivudine, and didanosine. The viral genome can be composed of either RNA or DNA and HIV.

The general potential targets of antiviral chemotherapy are:

(1) Attachment of virus to host cell,

(2) Penetration of the host cell by the virus,

(3) Viral particle uncoating, release, and transport of viral nucleic acid and transport proteins,

(4) Nucleic acid polymerase release/activation,

(5) Translation of mRNA (messenger RNA) to polypeptides (early proteins),

(6) Transcription of mRNA,

(7) Replication of nucleic acids,

(8) Protein synthesis (late proteins),

(9) Viral polypeptide cleavage into polypeptides necessary for maturation,

(10) Assembly of viral capsids and precursors,

(11) Encapsidation of nucleic acid,

(12) Envelopment, and

(13) Release.

Early antiviral research focused on compounds that inhibited viral DNA synthesis, purine, and pyrimidine nucleoside analogs. Today most current antiviral agents target RNA-based viruses and the inhibition of reverse transcriptase in order to block the transcription of the RNA genome to DNA. Such inhibition would prevent the synthesis of viral mRNA and proteins.

Protease inhibitors affect the synthesis of late viral proteins and viral packaging activity. There are no currently available drugs that target early viral protein synthesis. Antiviral compound research has included alkaloids, carbohydrates, chromones, coumarins, flavonoids, lignans, phenolics, quinines, xanthones, phenylpropanoids, tannins, terpenes, steroids, iridoids, thiopenes, polyacetylenes, lactones, butenolides, phospholipids, proteins, peptides, and lectins. While plants have been a common hunting ground, many other sources are now starting to be explored, especially the marine environment. The use of natural products in the field of antiviral research appears to be limited only by the imagination of the researcher. Natural products are indeed viable sources and resources for drug discovery and development (Artuso, 1997). Indeed, without natural products, medicine would be lacking in therapeutic tools in several important clinical areas such as neurodegenerative disease, cardiovascular disease, solid tumors treatment, and immunoinflammatory disease (Banerji, 2000; Harvey, 2000; Nisbet *et al.*, 1997). Furthermore, the continual emergence of new natural product chemical structure skeletons, with interesting biological activities along with the potential for chemical modification and synthesis bode well for the utility of natural products. Finally, the uses of natural products need to be by no means restricted to pharmaceuticals but can also be expanded to agrochemicals. For example, the use of pyrethrins obtained from *Chrysanthemum* sp. As insecticides has been very popular over the years and persists today. Research continues into the use of natural products as pesticides. The new medicinal uses of plants like *Achyranthes* (hypoglycaemic and estrogenic), *Aegle* (hypoglycaemic), *Phyllanthus* (contraceptive) and *Sida* (antiplaque activities) and new active components of *Sida*, *Aloe*, *Boerhavia*, *Eclipta* and *Phyllanthus* were taken as models and the role of minor components like phenolics has been emphasized.

Sikkim and Darjeeling Himalayan region is characterized by a rich floral diversity and an equally rich Ethnomedicinal tradition. Herbal medicine is the dominant system of medicine practiced by the local tribes of this region for the treatment of diabetes (Chhetri *et al.*, 2005).

Today there are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world. Where several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The original plant substance/chemical name is shown under the "Drug" column rather than the finished patented drug name. For example, many years ago a plant chemical was discovered in a tropical plant, *Cephaelis ipecacuanha*, and the chemical was named emetine (see **Table 1.3**). A drug was developed from this plant chemical called Ipecac which was used for many years to induce vomiting mostly if someone accidently swallowed a poisonous or harmful substance. Ipecac can still be found in pharmacies in many third world countries but has been mostly replaced by other drugs in the United States. Another example

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of this is the plant chemical named taxol shown in the drug column below. The name taxol is the name of the plant chemical originally discovered in the plant. A pharmaceutical company copied this chemical and patented a drug named Paclitaxel which is used in various types of tumors today in the U.S. and many other countries.

There are 120 substances which are sold as drugs worldwide but not in all countries. Some European countries regulate herbal substances and products differently than in the United States. Many European countries, including Germany, regulate herbal products as drugs and pharmaceutical companies prepare plant based drugs simply by extracting out the active chemicals from the plants. A good example is the plant substance, cynarin (**Table 1.3**). Cynarin is a plant chemical found in the common artichoke (*Cynara scolymus*). In Germany, a cynarin drug is sold for liver problems and hypertension which is simply this one chemical extracted from the artichoke plant or a plant extract which has been standardized to contain a specific milligram amount of this one chemical. These products are manufactured by pharmaceutical companies, sold in pharmacies in Germany and a doctor's prescription is required to purchase them. In the United States artichoke extracts are available as natural products and sold in health food stores. Some products are even standardized to contain a specific amount of the cynarin chemical. One can purchase these natural and standardized extracts over the counter without a prescription and one could not go to a pharmacy in the U.S. and obtain a cynarin drug with a prescription. Another similar example is the plant chemical, Silymarin, shown in **Table 1.1**. Silymarin is a chemical found in the milk thistle plant and natural milk thistle extracts standardized to contain specific amounts of Silymarin are found in just about every health food store in the United States. However in Germany, Silymarin drugs and milk thistle standardized extracts are sold only in pharmacies and require a doctor's prescription for liver problems.

Some of the drug/chemicals shown in **Table 1.3** are still sold as plant based drugs requiring the processing of the actual plant material. Others have been chemically copied or synthesized by laboratories and no plant materials are used in the manufacture of the drug. A good example of this is the plant chemical quinine, which was discovered in a rainforest tree (*Cinchona ledgeriana*) over 100 years ago. For many years the quinine chemical was extracted from the bark of this tree and processed into pills to treat malaria. Then a scientist was able to synthesize or copy this plant alkaloid into a chemical drug without using the original tree bark for manufacturing the drug. Today, all quinine drugs sold are manufactured chemically without the use of any tree bark. However, another chemical in the tree called quinidine which was found to be useful for various heart conditions couldn't be completely copied in the laboratory and the tree bark is still harvested and used to extract this plant chemical from it. Quinidine extracted from the bark is still used today to produce quinidine-based drugs. In the U.S. there

are four patented brand-name heart drugs sold in pharmacies containing bark-extracted quinidine: Cardioquin™, Quinaglute Dura-tabs™, Quinidex Extentabs™ and Quin-Release™. The National Cancer Institute (NCI) has several ongoing lavones tive programs which screen plants for the possiblility of new drugs and active plant chemicals for cancer and AIDS/HIV.

Because well over 50 percent of the estimated 250,000 plant species found on earth come from tropical forests, NCI concentrates on these regions. Plants have been collected from the African countries of Cameroon, the Central African Republic, Gabon, Ghana, Madagascar, and Tanzania. Collections are now concentrated in Madagascar (one of the most rapidly disappearing rainforest regions in the world), and collaborative programs have been established in South Africa and Zimbabwe.

In Central and South America, samples have been collected from Belize, Bolivia, Colombia, the Dominican Republic, Ecuador, Guatemala, Guyana, Honduras, Martinique, Paraguay, Peru, and Puerto Rico. The NCI has established collaborative programs in Brazil, Costa Rica, Mexico, and Panama. Southeast Asian collections have been performed in Bangladesh, Indonesia, Laos, Malaysia, Nepal, Pakistan, Papua New Guinea, the Philippines, Taiwan, Thailand, and Vietnam. Collaborative programs have been established in Bangladesh, China, Korea, and Pakistan. In each country, NCI contractors work in close collaboration with local botanical institutions.

Since 1986, over 40,000 plant samples have been screened, but thus far only five chemicals showing significant activity against AIDS have been isolated. Three are currently in preclinical development. Before being considered for clinical trials in humans, these agents must show tolerable levels of toxicity in several animal models. For AIDS, three agents are presently in preclinical or early clinical development. The following are plants and chemicals which are still under research for cancer and AIDS/HIV:

(+)-Calanolide A and (-)-Calanolide B (Costatolide) are isolated from *Calophyllum lanigerum* and *Calophyllum teysmanii*, respectively, trees found in Sarawak, Malaysia. Both these agents are licensed to Medichem, Inc., Chicago, which is developing them in collaboration with the Sarawak State Government through a joint company, Sarawak Medichem Pharmaceuticals, Inc. (+)-Calanolide A is currently in early clinical trials in the United States.

Conocurovone, isolated from the shrub species, *Conospermum incurvum* (Saltbush), found in Western Australia, has been licensed for development to AMRAD, a company based in Victoria, Australia.

Michellamine B, from the leaves of *Ancistrocladus korupensis*, a vine found in the Korup rainforest region of southwest Cameroon, has undergone extensive preclinical study, but is considered too toxic for advancement to clinical trials.

Prostratin, isolated from the wood of *Homolanthus nutans*, a tree found in Western Samoa, has been placed on low priority, largely due to its association with a class of compounds shown to be tumor promoters.

A tree native to China *Camptotheca acuminate* is the source of four promising anticancer drugs, two of which have been approved by the FDA and are described above. The other two chemicals still under research include:

(i) (9-aminocamptothecin): Currently in clinical trials for several types of cancer, including ovarian and stomach cancers and T-cell lymphoma.

(ii) Tothecin: While no clinical trials are being performed in the United States, trials are ongoing in China. Homoharringtonine from the Chinese tree, *Cephalotaxus harringtonia* are in early clinical trials. Perillyl alcohol, and flavopiridol, a totally synthetic compound based on a flavones isolated from are in early clinical trials.

Table 1.4 Currently used important drugs derived from plant sources as per WHO. (Muhammad *et al.*, 2005)

Drug/Chemical	Action/Clinical Use	Plant Source
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis vernalis*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Aesculetin	Anti-dysentery	*Frazinus rhychophylla*
Agrimophol	Anthelmintic	*Agrimonia supatoria*
Ajmalicine	Circulatory Disorders	*Rauvolfia sepentina*
Allantoin	Vulnerary	*Several plants*
Allyl isothiocyanate	Rubefacient	*Brassica nigra*
Anabesine	Skeletal muscle relaxant	*Anabasis sphylla*
Andrographolide	Baccillary dysentery	*Andrographis paniculata*
Anisodamine	Anticholinergic	*Anisodus tanguticus*
Anisodine	Anticholinergic	*Anisodus tanguticus*
Arecoline	Anthelmintic	*Areca catechu*
Asiaticoside	Vulnerary	*Centella asiatica*
Atropine	Anticholinergic	*Atropa belladonna*
Benzyl benzoate	Scabicide	*Several plants*
Berberine	Bacillary dysentery	*Berberis vulgaris*

Table 1.4 continued

Drug/Chemical	Action/Clinical Use	Plant Source
Bergenin	Antitussive	*Ardisia japonica*
Betulinic acid	Anticancerous	*Betula alba*
Borneol	Antipyretic, analgesic, antiinflammatory	*Several plants*
Bromelain	Anti-inflammatory, proteolytic	*Ananas comosus*
Caffeine	CNS stimulant	*Camellia sinensis*
Camphor	Rubefacient	*Cinnamomum camphora*
Camptothecin	Anticancerous	*Camptotheca acuminata*
(+)-Catechin	Haemostatic	*Potentilla fragarioides*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cissampeline	Skeletal muscle relaxant	*Cissampelos pareira*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Codeine	Analgesic, antitussive	*Papaver somniferum*
Colchiceine amide	Antitumor agent	*Colchicum autumnale*
Colchicine	Antitumor agent, anti-gout	*Colchicum autumnale*
Convallatoxin	Cardiotonic	*Convallaria majalis*
Curcumin	Choleretic	*Curcuma longa*
Cynarin	Choleretic	*Cynara scolymus*
Danthron	Laxative	*Cassia species*
Demecolcine	Antitumor agent	*Colchicum autumnale*
Deserpidine	Antihypertensive, tranquillizer	*Rauvolfia canescens*
Deslanoside	Cardiotonic	*Digitalis lanata*
L-Dopa	Anti-parkinsonism	*Mucuna sp*
Digitalin	Cardiotonic	*Digitalis purpurea*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis purpurea*
Emetine	Amoebicide, emetic	*Cephaelis ipecacuanha*
Ephedrine	Sympathomimetic, antihistamine	*Ephedra sinica*
Etoposide	Antitumor agent	*Podophyllum peltatum*
Galanthamine	Cholinesterase inhibitor	*Lycoris squamigera*
Gitalin	Cardiotonic	*Digitalis purpurea*
Glaucarubin	Amoebicide	*Simarouba glauca*
Glaucine	Antitussive	*Glaucium flavum*
Glasiovine	Antidepressant	*Octea glaziovii*
Glycyrrhizin	Sweetener, Addison's disease	*Glycyrrhiza glabra*

Table 1.4 continued

Drug/Chemical	Action/Clinical Use	Plant Source
Gossypol	Male contraceptive	*Gossypium species*
Hemsleyadin	Bacillary dysentery	*Hemsleya amabilis*
Hesperidin	Capillary fragility	*Citrus species*
Hydrastine	Hemostatic, astringent	*Hydrastis canadensis*
Hyoscyamine	Anticholinergic	*Hyoscyamus niger*
Irinotecan	Anticancer, antitumor agent	*Camptotheca acuminata*
Kaibic acud	Ascaricide	*Digenea simplex*
Kawain	Tranquillizer	*Piper methysticum*
Kheltin	Bronchodilator	*Ammi visaga*
Lanatosides A, B, C	Cardiotonic	*Digitalis lanata*
Lapachol	Anticancer, antitumor	*Tabebuia sp.*
a-Lobeline	Smoking deterrant, respiratory stimulant	*Lobelia inflata*
Menthol	Rubefacient	*Mentha species*
Methyl salicylate	Rubefacient	*Gaultheria procumbens*
Monocrotaline	Antitumor agent (topical)	*Crotalaria sessiliflora*
Morphine	Analgesic	*Papaver somniferum*
Neoandrographolide	Dysentery	*Andrographis paniculata*
Nicotine	Insecticide	*Nicotiana tabacum*
Nordihydroguaiaretic acid	Antioxidant	*Larrea divaricata*
Noscapine	Antitussive	*Papaver somniferum*
Ouabain	Cardiotonic	*Strophanthus gratus*
Pachycarpine	Oxytocic	*Sophora pschycarpa*
Palmatine	Antipyretic, detoxicant	*Coptis japonica*
Papain	Proteolytic, mucolytic	*Carica papaya*
Papavarine	Smooth muscle relaxant	*Papaver somniferum*
Phyllodulcin	Sweetner	*Hydrangea macrophylla*
Physostigmine	Cholinesterase Inhibitor	*Physostigma venenosum*
Picrotoxin	Analeptic	*Anamirta cocculus*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Pinitol	Expectorant	*Several plants*
Podophyllotoxin	Antitumor anticancer agent	*Podophyllum peltatum*
Protoveratrines A, B	Antihypertensives	*Veratrum album*
Pseudoephredrine*	Sympathomimetic	*Ephedra sinica*

Table 1.4 Continued

Drug/Chemical	Action/Clinical Use	Plant Source
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quisqualic acid	Anthelmintic	*Quisqualis indica*
Rescinnamine	Antihypertensive, tranquillizer	*Rauvolfia serpentina*
Reserpine	Antihypertensive, tranquillizer	*Rauvolfia serpentina*
Rhomitoxin	Antihypertensive, tranquillizer	*Rhododendron molle*
Rorifone	Antitussive	*Rorippa indica*
Rotenone	Piscicide, Insecticide	*Lonchocarpus nicou*
Rotundine	Analagesic, sedative, traquillizer	*Stephania sinica*
Rutin	Capillary fragility	*Citrus species*
Salicin	Analgesic	*Salix alba*
Sanguinarine	Dental plaque inhibitor	*Sanguinaria canadensis*
Santonin	Ascaricide	*Artemisia maritma*
Scillarin A	Cardiotonic	*Urginea maritima*
Scopolamine	Sedative	*Datura species*
Sennosides A, B	Laxative	*Cassia species*
Silymarin	Antihepatotoxic	*Silybum marianum*
Sparteine	Oxytocic	*Cytisus scoparius*
Stevioside	Sweetner	*Stevia rebaudiana*
Strychnine	CNS stimulant	*Strychnos nux-vomica*
Taxol	Antitumor agent	*Taxus brevifolia*
Teniposide	Antitumor agent	*Podophyllum peltatum*
a-Tetrahydrocannabinol	Antiemetic, decrease occular tension	*Cannabis sativa*
Tetrahydropalmatine	Analgesic, sedative, traquillizer	*Corydalis ambigua*
Tetrandrine	Antihypertensive	*Stephania tetrandra*
Theobromine	Diuretic, vasodilator	*Theobroma cacao*
Theophylline	Diuretic, brochodilator	*Theobroma cacao and others*
Thymol	Antifungal (topical)	*Thymus vulgaris*
Topotecan	Antitumor, anticancer agent	*Camptotheca acuminata*
Trichosanthin	Abortifacient	*Trichosanthes kirilowii*
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
Valapotriates	Sedative	*Valeriana officinalis*
Vasicine	Cerebral stimulant	*Vinca minor*

Table 1.4 Continued

Drug/Chemical	Action/Clinical Use	Plant Source
Vinblastine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Vincristine	Antitumor, Antileukemic agent	*Catharanthus roseus*
Yohimbine	Aphrodisiac	*Pausinystalia yohimbe*
Yuanhuacine	Abortifacient	*Daphne genkwa*
Yuanhuadine	Abortifacient	*Daphne genkwa*

1.6.1 Natural products and High Throughput Screening

Natural products are the most important anticancer and anti-infective agents. More than 60% of approved and pre-new drug application (NDA) candidates are either natural products or related to them, not including biologicals such as vaccines and monoclonal antibodies (Cragg *et al.*, 1997). Secondary metabolism has evolved in nature in response to needs and challenges of the natural environment which enables the nature continually carrying out its own version of combinatorial chemistry (Verdine, 1996) for the over 3 billion years during which bacteria have inhabited the earth (Holland,1998). During that time, there has been an evolutionary process going on in which producers of secondary metabolites evolved according to their local environments. If the metabolites were useful to the organism, the biosynthetic genes were retained, and genetic modifications further improved the process. Combinatorial chemistry practiced by nature is much more sophisticated than that in the laboratory, yielding exotic structures rich in stereochemistry, concatenated rings, and reactive functional groups (Verdine, 1996). As a result, an amazing variety and number of products have been found in nature. The total number of natural products produced by plants has been estimated to be over 500,000 (Mendelson *et al.*, 1995). One-hundred sixty thousand natural products have been identified (Chapman, 2001), a value growing by 10,000 per year (Henkel *et al.*, 1999). About 100,000 secondary metabolites of molecular weight less than 2500 have been characterized, half from microbes and the other half from plants (Fenical *et al.*, 1993; Berdy, 1995; Roessner *et al.*, 1996)

It is not generally appreciated that a number of synthetic products of wide medical use have a natural origin from microbial, plant, and even animal systems. The predecessor of aspirin has been known since the fifth century BC, at which time it was extracted from willow tree bark by Hippocrates. It probably was used even earlier in Egypt and Babylonia for fever, pain, and childbirth (Kiefer, 1997). Salicylic acid derivatives have been found in plants such as white willow, wintergreen, and meadowsweet. Synthetic salicylates were produced on a large scale in 1874 by the Bayer Company in Germany. In 1897, Arthur Eichengrun at Bayer discovered that

its acetyl derivative was able to reduce its acidity, bad taste, and stomach irritation (Shapiro, 2003); thus was born aspirin, of which 50 billion tablets are consumed each year. The drugs Acyclovir (Zovirax) used against herpes virus and Cytarabine (Cytostar) for non-Hodgkin's lymphoma were originally isolated from a sponge (Rayl, 1999). Drugs inhibiting human immunodeficiency virus (HIV) reverse-transcriptase and protease were derived from natural product leads screened at the National Cancer Institute (Yang *et al.*, 2001). Angiotensin-converting enzyme (ACE) inhibitors, widely used for hypertension and congestive heart failure, are chemicals based on peptides isolated from snake venom (Ondetti *et al.*, 1971; Patchett, 2002). In the last decade, some large pharmaceutical companies, emphasizing combinatorial chemistry, left natural products and attempted to fill the void with large numbers of synthetic molecules. Unfortunately, the chemistry employed did not create sufficiently diverse or pharmacologically active molecules. Fortunately, some small biotechnology companies have revitalized the interest in natural products. Approaches such as diversity-oriented synthesis, which mimics the structures of natural products, are emerging for drug discovery (Schreiber, 2000).

A few years ago, it was thought that combinatorial chemistry and high-throughput screening (HTS) would yield many new hits and leads, but the result has been disappointing, despite the extraordinary amount of money spent (Ausman, 2001; Horrobin, 2001). After it was developed in the early 1990s, HTS methods achieved speed and miniaturization but discovery of new leads did not accelerate. HTS methods allowed 100,000 chemicals to be assayed per day, and combinatorial and other chemical libraries of 1 million compounds were available commercially. Despite this, no drugs had been approved that resulted from HTS by 1999 (Fox, 1999). The advent of combinatorial chemistry, HTS, genomics, and proteomics has "not yet delivered the promised benefits" (Ernst, 2000). Investment in genomics and HTS has had no effect on the number of products in preclinical development or phase I clinical trials. The problems are that HTS has not been applied to natural product libraries, and combinatorial chemistry has not been applied to natural product scaffolds (Demain, 2002; Kingston *et al.*, 2002; Waldmann *et al.*, 2002). Natural product collections have a much higher hit rate in high-throughput screens than do combinational libraries (Breinbauer1 *et al.*, 2002; Breinbauer 2 et al. 2002) pointed out that the numbers of compounds in a chemical library are not the important point; it is the biological relevance, design, and diversity of the library, and that a scaffold from nature provides viable, biologically validated starting points for the design of chemical libraries. In the past few years, some companies have dropped the screening of their natural product libraries because they considered that such extracts were not amenable to HTS (Fox, 1999). Even worse, we hear that combinatorial chemistry is replacing natural product efforts for discovery of new drugs, and that most companies have even dropped their natural product programs to

support combinatorial chemistry efforts. This makes no sense, since the role of combinatorial chemistry, like those of structure–function drug design and recombinant DNA technology two and three decades ago, is that of complementing and assisting natural product discovery and development, not replacing them (Paululat *et al.*, 1999). Instead of downgrading natural product screening, there is real opportunity in combining it with HTS, combinatorial chemistry, genomics, proteomics, and new discoveries being made in biodiversity.

Although the performance of the pharmaceutical industry has been dismal recently because of poor decisions, the biotechnology industry is doing very well. Between 1997 and 2002, 40% of the drugs introduced came from biotechnology companies. The five largest pharmaceutical companies have in-licensed from 6 to 10 products from biotechnology or specialty pharmaceutical companies, yielding 28–80% of their revenue. The biotechnology industry had two drug/vaccine approvals in 1982, none in 1983–1984, one in 1985, rising to 32 in 2000! The number of patents granted to biotechnology companies rose from 1500 in 1985 to 9000 in 1999. Some biotechnology companies are entering the area of natural product screening and, in the end, may save this valuable resource from falling into obscurity (Arnold *et al.*, 2005).

1.7 Ethnomedicinals from the Eastern Himalayan region of India

In the North East India, each state contains a number of tribal groups. Sikkim is a state of N.E. India, which is the highest number of orchid species known from any single state of India. Such a rich biodiversity in the state has provided an initial advantage to its inhabitants for observing, and scrutinizing the rich flora and fauna for developing their own traditional knowledge. Most of the tribe economies have been historically engaged in subsistence agriculture or hunting and gathering. Over the years, they have developed a great deal of knowledge on the use of plants and plant products in curing various ailments. A review of the literature reveals that many tribal areas and tribal communities in the eastern Himalayan region of India are either under explored or unexplored with regard to their floral wealth used in curing diseases (Bhanumathi *et al*, 2000). Therefore, a need was felt to gather in-depth information on the plant species used by this tribal group and suggest that similar studies need to be carried out across the various groups of tribes for comparison as well as for documenting the knowledge which may be under threat due to the influence of modernization (Kala, 2005). The present study thus aims to highlight and record in detail the traditional knowledge of the local tribes on the use of medicinal plant species growing in and around their settlements.

1.7.1 Medicinal plants survey

A literature survey was carried out for compilation of existing information on the medicinal plants used by Majhitar villagers were undertaken during May and June 2005 to gather data on the indigenous uses of medicinal plant species. During the survey period, information was also gathered using semi-structured questionnaires on types of ailments cured by the traditional use of medicinal plants and plant parts used in curing different ailments. Cross-checking of data was made with the help of group discussions among different age classes. The data obtained is almost same as the earlier worker of this region (Kala, 2005) (**Table 1.6**).

1.8 Alternative medicine

Some of the alternative methods provide help by strengthening the body and controlling the side effects of conventional treatments. Other approaches, because of their gentle noninvasive nature, may in some cases be preferred over more orthodox treatments offered by modern medicine.

Alternative medicine is usually holistic in approach. Holistic means that the goal is to treat the whole body, rather than just the affected area. Many also aim to treat the individual on a number of different levels, including physical, mental, spiritual and emotional. Some scientist's reject the above classification and to varying degrees reject the term "alternative medicine" itself.

The following three commentators argue for classifying treatments based on the objectively verifiable criteria of the scientific method, not based on the changing curricula of various medical schools or social sphere of usage. They advocate a classification based on Evidence-based medicine, i.e., scientifically proven evidence of efficacy (or lack thereof). According to them it is possible for a method to change categories (proven vs. nonproven) in either direction, based on increased knowledge of its effectiveness or lack thereof:

George D. Lundberg, former editor of the Journal of the American Medical Association (JAMA), and Phil B. Fontanarosa, Senior Editor of JAMA, state: 'There is no alternative medicine. There is only scientifically proven, evidence-based medicine supported by solid data or unproven medicine, for which scientific evidence is lacking'.

Whether a therapeutic practice is 'Eastern' or 'Western,' is unconventional or mainstream, or involves mind-body techniques or molecular genetics is largely irrelevant except for historical purposes and cultural interest. As believers in science and evidence, we must focus on

fundamental issues-namely, the patient, the target disease or condition, the proposed or practiced treatment, and the need for convincing data on safety and therapeutic efficacy".

Richard Dawkins, Professor of the Public Understanding of Science at Oxford, defines alternative medicine as a"...set of practices which cannot be tested, refuse to be tested, or consistently fail tests. If a healing technique is demonstrated to have curative properties in properly controlled double-blind trials, it ceases to be alternative. It simply becomes medicine." He also states that "There is no alternative medicine. There is only medicine that works and medicine that doesn't work."

Other well-known proponents of evidence-based medicine, such as the Cochrane Collaboration and Edzard Ernst, Professor of Complementary Medicine at the University of Exeter, use the term "alternative medicine" but agree with the above commentators that all treatments, whether "mainstream" or "alternative", ought to be held to standards of the scientific method. Oxford University Press publishes a peer-reviewed journal entitled Evidence-based Complementary and Alternative Medicine (eCAM).

1.8.1 Alternative Medicine vs conventional Medicine

Alternative therapies provide some services not available from conventional medicine. Examples are patient empowerment and treatment methods that follow the biopsychosocial model of health.

Advocates of alternative medicine hold that the various alternative treatment methods are effective in treating a wide range of major and minor medical conditions, and contend that recently published research proves the effectiveness of specific alternative treatments. They assert that a PubMed search revealed over 370,000 research papers classified as alternative medicine published in Medline-recognized journals since 1966 in the National Library of Medicine database. Advocates of alternative medicine hold that alternative medicine may provide health benefits through patient empowerment, by offering more choices to the public, including treatments that are simply not available in conventional medicine: "Most Americans who consult alternative providers would probably jump at the chance to consult a physician who is well trained in scientifically based medicine and who is also open-minded and knowledgeable about the body's innate mechanisms of healing, the role of lifestyle factors in influencing health, and the appropriate uses of dietary supplements, herbs, and other forms of treatment, from osteopathic manipulation to Chinese and Ayurvedic medicine. In other words, they want competent help in navigating the confusing maze of therapeutic options that are

available today, especially in those cases in which conventional approaches are relatively ineffective or harmful."

Evidence-based medicine (EBM) applies the scientific method to medical practice, and aims for the ideal that healthcare professionals should make "conscientious, explicit, and judicious use of current best evidence" in their everyday practice. Prof. Edzard Ernst is a notable proponent of applying EBM to CAM.

Although advocates of alternative medicine acknowledge that the placebo effect may play a role in the benefits that some receive from alternative therapies, they point out that this does not diminish their validity. Researchers who judge treatments using the scientific method are concerned by this viewpoint, since it fails to address the possible inefficacy of alternative treatments.

Complementary and alternative medicine (CAM), as defined by the National Center for Complementary and Alternative Medicine (NCCAM), is a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine. Complementary medicine is used together with conventional medicine. Alternative medicine is used in place of conventional medicine.

The results of studies of CAM use have been inconsistent. One large-scale study published in the November 11, 1998, issue of the Journal of the American Medical Association found that CAM use among the general public increased from 33.8 percent in 1990 to 42.1 percent in 1997. However, an analysis of data from the 1999 National Health Interview Survey indicated that only 28.9 percent of U.S. adults (age 18 and over) had used at least one CAM therapy in the past year. These results were published in the journal Medical Care in 2002.

Several surveys of CAM use by cancer patients have been conducted with small numbers of patients. One study published in the February 2000 issue of the journal Cancer reported that 37 percent of 46 patients with prostate cancer used one or more CAM therapies as part of their cancer treatment. These therapies included herbal remedies, vitamins, and special diets.

A larger study of CAM use in patients with different types of cancer was published in the July 2000 issue of the Journal of Clinical Oncology. This study found that 69 percent of 453 cancer patients had used at least one CAM therapy as part of their cancer treatment. Additional information about CAM use among cancer patients can be found in a review article published in Seminars in Oncology in December 2002.

It is important that the same rigorous scientific evaluation used to assess conventional approaches be used to evaluate CAM therapies. The National Cancer Institute (NCI) and NCCAM are funding a number of clinical trials (research studies) at medical centers to evaluate CAM therapies for cancer.

Conventional approaches to cancer treatment have generally been studied for safety and effectiveness through a rigorous scientific process that includes clinical trials with large numbers of patients. Less is known about the safety and effectiveness of complementary and alternative methods. Some CAM therapies have undergone rigorous evaluation. A small number of CAM therapies originally considered to be purely alternative approaches are finding a place in cancer treatment—not as cures, but as complementary therapies that may help patients feel better and recover faster. One example is acupuncture. According to a panel of experts at a National Institutes of Health (NIH) Consensus Conference in November 1997, acupuncture has been found to be effective in the management of chemotherapy-associated nausea and vomiting and in controlling pain associated with surgery. In contrast, some approaches, such as the use of laetrile, have been studied and found ineffective or potentially harmful.

1.8.2 Use of Complimentary and Alternative Medicine worldwide

The Canadian Complementary Medical Association is a network of Canadian physicians, osteopaths, residents and medical students with a special interest or expertise in complementary medicine. It was founded at a meeting in Banff, AB in 1996 to promote the best in both unconventional and orthodox therapeutics in medical practice. Membership in the association is limited to those holding a recognized medical degree (M.D., D.O., or equivalent), or to retirees and students in these fields. CAMline is an evidence-based website on complementary and alternative medicine (CAM) for healthcare professionals and the public, with a Canadian perspective. It is funded by the Change Foundation of the OHA (Ontario Hospital Association) and the Richard Ivey Foundation, both of which are based in Toronto. It offers access to evidence-based information on natural health products, CAM therapies and practitioners and health conditions; at the moment (May 2002), there is not a lot of information available under some of the headings. It is to be hoped that the development of this site occurs rapidly because there is a great need for the kind of information promised by this site. The founders (5 partner institutions) and expert advisors are all listed; their credentials indicate that they are drawn mainly from the various disciplines of allopathic, traditional medicine and medical practice

In 1999, a document outlining the situation with respect to complementary and alternative health practices in Canada and their relationship to Canadian health system policies and

practices was prepared for Health Canada by the York University Centre for Health Studies in Toronto. The panel of distinguished authors/consultants includes faculty from universities in several provinces. Although the specific details may no longer be absolutely current, the document contains a wealth of information about CAM in Canada and is made available here for that reason.

The Complementary Medical Association is a British organization whose primary aim is to promote ethical, responsible, professional complementary medicine to the public and the medical profession. The public will find useful the five windows offering access to information about: Therapies, Remedies, Supplements, Conditions, and Drug-Herb Interactions.

1.8.3 Alternative Medicine Practice and Internet

The Internet Health Library offers a large collection of information about a great variety of alternative therapies and health problems; there is information about diet and lifestyle, environmental health, women's health, and so on, from the point of view of the complementary and alternative health practitioner. There is a collection of health news, updated regularly, and access to information also about professional organizations of practitioners and training in the various disciplines of CAM.

The Alternative Medicine Foundation is a U.S. charitable organization which is formed to provide evidence-based resources for health care professionals and reliable information on alternatives to conventional western biomedicine for patients and consumers. The programmes it has set up to deliver this information seem a bit thin at the moment. There is: Herb-Med, described as an "interactive, evidence-based herbal formulary", and **TibetMed,** a website devoted to Tibetan medicine. The herbal formulary has potential, but no text; under standard headings for each herb, the information seeker is directed to lists of references which are not summarized; they are merely cited. In some cases, an abstract of the article cited may be available from **PubMed,** but this is not always so. The foundation is the home of the Journal of Alternative and Complementary Medicine, but none of this material appears to be available via the website. The curious are directed to online subscription information at the site of the publisher Mary Ann Liebert, Inc. Alternative Health News Online is prepared by journalists for the public and aims at helping us to separate the hogwash from the promising therapies in alternative medicine. It is updated daily and covers topics such as diet and nutrition, mind/body control, alternative medical systems, manual healing and longevity. It offers a free, weekly e-mail newsletter, bulletins and alerts on health news and a column of reviewed and recommended books on alternative health care. It also offers links to other sources of health

information on the Internet. This is a great place to begin -- and to come back to -- when searching for alternative health information on the Internet.

The Alchemical Medicine Research and Teaching Association (AMRTA) maintained a complex and very helpful web site on alternative health care which used to be called: Natural Medicine, Complementary Health Care and Alternative Therapies, but has now become: healthwwweb: The Science of Nature. Their stated goal is to reunite the art of healing with the science of medicine. This site offers access to information regarding organizations in the field of alternative health care, training for practice, Internet resources of many kinds, mailing lists, professional journals and computer support for practice of alternative therapies.

The Health World Online Village, although not entirely composed of references to alternative and complementary therapies, has such a wide range and so many good explanations of unconventional therapies presented cheek-by-jowl with standard allopathic treatments and topics that it has to be presented here so that you won't miss it. Resources for consumers and health care professionals in the whole panorama of health care interests are available here: from acupuncture, through aromatherapy and flower medicine, to spas and retreats where you can rest and relax in a health-enhancing way. This is a site not-to-be-missed!

The Health Action Network Society, also known as HANS, of Burnaby, BC, is a non-profit society accepting individual memberships. Their website offers lots of information regarding alternative therapies and practitioners in Canada.

The Holistic Healing Web Page offers a great variety of web resources in alternative health care in a number of classes. The site can be used to present ideas about therapy that can be discussed with an individual's health care provider and is a great collection of diverse ideas and sources.

The Healing Spectrum offers access to resources that support healing from many different points of view. Both Alternative and Allopathic Medicine are profiled on this site, as well as other disciplines that can be used to promote healing. Creator Dianne Marcotte has pulled together a large range of Internet information sources to explain disease and to promote interest in a variety of routes to wellness.

The National Center for Complementary and Alternative Medicine, part of the U.S. National Institutes of Health, identifies and evaluates unconventional health care practices. The NCCAM supports and conducts research and research training on these practices and disseminates information. Their web site offers an overview of the six broad categories of alternative medicine: diet-nutrition-lifestyle changes, mind-body interventions, bioelectromagnetic

applications, alternative systems of medical practice, manual healing, pharmacological and biological treatments, and herbal medicine. There is a FAQ which explains Complementary and Alternative Medicine and the involvement of the National Center with alternative therapy.

The Research Council for Complementary Medicine (RCCM) is a British charity which carries out, promotes and evaluates rigorous research in complementary medicine to encourage safe, effective practice and improved patient care. The RCCM exists not just to educate, to inform or to research but primarily to ensure that the patients are offered treatments that have been show to be effective, treatment that is safe. It is the well-being of patients that is ultimately at stake. The RCCM believes that: research of the highest scientific rigour, supported by reliable information, is essential if complementary options of healthcare are to be made more widely available. The RCCM maintains a database -- the Centralised Information Service for Complementary Medicine (CISCOM) -- of over 60,000 references to the literature of complementary medicine which can be searched for you by contacting them; there is a charge for the service. A small collection of citations to randomised trials in various areas of complementary medicine is available on the website. The RCCM is also involved in creating and maintaining a registry and archive of randomised trials in complementary medicine for the Cochrane Collaboration Field in Complementary Medicine. This work is supported by the U.S. NCCAM, through the University of Maryland. The RCCM is active in medical education in the U.K., as well.

The famous Dr. Andrew Weil, author of the currently popular: 8 Weeks to Optimum Health and many other books and articles now answers your questions at his own web site, called: Ask Dr. Weil. Dr. Andrew Weil is a leader in the integration of Western medicine and the exploding field of alternative medicine.

Wellways.com is a large new collection of links to alternative healthcare resources on the web and to articles of topical interest. The Center for Alternative Medicine Research at Beth Israel Deaconess Medical Center (Harvard Medical School) has a website which offers some access to research and some information for the general public The Milbank Memorial Fund, an endowed national foundation that supports nonpartisan analysis, study, research and communication on significant issues in health policy, has recently published: Enhancing the Accountability of Alternative Medicine on the WWW. This study deals with the use of CAM (Complementary and Alternative Medicine) in the U.S. today, cost and reimbursement issues, and evaluation of the various therapies offered

NOAH: New York Online Access to Health offers a large collection of information on many topics in health and healthcare. One of these collections focuses on alternative medicine. Called

Complementary and Alternative Medicine, this collection offers access to a wide range of topics in the alternative healthcare arena. The collection draws heavily on other sources, but organizes its material in a useful fashion.

The Holistic Channel advertises itself as a source of alternative healthcare news from around the world, but it is also much more than a news source. In addition to news, it offers live radio on natural healthcare, "healing" music and live (but not free) consultations with various sorts of healthcare practitioners. I am not able to find any evidence of the availability of information in non-English languages, nor of the large database of scientifically-validated information, both of which are advertised. There is a lot of marketing of a whole variety of health-related (and not-so-closely-related) stuff going on at this site from a selection of links to online health stores. There is even a section for "deals", but, as always, it is up to you to decide whether the "deal" is real!

AlternativeDr.com offers information about a wide array of alternative medicine resources on the Internet. One can find information about alternative therapies (acupuncture, energy medicine, naturopathy, among others) that are commonly included on sites that deal with complementary and alternative medicine (CAM), and on others (beauty and spa, midwifery, pet therapy) that are not so commonly encountered on CAM sites. There is a discussion board, information about how the drugs are taken and possible interactions with the dietary supplements (herbs, minerals, and vitamins), special reports on CAM topics, and a directory of practitioners (mainly U.S.) One may want to consult. Consumers can access suppliers of alternative therapy supplies in the 'storefront', and exploring the site will uncover many features not mentioned here.

International Health News is a service based in Victoria, BC, which scans 50 respected medical and scientific journals every month and summarizes important medical, health and nutrition news. Our focus is on complementary and preventive medicine, specifically in regard to diet, supplements, vitamins, exercise and lifestyle. We also report on the latest in the fight against arthritis, cancer, heart disease and other degenerative conditions and keep a sharp eye out for warnings about medical procedures and side effects of pharmaceutical drugs. The full database and monthly updates are available only to subscribers, but a collection of essays on various topics, with extensive references to the latest published research, is available free of charge. Some of the essay topics are: Parkinson's disease, breast cancer prevention, prostate cancer prevention, vitamin B_{12}, and fish oils. There are also free excerpts from the database on various topics and sample issues of the monthly newsletter. This site is a real find for those interested in the progress of research into CAM topics.

The Consumer Health Organization of Canada is committed to making people aware of the "holistic" or "alternative" approach to health. They emphasize the prevention of disease through nutrition, whole foods, dietary supplements, herbs and other healing modalities. The organization believes in the philosophy that society benefits from healthy individuals. They work to prevent disease by sharing knowledge and aim to reduce health care costs by avoiding illness. Their website offers access to articles on topics of interest and directs readers to other websites in related areas

The PULSE of Oriental Medicine deals with alternative and traditional oriental medicine resource for the public. It aims to offer information about oriental medicine in easily understood language. EnerChihealth.com is the name of a web site which offers a wealth of information about complementary and alternative medicine in the Canadian context. Holly Gerrish, B. N. (from BC, now living in Alberta) is the founder of Enerchi Health Inc., a company dedicated to presenting objective, research based information on complementary and alternative health practices. On this site, one can find information about medical conditions, and complementary and alternative therapies. There are links to books, articles, related web sites, professional associations and training centers for each therapy. Drug/herb interactions, vitamins, nutritional information and recipes for special diets are available in the site. There is a FAQ which explains many of the terms involved and lots of information about questions to ask a practitioner before engaging her services. There is also a Calendar of Events which is currently blank, but offers to list complementary and alternative medicine events across the country. It is to be hoped that these features are developed soon because they look to be very helpful. The Continuum Center for Health and Healing, an initiative of Beth Israel Medical Center in New York city offers an expanded, integrative practice of health care, and a wonderful web site for anyone seeking information on alternative therapies. One don't have to travel to NYC to use their extensive collection of information; no attempt to be exhaustive is made. Instead, in the Health conditions in A-Z section, research and review articles were selected with a view to providing reliable information from scientific studies showing the complementary/alternative therapeutic approaches that have been studied for each health condition and the significant results to date. There are also collections of information available (and others are being developed) on Complementary/Alternative Therapies, and Traditional and Indigenous Healing Systems. Each entry in these sections follows a pattern which offers information such as history and philosophy of the practice, treatment approaches, and training and licensing of practitioners, among other things. There is an extensive section on professional education for the various alternative disciplines, with links to web sites and recommended resources. This is a thoroughly useful web site; it bears a lot of exploring to plumb its depths.

The Integrative Medicine Institute is a clinic in Calgary, AB, providing a combination of approaches to health care. Practitioners at the clinic bring the best of conventional western medicine and alternative therapies to the partnerships they form with their patients. The site offers information about the beliefs the practitioners have about healing and the services they offer. In addition to a family physician and a psychiatrist, the clinic staff includes naturopathic doctors, a dentist, a nurse, a physiotherapist, a registered holistic nutritionist, and others qualified in Chinese medicine and acupuncture, flower essence therapy, massage and a host of other alternative therapies. There is a list of courses offered at the clinic and information about the therapies used in the care of patients.

The Integrative Health Institute, also located in Calgary, integrates conventional, complementary and alternative health care practices to educate people and corporations interested in improving their personal health. They offer education and research services to support their goal. A site called: Herbal Healing, seems badly titled at first glance. While the site has a lot of information about ayurvedic herbs and herbal healing, it also has extensive space devoted to many other alternative modalities: reiki, yoga, religious healing (from a variety of faiths), tai chi, acupuncture, and more. There is no information about the compilers of this information or about who is responsible for it on the site, which always tends to make one cautious about any advice offered, but there is a lot of variety here not found elsewhere. Explore this site with the awareness that whoever has put this interesting site together does not, apparently, stand behind the information they offer.

Herbal medicines are the oldest remedies known to mankind. Herbs had been used by all cultures throughout history but India has one of the oldest, richest and most diverse cultural living traditions associated with the use of medicinal plants. In the present scenario, the demand for herbal products is growing exponentially throughout the world and major pharmaceutical companies are currently conducting extensive research on plant materials for their potential medicinal value. In many journals, national and international, we find an increasing number of research publications based on herbal drugs. Many analysis-based studies regarding pharmacological research in India have been conducted in the past. Out of these, one study has shown an upward trend in indigenous drug research but there are only few studies on the exclusive analysis of herbal drug research in India. Therefore, the present study was undertaken to analyze the recent trends of herbal drug research in India keeping the Indian Journal of Pharmacology as a marker. The issues of the Indian Journal of Pharmacology from 1995 to August 2003 were reviewed manually in the central library of Govt. Medical College, Jammu, and the Herbal Drug Research Trend Index (HDRTI) was worked out for presentations at IPS conferences as well as for paper publications in the Indian Journal of Pharmacology. Abstracts of the annual IPS conferences and articles (full communications/short

communications/letters/correspondence) published in the Indian Journal of Pharmacology were reviewed in the present study. HDRTI was worked out as a three-year average percentile of herbal drug research for both the parameters respectively. or this, yearly data were collected first and then a three-year average percentage of herbal drug research for the parameters was calculated for the years (1995-1997), (1998-2000) and (2001 to August 2003) by dividing the total percentage for three-year herbal drug research by number of years. Herbal medicines form a major part of remedies in traditional medical systems such as Ayurveda, Rasa Sidha, Unani, and Naturopathy. Hence, all animal and clinical studies on herbal medicines were reviewed. The data for the years 1981-1983 were taken as baseline for the comparison of recent herbal drug research trends.

1.9 Herbal drug research in India

The present study showed that interest has increased in herbal drug research in India, which supported the findings of Adithan (1996), its maximum utilization of the phytotherapeutic approach wherein crude plant preparations were used. The maximum work was observed with polyherbal preparations. To use herbal products, adequate precautions should be exercised. Herbal medicines are the oldest remedies known to mankind. Herbs had been used by all cultures throughout history but India has one of the oldest, richest and most diverse cultural living traditions associated with the use of medicinal plants (Bhatt, 1999).

Ashwagandha (*Withania somnifera*) is often described as the Indian equivalent of ginseng — the Chinese medicinal plant that has captured a huge chunk of the global market. Yet the Indian herb is not a commercial success. One of the reasons for this is the lack of standards, which has led to a wide variation in the chemical composition of ashwagandha-based drugs. The discrepancy, detected during a recent study, occurs in herbal products of even reputable companies.

These drugs are portrayed as potent healers with a wide range of benefits like improving memory, cognition, stamina, vigour and resistance to diseases, as well as relieving tension and depression. But experts feel that in view of the latest findings, a huge question mark hangs on the efficacy of these medicines.

The study was published in the February 2004 issue of Current Science, a journal brought out by the Indian Institute of Science, Bangalore. Entitled 'Phytochemical variability in commercial herbal products and preparations of *Withania somnifera*, it was conducted by three institutes of the Council of Scientific and Industrial Research (CSIR), the Central Institute of Medicinal and

Aromatic Plants (CIMAP) and National Botanical Research Institute, both in Lucknow, and the Regional Research Laboratory in Jammu.

During the study, the amount of withaferin A — one of the withanolides of ashwagandha was analysed in 10 products being sold in the market (**Table 1.5**). Withanolides are secondary chemicals produced by the plant. The scientists assumed withaferin a to be an indicator of the presence of ashwagandha. They found that the amount of the chemical per gramme of ashwagandha varied from 100 per cent to merely 0.9 per cent. In nine of the products, the quantity was less than 50 per cent.

The research highlights these inconsistencies to underscore the importance of standardizing herbal products. It also points out that the anomaly can only be corrected through stringent legislation, which doesn't exist in the country at present. Several factors like sources of raw materials, harvest and post-harvest conditions, and processing and manufacturing techniques have to be regulated for controlling the quality of herbal products. But the Drugs and Cosmetics Act, 1940, which governs the herbal medicine industry, only lays emphasis on making drugs in clean factories and testing raw materials for genuineness. G S Lavekar, director, Central Council for Research in Ayurveda and Siddha, under the Union ministry of Health and Family Welfare, says: "Parameters such as chemical and biological markers should be set so that an acceptable range can be established for chemical constituents."

R S Sangwan of CIMAP, a member of the study team, suggests that ashwagandha should be marketed as a single plant product and not a traditional medicine where a combination of plants is used. While modern scientists are in favour of identifying active ingredients in herbal products and using them as medicines, conventional practitioners believe that such isolates cease to be traditional medicines.

The report has evoked a mixed response from the industry. Some companies are of the opinion that the laws should be strengthened. At the same time, there are others who claim their products are up to the mark. Paranjay Sharma, president of Shree Baidyanath Ayurved Bhawan Private Limited, says a manufacturer can only be penalized if are not exist. He feels quality control should be introduced in the production process at the raw material stage itself. Significantly, the good manufacturing practices that are stipulated currently do not deal with this aspect.

S K Mitra of Himalaya Drug Company says his company uses chemical indicators to ensure that even products in different batches conform to a uniform standard. Further, it conducts trials on humans to ensure the efficacy of the drugs. A senior representative of another manufacturer

implicated in the study asserts: "The researchers did not find the withanolide because it could have been masked by other chemicals present in the product." Sharad Goel, spokesperson for Dabur, says: "We believe that the researchers should have estimated total withanolides, which is a widely accepted biological benchmark, for the purpose of comparing different products available in the market" (Anonymous, 2004).

Table 1.5 Ten herbal products that were subjected to scan test

(Anonymous, 2004)

Brand name	**Manufacturer**
Ashwagandharista	Baidyanath Ayurved Bhawan
Himalaya Ashwagandha	Himalaya Drug Company
Stresswin	Baidyanath Ayurved Bhawan
Stresscom	Dabur India Ltd
Himalaya massage oil	Himalaya Drug Company
Lovemax	BACFO Pharma Ltd
Vigomax	Charak Pharma Ltd
Vital Plus	Mukthi Pharma
Amrutha Kasthuri	Pankajakasthuri Herbals India Ltd
Brento	Zandu Pharmaceutical Works Ltd

1.10 Herbal technology – Concepts and scope

Herbal technology circumscribes all the advancing technical frontiers (except genes) meant to tap myriads of modes of manipulating plants around us. A large number of technologies have been developed to harvest the bountiful products that the plants manufacture, including natural dyes, biofertilizers, biopesticides and biofuel. Emphasis to be given on new medicinal plants (from ethnobotanical surveys), new uses of known medicinal plants, active components and biomarkers, viable substitutes and methods of cultivation, storage, extraction, formulations, efficacy and quality control. In India, it is reported that, more than 150 plants used as biopesticides (bacteria, fungi, virus and protozoa), sources of liquid resins (*Copaifera longdorfii*, *Hardwickia pinnata* and *Dipterocarpus turbinatus*) which can be used as biodiesel, petrocrops, sources of ethanol and a large number of plants yielding non-edible oils as well as production of biodiesel by methylating the oils and medicinal natural dyes.

In the last few decades there has been an exponential growth in the field of herbal medicine (Adhithan, 1996). It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects. In olden times, **vaidyas** used to treat patients on individual basis, and prepare drug according to the requirement of the patient. But the scene has changed now; herbal medicines are being manufactured on a large scale in mechanical

units, where manufacturers come across many problems such as availability of good quality raw material, authentication of raw material, availability of standards, proper standardization methodology of single drugs and formulations, quality control parameters, etc. Ambiguity of our own system of medicine – the Ayurveda, is reflected in the interpretation of names and description of drugs given in the books like Charaka Samhita and Sushruta Samhita, etc. Due to lack of scientific names in the original texts, under one name, different plants are known in different parts of the country as per the description, which makes the drug controversial, e.g. Jivanti, Brahmi (Harish, 2001). These controversies should be eliminated (Tandon *et al.*, 2006).

In the present scenario, the demand for herbal products is growing exponentially throughout the world and major pharmaceutical companies are currently conducting extensive research on plant materials for their potential medicinal value in India and many research articles regarding analysis based studies are published (Dandiya, 1974; Adhithan, 1996 and Singh, 2000).

There is an increased interest on herbal drug research in India (Adithan, 1996) with maximum utilization of the phytotherapeutic approach wherein crude plant preparations were used, mostly with polyherbal preparations. This inclination may be due to: Firstly, people all over the world is looking to various alternative systems of medicine, especially herbal drugs which are claimed to be safe, equally effective in comparison to allopathic drugs and which provide some answer to chronic diseases. Secondly, either these herbal drugs are marketed with exaggerated claims or in some cases are credited with innumerable pharmacological activities which are not mentioned in the text of various traditional systems of medicine. And lastly, as compared to the modern medicines, herbal medicines have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness but they lack an experimental base. In conclusion, it can be said that there is upward trend of herbal drug research in India recently.

Table 1.6 Medicinal plant species, parts used to treat ailments by tribal people (Kala, 2005) .

Sl No.	Species	Family	Part used	Uses
1	*Acorus calamus* L.	Araceae	Root	Cut, wounds, skin diseases, bone fracture
2	*Ageratum conyzoides* L.	Asteraceae	Leaf	Cut, wounds
3	*Allium cepa* L.	Liliaceae	Bulb	Eye pain
4	*Allium hookeri Thwait.*	Liliaceae	Bulb	Eruption of skin, cough, cold, wounds
5	*Alocasia fornículata (Roxb.) Schott.*	Araceae	Root	Crack of heels
6	*Alstonia scholaris* (L.) Br.	Apocynaceae	Leaf, bark	Headache, stomach disorder, menstrual disorder
7	*Amomum aromaticum* Roxb.	Zingiberaceae	Leaf, seed	Fever, abortion
8	*Amorphophallus paeoniifolius* (Dennst.) Nicolson	Araceae	Corn	Piles
9	*Andrographis paniculata* (Burm. f.) *Wall. ex Nees*	Acanthaceae	Leaf	Dysentery
10	*Anisomeles indica* (L.)	Lamiaceae	Shoot	Bodyache
11	*Angiopteris evecta* (Forst.) Hoffm.	Angiopteridacea e	Stem	Health tonic
12	*Antidesma acidum* Retz.	Euphorbiaceae	Leaf	Wounds
13	*Argemone mexicana* L.	Papaveraceae	Shoot	Skin diseases
14	*Artemisia indica* Willd.	Asteraceae	Leaf	Bodyache, asthma, skin diseases
15	*Artemisia maritima* L.	Asteraceae	Shoot	Blood purification
16	*Artemisia nilagirica* (Cl.) Pamp.	Asteraceae	Leaf	Cough, headache, sores
17	*Asplenium nidus* L.	Aspleniaceae	Leaf	Ulcer
18	*Barleria prionitis* L.	Acanthaceae	Leaf	Cough
19	*Begonia roxburghii* (Miq.) DC.	Begoniaceae	Leaf	Indigestion
20	*Berberis wallichiana* (Wall.) Brongn.	Berberidaceae	Fruit, root	Indigestion, bodyache
21	*Bergenia ciliata* (Haw.) Sternb.	Saxifragaceae	Root, leaf	Cut, wounds
22	*Brassiopsis glomarulata* (Bl.) Regel.	Araliaceae	Fruit	Cough
23	*Buddleja asiatica* Lour.	Buddlejaceae	Leaf	Inflammation
24	*Callicarpa macrophylla* Vahl	Verbenaceae	Leaf	Headache
25	*Callicarpa vastita* Roxb.	Verbenaceae	Leaf	Indigestion
26	*Calotropis gigantea* (L.) Br.	Asclepiadaceae	Root	Dog bite
27	*Canarium resiniferum* Brace ex King	Burseraceae	Fruit	Urinary complaints
28	*Capparis spinosa* Lam.	Capparaceae	Root	Rheumatic pain
29	*Cardamine hirsuta* L.	Brassicaceae	Leaf	Indigestion

Contnued Table 1.6.

S No.	Species	Family	Part used	Uses
30	*Castanopsis tribuloides* DC.	Fagaceae	Stem	Cough, goiter, indigestion
31	*Centella asiatica* L.	Apiaceae	Shoot	Constipation, gastritis, blood purification
32	*Chenopodium ambrosioides* L.	Chenopodiaceae	Leaf	Toothache
33	*Christella parasitica* (L.) Lev.	Thelypteridaceae	Fronds	Cut, Wounds
34	*Chromolaena odorata* (L.) King & Robinson	Asteraceae	Leaf	Cut, wounds, headache, fever
35	*Cirsium lapskyle* Petral.	Asteraceae	Shoot	Indigestion
36	*Cissampelos pareira* L.	Menispermaceae	Tuber	Health tonic
37	*Clerodendrum glandulosum* Coleb. ex Wall.	Verbenaceae	Leaf	Blood pressure, fever, cough
38	*Clerodendrum serratum* (L.) Moonb	Verbenaceae	Leaf	Eye disorders
39	*Coelogyne pectata* Lindl.	Orchidaceae	Pseudobulb	Burns
40	*Colocasia affinis* Schott	Araceae	Leaf	Fever, respiratory disorder
41	*Crassocephalum crepidioides* (Benth.) Moore	Asteraceae	Leaf	Indigestion, headache, stomachache, cut, wounds
42	*Crotolaria pallida* Ait.	Fabaceae	Root	Bodyache
43	*Croton roxburghii* Balak	Euphorbiaceae	Fruit	Indigestion
44	*Curcuma caesia* Roxb.	Zingiberaceae	Rhizome	Cough, asthma
45	*Curcuma aromatica* Salisb.	Zingiberaceae	Whole plant	Blood purification
46	*Curcuma zedoaria* Rosc.	Zingiberaceae	Rhizome	Cold, cough
47	*Cuscuta reflexa* Roxb.	Cuscutaceae	Whole plant	Purgative
48	*Cyathea gigantea* (Wall. ex Hk. f.)	Cyatheaceae	Leaf	Bodyache
49	*Cyathula prostrata* (L.) Bl.	Amaranthaceae	Shoot	Appetizer, dysentery, skin diseases
50	*Cymbidium aloifolium* (L.) Sw.	Orchidaceae	Tuber	Wounds
51	*Dendrocnide sinuta* (Bl.) Chew.	Urticaceae	Leaf	Urogenital disorder, toothache, dysentery
52	*Dicranopteris linearis* (Burm. f.) Und.	Gleicheniaceae	Whole plant	Indigestion
53	*Dicrocephala bicolor* (Roth) Sch.	Asteraceae	Shoot	Digestive problems

Contnued Table 1.6.

S No.	Species	Family	Part used	Uses
54	*Dillenia indica* L.	Dilleniaceae	Furit	Stomachache
55	*Dioscorea alata* L.	Dioscoraceae	Tuber	Indigestion
56	*Dioscorea bulbifera* L.	Dioscoraceae	Tuber	Indigestion
57	*Dioscorea hamiltonii* (Hk. f.)	Dioscoraceae	Tuber	Dysentery
58	*Diplazium esculentum* (Retz.) Sw.	Athyriaceae	Fronds	Constipation
59	*Ecbolium viride* (Forsk) Alston	Meliaceae	Root	Rheumatism
60	*Eclipta prostrata* (L.) L.	Asteraceae	Shoot	Cut, wounds
61	*Elaeagnus caudata* Sch. ex Momiyama	Elaeagnaceae	Fruit	Health tonic
62	*Elaeagnus pyriformis* Hk. f.	Elaeagnaceae	Fruit	Constipation
63	*Elatostema platyphyllum* Wedd.	Urticaceae	Root	Vomiting
64	*Elsholzia blanda* (Benth.)	Lamiaceae	Leaf	Itching
65	*Eluesine coracana* (L.) Gaertn.	Poaceae	Grains	Stomach disorder, tonic, cold
66	*Eupatorium odoratum* L.	Asteraceae	Leaf	Wounds, cut
67	*Erigeron bonariensis* L.	Asteraceae	Leaf	Nose block
68	*Eryngium foetidum* L.	Apiaceae	Seed	Madness, headache
69	*Ficus benjamina* L.	Moraceae	Stem	Stomach disorder
70	*Ficus hirta* Vahl	Moraceae	Fruit	Wounds, cut
71	*Gerbera piloselloides* (L.) Cass.	Asteraceae	Leaf	Rheumatic pain
72	*Gloriosa superba* L.	Liliaceae	Tuber	Killing lice in hairs
73	*Gmelina arborea* Roxb.	Verbenaceae	Leaf	Stomach disorders
74	*Gynostemma pedata* Bl.	Cucurbitaceae	Leaf	Throatache
75	*Gynura biscolor* (Roxb. ex Willd.) DC.	Asteraceae	Leaf	Intestinal worms
76	*Gynura nepalensis* DC.	Asteraceae	Leaf	Indigestion
77	*Hedychium coronarium* Koen.	Zingiberaceae	Rhizome	Bodyache
78	*Hedychium dekianum*	Zingiberaceae	Rhizome	Cut, wounds
79	*Hedychium spicatum* Buch.-Ham. ex Sm.	Zingiberaceae	Rhizome	Stomach disorder
80	*Hibiscus rosa-sinensis* L.	Malvaceae	Flower	Reproductive disorders

Contnued Table 1.6.

S No.	Species	Family	Part used	Uses
81	*Houttuynia cordata* Thunb.	Saururaceae	Shoot	Freshness, good sleep, heart disorders
82	*Hyptis suaveolens* (L.) Poit.	Lamiaceae	Leaf	Itching, cough, cold
83	*Hypericum japonicum* Thunb. ex Murr.	Hypericaceae	Stem	Cut, wounds
84	*Impatiens latifolia* L.	Balsaminaceae	Leaf	Headache, digestive disorder
85	*Impatiens racemosa* DC.	Balsaminaceae	Leaf	Digestive disorder
86	*Indigofera tinctoria* L.	Fabaceae	Root	Wound
87	*Jasminum humile* L.	Oleaceae	Root	Ringworm
88	*Laginaria siceraria* (Molina) Standl.	Cucurbitaceae	Fruit	Burns
89	*Leonotis nepetifolia* R. Br.	Lamiaceae	Seed	Burns
90	*Lithocarpus dealbatus* (Miq.) Rehder	Fagaceae	Fruit	Indigestion
100	*Mucuna pruriens* (L.) DC.	Lauraceae	Fruit	Cough, cold, hair tonic, indigestion, good sleep
101	*Murraya koenigii* (L.) Spr.	Lauraceae	Fruit	Bone fracture, stomach disorder
102	*Musa paradissica* L.	Musaceae	Fruit	Indigestion
103	*Myrica esculenta* Ham. ex D. Don.	Myricaceae	Fruit, bark	Indigestion, skin eruption
104	*Myrsine semiserrata* Wall.	Myrsinaceae	Seed	Skin diseases
105	*Oenanthe javanica* (Bl.) DC.	Apiaceae	Shoot	Indigestion
106	*Oroxylum indicum* (L.) Vent.	Bignoniaceae	Seed	Purgative, headache
107	*Osbeckia stellata* Buch.-Ham. ex D. Don	Melastomataceae	Leaf	Toothache
108	*Oxalis corniculata* L.	Oxalidaceae	Shoot	Appetizer, headache
109	*Paedaria foetida* L	Rubiaceae	Stem	Gastritis, diarrhea, stomach disorder
110	*Passiflora foetida* L.	Passifloraceae	Fruit	Respiratory disorder
111	*Photinia integrifolia* Lindl.	Rosaceae	Fruit	Indigestion
112	*Perilla frutescens* (L.) Britt.	Lamiaceae	Seed	Fever, headache
113	*Physalis angulata* L.	Solanaceae	Fruit	Gastric trouble

Contnued Table 1.6.

Sl No.	Species	Family	Part used	Uses
114	*Physalis minima* L.	Solanaceae	Fruit	Gastric trouble
115	*Physalis peruviana* L.	Solanaceae	Leaf	Pain in pregnancy
116	*Picrorhiza kurrooa* Benth.	Scrophulariaceae	Root	Cold, fever
117	*Pinus roxburghii* Sarg.	Pinaceae	Seed	Indigestion
118	*Piper brachystachyum* Wall.	Piperaceae	Seed	Cough
119	*Piper trioicum* Roxb.	Piperaceae	Root	Cough
120	*Plantago major* L.	Plantaginaceae	Leaf	Constipation
121	*Plectranthus japonicus* (Burm. f.) Koidz.	Acanthaceae	Leaf	Fever
122	*Polygonum nepalense* Meissn.	Polygonaceae	Leaf	Indigestion
123	*Polygonum perfoliatum* L.	Polygonaceae	Leaf	Indigestion
124	*Portulaca oleracea* L.	Portulacaceae	Stem, Leaf	Appetizer
125	*Pouzolzia hirta* (Bl.) Hassk.	Urticaceae	Root	Constipation
126	*Pterospermum acerifolium* Willd.	Sterculiaceae	Flower	Earache
127	*Rhus chinensis* Miller	Anacardiaceae	Fruit	Blood dysentery
128	*Rubia cordifolia* L.	Rubiaceae	Shoot	Stomachache
129	*Rubus calycinus* Wall.	Rosaceae	Fruit	Stomach disorder
130	*Rubus ellipticus* Sm.	Rosaceae	Fruit	Indigestion
131	*Rubus paniculatus* Sm.	Rosaceae	Fruit	Stomach disorder
132	*Rubus roseafolius* Sm.	Rosaceae	Fruit	Indigestion
133	*Rumex nepalensis* Spr.	Polygonaceae	Leaf	Indigestion
134	*Saurauria roxburghii* Wall.	Saurauriaceae	Leaf	Constipation
135	*Schefflera glomerata* L.	Araliaceae	Fruit	Indigestion
136	*Schizostachium capitatum* (Munro) *Majumdar*	Poaceae	Shoot	Diarrhea, dysentery, stomach disorder
137	*Senna alata* (L.) Roxb.	Caesalpiniaceae	Leaf	Skin diseases

Contnued Table 1.6.

Sl No.	Species	Family	Part used	Uses
138	*Senna tora* (L.) Roxb.	Caesalpiniaceae	Leaf	Low blood pressure
139	*Sphenomeris chinensis* (L.) Maxon	Lindsaeceae	Fronds	Sprains
140	*Solanum kurzii* Brace ex Prain	Solanaceae	Fruit	Cough, worms infestation
141	*Solanum myriacanthum* Dunal	Solanaceae	Seeds	Toothache
142	*Solanum nigrum* L.	Solanaceae	Leaf	Liver tonic, indigestion
143	*Solanum torvum* Sm.	Solanaceae	Fruit	Cough, skin diseases
144	*Sonchus asper* (L.) Hill	Asteraceae	Shoot	Indigestion
145	*Sonchus arvensis* L.	Asteraceae	Shoot	Stomachache, gastritis
146	*Spilanthus clava* L.	Asteraceae	Leaf	Throat pain
147	*Spilanthes paniculata* DC.	Asteraceae	Leaf	Constipation
148	*Stellaria media* (L.) Vill.	Caryophyllaceae	Leaf	Itching
149	*Stereospermum chelonoides* (L. f.) DC.	Bignoniaceae	Leaf	Sprain
150	*Strobilanthus helictus* T. Anders	Acanthaceae	Shoot	Indigestion
151	*Terminalia chebula* Retz.	Combretaceae	Fruit	Cough
152	*Toddalia aculeata* Pers.	Rutaceae	Fruit	Throat pain
153	*Urtica dioica* L.	Urticaceae	Leaf	Bone fracture
154	*Vernonia cinerea* (L.) Less	Asteraceae	Leaf	Indigestion
155	*Zanthoxylum acanthopodium* DC.	Rutaceae	Fruit	Dysentery
156	*Zanthoxylum armatum* DC.	Rutaceae	Fruit	Cold, cough, fever, appetizer
157	*Zanthoxylum oxyphyllum* Edgew.	Rutaceae	Fruit	Stomach disorder
158	*Zingiber officinale* Rosc.	Zingiberaceae	Rhizome	Cough

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CHAPTER 2

REVIEW OF LITERATURE

Urtica parviflora Roxb.

2.0 Introduction

Urtica parviflora Roxb. Often called as 'Bichu' in Punjab 'Shishoon' in Kumaon, 'Kaldiya' and 'Kandari' in Garhwal, it is known as 'Shisoona' or 'Shisno' in Nepal to Assam however, the common English names is 'Common Nettle' or 'Stinging Nettle'. *Urtica parviflora* belongs to family URTICACEAE (CHOPRA, 1956).

Fig 2.1 Photograph showing the herb of *Urtica parviflora* Roxb. Obtained from Majhitar, eastern part of Sikkim.

It is a monoecious, perennial herb consisting of long stoloniferous rhizomes found in forests and amongst taller herbaceous vegetation, 1700 - 2800 metres, partly shady, moist places of evergreen forests, along streams, roadsides (1500-2400 m) of Nepal, Bhutan, West China and in North India. In India it is mainly found in the Garhwal Himalayas, Kashmir, Assam and Sikkim (CHOPRA, 1956).

2.1 Morphology

Morphologically it is having slender stems, simple or few branched, 25.50 cm tall stems, petioles and both surfaces of leaf blade sparsely hirtellous and armed with stinging hairs. Stipules greenish, interpetiolar, connate, ovate or oblong-ovate 4.6 mm, membranous, with several ribs, apex shallowly 2-cleft or emarginated, petiole slender 2.5.7 cm, leaf blade broadly ovate or ovate-cordate, 2.5.8.5 × 2.7 cm, often membranous, 5-veined, lateral basal veins arcuate, reaching middle margin, secondary veins 2.4 each side, base rounded or shallowly cordate, margin doubly dentate, teeth increasing in size distally, apex acuminate, cystoliths botuliform or sometimes punctiform. Inflorescences unisexual, male inflorescences in distal axils, spicate, 4.7 cm, female ones in proximal axils, subspicate, slender, with a few short branches, or male flowers in middle axils, female flowers in proximal and distal axils, subequal to or shorter than petioles. Male flowers sessile or short pedicellate, perianth lobes connate 1/2 of length, densely hirtellous, then glabrescent. The female flowers have perianth lobes connate at base, unequal, dorsal and ventral lobes suborbicular, sparsely setulose, lateral lobes broadly obovate. The leaves of the plants have stinging hairs, causing irritation to the skin. Young cooked leaves are very nutritious food, high in vitamins and minerals, especially of α-tocopherol and vitamin C (Fritioff, 2005).

2.2 Phytochemical review and ethno-medicinal uses of nettles

- Methanolic extracts of stinging nettle (*Urtica diocia* L.) roots were investigated for aromatase inhibition. Inhibitory effects on aromatase have been known to date neither for pentacyclic triterpenes nor for secondary fatty alcohols (Dietmar *et al.*, 1995).

- *Urtica diocia* L. is most commonly used for the treatment of Prostatic Hyperplasia (BPH) (Brammer, 2001).

- The presence of histamine, 5-hydroxytryptamine (5-HT), and acetylcholine has been demonstrated in the Indian stinging nettle, *Urtica parviflora* (Roxb). In addition, the presence of a histamine liberating substance is strongly indicated (Saxena, 1965).

- Teng Fie *et al.,* isolated some compounds: beta sitosterol, trans-ferulic acid, dotriacotane, erucic acid, ursolic acid,scopoletin, rutin, quercetin and p-hydroxylbezalcohol. Dotriacotane, erucic acid, scopoletin, rutin and p-hydroxylbezalcohol were obtained from *Urtica* L. for the first time. Their structures were confirmed by modem spectral analysis (Teng Fei, 2007).

- Various types of flavonol glycosides have been isolated from flowers of *Urtica diocia* (Chaurasia *et al.*, 1987).

- Han *et al.* found oxalic acid and tartaric acid in the stinging hairs of *U. thunbergiana* and *U. diocia* (Han *et al., 2006*).

- Mohammed *et al.* found *Urtica diocia* has an antiplatelet action in which flavonoids are mainly implecated. This study supports the traditional use of *Urtica diocia* in the treatment and/or prevention of cardiovascular disease (Mohammed *et al., 2006*).

- *Urtica diocia* extract is an excellent substitute for synthetic COX 2 inhibitors as anti-inflammatory medication, because of its actions against the pathways of inflammatory cytokines (Whyte, 2005).

- From the water extract of the roots of *Urtica diocia* (stinging nettle) a polysaccharides fraction was isolated which revealed activity in the carrageenan rat paw edema model and lymphocyte transformation test (Wagner *et al.*, 1989).

- Isolated nettle polysaccharides promote tumor necrosis factor (TNF) production in-vitro, while whole plant extracts inhibit TNF (Anonymous3, 2007).

- Rhizomes of stinging nettle (*Urtica diocia*) contain a complex mixture of isolectins which exhibit agglutination (Peumans *et al.*, 1986).

- Root extract is used in toothache (Bhattacharjee, 2004).

- Ethnomedicinally it is used infrequently. Decoction of the root is used as febrifuge and as cleansing agent after parturition in females (Srivastava, 1993).

- Fresh root is used for boils in following manner: 500 g of fresh root is washed with water and 100 g wheat flour, 20 g turmeric with 50 g common salt are mixed together. The mixture is fried in mustard oil. Resulting paste is applied in boils for 2 to 3 times for 10 days (Singh, 1995).

2.3 Commercial uses

- Stems yield fibre used for making ropes (Anonymous, 1986).
- The whole plant is used for making nettle beer (Sing, 2005).
- The areal parts are used for making green colored dyes for cloths (Anonymous1, 1985).

Advance research on *Urtica parviflora* Roxb and related species.

Peumans et al., (1984) first isolated a lectin from *U. dioica* rhizomes. It is the first single chain lectin to be found in plants. UDA differs from all other known plant lectins with respect to its molecular structure. It interacts with cells in a specific way & induces γ-interferon production by human lymphocytes.

Shibuya et al., (1986) found UDA has two carbohydrate binding sites per molecule chain. Van Damme *et al* (1988) & Va damme & Peumans (1987) isolated six isororm isolectins from *Urtica* rhizomes. They all induce γ-interferon in fresh human lymphocytes.

Wagner et al., (1987) examined a polysaccharide fraction of the water extract of the roots of *U. dioica* & found anti inflammatory activity in the carrageenan rat paw edema model & lymphocyte transformation test. They also found UDA to stimulate the proliferation of human lymphocytes.

Le Moal et al., (1992) reported that the lectins found in *Urtica* used to stimulate thymocytes and spleen T lymphocytes in mouse.

Obertreis et al., (1996) studied the antiphlogistic effects from the extracts of *Urtica* leaves. They isolated caffeoylmalic acid and and studied its therapeutic effect on rheumatoid arthritis.

Taucher et al., (1996) studied the standardized preparation of *U.diocia* (IDS 23) and found to reduce TNFα, IL-1β after Lipopolyscharides (LPS) stimulation of these cytokines in blood.

Rhiehemann et al., (1999) suggested that ant-inflammatory effect of *Urtica* extracts may be related to inhibitory effect on NF- kappa β activation by IDS 23.

Callicarpa arborea Roxb.

2.4 Introduction

Callicarpa arborea Roxb. often called as 'Bormala', 'Kojo' in Bengali, 'Sunga' in Lepcha and 'Guenlo' in Nepali belongs to family VERBENACEAE.

It is a small, moderate sized tree about 12m in height found in the bamboo and deciduous or mixed evergreen and deciduous forests on mountain slopes(1000-2500) m. of Bangladesh, Bhutan, Cambodia, **India,** Indonesia, Laos, Malaysia, Myanmar, Nepal, Thailand and Vietnam. In India it is found in the the upper gangetic plains, lower hills of Kumaon to Sikkim, Assam, Bengal and Khasi hills (300-2000 meters) (Purkayastha *et al.*, 1982, 1985; Pearson *et al.*, 1932).

Fig 2.2 Photograph showing the leaves and inflorescence of *Callicarpa arborea* Roxb. obtained from Majhitar, eastern part of Sikkim.

2.5 Morphology

Morphologically it is having stout trunk with grey short bark with compressed four angled densly tomentose, branchlets, large ovate to ovate-lanceolate or ovate-oblong, acute or sub-acuminate, entire leaves 5.5" by 2.75" to 12" by 5.5", leathery, abaxially densely yellow-brown

stellate tomentose (both sides when young), adaxially dark green and shiny, base cuneate to rounded, marginentire. Cymes 6-11 cm across; peduncle 4-angled, longer than petioles. Calyx cup-shaped, truncate or nearly so, outside densely gray stellate tomentose. Corolla purple, ca. 3 mm. Stamens much longer than corolla. Ovary densely stellate tomentose. Fruit purple-brown, ca. 2 mm in diam. On peduncles 1-2" long which exceed the petioles. Drupe purple, bark-light or sand coloured, blaze soft, white yellowish streaks, hairs present. The bark of the plant is used for medicinal purposes. It mainly consists of aromatic oil containing hydrocyanic acid (Gurung *et al.,* 2002).

2.6 Phytochemical review and ethno-medicinal Uses

- Ethnomedicinally the bark is used as tonic and carminative applied in cutaneous diseases, rheumatism and gonorrhoea (Gurung, 2002)
- Paste of bark and leaf applied on scorpion sting area of skin (Anonymous4, 1976)
- The leaf is used as febrifuge and the bark is used in various skin diseases (Gurung, 2002)

2.7 Commercial uses

- Wood used for making oil, fuel and charcoal (Haines, 1965).

Advance research on *Callicarpa arborea* Roxb.

R. Chenphen et al., (2002) Investigated antimalarial principles from Callicarpa arborea Roxb. and Boesenbergia species.

Gurung 1, (2002) investigated the ethnic use of the plant as the Juice of fruit is used to relief fever.

Blakesley et al., (2002) investigated the ethnomedicinal importance. He examined the the seed germination characteristics of this species.

Morinda citrifolia Linn.

2.8 Introduction

2.8.1 Common Names

Indian Mulberry (India), Noni (Hawaii), Nono (Tahiti and Raratonga), Polynesian Bush Fruit, Painkiller Tree (Caribbean islands), Lada (Guam), Mengkudo (Malaysia (Malaysia), Nhau (South East Asia), Grand Morinda (Vietnam), Cheese fruit (Australia), Kura (Fiji), Bumbo (Africa).

The vernacular names for *Morinda citrifolia* are numerous, almost every island nation of the South Pacific and Caribbean has a term used for this plant (Rita, 1998). *Morinda citrifolia* Linn. often called as Surangi or Bartungi in Hindi, Bengali, Gujurati & Marathi. In Oriya it is called as Achu or Pindra and in Tamil called Nuna, in Nepali it's called as Hardikath, belongs to family RUBIACEAE.

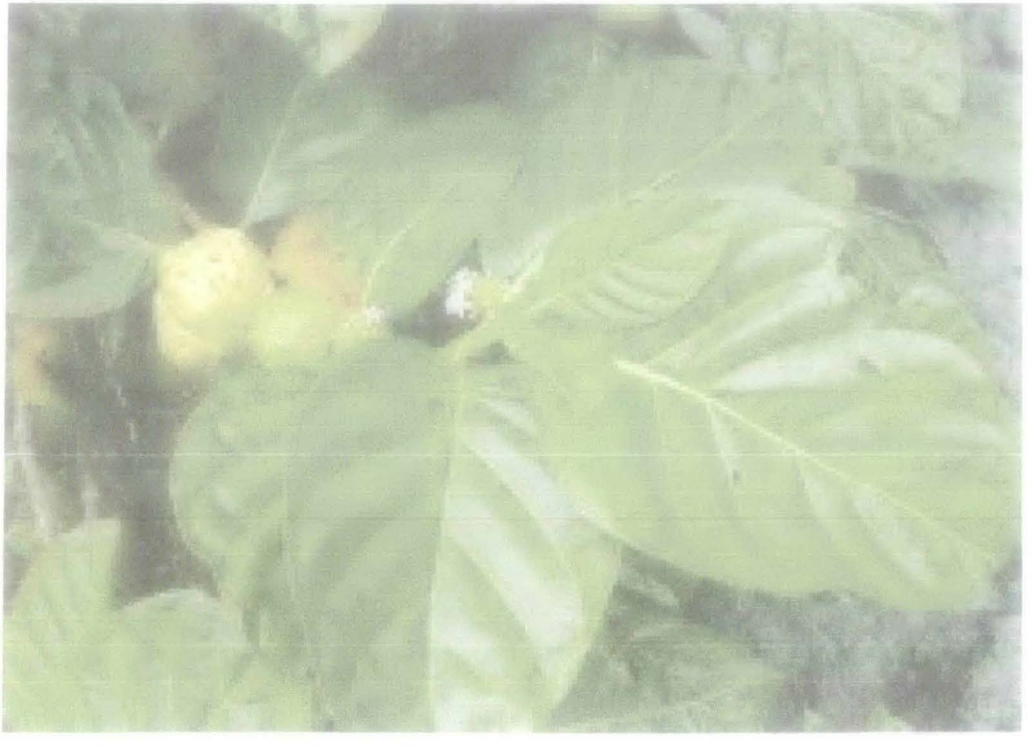

Fig 2.3 Photograph showing the fruit, leaves and inflorescence of *Morinda citrifolia* Linn.

Fig 2.4 Photograph showing the root bark of *Morinda citrifolia* Linn.

2.9 Morphology

A small tree with a straight trunk found throughout the greater part of India including Bengal, Bihar, and Orissa grown as a shade tree and support for pepper vine. Morphologically the plant has broadly elliptic, glabrous, bright green leaves, white flowers, ovoid, glossy white fruits and yellowish brown, fairly hard with close-grained wood. The coloring material resides maximum in root and bark mostly found in young plants. The coloring principle of Morinda root is Morindone (trihydroxymethyl anthraquinone), $C_{15}H_{10}O_5$, m.p= 281-282^{0}c. Morindone present in bark mainly as glucoside morindin ($C_{27}H_{30}O_{14}$). On hydrolysis morindin yields glucose, rhamnose and morindone.

2.10 Phytochemical Review and ethno-medicinal uses

- Noni fruit juice has been reported to have anticancer activity (Cowden, 2005).
- *Morinda citrifolia* found to have insulinotropic activity (Hamid *et al.*, 2008).

- *Morinda citrifolia* has very week esrogenic activity (Chearskul, 2004).
- The root is cathartic. The leaf act as febrifuge and tonic; heals wounds and ulcers. The baked fruit is given in asthma and dysentery. The leaf juice is applied to gout externally (Duduku *et al.,* 2007).
- The fruit is also used for leucorrhoea and sapraemia.
- Traditionally the tender leaves and fruits are used as food.

2.11 Commercial uses

- Commercially the leaves are used to rear silk worm.
- The pulp of the fruit used for cleansing hair.
- The wood is used for turning and for making plates and toys.

2.12 Advance research on *Morinda citrifolia* Linn.

Chafique et al., (1990) worked on the roots of plant for **analgesic** and **behavioral effects** on mice and found non toxic with a dose related effect in 1990. The extract did not exhibit any toxic effects but did show a significant dose related, central analgesic activity in the writhing and hotplate tests; this effect was confirmed by the antagonistic action of naloxone.

Leach et al., (1988) examined the **antibacterial activity** of some medicinal plants of Papua New Guinea. The widespread medicinal use of these plants would suggest they do contain pharmacologically active substances.

Levand et al., (1979) separated several compounds from dried fruit of *Morinda citrifolia* (asperuloside, glucose, caprolic and caprylic acids).

Moorthy et al., (1955) investigate the extract of the roots of this plant for preliminary phytochemical and pharmacological study. This was used as an effective **hypotensive** agent in a majority of patients treated by Dang Van Ho in 1955.

Bushnell et al., (1950) looked into the **antibacterial** properties of this plant found effective against the intestinal pathogens (*P. aeruginosa, M. pyogenes* and *E. coli*). The extract from the ripe fruit was shown to have moderate antibacterial properties against

Salmonella typhosa, *Salmonella Montevideo*, *Salmonella schottmuelleri*, *Shigella paradys*, BH and *Shigella paradys*.

Locher et al., (1995) investigated the **antimicrobial activity** and anticomplement activity of extracts obtained from this plant.

Lung et al., explored the **anticancer activity** of this plant on intraperitoneally implanted Lewis Lung carcinoma in syngeneic mice. The ethanol predipotate (noni-ppt) was not directly toxic to cancer cells. It acts indirectly by enhancing the host immune system involving macrophages or lymphocytes. Noni-ppt had a beneficial effect when combined with suboptimal doses of chemotherapeutic agents. This suggests a possibility of clinical application of noni-ppt in cancer treatment.

Sim et al., (1993) inspect the isolation and characterization of a fluorescent compound from the fruit of *Morinda citifolia* (Noni).

Hiramatsu et al., (1993) were isolated **anthraquinone** from the chloroform extract of the root of this plant and camnacanthal induced normal morphology and cytosketal structure modification in rat- transformed cancer cells.

Kikuzaki et al., were isolated three **new glycosides** from Morinda fruit. They are 6- O-(beta-D-glucopyranosyl)-1-O-octanoyl-beta-D glucopyranose, 6-O- (beta- D-glucopyranosyl)- 1- O-hexanoyl –beta- D glucopyranoseand 3- methylbut- 3-enyl 6-O-beta-D- glucopyranosyl- beta- D- glucopyranoside.

Mieller et al., (200) studied on noni juice for the hidden potential for hyperkalemia. The potassium concentration in noni juice was determined and found to be 56.3m Eq/l, similar to that in orange juice and tomato juice.

Hirazumi et al., (1999) investigated an **immunomodulatory** polysaccharide- rich substance from the fruit substance of *Morinda citifolia* (Noni) with **antitumour activity**. Therapeutic administration of noni-ppr significantly increased survival time of tumor bearing mice. Results also suggested the possibility, that mono-ppt may suppress tumor growth through activation of the host immune system. Noni-ppt was also capable of stimulating the release of several cytokines from immune cells.

Hiwase et al., (1999) inspected that damnacanthal, from *Morinda citrifolia* exhibited apoptosis (cell death) in cancer cells. Damnacanthal treated cancer cells showed more DNA fragmentation from ultraviolet irradiation, than cancer cells treated with ultraviolet radiation alone.

Kikuzaki et al., investigated novel trisaccharide fatty acid ester was 2, 6-di-O- (beta- D-glucopyranosyl) - 1-O-octanoyl-beta- D-glucopyranose identified from the fruits of morinda citrifolia (noni).

Gendong et al., (1998) analyze Jamu Gendong, a kind of traditional medicine in Indonesia for antibacterial and antifungal activities. The results showed the samples were heavily contaminated with bacteria, yeast and moulds.

Hirazumi et al., (1994) investigated antitumor studies of a traditional hawailan medicinal plant. *Morinda citrifolia* (noni-ppt) , in the Lewis Lung peritoneal carcinomatosis model. The pattern of release of these mediators suggested that mono-ppt may promote both a non-specific and th1 cell mediated antitumor response.

Konturek et al., showed that *Morinda citrifolia* (noni) inhibits gastric emptying in male rats via a mechanism involving stimulation of cholecystokinin and its receptor activation. Cholecystokinin is a peptide hormone of the gastrointestinal system responsible for stimulating the digestion of fat and protein. It delays gastric emptying and inhibits gastric acid and plasma gastrin responses

Pu et al., (2004) explored effect of juice from *Morinda citifolia* (Noni) on gastric emptying in male rats. The results suggest that oral noni inhibits gastric emptying in male rats via a mechanism involving stimulation of CCK secretion and CCK1 receptor activation.

Desmond et al., (2004) have been studied two clinical case-study reports on the effects of morinda citrifolia for immune responses pivotal to cancer patient's long term survival. The author concludes that these cases are valuable experiences and hope to stimulate interest in noni research as an important part of adjuvant immunotherapy for cancer.

Wang et al., (2000) were examined of synergistic effects of Tahitian noni juice and methylsulfonymethane on mammary breast cancer prevention at the initiation stage of chemical carcinogenesis induced by DMBA in female Sprague-dawley rats.

Siddiqui et al., (2003) have been isolated and elucidated structural determination of a **benzofuran** and **Bis-Nor- isoprenoid** from Aspergillus niger grown on the water soluble fraction of *Morinda citifolia* Linn. Leaves.

Stalman et al., (2003) were studied on cell cultures of *Morinda citrifolia* L. are capable of accumulating substantial amounts of anthraquinones. All study indicates that the main point of regulation in anthraquinone biosynthesis is located at the entrance of the specific secondary route.

Conrad et al., (2003) inspected the inhibition of angiogenic initiation and disruption of newly established human vascular networks by juice from *Morinda citrifolia* (noni). Noni in concentrations of 5% (V/V) or greater was highly effective in inhibiting the initiation of new vessel sprouts from placental vein explants, compared with initiation in control explants in media supplemented with an equivalent amount of saline.

Wang et al., (1999) reported that noni have antibacterial, antiviral, antitumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects.

Saludes et al., investigated antitubercular constituents from the hexane fraction of *Morinda citrifolia* L. and found the major constituents are E-phytol, cycloartenol, stigmasterool, sitosterol, campesta-5,7,22-trien-3-ol and the ketosteroids stigmasta-4-en-3-one and stigmasta-4-22-dien-3-one.

Xiong et al. studied **antioxidative activity** of extracts from morinda citrifolia L. root, fruit and leaf and found the activity in the roots may be due to both polar and non-polar compounds but, in the leaf and fruit, only to non-polar compounds.

Scott, (2006) evaluated the **antifungal activity** of extracts of *Morinda citrifolia* L. and found it exhibit significant antimicrobial and antifungal activity against various strains of fungi and bacteria A.niger, C.alvicans, E.coli, S.aureus and T. mentagrophytes.

Wang et al., (2002) investigated the protective effects of *Morinda citifolia* on hepatic injury induced by a liver carcinogen and found that as a selective COX-2 inhibitor, it may protect liver by suppressing COX-2 enzymes.

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CHAPTER 3

PHYTOCHEMICAL STUDIES

3.0 Phytochemical study

3.1 Introduction

It is very much essential that the freshly collected plant tissues and the plant materials under investigation should be plunged into boiling alcohol within minutes after collection for their phytochemical analysis. Alternatively, plants may be dried before extraction under controlled conditions or in shade to avoid any chemical changes occurring. It should be dried as quickly as possible, at low temperature, preferably in a good air draft. Indeed, analysis for flavonoids, steroids, alkaloids, quinines and terpenoids has been successfully carried out on herbarium plant tissues dating back many years (Harborne, 1983). Sterols and triterpenes are based on the cyclopentane per hydro-phenanthrene ring system. At one time, sterols were mainly considered to be animal substances, but in recent years, an increasing number of such compounds have been detected in plant tissue. These sterols occur both as free and simple glucosides.

Contamination of the plant tissue under study with other plants is an obvious point to watch. It is essential, to employ plants which are free from diseases, i.e., which are not affected by viral, bacterial or fungal infections, because, these may seriously alter plant metabolism and unexpected products could be formed, possibly in large amounts. The classical mode of extraction naturally depends on (i) the texture and water content of the plant material being extracted and (ii) the type of substance that is being isolated. The procedure for obtaining drug substances from dried plant tissue (whole plant, root, leaf and dried seeds) is the continuous hot percolation by soxhlet apparatus or cold percolation with a range of solvents like petroleum ether, methanol or rarely with diethyl ether. Methanol, in any case, is a good-purpose solvent for preliminary extraction and by extracting the same with different solvents; same or different compounds in varying proportions may be recovered in several fractions. The extract obtained is clarified by filtration through celite by a water pump and is then concentrated *in vacuo*. If the single component is present, it can be purified by crystallization and then the material should be used available for further analysis. In most cases, mixtures of components may be present and it is necessary to separate those compounds by chromatography techniques. As a standard precaution against loss of material, concentrated extract should be stored in the refrigerator and used for further investigations.

The isolation and purification of plant constituents is mainly carried out by using one or other, or a combination of four-chromatography technique viz. paper chromatography (PC), thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC). The choice of technique depends largely on the nature of the substances present. It is very important to note that, there is considerable overlap in the use of above techniques and often a combination of PC and TLC, and GLC, followed by HPLC may be

the best approach for separating a particular class of plant compounds. For preparative work, TLC is carried out and for isolation on large-scale coloumn chromatography is useful. This procedure yields very good quantity of purified components.

Triterpenoids are compounds with a carbon Skelton based on six isoprene units which are derived biosynthetically from the acyclic C_{30} hydrocarbon, squalene. They have relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids. Triterpenoids can be divided into four groups of compounds: true triterpenes, steroids, saponins and cardiac glycosides. The later two groups are essentially triterpenes or steroids which occur mainly as glycosides. These are also known as the steroidal alkaloids. Many triterpenes are known in plants and new ones are regularly being discovered and characterized (Connolly *et al.,* 1991). But only a few are known to be widely distributed. This is also true for the pentacyclic triterpenes α- and β-amyrin and the derived acids, ursolic acid and oleanolic acids. These and related compounds occur especially in the waxy coatings of leaves and fruits such as apple and pear and they may serve a protective function in repelling insect and microbial attack. Triterpene are also found in resins and barks of trees and in latex of many plants (Harborne, 1983).

3.2. Materials and Methods

3.2.1. Plant material

The leaf of *Urtica parviflora* (*U. parviflora*), leaf of *Callicarpa arborea* (*C. arborea*) and root bark of *Morinda citrifolia* (*M. citrifolia*) were collected from the southern and eastern district of Sikkim. They were authenticated at Botanical survey of India, Gangtok, Sikkim. The voucher specimens were preserved in our laboratory for future reference. The collected plant parts were dried in shade, pulverized in mechanical grinder and passed through 40-mesh sieve to get the powder.

3.2.2. Extraction procedure

The powdered plant materials were subjected to methanol extraction (70%) in a Soxhlet extractor fitted with a waterbath. The methanol extracts were concentrated, suspended in hot distilled water, cooled and the blast precipitate was filtered off. The water soluble component was fractionated by extracting it successively with petroleum ether, chloroform and acetone. The chloroform soluble fraction was subjected separately to chromatographic analysis in case of *U. parviflora* and *M. citrifolia.* Similarly, the acetone soluble fraction was taken for chromatographic analysis in *C. arborea.* The aqueous, and petroleum ether fraction did not show any positive pharmacological activities under perview of this investigation and was discarded. Flow chart of extraction has been shown in **Fig 3.0**.

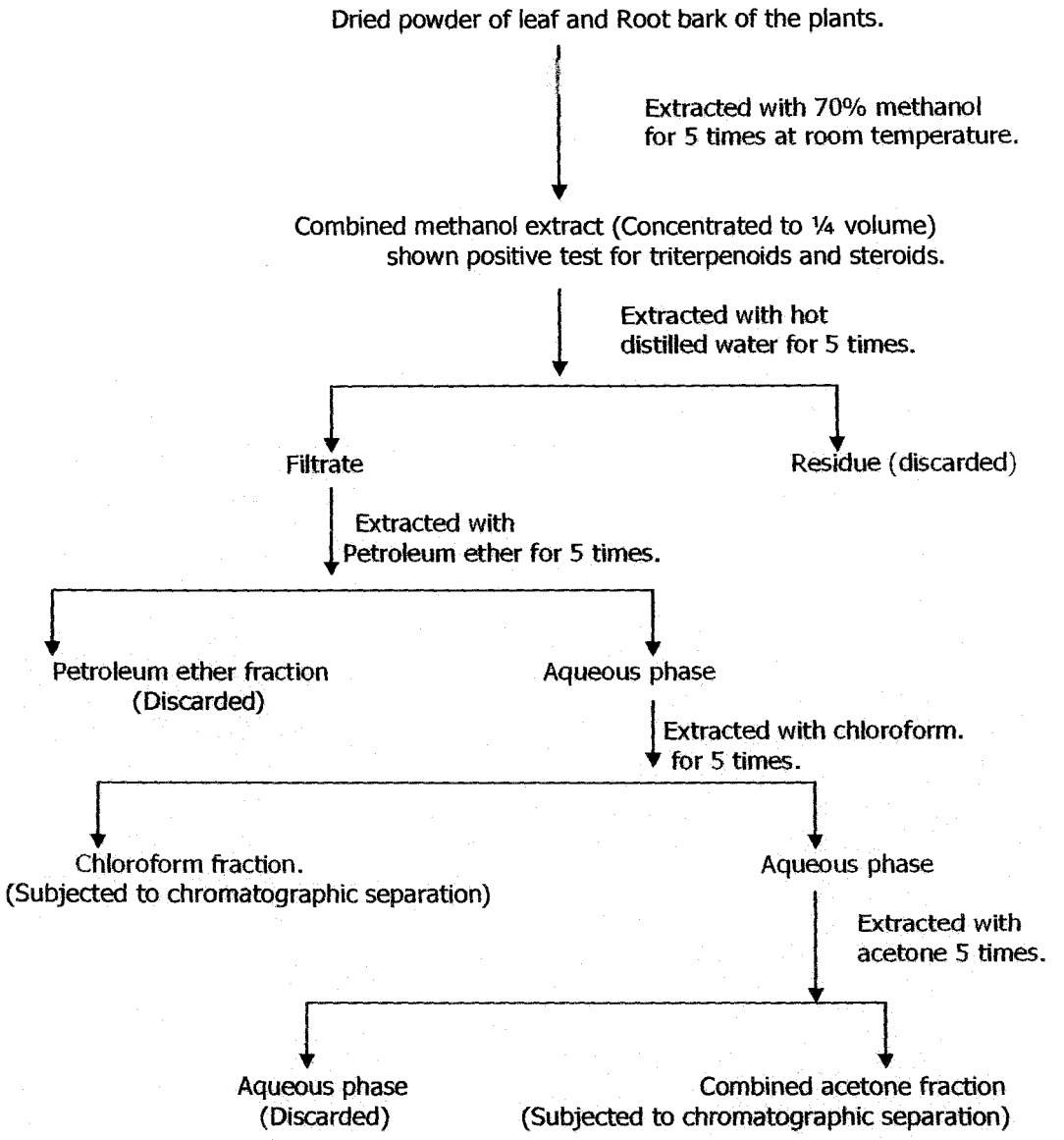

Fig 3.0 Flow chart showing the method of extraction of *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn.

3.2.3. Isolation and purification of phytoconstituent from the leaf of *U. Parviflora.*

3.2.3.1. Preliminary phytochemical Test

The preliminary phytochemical group test of *leaf* extract was performed by the standard methods (Plummer, 1985; Pollock *et al.*, 1965 and Trease *et al.*, 1966).

Tests for Alkaloids

- Small quantity of the leaf extract of *U. parviflora* was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent. Absence of yellowish buff colored precipitate indicated the absence of alkaloids.
- A small quantity of leaf extract of *U. parviflora* was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was then treated with Dragendroff"s reagent. Absence of orange brown precipitate indicated the absence of alkaloids.
- Small quantity of leaf extract of *U. parviflora* was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. Failure to produce reddish brown precipitate suggested the absence of alkaloids.
- Small quantity of leaf extract of *U. parviflora* was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Hager's reagent. Absence of yellowish precipitate demonstrated the negative test for alkaloids.

Test for amino acids and Proteins.

- Small quantity of leaf extract of *U. parviflora* was dissolved in a few ml of distilled water and treated with Ninhydrin reagent at pH 5.0. The presence of purple coloration suggested the positive test for amino acids.
- Small quantity of leaf extract of *U. parviflora* was treated with few drops of 10% sodium hydroxide solution. Few drops of 1% cupper sulphate solution was added and mixed. Formation of violet or purple colour demonstrated the presence of proteins.
- Small quantity of leaf extract of *U. parviflora* was treated with 1.0 ml of concentrated nitric acid. The sample was boiled and cooled. Few drop of 40% sodium hydroxide was added drop by drop. Appearance of orange colored solution indicated the presence of proteins.
- A small quantity of leaf extract of *U. parviflora* was treated with few drops of Millon's reagent. The samples were boiled and cooled. Few drop of 40% sodium nitrate solution was added to the sample drop by drop. Appearance of red precipitate confirmed the presence of proteins.

Test for reducing sugar

- A small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Benedict's reagent was added and heated for few minutes. Formation of brick red precipitate confirmed the presence of reducing sugars.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Fehling's A and B solution were added and heated for few minutes. Development of brick red colour demonstrated the presence of reducing sugars.

Test for steroids and triterponoids

- Libermann-Buchard test: 10mg of leaf extract of *U. parviflora* was dissolved in 1.0 ml of chloroform; 1.0 ml of acetic anhydride was then added to the mixture followed by 2.0 ml of concentrated sulphuric acid. A reddish violet ring at the junction of the two layers confirmed the presence of triterpenoids and steroids.
- Salkowski Test: 1.0 ml of concentrated sulphuric acid was added to 10 mg of leaf extract of *U. parviflora* and dissolved in 1.0 ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.

Test for flavonoids and their glycosides

- A small quantity of leaf extract of *U. parviflora* was dissolved in methanol and hydrolyzed with 10% sulphuric acid and cooled. Then, it was extracted with diethyl ether and divided into three portions in separate test tubes. 1.0 ml of diluted sodium carbonate solution, 1.0 ml of 0.1M sodium hydroxide and 1.0 ml of diluted ammonia solutions were added to the first, second and third test tubes respectively. Presence of yellow colour in all the tubes demonstrated the presence of flavonoids.
- A small quantity of leaf extract of *U. parviflora* was dissolved in methanol. One piece of magnesium followed by concentrated hydrochloric acid was added drop wise to the test sample and heated. Presence of magenta colour demonstrated the presence of flavonoids.

Test for Tannins

- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 10% aqueous potassium dichromate solution. Presence of yellowish brown precipitate demonstrated the presence of tannins.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrate was allowed to react with 10% lead acetate solution. Presence of yellow colour precipitate indicated the positive test for tannins.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrates were then allowed to react with 1.0 ml of 5% ferric chloride solution. Presence of greenish black coloration demonstrated the presence of tannins.
- Small quantity of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and filtered. The filtrates are then allowed to react with 1.0 ml of 1% gelatin and 1.0 ml of 10% sodium chloride solution. Presence of white buff coloured precipitate demonstrated the presence of tannins.

Test for Saponins

- Small quantities of leaf extract of *U. parviflora* was dissolved in minimum amount of distilled water and shaken in a graduated cylinder for 15 minutes. Absent of stable foam suggested the absence of saponins.
- A small quantity of leaf extract of *U. parviflora* was dissolved in methanol. 1.0 ml of extract solution was treated with 1% lead acetate solution. Absent of white precipitate indicated the absence of saponins.

Test for Anthraquinones

- Small quantity of leaf extract of *U. parviflora* was dissolved in methanol. 5.0 ml of the extract solution was hydrolyzed with dilute sulphuric acid and extracted with benzene. 1.0 ml of dilute ammonia was then added to the samples. Development of rose pink colouration indicated the presence of anthraquinones.

3.2.3.2. Thin Layer Chromatography of the methanol leaf extract of *U. parviflora*.

A layer of silica gel G (Loba) was used all along in the present investigation. Chromatographic plates were prepared according to the general method. Glass plates of 20cm × 10 cm were coated with slurry of silica gel prepared by mixing 6 gm of silica gel G with 12 ml of distilled water. The layer was allowed to set for 30 min at room temperature and was then activated at 110°C for 30 min.

The sample of methanol extract was spotted on the plates and chromatogram was developed in chromatographic chambers using selected solvent systems at a room temperature (28°C) and at an angle of 70°. Since the rate of migration of a compound on a given adsorbent depends upon the solvent used, the solvent system can be arranged in order of elutive power (Skoog, 1988; Fried *et al.*, 1994). Mixtures of two or three solvents of different polarities give better separation than the chemically homogeneous solvents. In the present investigation, several solvent systems were studied for effective separation of the components, but the under mentioned solvent systems showed better result. The mobile phase (solvent system) was allowed to run up to a distance of 10 cm from the origin. The time required for the development of chromatograms differed from 45 to 60 min. The plates were removed from the chamber after completion of the run and were allowed to dry in air. The resulting bands were located using UV-light spraying with spray reagents followed by heating in the oven for 5-10 min at 120°C.

3.2.3.2.1 Solvent systems used

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

3.2.3.2.2 Spray reagents used

Anisaldehyde sulphuric acid reagent (Dequeker, 1964)

10% Sulphuric acid reagent (Wagner *et. al.*, 1997)

3.2.3.3 Coloumn Chromatography of leaf extract of *U. parviflora*.

3.2.3.3.1 Coloumn

A glass coloumn, 25 cm in length, 3.5 cm dia. The bottom of the coloumn was plugged with glass wool.

3.2.3.3.2 Adsorbent

Sephadex LH-20 (E. Merck) and Silica gel 60-120 (Loba).

3.2.3.3.3 Solvents used

Hexane: ethyl acetate (with increasing amount of ethyl acetate), Benzene: acetone (with increasing amount of acetone).

3.2.3.3.4 Preparation of coloumn

The coloumn was washed with distilled water and finally rinsed with acetone to remove the impurities. It was fixed with a stand and packed with slurry of Sephadex LH-20 and Hexane. The solvent (Hexane) was adjusted to drip at the rate of 100 drops per minute and a level of 5 cm of the solvent was maintained on the top of the Sephadex layer. The coloumn was recycled with Hexane for several times to prevent any shrinkage and air bubble. The final dimension of the Sephadex coloumn was 3.5 × 15 cm.

3.2.3.3.5 Separation of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The excess solvent on the top of the coloumn was allowed to flow down and then the dried mixture of chloroform fraction of the leaf extract and Sephadex LH-20 was layered on the top of the coloumn. A thin layer of cotton was placed over it. The solvent was allowed to flow down slowly till the mixture was adsorbed on the top of the coloumn. Gradient elution was carried out using Hexane: ethyl acetate with step increasing the polarity by increasing the ratio of ethyl acetate from 10% to 100%. The rate of elution was adjusted at 30 drops per minute and fractions of 25 ml each were collected in 100 ml of serially numbered conical flasks. TLC was done for each fraction with the same solvent system, which was used as the main eluent in the coloumn chromatography.

The eluted fraction number 41-54 having identical hR_f values were pooled together and evaporated to dryness. It was rechromatographed in a silica gel 60-120 (Loba) coloumn. Gradient elution was carried out using Benzene and increasing the polarity with acetone in 10% stepwise elutions to 100% acetone. Fraction number 16-28 were combined and evaporated to dryness to provide an amorphous powder, which was crystallized from methanol to give fine, needle shaped, white crystals. The isolated crystalline material was further examined by different physico-chemical techniques for its structure elucidation.

3.2.3.4 Qualitative analysis of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The isolated compound was further subjected to chemical tests for confirmation of its chemical nature.

3.2.3.5 Physical Nature of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The isolated compound was subjected to various physicochemical parameters viz: physical appearance, solubility and melting point.

3.2.3.6 Thin Layer Chromatographic study of the compound isolated from chloroform fraction of *U. parviflora* leaf.

Thin layer chromatographic study of the isolated compound was carried out on silica gel G plates with different solvent systems. The plates were prepared as described in 3.2.3.2 and used for the study. The chromatograms were run with three solvent systems mentioned below in a chromatography chamber.

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene:acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

The mobile phase was allowed to run up to 10 cm (solvent font) and the plates were dried. The spots were observed under UV light at 366 nm (long wave) before and after spraying the spraying reagents.

3.2.3.7 Ultraviolet-Visible absorption spectral analysis of the compound isolated from chloroform fraction of *U. parviflora* leaf.

Ultraviolet-visible absorption spectroscopy is the most useful technique available for triterpenoids and steroids structure analysis and is used to aid both identification of the type

and definition of the oxygenation pattern. The crystalline isolated compound was dissolved in spectroscopic grade methanol and the absorption spectra were taken in Shimadzu 1601 double beam UV-Visible spectrophotometer.

3.2.3.8 Infrared spectrum of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The infrared (IR) absorption spectra of the isolated compound were taken with Perkin Elmer FTIR spectrophotometer, in potassium bromide discs. The spectra were recorded in the region of 4000 cm^{-1} to 400 cm^{-1}. The spectra of the isolated compound with absorption bands were recorded and tabulated.

3.2.3.9 Nuclear Magnetic Resonance (NMR) spectra of the compound isolated from chloroform fraction of *U. parviflora* leaf.

The ^{1}H and ^{13}C NMR spectra of the isolated compound were undertaken in Brucker WM 400 Spectrophotometer in DMSO-d_6 (Dimethyl sulphoxide) solution. The spectra are recorded and tabulated.

3.2.3.10 Mass Spectrometry of the compound isolated from chloroform fraction of *U. parviflora* leaf.

GC-MS spectra were recorded using a Finnigan Matt GCQ Mass Spectrometer.

3.2.4 Isolation and purification of phytoconstituent from the leaf of *Callicarpa arborea.*

The methods used for isolation and purification of phytoconstituent from the leaf of *Callicarpa arborea* were as per the procedure followed for the isolation and purification of phytoconstituents from the leaf extract of *U. parviflora* described in 3.2.3.1 to 3.2.3.10 except the following points:

1. The identical eluted fractions number 32-46 in coloumn chromatographic separation in 3.2.3.3.5 were rechromatographed by using Benzene: methanol as eluent. Gradient elution was carried out by increasing the polarity with methanol in 10% stepwise elutions to 100% methanol.
2. The rechromatographed fractions numbers 18 to 26 were combined and evaporated to dryness followed by crystallization in hexane-ethyl acetate to result a triterpenoid.

3.2.5 Isolation and Purification of Phytoconstituent from the root bark of *Morinda citrifolia*

The methods used for isolation and purification of phytoconstituents from the root bark of *Morinda citrifolia* were as per the procedure followed for the isolation and purification of phytoconstituents from the leaf extract of *U. parviflora* described in 3.2.3.1 to 3.2.3.10 expect the following points:

1. The TLC study was carried out by using the spraying reagents: bromine water and dilute ammonia solution. (Rajendran *et.al.,* 2007)
2. The identical eluted fractions number 48-59 in coloumn chromatographic separation in 3.2.3.3.5 were rechromatographed by using Benzene: methanol as eluent. Gradient elution was carried out by increasing the polarity with methanol in 10% stepwise elutions to 100% methanol.
3. The rechromatographed fractions numbers 22 to 30 were combined and evaporated to dryness to result an anthraquinone derivative.

3.3 Results

3.3.1 Phytochemical Study of *U. parviflora* leaf.

The concentrated methanol extract obtained from the shade-dried leaf of *U. parviflora* was fractionated successively petroleum ether, chloroform and acetone. It was observed that only the chloroform fraction exhibited significant pharmacological activities under perview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of amino acids, proteins, steroids and triterpenoids (**Table 3.1**). The thin layer chromatographic study of the chloroform fraction showed the presence of three components with selected mobile phase (**Table 3.2-3.3**). This fraction was subjected to coloumn chromatographic separation on Sephadex LH 20 coloumn in which fifty-eight fractions were collected. The fractions having identical results were mixed together (**Table 3.4**). They were purified with a silica gel (60-120) coloumn, which yielded a steroidal compound (compound I). It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound I, which are depicted in **Table 3.5 and Table 3.6**. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (IR, GC-MS, ^{13}C and ^{1}H NMR) data (Faizi *et al.,* 2001; Peirs *et al.,* 2006).

Compound I was obtained as white shining, needle shaped crystal. It is insoluble in aqueous solvent and sparingly soluble in ethyl acetate. The compound was melted at 128.5^{0}- 129. 2^{0}C. The UV absorption spectrum of compound I showed strong absorption at 492 nm in its spectrum, which implied the presence of steroidal ring in its structure. The IR spectrum of the

compound is presented in **Fig 3.1**. The IR spectrum shows the presence of absorption bands at 3430, 2959, 2935, 2868, 1667, 1708 cm^{-1} The IR spectrum confirmed the presence of C=C (1667 cm^{-1}) and hydroxyl group (3430 cm^{-1}) in compound I.

The ^{1}H NMR spectrum of compound I is presented in **Fig 3.2**. Comparisons of ^{1}H and ^{13}C NMR spectra of the isolated compound facilitated the identification of the structure (**Table 3.7**). The ^{1}H NMR spectrum displayed H-6 at δ 5.4 as a multiplet. The same spectrum showed signals for H-3 at δ 3.5 (m, H-18 at δ 0.68 (s), H-19 at δ 0.98 (s), C-21 at δ 0.9 (d, J= 6.5 Hz). The ^{13}C NMR spectrum (**Fig 3.4**) showed C-5 and C-6 double bond carbons at δ 122.09 and 138.29 suggesting the sitosterol structure. The fragmentation ion at m/z 414 in its mass spectrum, which is presented in **Fig 3.3**, inferred the compound is corresponding to the molecular formula $C_{29}H_{50}O$. It was unambiguously identified as **β-sitosterol** on the basis of all the spectral data (**Fig 3.5**) (Faizi *et al.*, 2001).

Table 3.1 Preliminary phytochemical test of methanol extract of *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn.

Phytoconstituents	*Urtica parviflora* (Leaf)	*Callicarpa arborea* (Leaf)	*Morinda citrifolia* (Root bark)
Alkaloids	—	—	+
Amino acids	+	—	+
Proteins	+	+	—
Reducing sugars	—	—	+
Steroids and Triterpenoids	+	+	+
Flavonoids	+	+	+
Tannins	+	—	—
Saponins	—	—	—
Anthraquinones	—	—	+
Gums and Mucilages.	—	—	—

+ ***ve*** indicates presence and – ***ve*** indicates absence of the phytoconstituents.

Table 3.2 Thin Layer Chromatography study of chloroform fraction of *Urtica parviflora* Roxb.

No of spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagent			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Blue	Blue	Blue	Bright blue	Bright blue	Bright blue	83	59	58
2	Blue	Blue	Blue	Blue Florescence	Blue Florescence	Blue Florescence	64	29	52
3	–	–	Blue	–	–	Blue Florescence	–	–	41

Spray reagent: Anisaldehyde sulphuric acid reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (4:1 v/v)

Table 3.3 Thin Layer Chromatography study of chloroform fraction of *Urtica parviflora* Roxb.

No of spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagents			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	83	59	58
2	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	64	29	52
3	–	–	Dark Violet	–	–	Violet florosce nce	–	–	41

Spray reagent: 10% sulphuric acid solution.

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene: acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (4:1 v/v)

Table 3.4 Coloumn and Thin layer chromatography separation of chloroform fraction of *Urtica parviflora* Roxb.

Eluent Hexane: ethyl acetate	Fraction number	Residue of selective fraction (gm)	Colour of TLC spots with hR_f values.	Inference
100:0	1-12	5.2	NIL	Fatty oil
90:10	13-17	4.6	NIL	Fatty solid
80:20	18-25	3.0	NIL	Fatty solid
70:30	26-30	6.7	NIL	Fatty solid
60:40	31-34	16.1	NIL	Greenish semisolid
50:50	35-39	14.3	NIL	Dark green semisolid
40:60	41-43	6.5	Two blue spots. (59, 54)	Mixture of compounds
30:70	44-51	6.0	Two bright blue spots. (58, 54)	Mixture of compounds
20:80	52-54	4.5	Two blue spots (59, 54)	Mixture of compounds
10:90	55-56	3.5	Three bright blue spot (84, 57, 29)	Mixture of compounds
0:100	57-58	2.0	Nil	Colourless solid

Spray reagent: Anisaldehyde sulphuric acid reagent

Table 3.5 Qualitative analysis of the compound I isolated from chloroform fraction of *Urtica parviflora* Roxb.

S.no	Treatment	Observation	Inference
1	1 mg of the crystalline solid was dissolved in 0.5 ml of chloroform 1.0 ml of acetic anhydride was then added to the mixture followed by 2.0 ml of concentrated sulphuric acid.	A reddish violet ring at the junction of the two layers.	Presence of steroids.
2	1.0 ml of concentrated sulphuric acid was added to 1 mg of isolated crystalline solid and dissolved in 1.0 ml of chloroform.	A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer.	Presence of steroids.

Table 3.6 Thin layer chromatography of the compound I isolated from chloroform fraction of *Urtica parviflora* Roxb.

Solvent system	**hR_f values**	**Colour of fluorescent produced**		
		UV_{366nm}	UV_{366nm} +Ansl	UV_{366nm} +10% H_2SO_4
TLC 1	64	Blue	Bright blue	Bright blue
TLC 2	59	Blue	Bright blue	Bright blue
TLC 3	41	Blue	Bright blue	Bright blue

Ansl: Anisaldehyde sulphuric acid reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.7 ^{1}H and ^{13}C data for compound I, isolated from chloroform fraction of *Urtica parviflora* Roxb.

Position	δ_H, 400 MH_Z, $CDCl_3$	δ_C, 400 MH_Z, $CDCl_3$
1	–	37.11
2	–	30.10
3	3.5, m	16.12
4	–	41.48
5	–	139.29
6	5.4, m	122.69
7	–	32.86
8	–	32.86
9	–	51.08
10	–	46.79
11	–	21.1
12	–	39.7
13	–	42.5
14	–	57.1
15	–	24.2
16	–	28.1
17	–	57.06
18	0.68, s	12.1
19	0.98, s	–
20	–	36.3
21	0.9, d (6.5)	21.2
22	–	33.9
23	–	26.0
24	–	–
25	–	29.8
26	0.81, d (6.6)	19.0
27	–	–
28	–	22.1
29	0.82, t (6.4)	

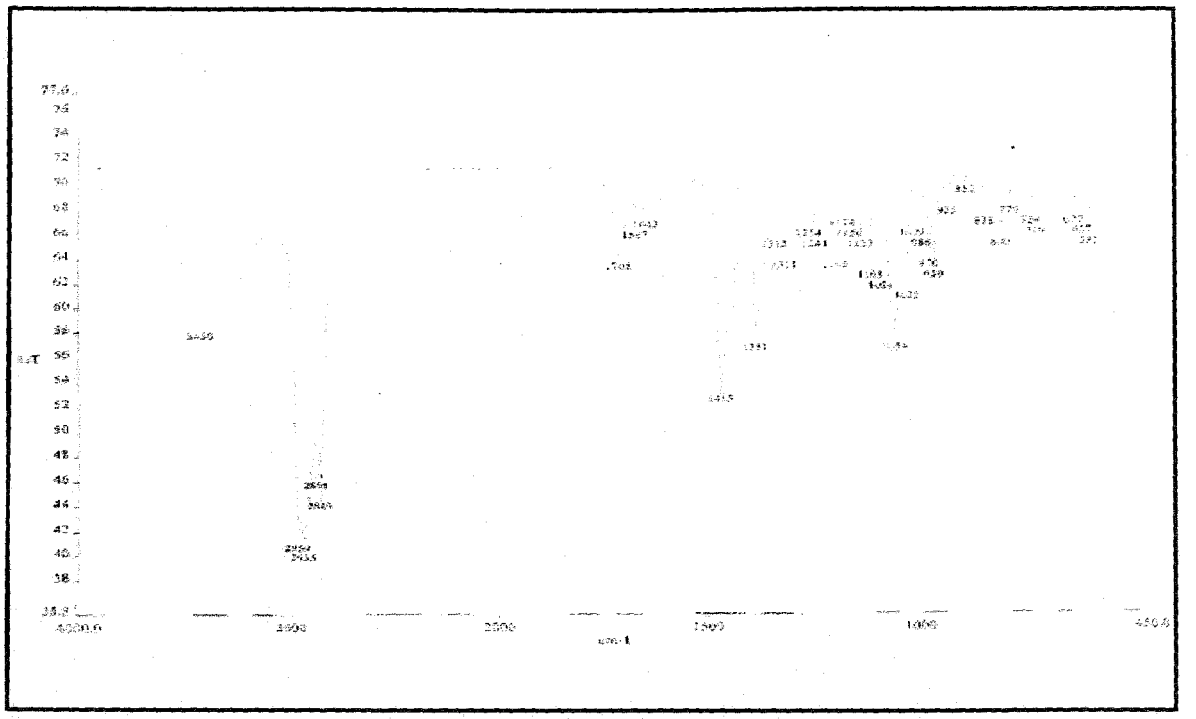

Fig 3.1 IR Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

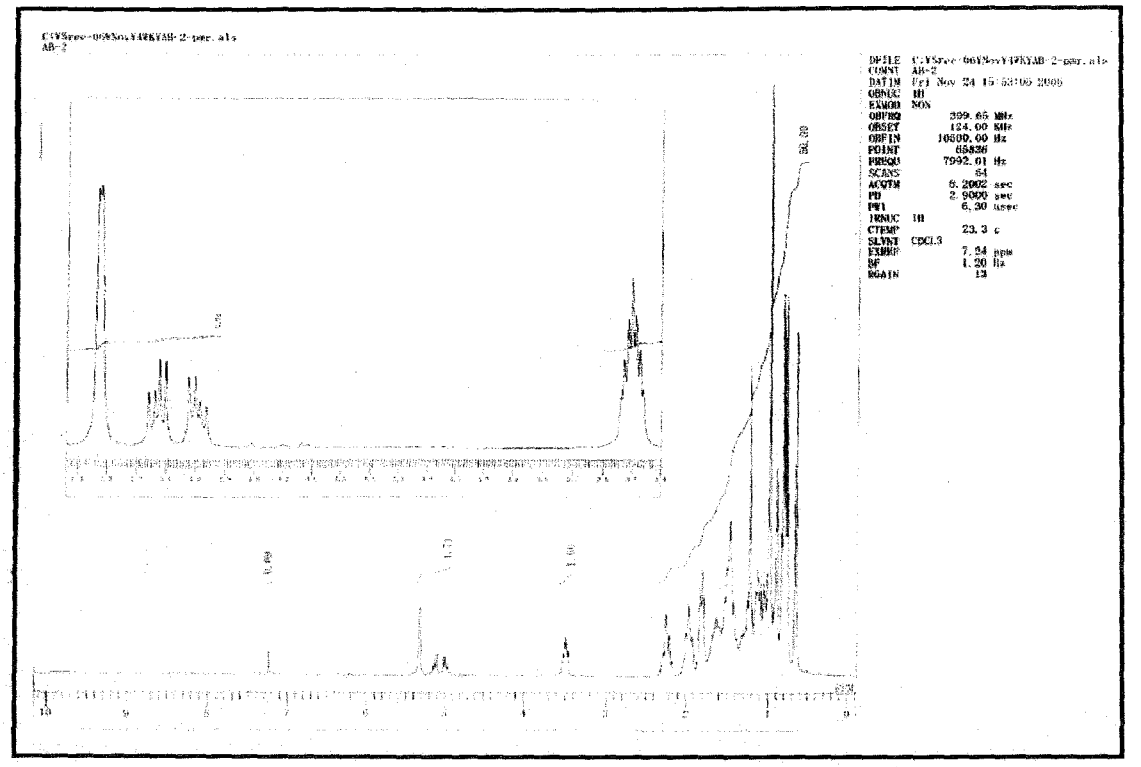

Fig 3.2 ^{1}H NMR Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

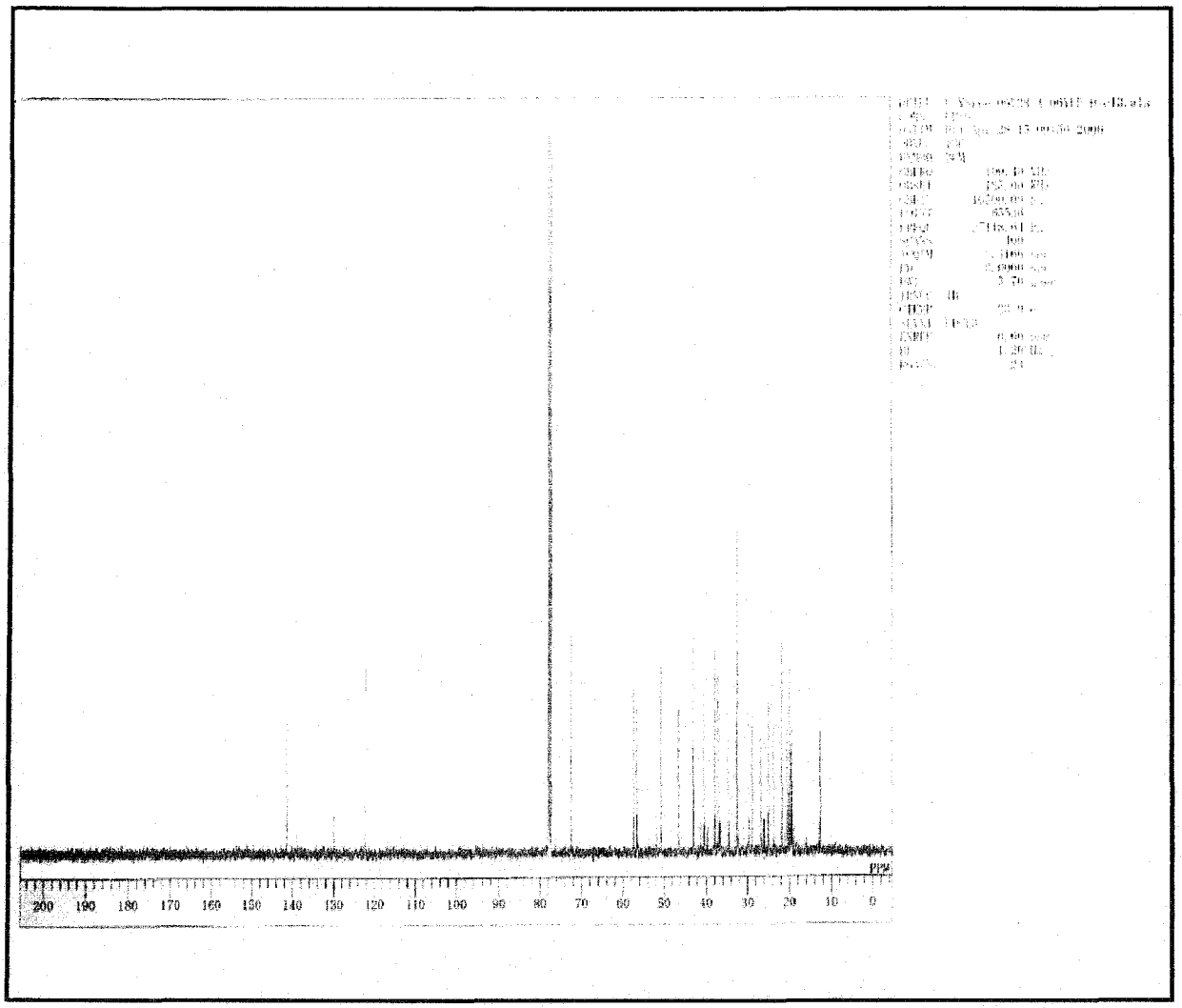

Fig 3.3 ^{13}C NMR Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

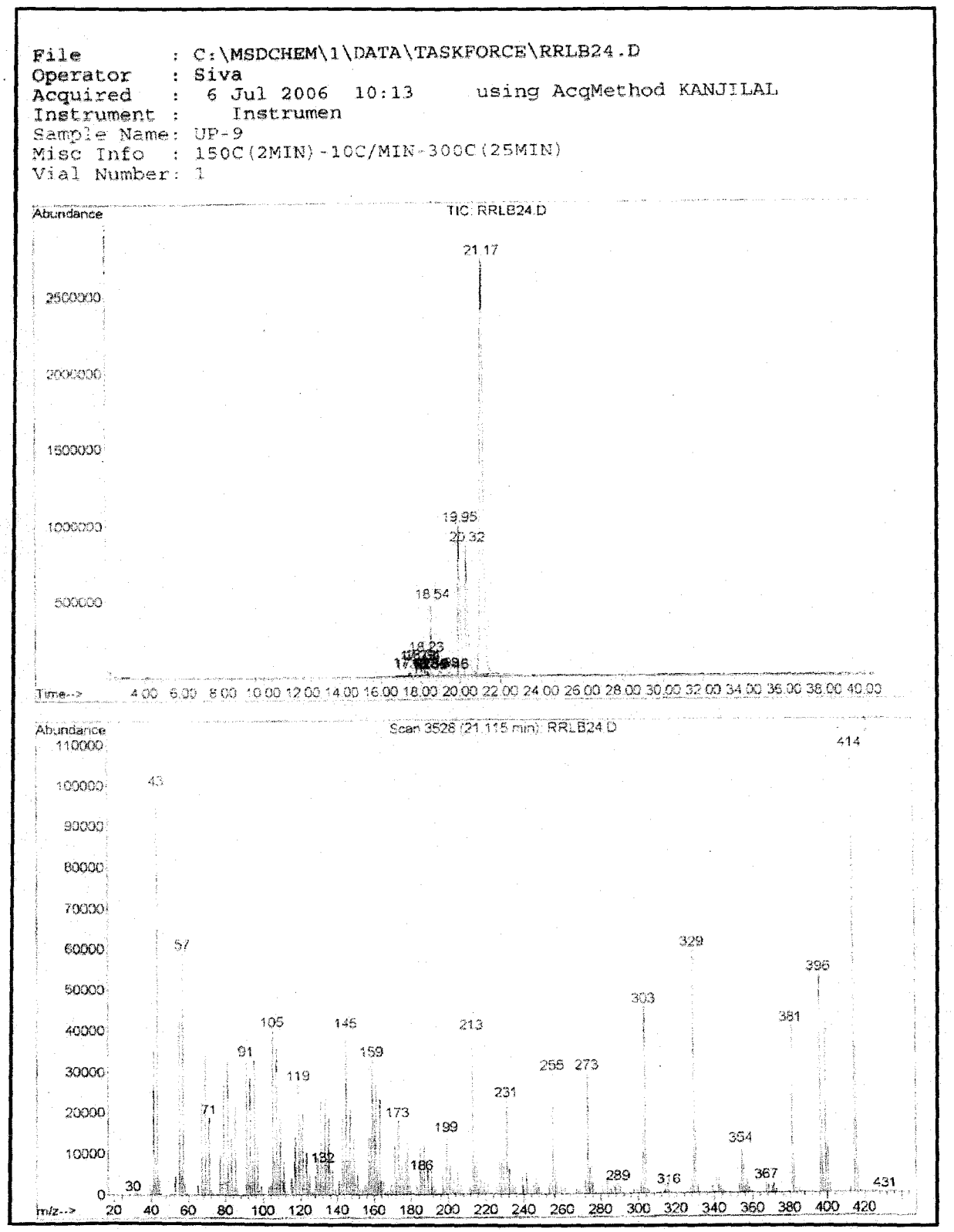

Fig 3.5 GCMS Spectrum of Compound I isolated from *Urtica parviflora* leaf extract.

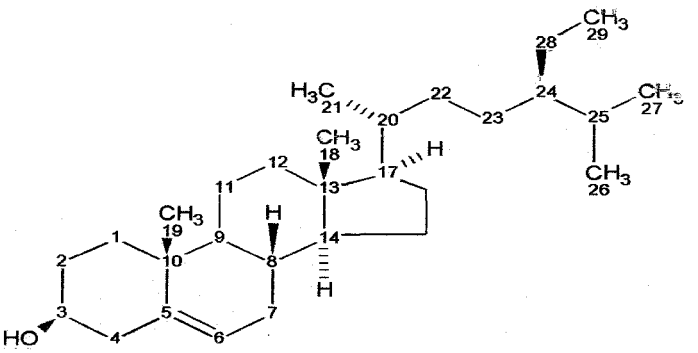

Fig 3.6 Compound I (β-sitosterol)

3.3.2 Phytochemical Study of *C. arborea* leaf.

The concentrated methanol extract prepared from the shade-dried root of *C. arborea* was fractionated successively with petroleum ether, chloroform and acetone. Different fractions of the extract were undertaken for preliminary pharmacological studies. It was observed that the acetone fraction exhibited significant pharmacological activities under perview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, proteins, (**Table 3.1**). The thin layer chromatographic study of acetone fraction of methanol extract showed the mixture of four components with selected mobile phase and spraying reagents (**Table 3.8-3.9**). This fraction was subjected to column chromatography, which yielded sixty-two fractions. The fractions having identical hRf values were mixed together (**Table 3.10**). They were purified with a silica gel column, by using benzene: methanol as mobile phase, which yielded a triterpenoid glycoside (compound II). It was further subjected to chemical tests and TLC to confirm the chemical nature and was depicted in **Table 3.11** and **3.12**. The chemical nature of the isolated compound was further characterized by comparison of its physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data with that of the reported values of triterpenoid glycoside (Alvarez *et al.,* 2003; Abe *et al.,* 2002 and Yoshida *et al.,* 2005).

Compound II was obtained as colorless amorphous powder, partially soluble in water and soluble in organic solvent. The compound was melted at $139^0 - 143^0C$. The UV analysis (**Fig 3.6 a, b**) showed a significant absorption at 242 nm. It indicates that the compound is an isoprene derivative (Yu Ping Lin *et al.,* 2003). The IR spectrum (**Fig 3.7**) shows the presence of absorption bands for the groups hydroxyl (2928 cm^{-1}), carbonyl (1691 cm^{-1}), double bond (1459 cm^{-1}) and ester (1030 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of compound II which are presented in **Table 3.13** showed that most of the signals of the aglycone were in good agreement with literature data of oleanolic acid (Kubota *et al.,* 1968). The ^{13}C NMR (**Fig 3.8**) showed the presence of cabonyls at δ C 144.17 (C), δ C (122.92) and δ H 5.25 (1 H, C=12). The spectra also showed the presence of hydroxyl at δ C 78.76 to 47.37 and δ H 5.236 to 3.13 (**Fig 3.9 a, b, c, d**). The fragmentation ion at 248 in its mass spectrum (**Fig 3.10**) inferred the compound having the molecular formula $C_{30}H_{48}O_3$. From these data it is concluded that the structure of the isolated triterpenoid is **Oleanolic acid**.

Table 3.8 Thin Layer Chromatography study of acetone fraction of *C. arborea.*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spray reagent			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Blue	Blue	Blue	Bright blue	Bright blue	Bright blue	68	54	69
2	Blue	Blue	Blue	Blue Florescence	Blue Florescence	Blue Florescence	64	28	49
3	–	Blue	Blue	–	Blue Florescence	Blue Florescence	–	29	41
4	–	–	Blue	–	–	Blue Florescence	–	–	53

Spray reagent: Anisaldehyde sulphuric acid reagent
A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene:acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.9 Thin Layer Chromatography study of acetone fraction of *C. arborea.*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagents			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	68	54	69
2	Dark Violet	Dark Violet	Dark Violet	Violet florosce nce	Violet florosce nce	Violet florosce nce	64	28	49
3	–	–	Dark Violet	–	–	Violet florosce nce	–	–	41
4	–	–	Dark Violet	–	–	Violet florosce nce	–	–	53

Spray reagent: 10% sulphuric acid solution.
A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene: acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.10 Coloumn and Thin layer chromatography separation of acetone fraction of *C. arborea.*

Eluent Benzene: methanol	Fraction number	Residue of selected fractions (gm)	Colour of TLC spots with hR_f values	Inference
100:0	1-14	4.6	NIL	Fatty oil
90:10	15-20	4.2	NIL	Fatty solid
80:20	21-24	2.7	NIL	Fatty solid
70:30	25-28	6.3	NIL	Fatty solid
60:40	29-30	14.5	NIL	Greenish semisolid
50:50	31-32	12.3	NIL	Dark green semisolid
40:60	32-38	6.3	Two yellowish spots. (68, 41)	Mixture of compounds
30:70	38-42	6.5	Two bright yellow spots. (68, 41)	Mixture of compounds
20:80	42-46	4.6	Two yellowish spots (69, 42)	Mixture of compounds
10:90	47-56	3.2	Three red spot (67, 52, 27)	Mixture of compounds
0:100	57-62	3.0	Nil	Colourless solid

Spray reagent: Anisaldehyde sulphuric acid reagent

Table 3.11 Qualitative analysis of the compound II isolated from acetone fraction of *C. arborea.*

S.no	Treatment	Observation	Inference
1	1 mg of the crystalline solid was dissolved in 0.5 ml of chloroform 1.0 ml of acetic anhydride was then added to the mixture followed by 2.0 ml of concentrated sulphuric acid.	A reddish violet ring at the junction of the two layers	Presence of triterpenoids
2	1.0 ml of concentrated sulphuric acid was added to 1 mg of isolated crystalline solid and dissolved in 1.0 ml of chloroform.	A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer.	Presence of triterpenoids

Table 3.12 Thin layer chromatography of the compound II isolated from triterpenoids.

Solvent system	hR_f values	Colour of fluorescent produced		
		UV_{366nm}	UV_{366nm} +Ansl	UV_{366nm} +10% H_2SO_4
TLC 1	64	Blue	Bright blue	Bright blue
TLC 2	28	Blue	Bright blue	Bright blue
TLC 3	49	Blue	Bright blue	Bright blue

Ansl: Anisaldehyde sulphuric acid reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).
B. TLC 2. Benzene:acetone (95:5 v/v)
C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.13 ^{1}H and ^{13}C data for compound isolated from acetone fraction of *C. arborea*.

Position	δ_H, 400 MHz, $CDCl_3$	δ_C, 400 MHz, $CDCl_3$
1	0.55, 1.59	38.89
2	1.10, 1.70	27.69
3	2.21, dd	78.76
4	–	37.15
5	0.64	55.74
6	1.32, 1.43	18.53
7	1.29, 1.61	37.11
8	–	39.37
9	1.49	47.65
10	–	26.91
11	1.89	23.43
12	5.25 t (3)	122.92
13	–	144.171
14	–	39.43
15	1.30, 1.71	23.32
16	1.32, 1.54	20.76
17	–	47.37
18	2.21	47.93
19	1.34, 162	48.04
20	–	48.22
21	1.32, 1.40	48.50
22	1.33, 1.89	48.79
23	0.91	27.92
24	0.74	16.81
25	0.88	15.51
26	0.76	16.90
27	1.12	26.91
28	–	180.70
29	0.89	49.07
30	0.88	15.21

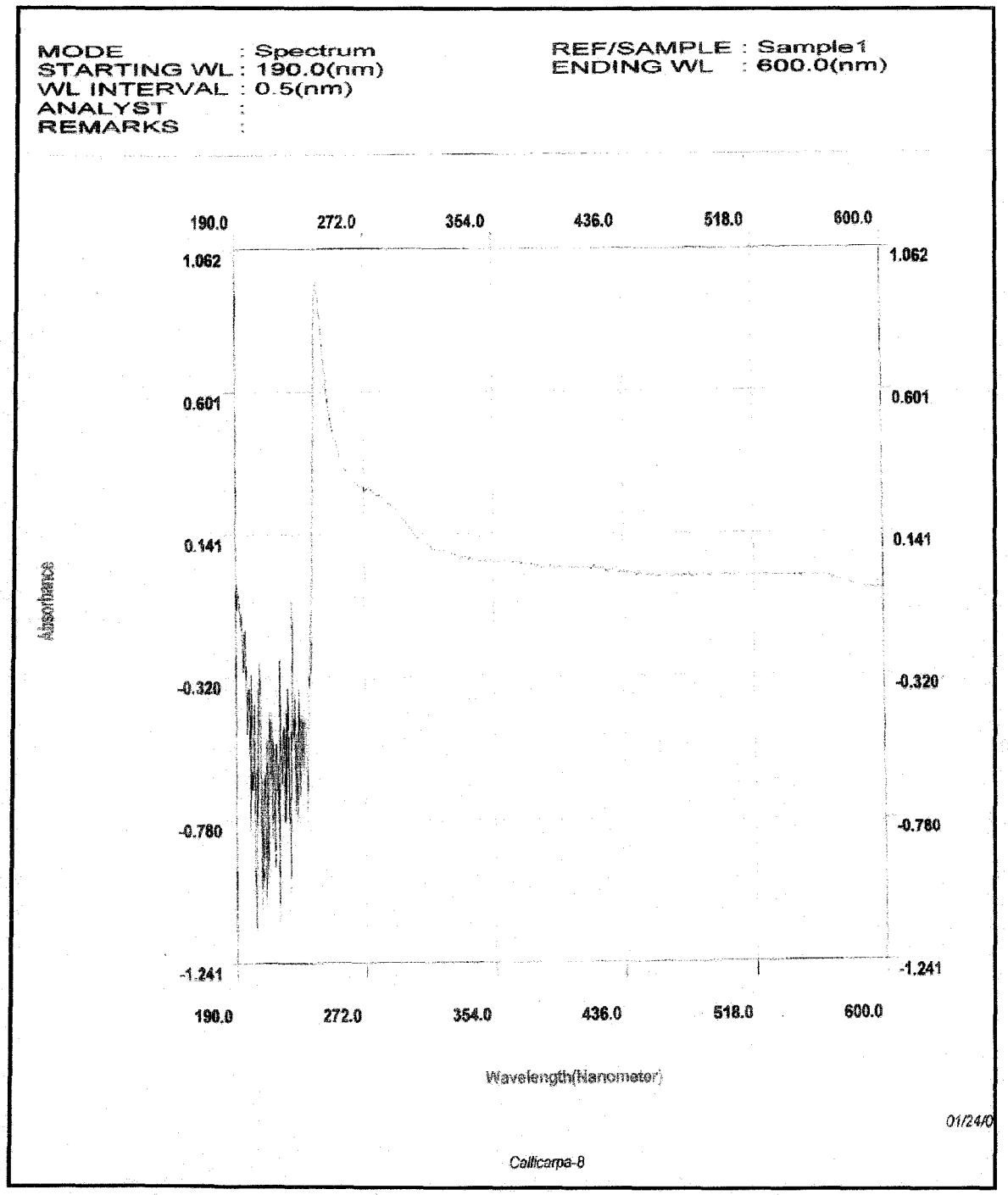

Fig 3.6 (a) UV Spectrum of Compound II isolated from *C. arborea* leaf extract.

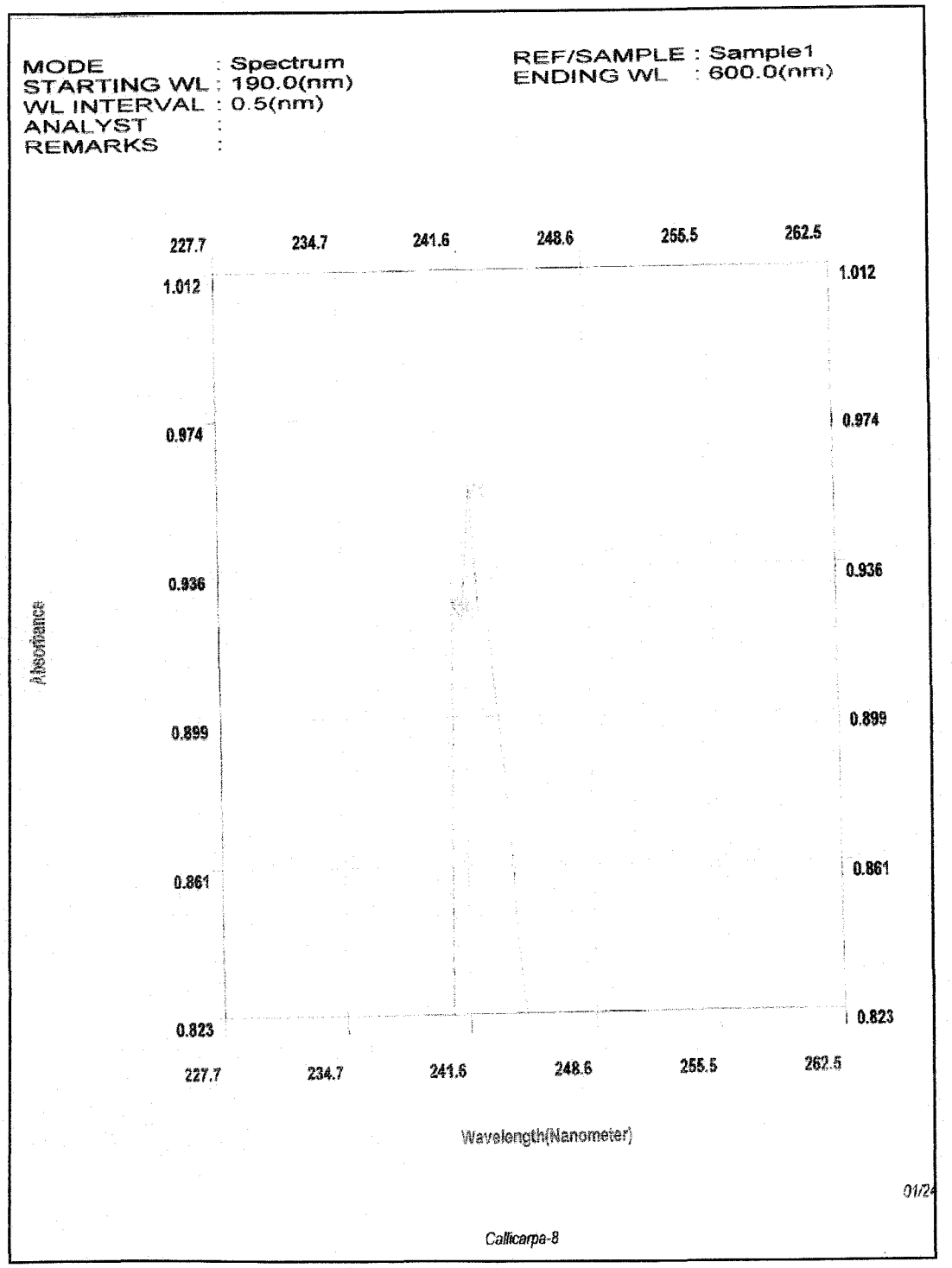

Fig 3.6 (b) UV Spectrum of Compound II isolated from *C. arborea* leaf extract.

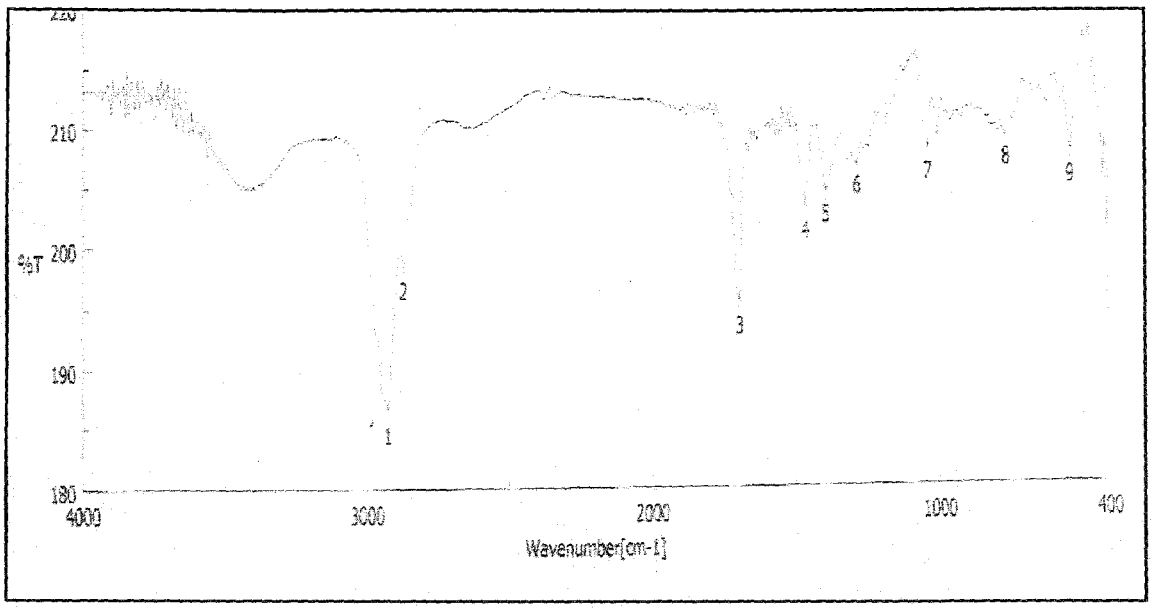

Fig 3.7 IR Spectrum of Compound II isolated from *Callicarpa arborea* leaf extract.

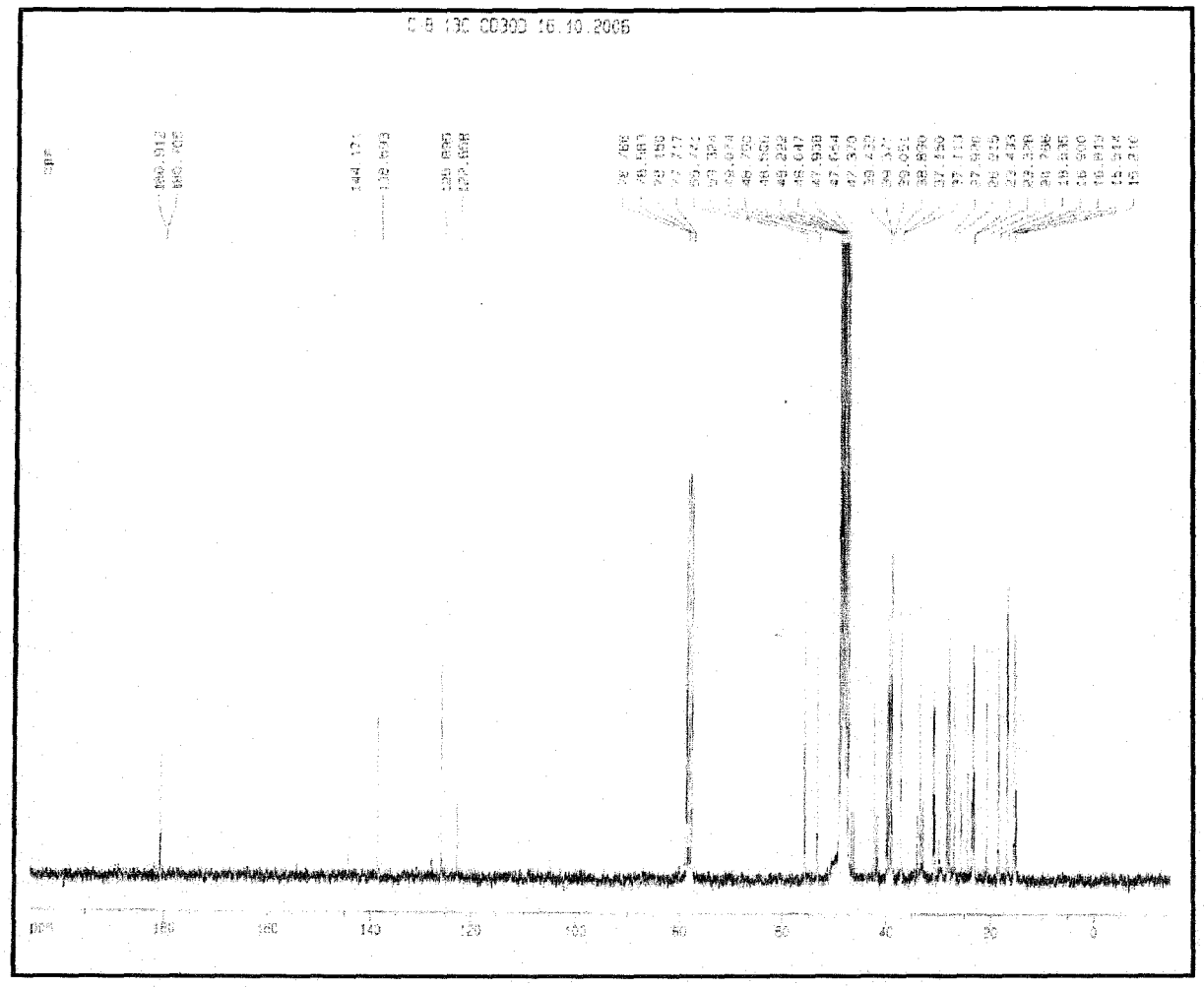

Fig 3.8 ^{13}C NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

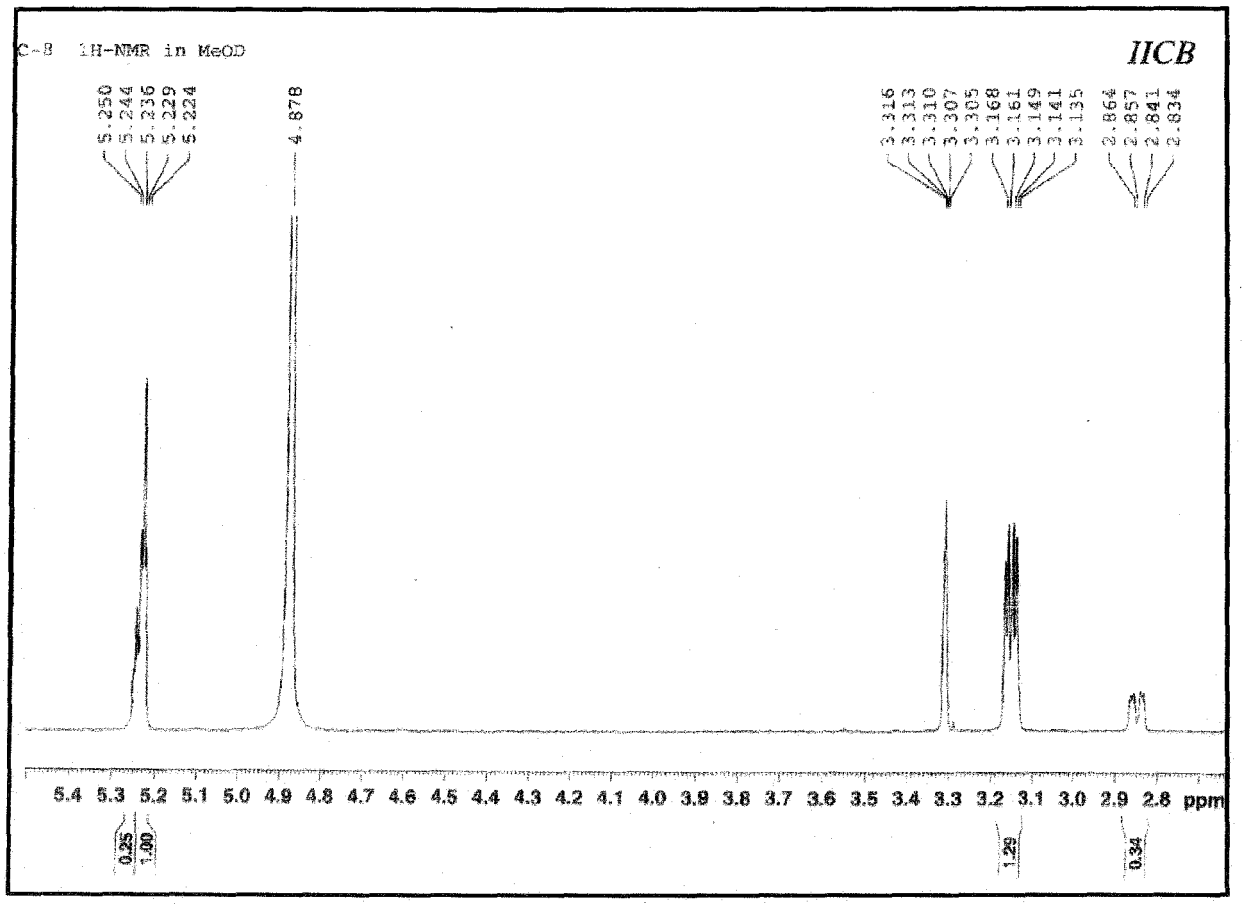

Fig 3.9 (a) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

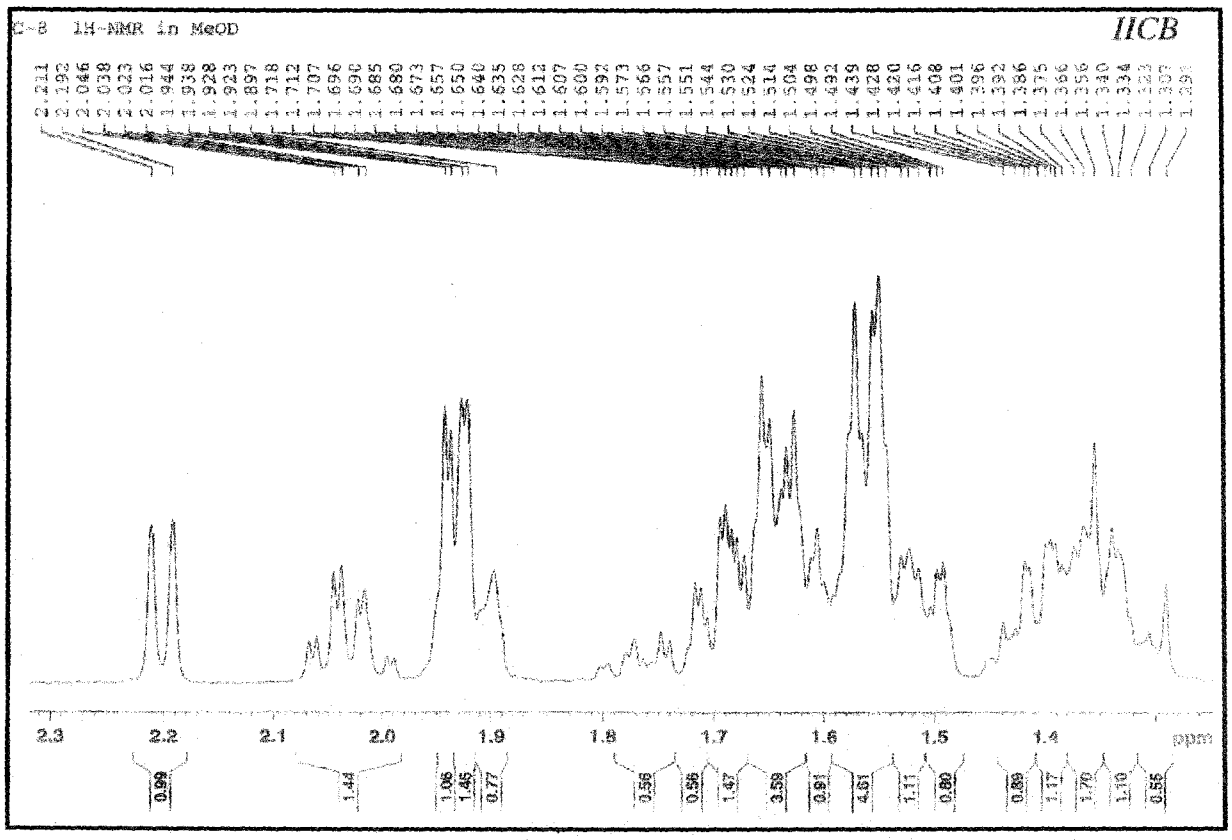

Fig 3.9 (b) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

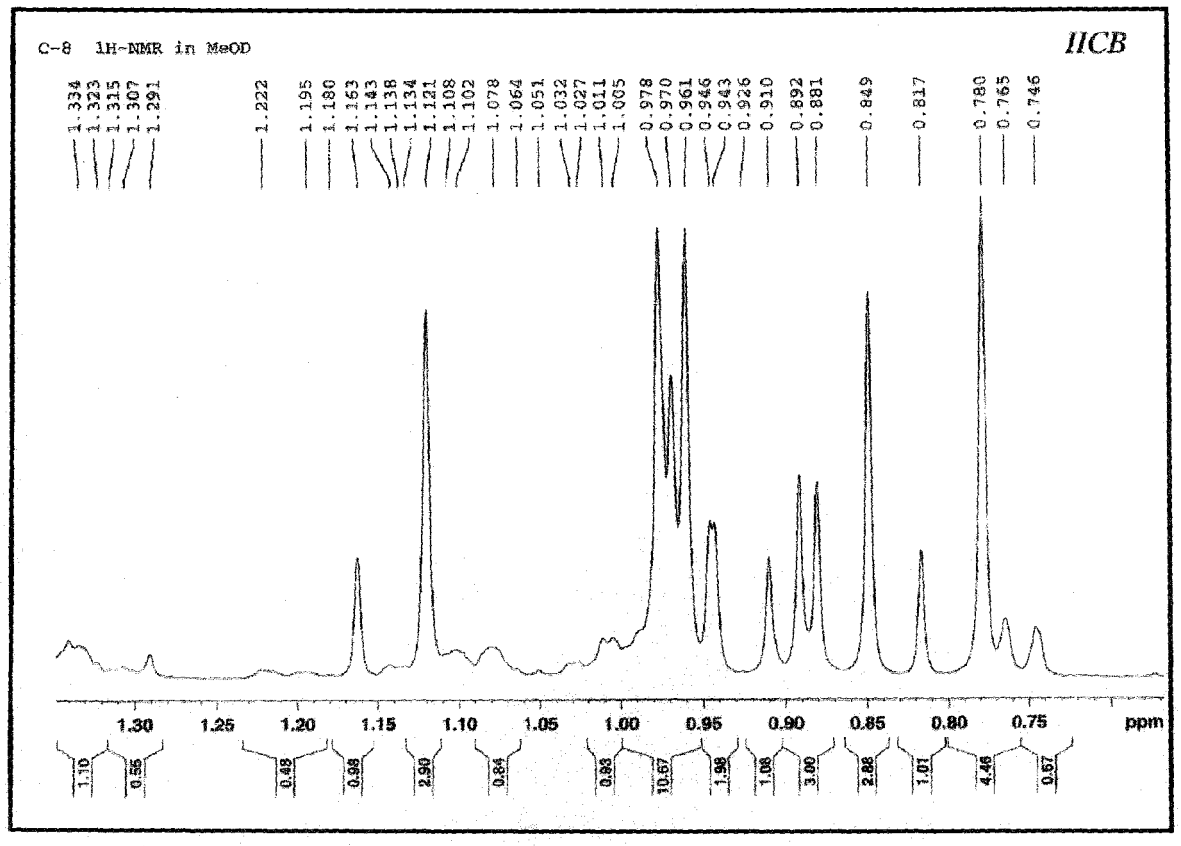

Fig 3.8 (c) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

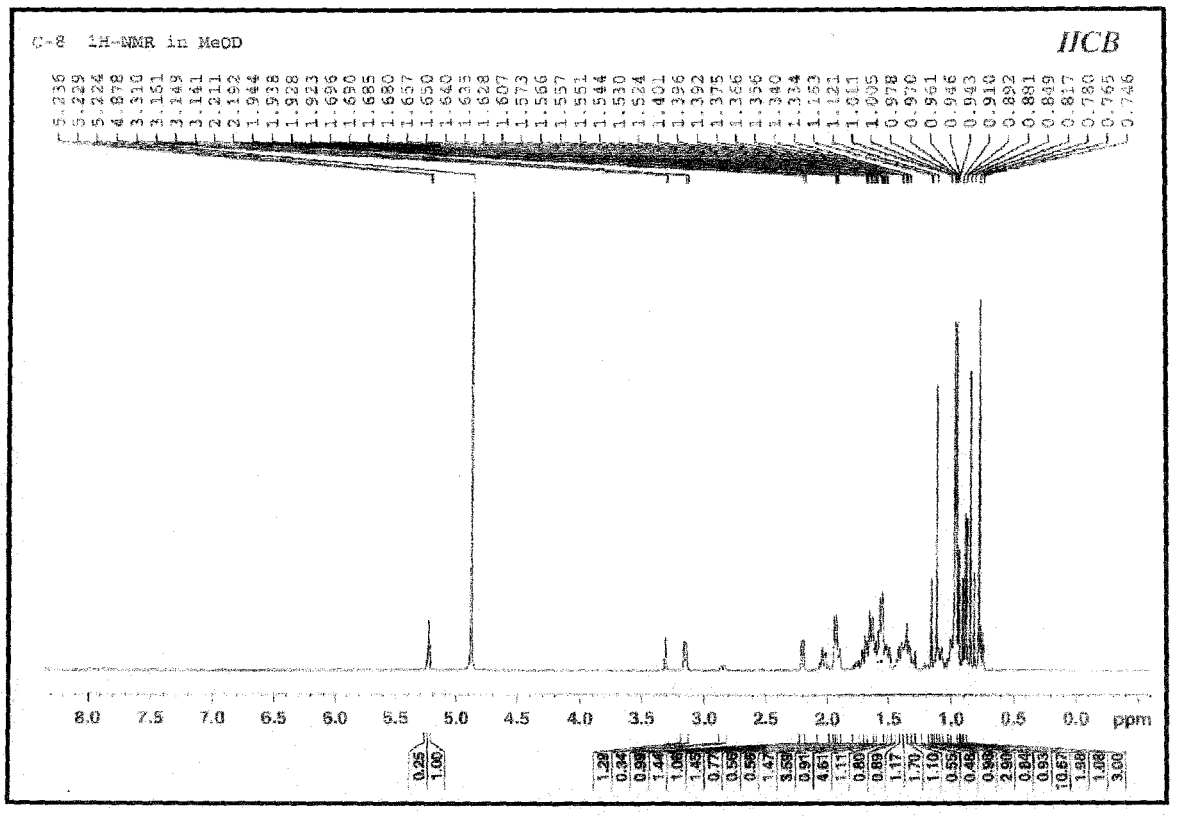

Fig 3.8 (d) ^{1}H NMR Spectrum of Compound II isolated from *C. arborea* leaf extract.

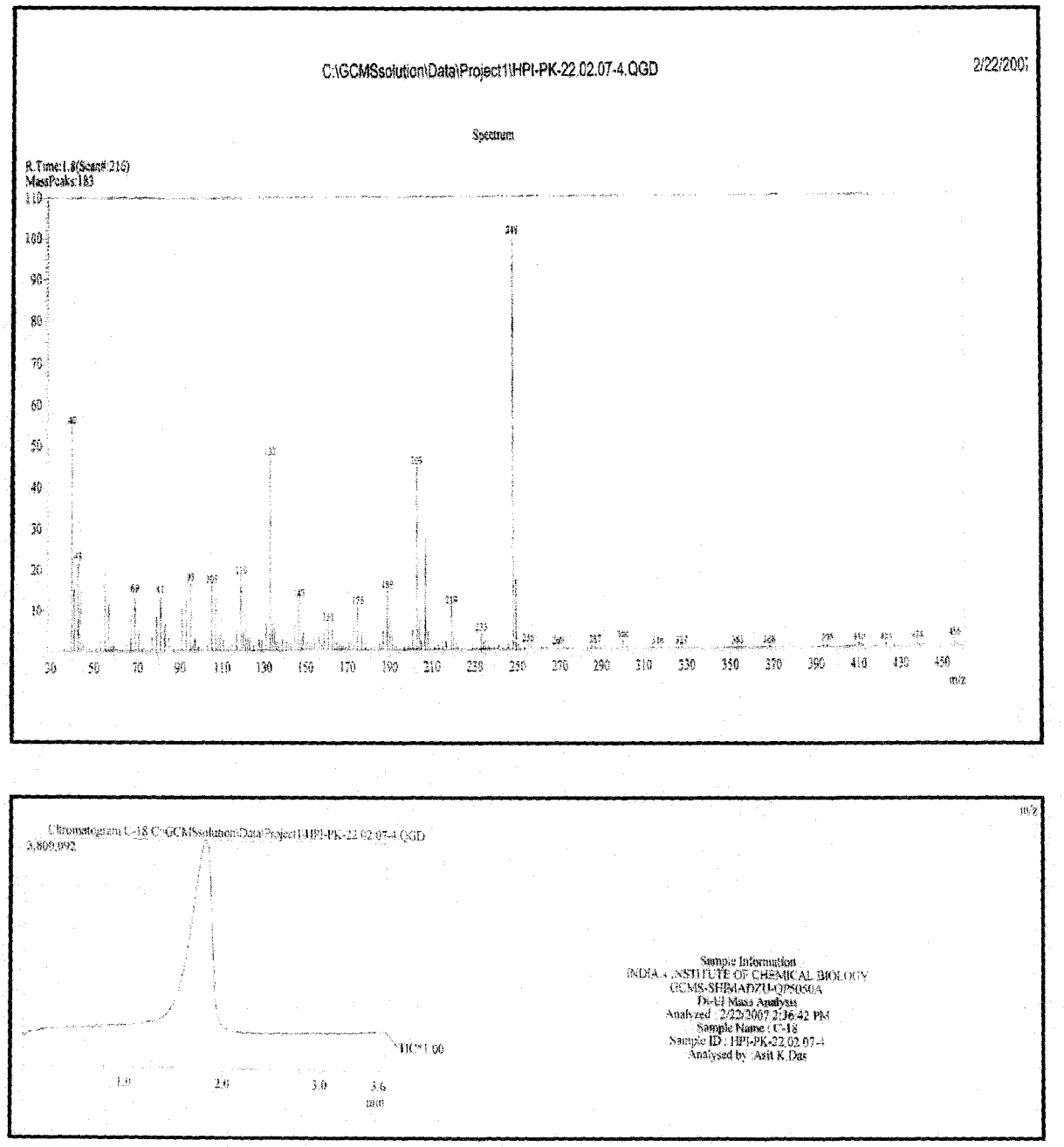

Fig 3.10 GCMS Spectrum of Compound II isolated from *C. arborea* leaf extract.

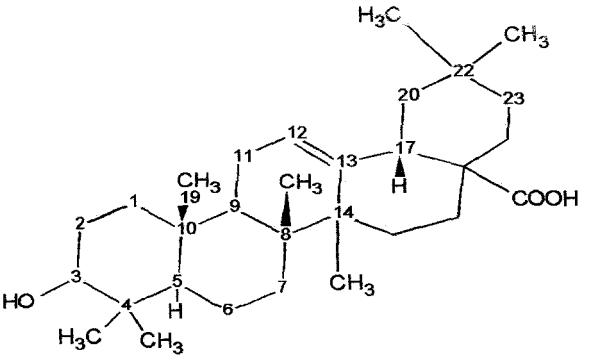

Fig 3.11 Compound II (Oleanolic acid)

3.3.3 Phytochemical Study of *M. citrifolia* root.

The concentrated methanol extract obtained from the root bark of *M. citrifolia* was fractionated successively petroleum ether, chloroform and acetone. It was observed that only the chloroform fraction exhibited significant pharmacological activities under perview of this investigation. So this fraction was subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of alkaloids, amino acids, reducing sugars, steroids and triterpenoids and anthraquinones (**Table 3.1**). The thin layer chromatographic study of the chloroform fraction showed the presence of five components with selected mobile phase (**Table 3.13-3.14**). This fraction was subjected to coloumn chromatographic separation on Sephadex LH 20 coloumn in which seventy-two fractions were collected. The fractions having identical results were mixed together (**Table 3.15**). They were purified with a silica gel (60-120) coloumn, which yielded a anthraquinone derivative (Compound III). It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound III, which are depicted in **Table 3.16** and **Table 3.17**. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, MS, ^{13}C and ^{1}H NMR) data. (Rajendran *et al.,* 2007; Ling *et al.,* 2002)

Compound III was obtained as orange, needle shaped crystal with melting point at 200.5^{0}C. The UV absorption spectrum of compound III is shown in **Fig 3.12**. The compound showed strong absorption at 426 nm in its spectrum, which implied the presence of conjugated chromophoric group in its structure. The IR spectrum of the compound is presented in **Fig 3.13**. The IR spectrum shows the presence of absorption bands at 1633, 1614, 1035, 3572, 3636 and 2984 cm^{-1}. The IR spectrum confirmed the presence of hydroxyl group (3572, 3636 cm^{-1}), carbonyl group (1633 cm^{-1}) in compound III.

The ^{1}H NMR and ^{13}C NMR spectrum of compound I is presented in **Fig 3.14** and **Fig 3.15** respectively. Comparisons of ^{1}H and ^{13}C NMR spectra of the isolated compound facilitated the identification of the structure (**Table 3.13**). The ^{13}C NMR spectrum displayed C-3 at δ 26.69 and C-6 δ 61.27 respectively. Peaks of C-8. The rest of the aromatic carbons appears from δ 113.39 to 161.07. The fragmentation ion at m/z 291 in its mass spectrum, which is presented in **Fig 3.16**, inferred the compound is an anthraquinone derivative. By comparing the spectral data of the literature survey, it is identified as **1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone** (**Fig 3.17**) (Kamiya, *et al.,* 2005; Chan *et al.,* 2005 and Wab *et al.,* 2007).

Table 3.13 Thin Layer Chromatography study of chloroform fraction of the of *M. citrifolia.*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagent			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Red	Red	Red	Bright red	Red Florescence	Red Florescence	74	43	25
2	Red	Red	–	Red Florescence	Red Florescence	–	52	32	–
3	–	Red	Red	–	Red Florescence	Red Florescence	–	24	41
4	–	Red	Red	–	Red Florescence	Red Florescence	–	18	33
5	Red	Red	Red	Red Florescence	Red Florescence	Red Florescence	28	12	25

Spray reagent: Dilute ammonia reagent

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.14 Thin Layer Chromatography study of chloroform fraction of the of *M. citrifolia*

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after spraying the spraying reagents			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Red	Red	Red	Bright red	Red Florescence	Red Florescence	73	43	25
2	Red	Red	–	Red Florescence	Red Florescence	–	52	32	–
3	–	Red	Red	–	Red Florescence	Red Florescence	–	23	41
4	–	Red	Red		Red Florescence	Red Florescence	–	18	31
5	Red	Red	Red	Red Florescence	Red Florescence	Red Florescence	28	12	25

Spray reagent: Bromine water.

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene: acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.15 Coloumn and Thin layer chromatography separation of chloroform fraction of the of *M. citrifolia.*

Eluent Benzene: Methanol	Fraction number	Residue of selective fraction (gm)	Colour of TLC spots with hR_f values.	Inference
100:0	1-12	5.2	NIL	Fatty oil
90:10	13-17	4.6	NIL	Fatty solid
80:20	18-25	3.0	NIL	Fatty solid
70:30	26-30	6.7	NIL	Fatty solid
60:40	31-34	16.1	NIL	Greenish semisolid
50:50	35-39	14.3	NIL	Reddish green semisolid
40:60	40-47	6.5	Two blue spots. (58, 32)	Mixture of compounds
30:70	48-52	6.0	Two bright blue spots. (58, 24)	Mixture of compounds
20:80	53-55	4.5	Two blue spots (59, 24)	Mixture of compounds
10:90	56-59	3.5	Three bright blue spot (64, 57, 27)	Mixture of compounds
0:100	60-72	2.0	Nil	Colourless solid

Spray reagent: Bromine water.

Table 3.16 Qualitative analysis of the compound III isolated from chloroform fraction of the of *M. citrifolia.*

S.no	Treatment	Observation	Inference
1	Small quantity of isolated compound was dissolved in methanol. 5.0 ml of the solution was hydrolyzed with dilute sulphuric acid and extracted with benzene. 1.0 ml of dilute ammonia was then added to the samples.	Development of rose pink colouration.	Presence of anthraquinones.

Table 3.17 Thin layer chromatography of the compound III isolated from chloroform fraction of the of *M. citrifolia.*

Solvent system	hR_f values	Colour of fluorescent produced		
		UV_{366nm}	UV_{366nm} +BW	UV_{366nm} +DA
TLC 1	58	Red	Bright red	Bright red
TLC 2	42	Red	Bright red	Bright red
TLC 3	32	Red	Bright red	Bright red

BW: Bromine water, DA: Dilute ammonia

A. TLC 1. Methylene Chloride: acetone (85:15 v/v).

B. TLC 2. Benzene:acetone (95:5 v/v)

C. TLC 3. Cyclohexane: Ethyl acetate (80:20 v/v)

Table 3.18 ^{1}H and ^{13}C data for compound isolated from chloroform fraction of *M. citrifolia.*

Position	δ_H, 400 MH_Z, $CDCl_3$	δ_C, 400 MH_Z, $CDCl_3$
1	–	163.36
2	7.73 (d,d)	134.25
3	–	29.69
4	7.79 (d)	134.37
5	8.10 (d)	126.55
6	–	61.27
7	7.36 (d)	119.24
8	–	183.45
9	–	186.97
10	–	161.07
11	–	126.86
12	–	126.55
13	–	113.17
14	–	133.39
6- OMe	7.26	–

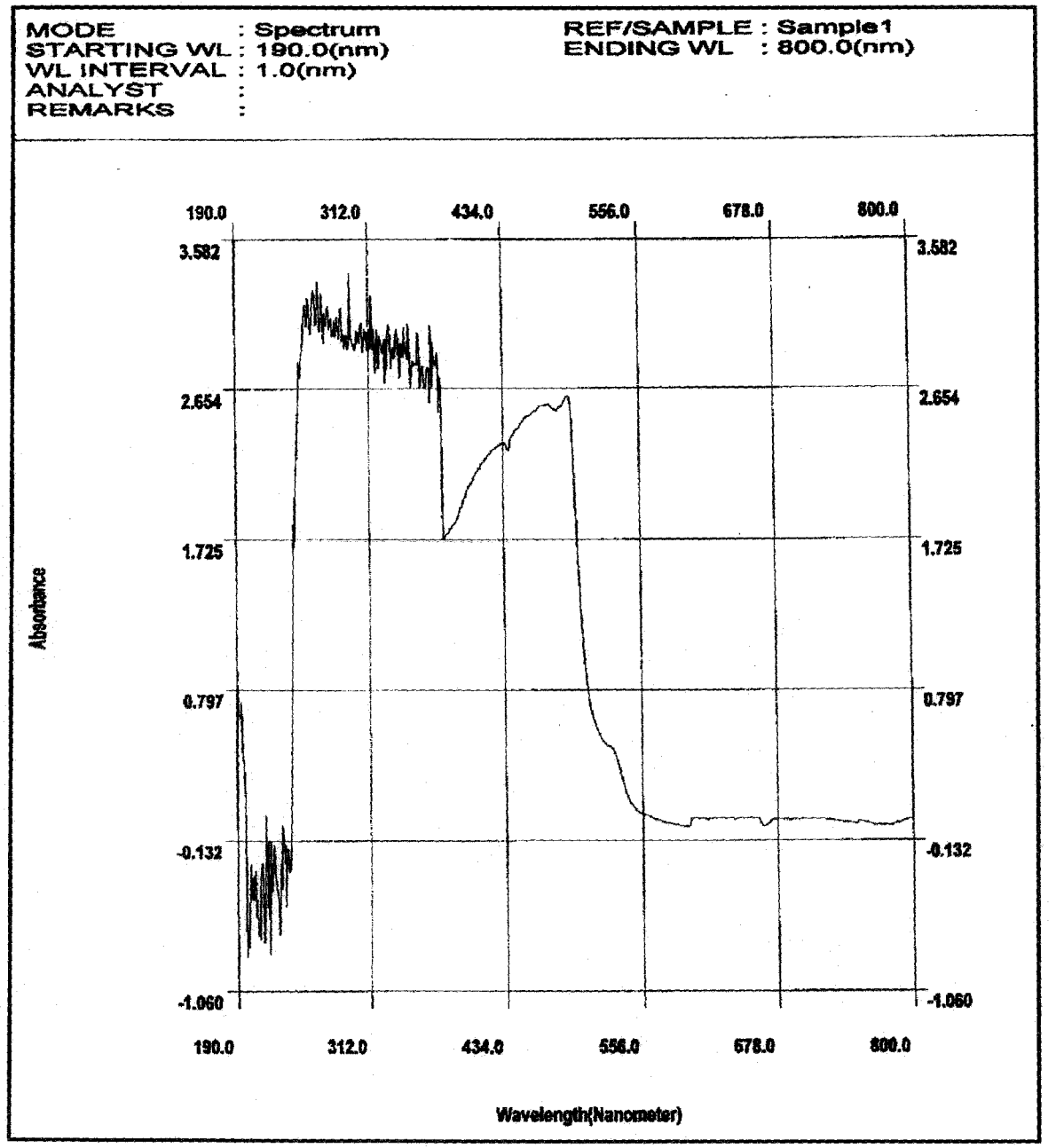

Fig 3.12 UV Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

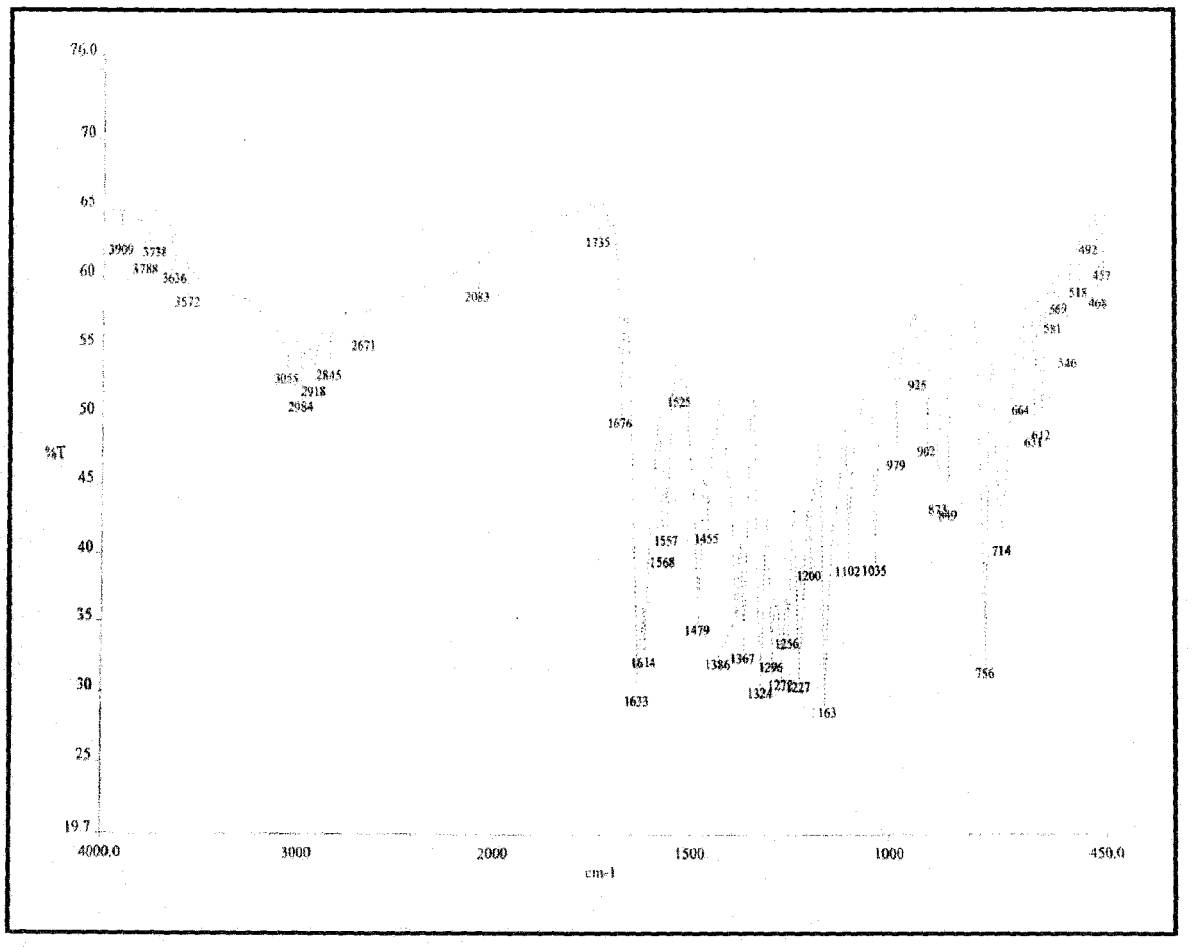

Fig 3.13 IR Spectum of Compound III isolated from *Morinda citrifolia* root bark extract.

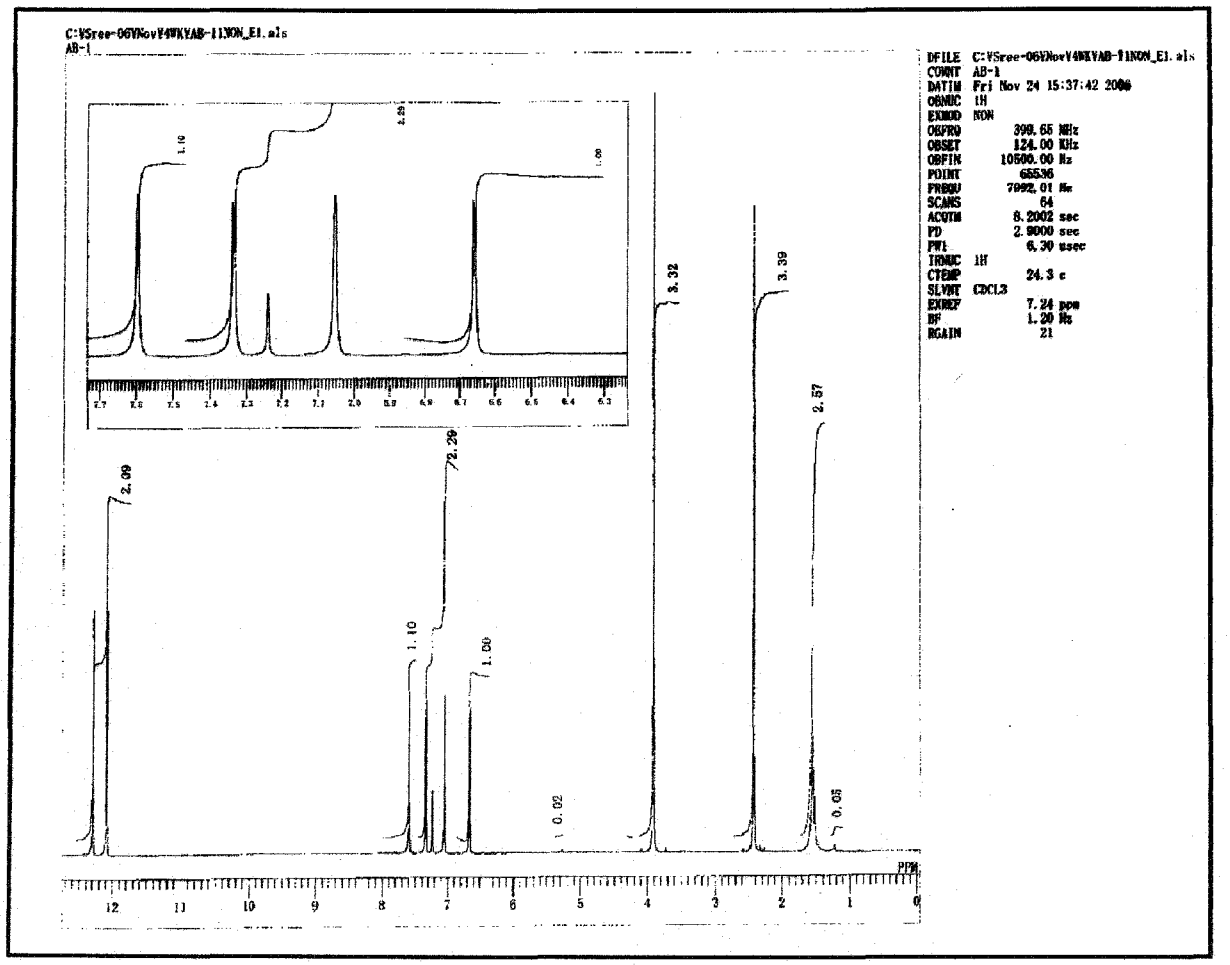

Fig 3.14 ^{1}H NMR Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

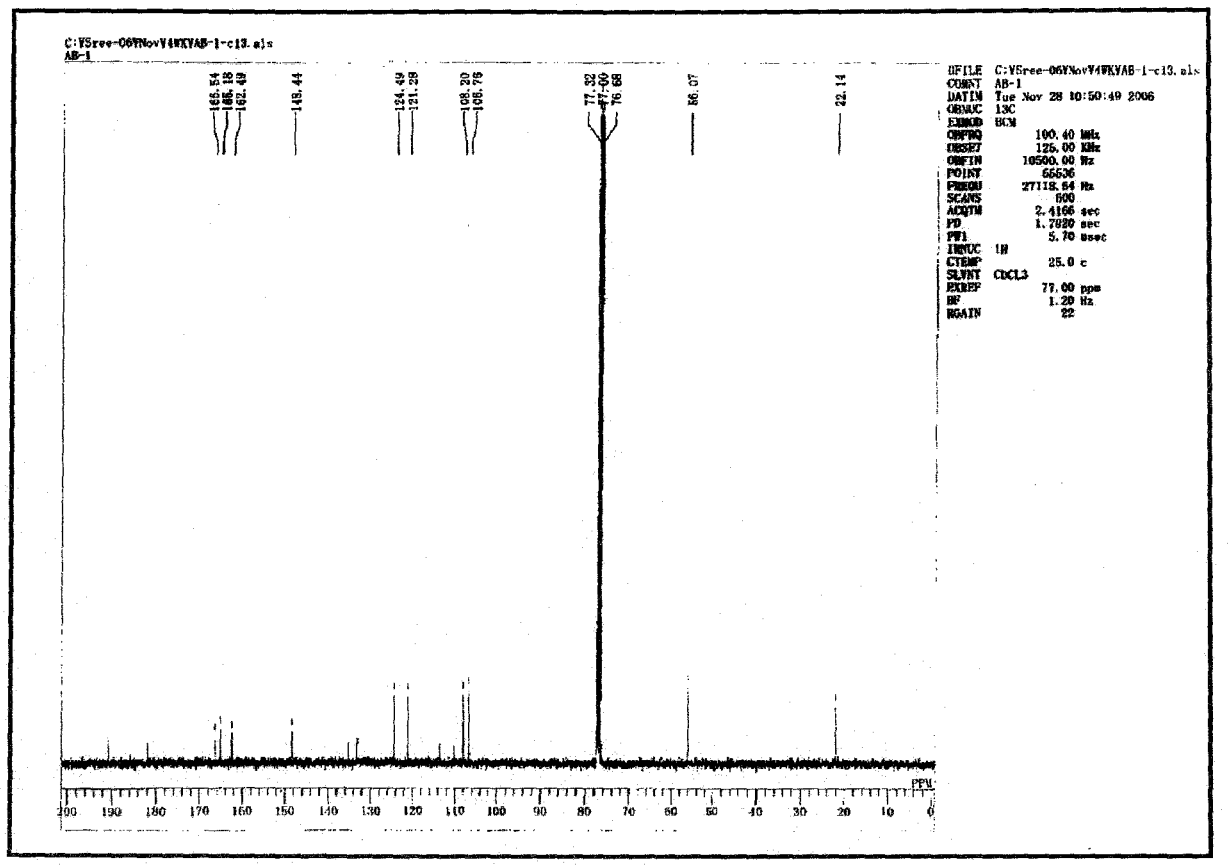

Fig 3.15 ^{13}C NMR Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

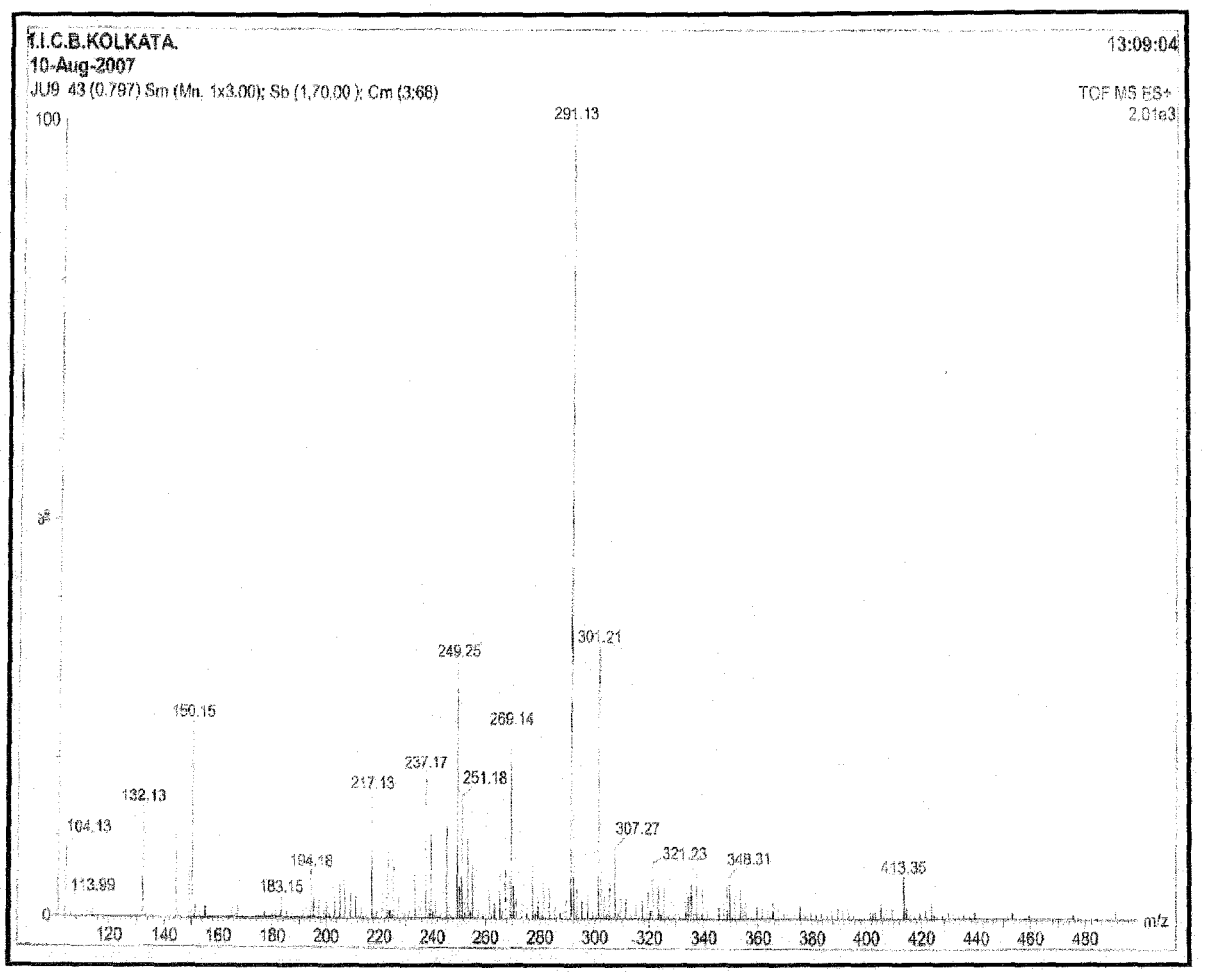

Fig 3.16 ESMS Spectrum of Compound III isolated from *Morinda citrifolia* root bark extract.

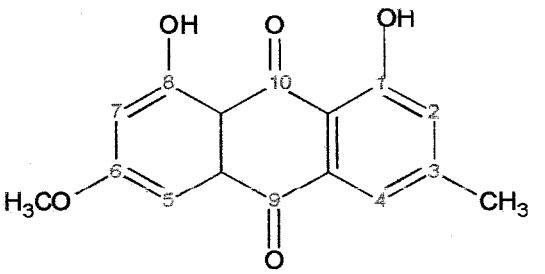

Fig 3.17 Compound III (1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone)

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CHAPTER 4

ACUTE TOXICITY STUDY

4.0 INTRODUCTION

Toxicology (Sood 1, 1999) is the study of the adverse effect of the chemicals on living organisms. It is unique form of science because it has more than a single discipline with one objective. It is concerned with the assessment and subsequent management of potential chemical hazards to man, animals, and the environment. In order to achieve this objective, the toxicologist need a detail knowledge, not only a chemical's primary and cumulative toxicity, but also of its 'no observed effect' level, as well as knowledge concerning its' teratogenic, mutagenic and carcinogenic potential.

4.1 Objective

I. Hazard/risk assessment

(A) Detection of causative factor of both acute and chronic illness due to chemicals.

(B) Exclusion or minimization of adverse effects to maintain balance between economic benefits against the risk to protect the manufacturer, worker, consumer, environment and public.

II. Aid the selection and development of therapeutic agents.

III. Aid basic science or Knowledge of life processes.

4.2 Duration of Toxicity Studies

Essentially three types of study have become mandatory in the course of safety evaluation of chemical. These are as follows:

4.2.1 Acute toxicity studies:

Acute studies demonstrate the adverse effects occurring within a short time, usually up to 2 months, following administration of a single dose of a substance or multiple doses given within 24 hr.

4.2.2 Repeated-dose (subacute/subchronic) studies: The definition of subchronic toxicity is confusing, as opinions differ as to the length of exposure that constitutes a sub acute study. However, their purpose is the same– namely to demonstrate adverse effect occurring as a result of repeated daily dose of a chemical for part, not exceeding 10 percent, of the life span of animal. Thus, 14, 21, and 28 day studies in rats are generally referred to as 'subacute' studies, while 90 days and upto 150 days' study constitute 'subchronic' tests.

4.2.3 Chronic toxicity study:

Chronic Toxicity Test or Long term toxicity test is defined as study of longer than 3 months duration, i.e., approximately 10% of life span in the laboratory rats. These types of studies are conducted in all species of laboratory animals and in some economically important animals, wild and domestic. The results of a chronic toxicity study should suggest signs and symptoms of adverse reactions to look for in man. With the exception of idiosyncratic reaction and hypersensitivity, many of the systematic and organismic response are predictable from laboratory animal to man (Hayes, 1982). Ideal duration for different types of toxicity studies are given in the **Table 4.1** (Witthawaskyl *et al.*, 2003).

Table-4.1 Duration for various toxicity tests (OECD guidelines)

TYPE OF STUDY	OECD GUIDELINE	MAX STUDY DURATION
ACUTE TOXICITY STUDY		
Acute oral toxicity study in rats	423	2 Months
Acute dermal toxicity study in rats	402	2 Months
Acute skin irritancy study in rabbits	404	2 Months
Acute oral toxicity study in mice	401	2 Months
Acute inhalation toxicity study in rats	403	2 Months
SUB CHRONIC TOXICITY STUDY		
Repeated Dose-28 Days Sub-acute Oral Toxicity Study in rats	407	5 Months
Repeated Dose-28 Days Sub-acute Oral Toxicity Study in Rabbit	410	5 Months
Repeated Dose-28 Days Sub-acute Oral Toxicity Study in dogs	409	5 Months
CHRONIC TOXICITY STUDY		
Repeated Dose-90 Days Chronic Oral Toxicity Study in rats	408	7 Months
GENOTOXICITY STUDY		
Ames test	471	2 Months
In vitro human lymphocyte	473	2 Months
Micronucleus test in mice	474	4 Months
Chromosomal aberration in mice	475	
In vitro mammalian gene mutation test	476	2 Months
REPRODUCTION		
Teratology/prenatal development in Rats	414	-
Teratology/prenatal development in Rabbits	414	-
CARCINOGENICITY		
Carcinogenicity in rats	451	-
Carcinogenicity in mouse	451	-
Combined chronic carcinogenicity in rats	453	-

4.3 Animal Husbandry and Observations

The most important facet of any toxicological experiment is the condition of the animals. Accordingly, all toxicity studies should be conducted in a controlled environment, at a temperature of 22 ±3 °C with adequate ventilation (i.e. 10 changes of air per hour), relative humidity between 30% and 70 %, and a twelve hours light/dark cycle. The diet and quality of drinking water should be of standard and maintained through out the experiment and this

should be carried out to Good Laboratory Practice standards to ensure reproducibility/validity of data.

4.4 Protocol Design

The protocol design (Sood 2, 1999) depends upon the type of chemical substances and the country in which it is used. Often both sexes of two species, employing a rout of exposure, which is anticipated to be the most probable rout of exposure for man is necessary for regulatory purposes. The laboratory mice and rats are the species typically selected. Additional species are required by some regulations and in these cases, probe studies are often used to select an appropriate dose range and species.

The experimental design for acute systemic toxicity assessment has for many years been a modification of the Travan approach of interval dose levels applied to groups of experimental animals such that an incidence of response can be achieved varying from zero incidence to 100 percent response, and median lethal dose can be derived (LD_{50}). The number of replicates and size of sample population will dictate whether the experimentally derived curve reflect the actual response. A wide variety of intrinsic and extrinsic factors can influence the outcome of a test. Many investigations in to the source of variability of acute toxicity testing have been conducted recently. In order to establish a dose response relationship, the same species / strain, sex and age should be divided in to equivalent size groups, with the different subgroups and the different subgroups treated at the same time of day with different doses by the same route and observed for a set and consistent period of time.

All protocols should state the ceiling or limit doses. Small differences in protocols are probably the major cause of the considerable laboratory to laboratory variations in result achieved (Lorke, 1983). There is some question concerning the utility of extensive pathological assessments as part of an acute study. Gross necropsies are the minimum requested by most regulatory bodies. Protocols include necropsies on all animals found dead and those killed following the two week post-dosing observation period. Body weights are determined on day 1 (prier to dosing), day 7 and day 14, as required by most regulatory guidelines. Animals should not differ in age by more than 15 percent.

Toxicological data help to make decision whether a new drug should be clinically used or not.

4.5 Acute toxicity

Acute toxicity is the toxicity produced by a pharmaceutical when it is administered in one or more doses during a period not exceeding 24 hours. The objective of an acute study are to define the intrinsic toxicity of a chemical, to assess the susceptible species, to identify the target organ of toxicity, to provide information for risk assessment after acute exposure to the chemical, and to provide information for the design and selection of dose levels for more prolonged studies. In the absence of data on the toxicity of a chemical, acute study also help in

formulating safety measure/ monitoring procedures for all workers involved in the development and testing of a chemical. Accordingly, a battery of tests under different conditions and exposure routes should be conducted.

From a regulatory viewpoint, acute toxicity data are essential in the classification, labeling and transportation of chemical (van den Hauvel *et al.*, 1987). Investigation of acute toxicity has led to the identification of selective toxic action and the beneficial use of substances as pesticides in controlling the environment and as drugs for therapeutic use in domesticated animals and man. The most frequent performed test is an acute systemic toxicity assessment, the number of animals used in the protocol designs is far fewer than for chronic studies and form a relatively small proportion of the total number of animals used in experimental studies. Traditionally, the emphasis in these types of studies was on determining the LD_{50}, time to death, the slope of the lethality curve, and the prominent clinical sings; however, non lethal parameters of acute toxicity testing should also be considered. Acute lethality testing designed to determine the amount of a chemical that cause death as the only end point, has come under extensive criticism. Acute toxicity studies have achieved a level of notoriety in the public domain due to the efforts of animals welfare groups. A primary focus has been the 'LD_{50}' test. While acute toxicity generally deals with the adverse effects of single doses, delayed effect may occur due to accumulation of the chemical in tissues or other mechanism, and it is important to identify any potential for these by repeated dose testing. Dosing periods distributed between single dose and 10% of life span doses are often called subacute.

The term 'subchronic' has been use to embrace the toxic effects associated with repeated doses of a chemical over greater than a 10% part of an average life- span of experimental animals.

4.5.1 Objective of acute toxicity test

(i) To determine the therapeutic index, i.e. ratio between the lethal dose and the pharmacologically effective dose in the same strain and species (LD_{50}/ED_{50}).

(ii) The greater the index, safer is the compound. LD_{50} with confidence limits is to be established on one common laboratory species such as mouse/rat using the standard method. The LD_{50} dose thus found was administered to guinea pigs, rabbits, cats or dogs on weight basis (on basis of relative surface area gives better results).

(iii) To determine the absolute dose for a species in the column, the absolute dose given to the species in a row was multiplied by the factor given at intersection of the relevant row and column (Table 4.2). Because of species variation, several species of animals (one rodent and one non-rodent) were used to determine LD_{50}.

(iv) When a clearly different response was observed in any of these species, a larger number of that species needs to be tested to establish the approximate LD_{50} value (Ghosh, 1984).

Table 4.2 Surface area ratios of some common laboratory species and man (Paget *et al.*, 1964)

	Mouse 20 g	Rat 200 g	Guinea-pig 400 g	Rabbit 1.5 kg	Cat 2 kg	Monkey 4 kg	Dog 12 kg	Man 70 kg
20 g Mouse	1.0	7.0	12.25	27.8	29.7	64.1	124.2	357.9
200 g Rat	0.14	1.0	1.74	3.9	4.2	9.2	17.8	56.0
400 g Guinea-pig	0.08	0.57	1.0	2.25	2.4	5.2	10.2	31.5
1.5 kg Rabbit	0.04	0.25	0.444	1.0	1.08	2.4	4.5	14.2
2 kg Cat	0.03	0.23	0.41	0.92	1.0	2.2	4.1	13.0
4 kg Monkey	0.016	0.11	0.19	0.42	0.45	1.0	1.9	6.1
12 kg Dog	0.008	0.06	0.10	0.22	0.24	0.52	1.0	3.1
70 kg Man	0.0026	0.018	0.031	0.07	0.076	0.16	0.32	1.0

4.6 Parameters in acute Systemic Toxicity Assessments

Establishing a dose- response relationship for exposures at which the probability of a known fraction of a population of a species under study will show lethality will not the objective of acute systemic toxicity studies. In summary, acute studies establish the following:

- Dose range for subsequent studies.
- Potency, ranking from extreme to non-toxic.
- Identifying probable physiological systems/target organs being affected.
- Extent or degree of effect—e.g. subdued behavior, coma and death.
- Minimal regulatory guideline requirements.

The following illustrates the additional data that can be obtained with appropriate protocol design:

Clinical sign: Time of onset, duration and recovery.

Morbidity: agonal changes; reflexes; pharmacological effects; dose responses curve (ED_{50})

Lethality: dose response (LD_{50} with confidence limit); shape and slop of dose- response curve; estimation of median lethal dose (LD_{50}); estimation of minimum lethal dose (LD_{01}); estimation of certain lethal dose (LD_{100}).

Body weight: decreased body weight gain; body weight loss; reduced food consumption.

Target organ identification: necropsy and gross tissue examination; histological examinations; blood clinical chemistry; hematology.

Physiological function: immunology; neuromuscular reflexes; behavioral screening; electrocardiogram; electroencephalogram.

Pharmacokinetic: therapeutics index; bioavailability (AUC, volume of distribution, half life).

Pharmacodynamics: relationship between plasma and tissue levels and occurrences of clinical sign.

Identification of the probable physiological systems and target organs involve in acute systemic toxicity are important objecting when conducting these types of assessments. Often in the

past, only a small selected amount of data from an acute toxicity study has been reported and recorded on informational databases. The data selected are often only the median lethal dose (LD_{50}), or median lethal concentration (LC_{50}) (Sood 2, 1999).

4.7 LD_{50}

LD stands for "Lethal Dose". LD_{50} is the amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals. The LD_{50} is one way to measure the short-term poisoning potential (acute toxicity) of a material. Most often acute toxicity test is done with rats and mice. It is usually expressed as the amount of chemical administered (e.g., milligrams) per 100 grams (for smaller animals) or per kilogram (for bigger test subjects) of the body weight of the test animal. The LD_{50} can be found for any route of entry or administration but dermal (applied to the skin) and oral (given by mouth) administration methods are the most common.

4.7.1 LC_{50}

LC stands for "Lethal Concentration". LC values usually refer to the concentration of a chemical in air but in environmental studies it can also mean the concentration of a chemical in water. For inhalation experiments, the concentration of the chemical in air that kills 50% of the test animals in a given time (usually four hours) is the LC_{50} value.

4.7.2 Reason for study of LD_{50} and LC_{50}

Chemicals can have a wide range of effects on our health. Depending on how the chemical will be used, many kinds of toxicity tests may be required. Since different chemicals cause different toxic effects, comparing the toxicity of one with another is hard. We could measure the amount of a chemical that causes kidney damage, for example, but not all chemicals will damage the kidney. We could say that nerve damage is observed when 10 grams of chemical A is administered, and kidney damage is observed when 10 grams of chemical B is administered. However, this information does not tell us if A or B is more toxic because we do not know which damage is more critical or harmful. Therefore, to compare the toxic potency or intensity of different chemicals, researchers must measure the same effect. One way is to carry out lethality testing (the LD_{50} tests) by measuring how much of a chemical is required to cause death. This type of test is also referred to as a "quantal" test because it is measures an effect that "occurs" or "does not occur". The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity. Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in humans.

4.7.3 Some other toxicity dose terms in common usage

LD_{nt} Lethal dose for 1% of the animal test population
LD_{100} Lethal dose for 100% of the animal test population

LD_{LO} The lowest dose causing lethality

TD_{LO} The lowest dose causing a toxic effect

4.8 Acute toxicity studies

4.8.1 Methods

The bioassays can be conducted according to the World Health Organization guideline for the evaluation of the safety and efficiency of herbal medicines and other drugs (WHO, 1992). The visual observations included skin changes, mobility, aggressiveness, sensitivity to sound and pain, excitation, tremors, twitches, motor coordination, righting reflex as well as respiratory movements and the number of survivors is noted after 24 hr. Also the animals are observed for next 14 days where their weights are recorded. The LD_{50} was then determined at the end of the experiment. The acute toxicity can be evaluated by Lorke method (1983), Turner method, Karber method, Lichtfield and Wilcoxon method (Lichtfield-Wilcoxon, 1949) etc.

4.8.2 Testing Procedures

The test compound should be administered to animals to identify doses causing no adverse effect and doses causing major (life-threatening) toxicity. The use of vehicle control groups should be considered. For compounds with low toxicity, the maximum feasible dose should be administered. Acute toxicity studies in animals should ordinarily be conducted using two routes of drug administration: The route intended for human administration, and intravenous administration, if feasible. When intravenous dosing is proposed in humans, use of this route alone in animal testing is sufficient. Studies should be conducted in at least two mammalian species, including a non rodent species when reasonable. The objectives of acute studies can usually be achieved in rodents using small groups of animals (for instance, three to five rodents per sex per dose). Where non rodent species are appropriate for investigation, use of fewer animals may be considered. Any data providing information on acute effects in non rodent species, including preliminary dose-range finding data for repeat-dose toxicity studies, may be acceptable.

4.8.3 Observation

Animals should be observed for 14 days after pharmaceutical administration. All mortalities, clinical signs, time of onset, duration, and reversibility of toxicity should be recorded. Gross necropsies should be performed on all animals, including those sacrificed moribund, found

dead, or terminated at 14 days. In addition, if acute toxicity studies in animals are to provide the primary safety data supporting single dose safety/kinetic studies in humans (e.g., a study screening multiple analogs to aid in the selection of a lead compound for clinical development), the toxicity studies should be designed to assess dose-response relationships and pharmacokinetics. Clinical pathology and histopathology should be monitored at an early time and at termination (i.e., ideally, for maximum effect and recovery). Corrosive characteristics should not be administered in concentrations that produce severe toxicity solely from local effects.

4.8.4 Karber's method

For calculating LD_{50} by this method, find out the least tolerated (smallest) dose (100% mortality) and most tolerated (highest) dose (0% mortality) by hit and trial method. Once these two doses are determined, select at least 5 doses in between the least tolerated and most tolerated dose, and observe the mortality due to these doses. Generally, Rats are use for this purpose and each dose group should consist of 10 animals. One can determine the LD_{50} value by different rout of administration. The LD_{50} value of a new drug is determined by oral as well as by one of the parenteral routes (ip, iv or im) of administration.

4.8.5 Acute toxicity studies and determination of median lethal dose

Whenever an investigator administers a chemical substance to a biological system, different types of interactions can occur and a series of dose-related responses result. In most cases these responses are desired and useful, but there are a number of other effects which are not advantageous. These may or may not be harmful to the patients. The types of toxicity tests which are routinely performed by pharmaceutical manufactures in the investigation of a new drug involve acute, sub-acute and chronic toxicity. Acute toxicity is involved in estimation of LD_{50} (the dose which has proved to be lethal (causing death) to 50% of the tested group of animals). Determination of acute oral toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. In screening drugs, determination of LD_{50} (the dose which has proved to be lethal (causing death) to 50% of the tested group of animals) is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. It is an initial assessment of toxic manifestations (provides information on health hazards likely to arise from short-term exposure to drugs) and is one of the initial screening experiments performed with all compounds. Data from the acute study may: (a) Serve as the basis for classification and labeling; (b) Provide initial information on the mode of toxic action of a substance; (c) Help arrive at a dose of a new compound; (d) Help in dose determination in animal studies; (e) Help determine LD_{50} values that provide many indices of potential types of drug activity.

4.8.6 Animal selection:

(i) Species and strain – Two species are selected, one rodent and other non-rodent, because species differ in their response to toxic agents. Animals are obtained from random breeding in a closed colony, because the aim was to discover new and unexpected effects of a drug in groups of animals of wider variability or F/1 hybrids of two inbred strains.

(ii) Number and sex of animals – At least five rodents are used at each dose level. They are all of the same sex. After completion of the study in one sex, at least one group of five animals of the other sex is dosed (Ghosh, 1984).

The females are nulliparous and non-pregnant. In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers may be considered. A drug effect that is seen in say, both a rat and a dog, probably involves a common physiological mechanism that is likely to be present in humans. Whereas an effect seen only in one of the two species indicates that it is peculiar to that species and is less likely to be present in the third species (Ghosh, 1984).

(iii) Age – If a compound is to be administered in infants under six months of age, the LD_{50} values in newborn rats under 24 h of age, are compared with those of mature rats in order to assess any difference in sensitivity due to age.

Assignment of animals – Each animal is assigned a unique identification number. A system to assign animals to test groups and control groups randomly is required.

Housing – Animals are group-caged by sex, but the number of animals per cage must not interfere with clear observation of each animal. The biological properties of the test substance or toxic effects (e.g. morbidity, excitability, etc.) may indicate the need for individual caging.

Administration – The compound is administered once (Ghosh, 1984), orally or parenterally, to rats that have been fasted for 18 h.

Dose levels and dose selection: The substance used in the toxicity tests should be as pure as the material eventually to be given to humans. At least three to four dose levels are used, spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and permit (Ghosh, 1984; Paget *et al.*, 1964) an acceptable estimation of LD_{50}.

- If the lethality of the groups is such that only one group has a lethality falling between 4 and 6 probits, more groups may be required.
- Solvent – Where necessary, the test substance is dissolved or suspended in a suitable solvent.

Volume: This depends on size of the test animal. In rodents (Ghosh, 1984; Turner 2, 1965) it should not exceed 1 ml/100 g body weight maximum of 50 ml/kg. Injection is given slowly and uniformly. This will avoid undue killing by a drug having predominant action on the CNS/heart (Ghosh, 1984).

Route of administration: The LD_{50} value depends on the route of administration. Usually the values are found to increase with the following sequences of routes: intravenous, intraperitoneal, subcutaneous and oral. The intravenous route is preferable to the intraperitoneal route (because many drugs get detoxified by the liver if the intraperitoneal route is employed).

The signs recorded during acute toxicity studies: These are increased motor activity, anaesthesia, tremors, arching and rolling, clonic convulsions, ptosis, tonic extension, lacrimation, Straub reaction, exophthalmos, pilo-erection, salivation, muscle spasm, opisthotonus, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia. After the test, the animal is the sole occupant of the cage, with free access to food and water during the observation period of 1–2 h, and thereafter at intervals.

At the end of the test surviving animals are weighed and sacrificed. A gross necropsy may be performed, all gross pathology changes are observed. If necropsy cannot be performed immediately after the death of the animal it should be refrigerated to minimize autolysis. Necropsies must be performed no later than 16 h after death (Ghosh, 1984).

Before the actual LD_{50} determination, a pilot study also can be done in a small group of animals, to select the dose ranges for the subsequent study.
The maximum non lethal and the minimum lethal doses are thus determined using about minimum number of animals (Ghosh, 1984). Once the approximate LD_{50} or the range between the maximum non lethal and minimum lethal doses is found, a final and more reliable LD_{50} assay can be planned using at least three or four dose levels within this range, with a larger number of animals in each group (Ghosh, 1984).

4.8.7 Arithmetical method of Karber

In this method of acute toxicity study, the interval mean of the number dead in each group of animals are used as well as the difference between doses for the same interval. The product of interval mean and dose difference is obtained. The sum of the product is divided by the number of animals in a group and the resulting quotient is subtracted from the least lethal dose in order to obtain LD_{50} value (Turner, 1965).
LD_{50} = The apparent least dose lethal to all in a group is $\Sigma (a \times b)/ N$

Where, N is the number of animals in each group, 'a' the dose difference and 'b' the mean mortality.

4.9 Subacute toxicity studies

The subacute toxicity studies can be carried out by Lorke (1983) method, Lichtfield and Wilcoxon method, where biochemical estimation of serum as well as of various orgasns, haematological parameters and histopathological changes are observed.

Liver enzymes such as Aspartate aminotransferase (AST), alanine aminotransferase (ALT); alkaline phosphatase (ALP), creatinine, protein, cholesterol, glutathione were assayed using various method used for biochemical estimation. Haematological parameters such as count of white blood cells (WBC), red blood cells (RBC) and platelets; histopathological investigation such as liver, kidney etc can be done with the help of microtomy.

4.10 Regulatory affaires

It is essential that any new method that is considered to be adequately validated as a replacement for an existing method receives as widespread international recognition as possible. For example, the OECD (The Organisation for Economic Cooperation and Development) test guidelines are particularly important in this respect, since they are used for tests conducted in member countries in Europe and North America, and in Japan, Australia and New Zealand. Furthermore, under the OECD Mutual Acceptance of Data Agreement, member countries have agreed to accept data from tests performed according to OECD test guidelines, provided that the principles of Good Laboratory Practice (GLP) are observed. The OECD has established a procedure for updating test guidelines and for the introduction of new test methods (Koeter, 1994). This takes into account both advances in science and proposals that are based on animal welfare considerations.

According to Schorderet (1992), subtances with LD_{50} values greater than 5 g/kg of body weight are considered to show low toxicity. According to the OECD guideline, if an acute toxicity test at one dose level of at least 500 mg/kg body weight produced no observable toxic effects (Witthawaskyl et al., 2003), the full study at a dose of 1000 mg/kg given once daily for 14 days can be used to evaluate subacute toxicity.

4.11 MATERIALS AND METHODS

4.11.1 Preparation of extract

The plant material used in this study i.e. leaves of *Urtica parviflora (U. parviflora), Callicarpa arborea (C. arborea)* and root bark of *Morinda citrifolia (M. citrifolia)* were collected at Majhitar, East Sikkim and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131, 132 and 133).

The leaves of *U. parviflora* and *C. arborea,* free from dirt were separated and shade dried for ten days and made to powder by a mechanical grinder. The powdered drugs (500g) were

extracted with ethanol by continuous hot extraction process (soxhelation). The solvent was recovered and the extracts were concentrated under reduced pressure. In case of *Morinda citrifolia* the clean roots are shade dried for twenty days and then subjected to soxhelation using ethanol as the solvent. The extract yield was found to be 5% for *U. parviflora,* 7.5% for *C. arborea* and 11.0% for *M. citrifolia.*

4.11.2 Animal stock

Adult albino rats (Wistar strain) weighing 160-200g were used in this study. All the animals were housed in a cross-ventilated room (22 ± 2.5°C), 12h light 12h dark cycle, fed with standard Local animal feed and water *ad libitum.* The animal studies were approved by Institutional Animal Ethics Committee IAEC No. HPI/07/60/IAEC/0005.

4.11.3 Acute toxicity study

The animals were divided into five groups of six rats per cage. The plant drugs were made in to slurry by adding Sodim Carboxy Methyl Cellulose (q.s) and fed orally with the help of animal feeder needle to each animal. The animals were kept fasted for 18 hrs before the experimentation. The doses were administered once to the animals. After administration, the animals were observed for next two hours for appearance of any sign and symptoms and then observed at the end of the 24^{th} hr. The animals dead or alive were counted and the data was tabulated. The LD_{50} was determined by the formula LD_{50} = Maximum given dose – (Maximum product / N). Before the experimentation a pilot study was performed to know the nearby doses to LD_{50}. The procedure was repeated for different extracts.

4.12 Results of the acute toxicity study

The results of acute toxicity study of the ethanolic leaf extract of *U. parviflora* and *C. arborea* in rat is presented in **Table 4.3** and **4.4** respectively. The result of the acute toxicity of ethanolic root extract of *M. citrifolia* is presented in **Table 4.5**. The LD_{50} (Median Lethal Dose) of orally administered ethanolic extracts of leaves of *U.parviflora, C. arborea* and root bark of *M. citrifolia* was found to be **3500 mg/kg, 1666.67 mg/kg** and **950 mg/kg** body weight per oral respectively.

Table 4.3 LD_{50} Determination by Karber's method of the alcoholic extracts of *U. parviflora*

Sl. No	Dose mg/kg	No. of animals	Dose difference (a)	Mortality	Mean Mortality (b)	Product (a × b)
1	1050	6	0	0	0	0
2	2100	6	1050	1	0.5	525
3	3150	6	1050	2	1.5	1575
4	4200	6	1050	4	3	3150
5	5250	6	1050	6	5	5250
TOTAL						10500

LD_{50} = Maximum given dose – (Maximum product / N)

=5250-(10500/6)

= 3500 mg/kg

Where N is the number of animals in each group, "a" is the dose difference and "b", the mean mortality.

Table 4.4 LD_{50} Detrmination by Karber's method of the alcoholic extracts of *C. arborea*

Sl. No	Dose mg/kg	No. of animals	Dose difference (a)	Mortality	Mean Mortality (b)	Product (a × b)
1	500	6	0	0	0	0
2	1000	6	500	1	0.5	250
3	1500	6	500	2	1.5	750
4	2000	6	500	4	3	1500
5	2500	6	500	6	5	2500
TOTAL						5000

LD_{50} = Maximum given dose – (Maximum product / N)

=2500-(5000/6)

= 1666.67 mg/kg

Where N, is the number of animals in each group, "a" is the dose difference and "b", the mean mortality.

Table 4.5 LD_{50} Detrmination by Karber's method of the alcoholic extracts of *M. citrifolia*

Sl. No	Dose mg/kg	No. of animals	Dose difference (a)	Mortality	Mean Mortality (b)	Product (a × b)
1	650	6	0	0	0	0
2	1300	6	650	1	0.5	325
3	1950	6	650	2	1.5	975
4	2600	6	650	4	3	1950
5	3250	6	650	6	5	3250
TOTAL						6500

LD_{50} = Maximum given dose – (Maximum product / N)

=3250 -(6500/6)

= 950 mg/kg

Where N, is the number of animals in each group, "a" is the dose difference and "b", the mean mortality.

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CHAPTER 5

HEPATOPROTECTIVE ACTIVITY

5.0 INTRODUCTION

Liver injury caused by toxic chemicals and certain drugs has been recognised as a toxicological problem. Herbal drugs are playing an important role in health care programmes world wide, and there is a resurgence of interest in herbal medicines for treatment of various ailments including hepatopathy (Venukumar *et al.,* 2004). Liver is the main organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation (Gram *et al.,* 1971).

5.1 Functions of Liver

The functions of the liver are:

a) Detoxication of bilirubin by conjugating it as bilirubin diglucuronides and excreting through bile.
b) Epimerisation of galactose as UDP derivative.
c) Synthesis of proteins like albumin.
d) Synthesis of prothrombin.
e) Control of enzymes alkaline phosphatase and release of transaminases i.e. Aspartate amino transferase and Alanine aminotransferase.

From practical point of view bilirubin, total and conjugated, alkaline phosphatase activity, and ALT (GPT) are the important investigations generally carried out in blood (Waugh *et al.,* 2006).

5.1.2 Detoxication of bilirubin

Bilirubin is formed daily from hemoglobin (Hb) in the reticulo-endothelial system chiefly of bone marrow and spleen. About 6.25 g of haemoglobin is degraded every day. Bilirubin is transported in the plasma in combination with albumin to the liver, where it is conjugated with UDP-glucuronic acid to form bilirubin glucuronide. Bilirubin is insoluble in water but bilirubin diglucuronide is soluble in water. This is an important difference in physical property. The glucuronide is excreted in the bile and through the bile goes to the intestine. There, it is reduced by bacterial enzymes to urobilinogen. Some urobilinogen is oxidized to urobilin. The urobilinogen not oxidized in the intestine, is returned to the liver (entero-hepatic circulation) and is oxidized to bilirubin which is re-excreted in to the bile. Normal urine therefore contains very little urobilinogen, i.e., 1 to 4mg per 24 hours. Bilirubin metabolism is deranged in three important diseases. They are (i) hemolytic jaundice, (ii) hepato-cellular jaundice, and (iii) obstructive jaundice.

5.1.2.1 Hemolytic jaundice

The liver is normal in this disease and can conjugate the usual amounts of bilirubin efficiently. But when there is hemolysis, there is extensive degradation of heme and over-production of bilirubin. There is subsequently a rise of bilirubin in the blood as the liver cannot remove it efficiently. This bilirubin is not soluble in water. Hence it will not give a direct positive reaction with the van den Bergh reagent (The van den Bergh reagent is a mixture of equal volumes of van den Bergh solution A, i.e. sulthanilic acid in dilute HCL, and van den Bergh solution B, i.e., Sodium nitrite). If there is bilirubin diglucuronide which is water-soluble, it will give a pink colour immediately. Such a reaction is called the van den Bergh direct positive reaction. There may be no positive reaction but on adding methanol, the serum may respond to the van den Bergh test. This is because methanol dissolves bilirubin. Once a solution is obtained with methanol, the serum will give a pink colour with the van den Bergh reagent. This is called the van den Bergh indirect test. In hemolytic jaundice, the van den Bergh test will be indirect positive. As the liver is normal, there is no regurgitation of whatever bilirubin that has been conjugated. Hence there will be relatively greater amounts of bilirubin than normal going to the intestine. As a result, there will also be an increased excretion of urobilinogen in urine. There will not be any direct-reacting bilirubin (i.e. bilirubin glucuronide) in hemolytic jaundice as there is no regurgitation of bile into the blood. The jaundice therefore is due to increased water-soluble bilirubin in blood.

In short the following are the results of the blood and urine tests in hemolytic jaundice:

(I) Bilirubin total elevated; normal values are 0.1-0.8mg/dL;
(ii) Bilirubin glucuronides not detectable;
(iii) Van den Bergh test – indirect positive; and
(iv) Urinary urobilinogen – increased.

5.1.2.2 Obstructive jaundice

The increased level of bilirubin is due to regurgitation of bile. The regurgitation takes place due to obstruction in the bile duct (Cholestasis). So, the bile instead of flowing into the gastro-intestinal tract regurgitates into the blood. As the obstruction is outside the liver, the condition is called extra–hepatic or post–hepatic obstruction. As the liver is normal except in chronic conditions, conjugation goes on so that there is both water-insoluble bilirubin and water-soluble bilirubin glucuronides in the blood. The later gives the van den Bergh direct positive reaction. There is increased bilirubin glucuronides and total bilirubin. In view of the obstruction in the bile duct, there is no formation of urobilinogen. Hence the urine does not contain urobilinogen. Due to the regurgitation of bile, the activity of the enzyme alkaline

phosphatase is quite high (<30-35 KA units/dL) in the blood. The transaminase like Alanine aminotransferase may not be elevated significantly as there is no damage of the liver except in chronic conditions.

The following are the diagnostic findings generally observed in obstructive jaundice:

(i) Total bilirubin – elevated;
(ii) bilirubin glucuronides (direct-reacting bilirubin increased);
(iii) van den Bergh test direct positive;
(iv) alkaline phosphatase activity-above 30 – 35 KA units /dL.]
(v) Serum Alanine amino transferase- slightly elevated; and
(vi) Urinary urobilinogen negligible.

5.1.2.3 Hepato cellular jaundice

In this condition the jaundice is due to the increased level of bilirubin and bilirubin glucronides in blood. This also is due to the regurgitation of bile but such regurgitation is not due to post-hepatic obstruction but to intra- hepatic obstruction (obstruction from within the liver) because of inflammation caused by infection. The presence of bilirubin glucuronide in serum renders the van den Bergh test direct positive. Total bilirubin is elevated. Alkaline phosphatase activity is also increased but is less than 30-35 KA units /dL. Normal values are 3-8 KA units/ dL).

So long as the obstruction is there, urinary urobilinogen is negligible. This is because the obstruction, intra hepatic or post- hepatic, prevents bilirubin from reaching the intestines for reduction to uroblinogen. However, when the inflammation subsides during recoverythere is no obstruction. Under such circumstances urinary urobilinogen excretion may increase.

In view of infection, there is liver damage. Hence, the enzyme Alanine amino transferase from the necrosed cells mixes with the blood. So, there is increase of this enzyme Alanine amino transferase (normal value is 2 – 15 IU/L); double if assay is done at 37°C.

The following are the diagnostic findings in hepato-cellular jaundice:

(i) Total bilirubin – increased;
(ii) Direct reacting bilirubin, i.e., bilirubin glucuronides increased;
(iii) Van den Bergh test direct positive;
(iv) Akaline phosphatase activity increased upto 30-35KA units/dL;
(v) Urinary urobilinogen increased or decreased depending on the condition; and
(vi) Alanine amino transferase- increased.

Table 5.1 The blood and urine tests for the differential diagnosis of the three types of jaundice

Condition	Total bilirubin	Bilirubin Glucuro-nide	Van den Berg test	Alkaline Phosphatase	Alanine Amino transfer ase	Urinary Urobiiinogen
Hemolytic Jaundice	Increased	Nil	Indirect positive	Normal	Normal	Increased
Obstructive Increased	Increased	Increased	Direct positive	Increased above 30-35 KA units/dL.	Slightly elevated	Negligible
Hepato-Cellular Jaundice	Increased	Increased	Direct positive	Increased but below 30-35 KA units/dL	Significa ntly elevated	Initial increase, but is lowered as the disease progresses

As hepato-cellular jaundice is due to infections like viral infection, it can be treated with medicines, and no surgery is needed. Hence, it is called medical jaundice. On the other hand, obstructive jaundice, if the obstruction is due to stone in the bile duct that cannot be cured with medicines. Surgery is the only way and hence it is called surgical jaundice.

The van den Bergh's test is based upon the formation of purple colour due to azobilirubin obtained by the reaction between bilirubin and van den Bergh's diazo reagent. The reagent A is sulphanilic acid in hydrochloric acid while the reagent B is dilute sodium nitrite solution. The reagents are mixed fresh in the prescribed proportions just prior to the performance of the test. A purple colour formed within thirty seconds of mixing indicates direct reaction. Sometimes it may be delayed direct, if there is a partial conjugation defect. If the reaction is not direct positive, methanol is used as a solvent to dissolve the water- insoluble bilirubin and the colour developed is measured after letting it stand for thirty minutes (indirect positive).

In hemolytic conditions the load of bilirubin is beyond the capacity of the conjugating ability of the liver, while in hepato-cellular jaundice, since the liver itself is diseased; it is not able to handle even the normal amounts of bilirubin reaching it.

The normal serum bilirubin level ranges between 0.1 to 0.8 mg/dL. The normal urinary urobilinogen output ranges between 1–4 mg per twenty four hours. A collection of postprandial urine is necessary for the estimation of urobilinogen. Icteric index done on earlier days is not estimated as the bilirubin levels are precisely analyzed.

5.1.3 Alkaline Phosphatase

The normal serum level of this enzyme ranges between 3-11 KA units/dL. The enzyme is excreted by the liver via the bile and hence when the liver is in disorder, the serum enzyme level goes up due to defective, excretion but all the non hepatic conditions which can cause a similar rise have to be scrupulously eliminated. Such conditions are rickets, osteomalacia, hyper-parathyroidism, post hepatic obstruction, Paget's disease and bone tumours. In infective hepatitis with intrahepatic obstruction when van den Bergh's reaction with serum is direct positive, the serum alkaline phosphatase levels are moderately raised up to about 35 KA units / dL. Though not as high as in post hepatic obstruction in which it will be greater than 35 KA units/dL.

5.1.4 Transaminases and dehydrogenases

Jaundice is a contra indication for the determination of serum transaminase levels as high values are obtained even in the absence of liver damage. Transamination is not the monopoly of any particular tissue in the body, so that in the absence of second tissue damage, it is valuable in the diagnosis of liver dysfunction. Serum Alanine amino transferase (originally called serum glutamate pyruvic transaminase) raised early in liver disease even in the preicteric phase. Serum Aspartate amino transferase (originally called serum glutamate oxalo-acetate transaminase) though diagnostic of myocardial infarction, can also be a useful liver function test, provided the liver involvement is not secondary to the involvement of the heart. Determination of serum isocitrate dehydrogenase is believed to be a specific liver function test. Specificity is also seen in the estimation of the serum levels of the heat-labile hepatic isozymes of lactate dehydrogenase, i.e. LD4 and LD5. The serum amylase levels are low in liver disease. The activity of pseudo – choline esterase may also be lowered. High levels of serum leucine amino peptidase are seen in liver damage though it is not specific for liver dysfunction.

5.1.5 Plasma protein levels and liver dysfunction

Hypoalbuminemia and hence hypo-proteinemia as well as hypo-fibrinogenemia are met within gross liver diseases, as the liver is concerned with the biosynthesis of these proteins. However, hypo-proteinemia can be seen in protein malnutrition as well as after hemorrhage so that a reversal of the ratio between albumin and globulin in serum is a more useful diagnostic test, the normal ratio being 3:2. A reversal of the ratio, which is a compensatory effort on the part of the extra-hepatic situations where 20 percent of globulins are synthesized normally, is the basis of the several tests for liver function though it is said that

the quality of albumin in liver disease also differs from the albumin in normal serum. Electrrophoretic separation of serum proteins can, for example, establish conditions like cirrhosis of liver characterized by hypo-albuminemia and hyper-gamma globulinemia. However, there are non hepatic conditions where one finds a similar feature.

5.1.6 Bromosulphalein excretion test

This is the dye of choice for assessing liver function. It is non-toxic and is practically and almost exclusively excreted by the liver. Occasionally, allergy to this substance may be encountered. Extra vacation of the dye outside the vein should be avoided as it is a bad irritant. The dye is injected slowly intravenously, in a dose of 5 mg per kg body weight in a five per cent solution. Blood is drawn through the opposite median cubital vein, 30-45 minutes after the injection of the dye. With a normal liver, the retention of the dye is less than ten percent at 30 minutes and less than six percent at 45 minute. The presence of jaundice vitiates the results. Hence this test is not to be done in the presence of jaundice but is valuable in the screening of pre icteric phase of conditions like infective hepatitis and it also has prognostic value in the assessment of residual damage of the liver, if any, during the convalescent period even after the patient's bilirubin in serum reaches normal levels and the patient is apparently clinically normal. This test is superior to all other tests as it caters to specificity as well as to sensitivity.

5.1.7 Prothrombin time of Plasma

This is a very useful liver function test not only for diagnostic purposes but slso as a measure of safety before one undertakes liver biopsy and operative procedures. Obviously, all the non-hepatic causes of prolongation of prothrombin time must be scrupulously excluded. The conditions are: deficient intake of vitamin K, defective absorption of vitamin K, Post-hepatic obstruction, steatorrhoea, administration of anticoagulants and administration of antibiotics. Isotonic sodium citrate solution (3.8 percent) is the anticoagulant of choice for the collection of blood as it serves the purpose of converting ionic into the non-ionic form of calcium. Suitable thromboplastic material is used, and controls are done simultaneously along with every test. The normal values vary according to the type of the thromboplastic material used.

5.1.8 Serum enzymes and isozymes in diagnosis of diseases

Clinical Enzymology is a branch of biochemistry dealing with the diagnostic value of enzyme estimation in serum and tissues in diseases. Rona was the first to introduce serum lipase estimation in pancreatic disease. Subsequently, estimations of many serum enzymes have

become popular in the clinical biochemical laboratory. In fact, estimations of alkaline phosphatase and Alanine amino transferase have become almost a daily routine.
The enzymes present in circulating plasma are of two types: 1) the plasma specific or functional plasma enzymes, and 2) the plasma non specific or non – functional plasma enzymes. The former type of functional plasma enzymes has their substrates in the plasma. These enzymes, though synthesized in the liver, are found in circulating plasma at higher concentrations than in tissues. Examples of functional plasma enzymes are lipoprotein lipase, pseudo-choline esterase, and enzymes associated with the clotting of blood. In these cases the blood levels are greater than the tissue levels.

There are many other enzymes circulating in plasma but do not have any substrate in plasma and have no role. These are usually present in small amounts but their levels are altered in pathological conditions. In many cases there is increase in their concentration and only in a few cases there is a decrease; examples of plasma non–specific enzymes are alkaline phosphatase, acid phosphatase, transaminases, viz., glutamate-oxalo acetate-transaminase (G.O.T., presently known as AST, Aspartate aminotransferase) glutamate-pyruvate-transaminase (G.P.T presently known as ALT. Alanine amino transferase), creatine phosphokinase (presently known as creatine kinase), lipase, amylase, etc. In these cases, the tissue levels are greater than the blood levels.

The increase in serum levels of an enzyme may be due to (1) obstruction in the normal flow and regurgitation; (2) Seepage of the enzymes from necrosed cells and (3) Slow rate of renal or other modes of clearance of serum enzyme.

Whatever may be the mechanism, a rise or fall in serum enzyme levels has been of immense value to a clinician. Some serum enzymes which are altered in diseased conditions are listed in the **Table 5.2**.

In any particular disease before arriving at a diagnosis, it is worthwhile to estimate three or four enzymes in serum rather than a single enzyme. Thus in heart disease, it is advisable to estimate AST, creatine kinase, CK-MB and hydroxyl butyrate dehydrogenase activity (HBD) (which reflects the levels of LDH_1 and LDH_2) while in liver diseases, the enzymes alkaline phosphatase, ALT, isocitrate dehydrogenase, LDH 4 and LDH 5 isozymes could be investigated. Likewise in pancreatic diseases, serum amylase and lipase estimations (also urine amylase) will be of value. In primary myopathies, aldolase, creatine kinase (CK) and CK-MM might be investigated.

While interpreting the values of the serum enzyme levels, it has to be taken into account that some enzymes are altered even in physiological conditions. Amylase is absent in the new born. CK is low at rest but increases after exercise; alkaline phosphatase is raised in the newborn and during pregnancy. Acid phosphatase, aldolase, and AST are raised in children and infants.

In addition to variation of serum enzymes and urine enzyme (e.g., amylase) there are reports about variation of enzymes in cerebrospinal fluid and in the tissues and about the usefulness of their studies in diseases.

5.1.9 Alkaline Phosphatase

There are six isozymes of alkaline phosphatase. They are from liver, bone, skeletal muscle, kidneys small intestines and placenta. Adult serum contains the isozyme of liver while in children; it is a mixture of liver and bone isozymes. Placental isozyme behaves like that of bone.

A distinct form was noticed in lymphatic leukemia and infectious mono nucleosis and new bands in malignancies. Electrophoretically, the isozymes are designated as alpha, beta, pre beta and gamma.

Alpha is elevated in liver damage, pre beta and beta increased in osteoblastic activity and gamma in intestinal involvement. Alpha is divisible into alpha 1 and alpha 2. Alpha 1 increase when there is an element of obstruction eg. Regurgitation of bile and alpha 2 in liver cell damage. Alpha 1 band is found only rarely in viral hepatitis. It is elevated in metastatic carcinoma of liver, obstructive jaundice and cholangitis.

Absence of alpha-1 with exaggerated alpha-2 suggests hepatitis. A sudden decrease of placental isozyme suggests placental insufficiency.

Table 5.2 Name of the enzyme with their commission number and normal levels in serum (Ramakrishnan *et al.*, 1995)

Name of the Enzyme with Enzyme commission Number and normal levels in serum	Clinical conditions in which its levels are altered	Remarks
1. Asparatate amino transferase AST (G.O.T)E.C.No.2.6.1.1 4 to 17U/1(25'C) 8 to 34 U/1 (37'C)	Myocardial infaraction (specific), liver diseases, crushing muscle injury, muscular dystrophy (but not in muscular disease of neurogenic orgin) acute pancreatitis and pulmonary embolism.	(I) increase begins at 3-8 hours after the onset of the episode; highest values at about 24hrs, returns to normal by 3 to 6 days (ii) normal in angina pectoris
2. Alanine amino transferase A.L.T(G.P.T) E.C.No.2.6.1.2 3 to 15U/1(25'C) 6 to 30U/1(37'C)	Infective hepatitis and toxic hepatitis (specific), myocardial infarction, infarction, infectious, mononucleosis, malignancy	(i) In hepatitis and other diseases associated with liver necrosis, ALT and AST increase as both of them are in high concentration in liver. ALT rises even before the onset of jaundice; its increase is parallel to necrosis. (ii) ALT content of content of heart is 1/20th of AST hence rise of ALT is less marked than AST in myocardial infarction (iii) Normal in cirrhosis of liver, obstructive jaundice without necrosis and hemolytic jaundice.
4. Alkaline phosphatase (bone forming enzyme) E.C.No.3.1.3.1 3 TO 13 KA units/dL (22 to 92 U/1) for adults 7.5 to 33.5 KA units for children (two and a half times adult value)	Physiological rise (i) in children due to osteoblastic activity (ii) in pregnant women from placental origin. Pathological : (iii) highest level in Paget's disease and hyperpara-thyroidism with skeletal muscle involvement, moderate rise in osteomalacia, considerable increase in renal rickets associated with secondary hyper para thyroidism, in bone damage due to metastatic carcinoma, myeloma, Hodgkins disease if the bones are invaded, Gauchers disease with bone resorption. (iv) increased in hepato cellular disease and bile duct abnormalities as serum alkaline phosphatase is cleared by liver. It is less than 30-35KA/dL in infective hepatitis but more than 30-35 KA/dL in obstructive Jaundice. Alkaline phosphatase increases in early obstructive disease even before bilirubin levels are increased. Considerable increase in inflammatory disease and metastatic carcinoma of the liver, bilary cerrhosis and cholangiolytic hepatitis. (v) Decrease in childhood hypothyroidism.	(i) Very helpful in differential diagnosis of jaundice; below 30-35 KA/dL in hepato cellular due to inflammation and regurgitation in to blood; above 30-35 KA/dL in obstructive jaundice due to regurgitation of bile into blood caused by post hepatic obstruction say due to stone and normal in hemolytic . (ii) 1 KA unit /dL = 7.1 U/1

5.1.10 Michaelis menten constant (Km value) of serum enzymes in differential diagnosis of disease

Km value is a fundamental characteristic of all enzymes and isozymes. It shows the efficiency of binding of the substrate to the active centres of the enzyme.The greater the Km value the less the efficiency and vice versa as Km is given by substrate concentration at half the velocity maximum.

Serum contains mixture of isozymes of an enzyme. For example, the alkaline phosphatase of serum is a mixture of isozymes of alkaline phosphatase from the liver and the bone with a little contribution from the kidneys and small intestines (and placenta during pregnancy). Each isozyme has a definite Km. In the usual course of analysis in a clinical biochemistry laboratory, total alkaline phosphatase activity is assayed in serum. If it is increased, it could be infective hepatitis (Moderate elevation) obstructive jaundice (high) or bone diseases like Paget's disease osteomalacia. How to get the differential diagnosis between these diseases, If, now the Km value of serum alkaline phosphatase (hereinafter referred to apparent Km value, as serum contains a mixture of isozymes each having a definite Km) is estimated having different substrate concentrations and drawing Line weaver-Burk plots, if the apparent Km is close to that of the isozyme of bone, it is bone disease. If it is close to that of liver isozyme, it is liver disease. i.e., what an estimation of total enzyme activity can not reveal, Km determination will help (Ramakrishnan *et al.*, 1995). It was found that apparent Km of serum alkaline phosphatase was higher in cirrhosis of liver but normal in infective hepatitis.

Aspartate amino transferase (AST or SGOT) of serum could have isozymes of heart and liver. The total enzyme activity is increased in myocardial infarction and infective hepatitis and can not clinch the diagnosis. But apparent Km of serum AST is increased in advanced infective hepatitis with bilirubin above 13mg .dL while it was not elevated in myocardial infarction.

Alanine amino transferase (ALT or SGPT) of serum could be a mixture of isozymes of liver and the heart. Estimation of total enzyme activity could not help in differential diagnosis. Apparent Km of serum ALT is increased in infective hepatitis but normal in myocardial infarction (Ramakrishnan *et al.*, 1995).

Increase of apparent Km of serum AST and ALT in infective hepatitis might be due to the interaction of toxic metabolites with the active sites of the enzymes and decreasing the efficient binding of the enzyme and the substrate.

Lactate dehydrogenase of serum has isozymes of heart, liver, etc. Cancerous tissues synthesize isozyme and release into the blood. Total LDH is increased in myocardial infarction infective hepatitis, carcinomas and hematogical disorders and is not of help in differential diagnosis. But the apparent Km of serum LDH is increased in all cases of Carcinomas but not in myocardial infarction or infective hepatitis (Ramakrishnan *et al.*, 1995).

Thus the determination of apparent Km value of serum enzymes is helpful in differential diagnosis of diseases while just an assay of total enzyme activity might record an elevation in more than one disease. Relating Km with diagnostic biochemistry is a new field with prospects of tremendous advancement.

Hepatotoxicity caused by drugs or chemicals can occur due to occupational, environmental or dietary exposure. Such toxicities appear to be the consequence of the unique vascular, secretary, synthetic and metabolic features of the liver (Beris, 1991). Hepatotoxins can affect biological macromolecules such as proteins, lipids, RNA, DNA and induce several types of lesions (Yerra, 2005), of which genotoxic alterations may lead to carcinogenesis.

Diethylnitrosamine (NDEA), a known toxin as well as a potent carcinogen found in air, water and soil (Murray *et al.*, 2000; Malila *et al.*, 2002), belongs to nitrosamine group that has been established to cause hepatotoxicity in human beings. The variety of products that would result in human exposure, include mainstream and sidesstream tobacco smoke (Jayaprakash *et al.*, 2001), meat and whiskey (Kirtikar *et al.*, 1975). It can also be generated from metabolism of certain therapeutics drugs (Dutt, 1995). NDEA is extensively used as a solvent in the fiber industry, as a softener for co-polymers, as an additive for lubricants, in condensers to increase the dielectric constant and for the synthesis of 1,1-diethylhydrazine (Nadkarni, 1976; Kholkute *et al.*, 1976). NDEA has been shown to cause cancer in liver and other organs in various experimental animals (Bandara *et a.*, 1989). NDEA is known to be bio-activated by cytochrome P450, following which it forms DNA adducts rapidly, bringing about mutation and fragmentation that may lead to micronuclei formation.

In order to intervene the earlier stages of chemical mediated toxicity, use of chemopreventive agents may be considered as an effective alternate. Among various agents with chemopreventive effects, phytoproducts are gaining more and more attention gradually due to their decreased toxicity and high efficacy. Studies of natural products provide opportunities to reveal interesting biology and generate leads pertaining to specific cellular targets, activities, and therapeutic manipulations (Prashanth *et al.*, 2001; Koul *et al.*, 2007).

Liver, the major site of intense metabolism is prone to various disorders as a consequence of exposure to toxins of extrinsic as well as intrinsic origin. Most of the hepatoprotective drugs now available in the market cost much, and hence a genuine need is felt to devise some cost-effective drugs based on plant principles in this regard (Venukumar *et al.*, 2004).

CCl_4 intoxication in rats is an experimental model widely used to study necrosis and steatosis. Hence, the same method is adopted to induce hepatopathy in this experiment.

Carbon tetrachloride and Paracetamol are known to cause liver damage (Recknagel, 1983; James *et al.*, 2003). When administered to rats, they act by inducing oxidative damages to liver cells which leads to cellular necrosis, resulting in increase in serum enzymes SGOT and SGPT. These models of hepatotoxicity has been widely used to study the antihepatotoxic activities of exogenous drugs in experimental animal models (Shenoy *et al.*, 2001; Bisshayi *et al.*, 2002; James *et al.*, 2003)

Paracetamol-and CCl_4 – induced hepatitis are usually used as experimental models in the search for new antihepatotoxic compounds (Parthasarathy *et al.,* 2004). Once introduced in the organism, CCl_4 is converted in the liver into a radical which reacts with molecular oxygen to form a trichloromethyl peroxyl radical. This compound attacks membrane polyunsaturated fatty acids and causes membrane lipid peroxidation (Recknagel, 1983) which leads to impairment of membrane function. When Paracetamol is introduced in excess in the body, this compound is also metabolized in the liver to a reactive metabolite which reacts with enzymes and membrane components of liver cells which results in cellular lesion (Rang *et al.*, 1999). In both cases, an increase in the serum of some liver enzymes such as ALT and AST is observed. An extract is said to be antihepatotoxic if it prevents the increase in the level of these serum enzymes in animals in which hepatitis has been experimentally induced.

Biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (ALP), bilirubin, total serum protein, lipid peroxide and glutathione content of the liver were estimated to determine liver function and metabolism.

5.1.11 Biochemical estimation

The AST, ALT (Oser, 1965), ALP (Bergmeyer, 1974), bilirubin (Oser, 1965) and total serum protein (Lowry *et al.*, 1951) can be estimated in serum of rats. Lipid peroxide (Ohakawa *et al.*, 1979) and glutathione (Mulder *et al.*, 1995) content of the liver are measured in liver tissue to determine liver function and metabolism.

5.1.11.1 Hepatotoxicity in laboratory animals

Paracetamol induced hepatotoxicity in rodents is a widely used animal model to assess the hepatoprotective activity of new compounds (Parthasarathy *et al.,* 2007). The rise in serum levels of SGOT, SGPT and ALP has been attributed to the damaged structural integrity of the liver. Pretreatment with plant extracts prior to the administration of Paracetamol significantly prevent the increase in ALP and aspartate aminotransferase activity in a dose dependent manner (Kyung *et al.*, 2001). The reversal of increased serum enzymes in paracetamol induced liver is observed in hepatoprotectve plant extracts may be due to prevention of leakage of the intracellular enzymes by its membrane stabilizing activity (Thabrew *et al.*, 1987).

The development of fibrosis is critical for progression of all chronic liver diseases. In the last 20 years, the molecular and cellular mechanisms regulating liver tissue scarring have been investigated in detail, and additional pieces of the puzzle are being added now that several research groups are investigating this aspect of liver pathophysiology. A major acquisition has been the identification of liver fibrosis as a tissue-specific counterpart of the "wound-healing" response, a process whereby damage triggers a series of events, involving multiple cell types, aimed to limit damage and to preserve tissue function and integrity (Friedman *et al.*, 2000). These events include inflammation, activation of fibrogenic myofibroblasts (e.g., hepatic stellate cells, HSC), deposition of fibrillar extracellular matrix, and possibly neo-angiogenesis. As data on the role of leptin in the regulation of these steps accumulate, it is important to establish their possible relevance to the pathophysiology of human chronic liver diseases (Fabio, 2007).

5.1.12 Plants in the treatment of liver and biliary tract diseases

Liver is the principal organ of metabolism and excretion in subject to a number of diseases which may be classed as liver cirrhosis (cell destruction and increase in fibrous tissue), acute chronic hepatitis (inflammatory disease) and hepatitis (non-inflammatory condition).

In 1989 it was estimated that there were some 200 million chronic carriers of the hepatitis B virus of which 40% were expected eventually to die of hepato-cellular carcinoma and 15% of cirrhosis. Causative factors for liver disorders include virus infection; exposure to, or consumption of, certain chemicals, chemotherapeutic agents and possibly plants materials such as those containing pyrrolizidine alkaloids, ingestion of industrial pollutants, including radioactive materials.

In such liver damage the serum level of the liver enzymes, particularly serum glutamic pyruvic transminase is raised and extent of its control by the antihepatotoxic drug used as a basis for estimation. Other effects of included liver damage which can also be used in the evaluation of the plant extracts are: the prolonged lengthening of the time of lost reflex induces by short acting barbiturates, reduction of prothrombin synthesis giving an extended prothrombin time.

Some researchers reported that about 170 phytocostituents isolated from 110 plants belonging to 55 families were stated to possess liver protective activity; about 600 commercial herbal formulations with claimed hepatoprotective activity are being marketed world-wide. The terminal events in the attack on the liver by carbon tetrachloride involved in the production of a highly reactive radical leading to lipid oxidation and the inhibition of the calcium pump of the microsome giving rise to liver lesions.

A number of plant drugs used for treating biliary disorders are cholagogues. Herbalists prescribe such drugs either singly or more commonly as mixtures; a cholagogue tea, for example, may consists of a mixture of Peppermint leaves 50.0%, Melissa leaves 20.0% (sedative adjuvant), Fennel fruits 20.0% (complementary carminative), Frangula bark 10.0% (gentle laxative).

Some antihepatotoxic and cholagogue drugs are listed below: (Evans, 2006)

1. *Silybum marianum*: This plant, syn. *Carduus marianus* is one of the milk thistles. Indigenous to the Mediterranean region, it has been introduced to most areas of Europe, North and South America and Southern Australia.
2. *Peumus boldus*: Leaves of this plat are collected from the small tree, *Peumus boldus* (Bolodo), indigenous to Chile.
3. *Taraxacum officinale* root (Compositae)
4. *Chionanthus virginicus* (Oleaceae)
5. *Euonymus atropurpureus* (Celastraceae)
6. *Hydrasitis canadenis* (Ranunculaceae)
7. *Juglans cinereae* (Juglandaceae)
8. *Veronicastrum virginicum* (Scrophulariaceae)
9. *Aralia elata* (Araliaceae)
10. *Eclipta alba* (Compositae)
11. *Picrorrhiza kurroa* (Scrophulariaceae)
12. *Schisandra chinenis* (Magnoliaceae)
13. *Javanese turmeric* (*Curcuma xanthorrhiza*)
14. *Wendelia calendulaceae* (Compositae)

Medicinal herbs are significant source of plant drugs. Latest trends have shown increasing demand of phyto-drugs and some medicinal herbs have proven hepatotprotective potential. Silymarin, a flavonol lignan mixture extracted from the *Silybum marianum* (Milk thistle) is a popular remedy for hepatic diseases (Pradhan *et al.,* 2006). Today every herbal company is marketing formulations for liver disorders but the actual scene is that only selected medicinal herbs have been tested for hepatotprotective activity. Some herbal formulations claiming to be hepatoprotective may actually contain chemical constituents having hepatotoxic potential. Andrographolide (*Andrographis paniculata*), Glycyrrhizin (*Glychyrrhiza glabra*), Picrrorihzin (*Picrorrhiza kurroa*) and Hypo-phyllanthin (*Phyllanthus niruri*) are potential candidates with hepatoprotective activity.

Alternative systems of medicine viz. Ayurveda, Siddha, and Traditional Chinese Medicine have become more popular in recent years in treating liver diseases (Eisenberg *et al.*, 1993). Medicinal herbs and extracts prepared from them are widely used to treat hepatitis, cirrhosis and loss of appetite (Cupp, 1999). Medicinal herb is a biosynthetic laboratory, for chemical compounds like glycosides, alkaloids, resins, oleoresins, etc. These exert physiological and therapeutic effect (PDR, 1998). The compounds that are responsible for medicinal property of the drug are usually secondary metabolites.

A number of recent reviews have focused on the adverse effects of herbal products (De Smet, 1997). Some herbs known to be hepatoprotective, their mechanisms of hepatoprotectivity and clinical documentation are available (Malhotra, 2001). In fact some herbal products claiming to be hepatoprotective may actually be having some components with hepatotoxic potential. *Silybum marianum, Picrrorhiza kurroa, Andrographis paniculata, Phyllanthus niruri,* and *Eclipta alba* are proven hepatoprotective medicinal herbs, which have shown genuine utility in liver disorders (Bisset, 1994). These plants are used widely in hepatotprotective preparations and extensive studies have been done on them (Malhotra *et al.*, 2007). Some of the medicinal plants with proven hepatoprotective activity are:

Taraxacum officinale

Traditionally, *Taraxacum officinale* has been used as a remedy for jaundice and other disorders of the liver and gallbladder, and as a remedy for counteracting water retention. Generally, the roots of the plant have the most activity regarding the liver and gallbladder. Oral administration of extracts from the roots of *Taraxacum officinale* has been shown to act as a cholagogue, increasing the flow of bile. Bitter constituents like taraxecerin and taraxcin are active constituents of the medicinal herb (Cordatos, 1992).

Cichorium intybus

Cichorium intybus is a popular Ayurvedic remedy for the treatment of liver diseases. It is commonly known as Kasni and is part of polyherbal formulations used in the treatment of liver diseases. In mice, liver protection was observed at various doses of *Cichorium intybus* but optimum protection was seen with a dose of 75 mg/kg given 30 minutes after CCl_4 intoxication.In preclinical studies an alcoholic extract of the *Cichorium intybus* was found to be effective against chlorpromazine-induced hepatic damage in adult albino rats. A bitter glucoside, Cichorin ($C_{32}H_{34}O_{19}$) has been reported to be the active constituent of the herb (Luczaj *et al.,*2007).

Solanum nigrum

In *Ayurveda*, the drug is known as *Kakamachi.* Aromatic water extracted from the drug is widely prescribed by herbal vendors for liver disorders. Although clinical documentation is scare as far as hepatoprotective activity is concerned, but some traditional practitioners have reported favorable results with powdered extract of the plant (Sultana *et al.,* 1995).

Glychyrrhiza glabra

Glychyrrhiza glabra, commonly known as liqoirice contains triterpene saponin, known as glycyrrhizin, which has potential hepatotprotective activity. It belongs to a group of compounds known as sulfated polysaccharides. Several studies carried out by Japanese researchers have shown glycyrrhizin to be for anti-viral and it has potential for therapeutic use in liver disease (Sanwa, 1985).

Experimental hepatitis and cirrhosis studies on rats found that it can promote the regeneration of liver cells and at the same time inhibit fibrosis. Glycyrrhizin can alleviate histological disorder due to inflammation and restore the liver structure and function from the damage due to carbon tetrachloride. The effects including: lowering the SGPT, reducing the degeneration and necrosis and recovering the glycogen and RNA of liver cells. Effect of glycyrrhizin has been studied on free radical generation and lipid peroxidation in primary cultured rat hepatocytes (Zhao, 1983). Favorable results have been reported in children suffering from cytomegalovirus after treating with glycyrrhizin (Numazaki, 1994).

Wilkstroemia indica

W. indica is a Chinese herb and has been evaluated in patients suffering from hepatitis B. A dicoumarin, daphnoretin is the active constituent of the herb. The drug has shown to suppress HbsAG in Hep3B cells. It is said to activator of protein kinase C (Chen *et al.*, 1996).

Curcuma longa

Like *Silymarin*, turmeric has been found to protect animal livers from a variety of hepatotoxic substances, including carbon tetrachloride, (Srinivas *et al.*, 1991) galactosamine, pentobarbitol, 1-chloro-2,4-dinitrobenzene,7 4-hydroxy-nonenal, (Selvam *et al.*, 1995) and paracetamol. Diarylhepatonoids including Curcumin is the active constituent of the plant.

Tephrosia purpurea

In Ayurveda, the plant is known as Sharpunkha. Alkali preparation of the drug is commonly used in treatment of liver and spleen diseases. In animal models, it offered protective action against carbon tetrachloride and D-galalactosamine poisoning (Murthy *et al.*, 1993). The roots, leaves and seeds contain tephrosin, deguelin and quercetin. The hepatoprotective constituent of the drug is still to be proved.

5.1.13 Use of hepatoprotective plants in traditional medicine

***Treadway,* (1998)** reported the traditional use of medicinal plants like *Terminalia chebula, Cichorium intybus, Piper longum, Terminalia arjuna, Emblica officinalis, Boerhaavia diffusa* and *Phyllanthus niruri* in revitalizing the liver and treating liver dysfunction and disease. Many of these herbs have been evaluated in clinical studies and are currently being investigated phytochemically for better understanding of their actions.

***Gupta et al.,* (2006)** investigated the effect of aqueous ethanolic extract of *Chamomile capitula* on blood and liver glutathione, Na+ K+- ATPase activity, serum marker enzymes, serum bilirubin, glycogen and thiobarbutiric acid reactive substances against paracetamol induced liver damage in rats.

***Madani et al.,* (2008)** had investigated the hepatoprotective effect of polyphenolic extracts of *Silybum arianum* and *Cichorium intybus* on thioacethamide-induced hepatotoxicity in rat.

***Deepak et al.,* (2007)** reported that the chloroform and methanolic extracts of *Ichnocarpus frutescens* have hepatoprotective and antioxidant effects on paracetamol (750mg/kg) induced acute liver damage in Wistar albino rats.

***Samudram et al.,* (2008)** evaluated the hepatoprotective effect of biherbal formulation of ethanolic extracts of *Eclipta alba* leaves and of *Piper longum* seeds at a dose level of 50 mg/kg body weight, administered orally daily once for 14 days against CCl_4 induced hepato-toxicity in albino rats.

5.2 MATERIALS AND METHODS

5.2.1 Plant Materials

The leaves of *Urtica parviflora* Roxb. was collected from Majhitar,East Sikkim, India in March 2006. The plant was identified by the Botanical Survey of India (BSI), Gangtok, Sikkim. The voucher specimen has been retained in our laboratory for future reference. The collected leaves were air dried and pulverized in a mechanical grinder. In the pilot study the other two plants namely methanol fraction of, leaf of *Callicarpa arborea* and root bark of *Morinda citrifolia* did not showed any hepatoprotective activity, thus excluded from the study. Also in this study the ethanolic fraction of *Urtica parviflora* was chosen because of its higher hepatoprotective activity as compared to other fractions.

5.2.2 Preparation of extracts and phytochemical study

The leaves (500 g) were coarsely powered and subjected to successive solvent extraction with petroleum ether (60-80°C), benzene, chloroform, ethanol and water. The solid extracts obtained after complete removal of the solvents under reduced pressure were stored in desicator. The ethanolic extract was suspended in aqueous Tween 80 solution (0.5 %). The chemical constituents of the extracts was identified by qualitative chemical tests and further confirmed by thin layer chromatography for the presence of alkaloids, sterols, tannins, reducing sugars and flavonoids (Trease, 1996).

5.2.3 Animals

Swiss Albino male rats of Sprague Dawley strain, weighing 150-175 g each, were used. They were housed under standard conditions of temperature (23 ±10°C) and relative humidity (55±10%); 12h/12h light/dark cycle and fed with standard pellet feed and water *ad libitum*. The Institutional Animal Ethics Committee reviewed the entire animal protocols prior to the experiments.

5.2.4 Experimental induction of liver damage and treatment

Liver damage was induced in rat by administering CCl_4 subcutaneously (SC) in the lower abdomen at the dose of 1ml /kg body weight except the animals of first group. CCl_4 was administered on every first and fourth day of the week up to 13 weeks (Venukumar *et al.*, 2004).

The rats were divided into 6 groups, 8 animals in each. Group I served as control, receiving Tween 80 solution (0.5%) orally. Group II received only CCl_4. The ethanol extract of *U. parviflora* Roxb leaves was administered orally to groups III, IV and V at a dose of 250, 500 and 750 mg/kg body weight respectively. Reference drug Silymarin (100 mg/kg) was administered orally to Group VI animals in Tween 80 solution (0.5%) (Pradhan *et al.*, 2006). Every day at 9.00 am a known quantity of food was replenished. The animals kept starved

over night one day before the last day of the experiment. On the next day they were sacrificed and blood was collected making an incision on jugular vein.

5.2.5 Enzyme assay

The serum was separated from blood for biochemical estimation by centrifugation at 2500-3000rpm. Different parameters like serum alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) activity were measured according to method of Reitmen and Franckel (Reitman 1957).

5.2.6 Estimation of total protein and bilirubin

The level of total protein (TP) was estimated in serum of the animals by Biuret method (Kingsley *et al.*, 1964). The level of bilirubin was estimated by the method of Mallory *et al.*, (1939).

5.2.7 Histopathologic examination

Liver lobes of the animals were removed and washed with normal saline. Small pieces of liver tissue were preserved in 10% formalin solution for histological analysis. The pieces were dehydrated with 90% ethanol, embedded in paraffin, cut into thin sliced sections (7 µm thick), stained with haematoxylin-eosin dye and observed under a light microscope, for cell necrosis, vascular degenerative changes, inflammation and fibrosis.

5.2.8 Statistical analysis

The data were analyzed statistically using one-way analysis of variance followed by Dunnett's't' test. The data are expressed as mean ± SEM. P values less than 0.05 indicate significance.

5.3 Results of hepatoprotective activity

The microphotographs of the histopathological examination of the liver of the animals under study are presented in the **Fig 5.1 to 5.6**. **Fig 5.1**, exhibits normal architecture of group I rat liver. Single dose of CCl_4 caused centrolobular necrosis extending to midzone with neutrophilic collection in liver of group II animals shown in **Fig 5.2**. Central to central bridging was seen. The cells of centrolobular region showed vacuolated cytoplasm. Vacuolar size showed variations from spherical to large droplet like structures. In most of the necrotic cells centrally placed nuclei were suspended in small amount of cytoplasm, which remains continuous by cytoplasmic strands that traverse through the vacuoles connecting peripheral of cytoplasm. Many of them were dead. Kupffer cells and sinusoidal cells showed arrest in distribution. The administration of ethanol extract at 250 mg/kg body weight protected the

liver partially which is shown in **Fig 5.3**. The extent of the necrotic region was reduced significantly. Numbers of necrotic cells located in this region were considerably reduced and were retained in immediate vicinity of the vein. Most of the cells on the boundary of the necrotic region showed small vacuoles indicating preliminary stage of necrosis. Necrotic region showed the pathological architecture as described above and in the region of healthy cells normal histological structure was evident. Centrolobular region of rats treated with ethanol extract at 500 mg/kg body weight along with CCl_4 showed normal cellular architecture without any necrotic cells that show any type of stress, **Fig 5.4**. Clear bile canaliculi were noted. Distribution of Kupffer cells and sinusoidal cells was normal. The livers of rats were totally normal when treated with ethanol extract and standard drug Silymarin at 750 mg/kg and 100 mg/kg body weight respectively, **Fig 5.5** and **5.6**.

To elucidate the biochemical mechanism of the hepatoprotective activities of the *U. parviflora* extract, the levels of ALT, AST, ALP, total protein and bilirubin were estimated. CCl_4 extensively studied liver toxicants and its metabolites such as trichloromethyl peroxy radical ($CCl_3O_2^-$) are involved in the liver damage (Kamalakkannan, 2005; Sherlock, 2002; Subramonium, 1999). The toxic chemical caused peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes (Mankani *et al.*, 2005). The increase in the levels of serum bilirubin reflected the depth of jaundice and the increase in the transaminases and alkaline phosphatases was clear indication of cellular leakage and the loss of cellular integrity of the cell membrane (Sarawat *et* al., 1993). The results depicted in **Table 5.3** showed that administration of animals with the test formulation at dose of 750 mg/kg body weight returned the enzymes levels, total protein and bilirubin to near normal, which were significantly lower than only CCl4 and close to Silymarin treated animals. Oral administration of *U.parviflora* ethanol leaves extract seems to reverse the hepatic cell damage in a dose dependent manner providing significant protection with a dose 750 mg/kg body weight.

The findings of our study provide some scientific basis for traditional use of *U. parviflora* leaves for managing hepatic disorders. The data obtained are consistent with literature report on hepatoprotective activity of *U.parviflora* leaf using enzyme assays and histopathological examination of liver in rats (Gurung, 1999; Kar *et al.,* 2007).

Table 5.3 Effect of ethanol leaves extract of *U. Parviflora* on CCl_4 induced hepatotoxicity in rats.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
ALT (IU/l)	30.63 ±3.32	120 ± 8.81[a]	118.3 ± 6.44[b]	96.7 ± 6.43[b]	47.9 ±4.98[b]	30.23 ± 1.82[b]
AST (IU/l)	60.12 ± 0.05	65 ± 6.40[a]	148.9 ± 7.76[b]	81.2 ± 5.18[b]	58.3 ±7.41[b]	58.1 ± 4.56[b]
ALP (IU/l)	58.29 ± 0.12	108.2 ± 3.39[a]	89.7 ± 1.73[b]	71.4 ± 4.81[b]	63.7 ±3.67[b]	57.9 ± 1.89[b]
Bilirubin (IU/l)	0.49 ± 0.05	2.67 ± 0.13[a]	1.15 ± 0.12[b]	0.86 ± 0.09[b]	0.74 ± 0.04[b]	0.56 ± 0.02[b]
Total protein (g/dl)	8.15 ± 0.27	5.21 ± 0.13[a]	6.17 ± 0.27[b]	7.56 ± 3.32[b]	9.54 ± 2.13[b]	9.23 ± 1.13[b]

Values are expressed as mean ± SEM from 6 rats in each observation. [a]$P<0.05$ compared to control group, [b]$P<0.05$ compared to CCl_4 treated group.

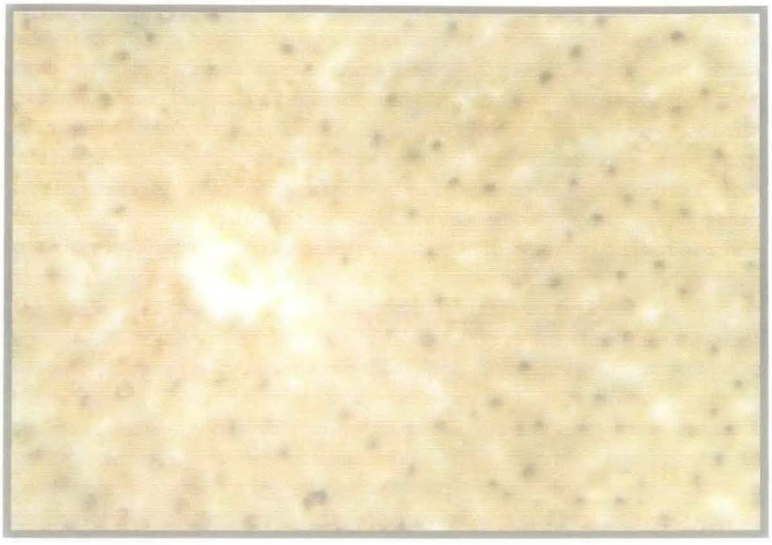


Fig 5.1 Normal rat liver section (M×400), haematoxylin–eosin stain. Liver section of the rat showing normal cellular architecture with distinct hepatic cells, sinusoidal spaces, central vein and vacuole.

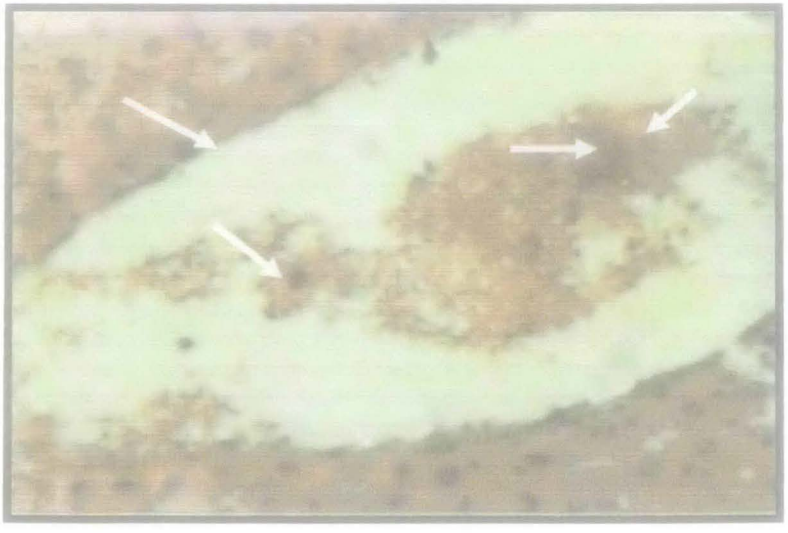


Fig 5.2 Liver section of rat intoxicated with CCl_4, (M×400), haematoxylin–eosin stain. Liver section of the rat showing disarrangement and degeneration of normal hepatic cells with centrilobular necrosis extending to midzone and sinusoidal haemorrhages and dilation.

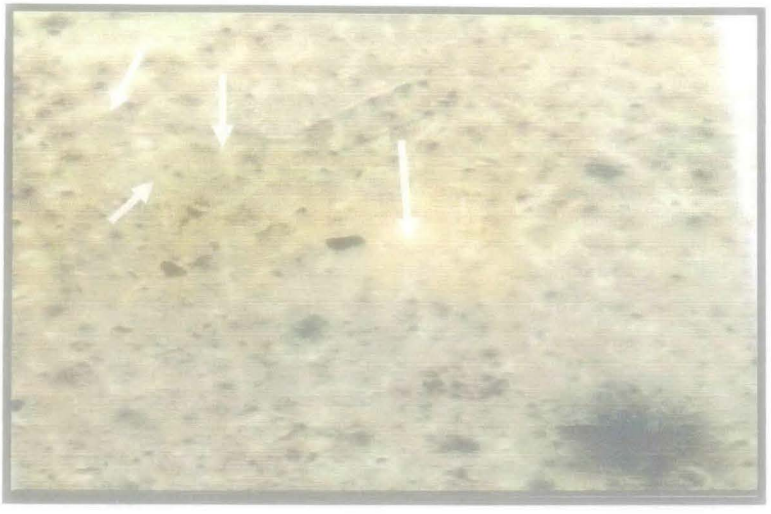


Fig 5.3 Liver section of rat treated with ethanolic extract and intoxicated with CCl_4. (M×400), haematoxylin–eosin stain. Liver section of the rat showing less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes.

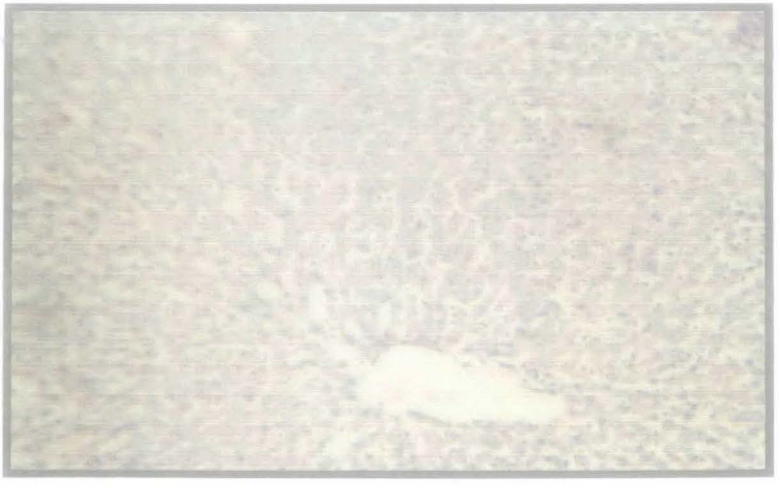
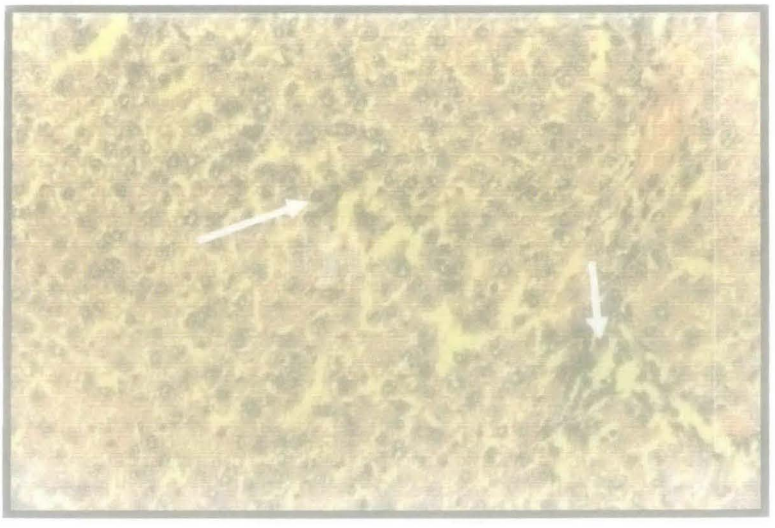


Fig 5.4 Liver section of rat treated with silymarin and intoxicated with CCl_4, (M×400), haematoxylin–eosin stain. Liver section of the rat showing less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes.



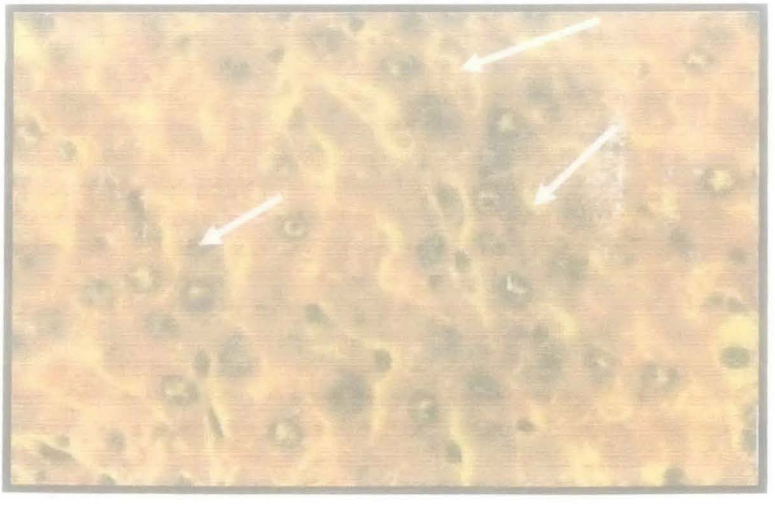


Fig: 5.5 and 5.6. Liver section of rat treated with aqueous extract and intoxicated with CCl_4, (M×400), haematoxylin–eosin stain. Liver section of the rat shows less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes.

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CHAPTER 6

WOUND HEALING ACTIVITY

6.1 INTRODUCTION

6.1.1 Wound Healing

Injury to tissue may result in cell death and tissue destruction. Healing on the other hand, is the body's response to injury in an attempt to restore normal structure and function. Also wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue (Puratchikody, 2006).

Wound care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1–3% of modern drugs (Mantle *et al.*, 2001).

Reports about medicinal plants affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelization and wound contraction are abundant in the scientific literature (Ulubelen *et al.*, 1995; Hemmati *et al.*, 2000; Choi *et al.*, 2001; Bairy, 2002). Still, one should keep in mind that plants have not only beneficial effects in promoting the healing process of wounds and burns or protecting the skin from fungal and bacterial infection or anti-tumor activity against skin cancer, but can also be involved in different allergic, photoallergic and irritant skin reactions (Mantle *et al.*, 2001). Many traditional remedies are based on systematic observations and methodologies and have been time-tested but for many of them, scientific evidence is lacking. There are only few prospective randomized controlled trials that have proved the clinical efficacy of these traditional wound healing agents. **Table 6.1** Includes some plants used in the process of wound healing.

Wounds are referred to as disruption of the normal anatomic structure and function (Gerald *et al.*, 1994). Skin wounds could happen through several causes like physical injuries resulting in opening and breaking of the skin. The most common symptoms of wounds are bleeding, loss of feeling or function below the wound site, heat and redness around the wound, painful or throbbing sensation, swelling of the tissues in the area and puslike drainage (Rashed *et al.*, 2003).

Wound healing is a very complex, multifactor sequence of events involving several cellular and biochemical processes. The aims in these processes are to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin to injury, initiates immediately after wounding and occurs in four stages. The first phase is coagulation, which controls excessive blood loss from the damaged vessels. The next stage of the healing process is inflammation and debridgement of the wound followed by re-epithelialization, which includes proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In the

final stage of the healing process collagen deposition and remodeling occurs within the dermis (Chettibi *et al.*, 1997; Hj Baie *et al.*, 2000).

Table 6.1 Use of following plants is reported in literature for the wound healing process: (George *et al.*, 1999; Hakeem *et al.*, 1989)

Plants	Family	Parts Used
1. *Curcuma longa*	Zingiberaceae	Rhizome
2. *Lawsonia inermis*	Lythraceae	Leaves juice
3. *Terminalia-chebula*	Combretaceae	Flowers
4. *Mallotus philippinensis*	Euphorbiaceae	Fruit
5. *Bambusa arundinaceae*	Graminae	Bamboo
6. *Agave Americana*	Amaryllidaceae	Leaves
7. *Aleo vera*	Lillaeceae	Leaves
8. *Artocarpus communis*	Integrifolia	Fruit
9. *Mirabillis jalapa*	Convolvulaceae	Root
10. *Solanum nigram*	Solanaceae	Leaves
11. *Sterculia urens*	Sterciliaceae	Root and bark
12. *Hibiscus rosa sinensis*	Malavaceae	Flower
13. *Daucus carota*	Umbelliferae	Rhizome
14. *Geum urbanum*	Rosaceae	Plant
15. *Olea europaea*	Oleaceae	Oil
16. *Glycyrrhiza glabra*	Leguminoseae	Root
17. *Plantago major*	Plantaginaceae	Leaves
18. *Ficus carica*	Moraceae	Friuts and leaves
19. *Cinchona officinalis*	Rubiaceae	Bark
20. *Solanum dulcamara*	Solanaceae	Plant
21. *Abutilon indicum*	Malvaceae	Whole plant
22. *Ricinus communis*	Euphorbiaceae	Oil and seeds
23. *Calendula officinalis*	Compositae	Whole plant
24. *Pistacia lentiscus*	Anacardiaceae	Resin
25. *Shorea robusta*	Dipterocarpaceae	Resin
26. *Blessed thistle*	Compositae	Whole plant
27. *Tridax procumbens*	Asteraceae	Whole plant
28. *Areca catechu*	Palmaceae	Seed
29. *Sausurrea lappa*	Sausurreae	Root

6.1.2 Tissue injuries

The injuries are of various types:

(1) Diabetic foot and leg ulcerations, including neuropathic ulcerations, decubitus lesions, and necrobiosis lipoidica diabeticorum (Michell *et al.*, 1997).

(2) Vascular ulcerations, including venous stasis ulceration, arterial ulcerations, varicose vein ulcerations, post-thrombotic ulcerations, atrophie blanche ulcerations, congenital absence of veins/ulcerations, congenital or traumatic arteriovenous anastomosis, temporal arteritis, atherosclerosis, hypertension (Martorell's ulcerations), thrombosis, embolism, platelet agglutination, ankle blow-out syndrome, or hemangiomas.

(3) Decubitus ulcers or pressure sores (e.g., on lying on bed for long time)

(4) Traumatic ulcerations, such as those caused by external injuries, burns, scalds, chemical injuries, post-surgical injuries, self-inflicted injuries, lesions at an injection site, neonatal or perinatal trauma, or sucking blisters.

(5) Infestations and bites, such as those caused by spiders, scorpions, snakes, or fly larvae (mydriasis).

(6) Cold injury, such as perniosis (erythrocyanosis frigida), or cryoglobulinemic ulcerations.

(7) Neoplastic ulceration, such as those caused by basal cell carcinomas, squamous cell carcinomas, malignant melanomas, lymphoma, leukemia, Kaposi's sarcoma, tumor erosion, midline lethal granuloma, or Wegener's granulomatosis.

(8) Blood diseases with ulcerations, such as polycythemia, spherocytosis, or sickle cell anemia.

(9) Skin diseases with ulcerations, such as tinea, psoriasis, pemphigoid, pemphigus, neurotic excoriations, trichotillomania, erosive lichen planus, or chronic bullous dermatosis of childhood.

(10) Metabolic disease ulcerations, such as those associated with diabetes mellitus or gout (hyperuricemia).

(11) Neuropathic ulcerations, such as those associated with diabetes mellitus, tabes dorsalis, or syringomyelia.

(12) Ischemic ulcerations, such as those associated with scars, fibrosis, or radiation dermatitis.

(13) Vasculitis ulcerations, such as those associated with lupus erythematosus, rheumatoid arthritis, scleroderma, immune complex disease, pyoderma gangrenosum, or ulceration associated with lipodermatosclerosis.

(14) Infectious ulcerations, such as: (a) viral ulcerations, e.g. those associated with Herpes simplex or Herpes zoster in an immunocompromised or normal individual; (b) bacterial infections with ulcerations, such as those associated with tuberculosis, leprosy, swimming pool granuloma, ulceration over osteomyelitis, Buruli ulcer, gas gangrene, Meleny's ulcer, bacterial gangrene associated with other bacterial infection (e.g., streptococcal infection), scalded skin syndrome, ecthyma gangrenosum (such as occur in children infected with Pseudomonas aeruginosa), and toxic epidermal necrolysis; (c) mycotic ulcerations, such as those associated with superficial fungal infection or deep fungal infection; (d) spirochetal ulcerations, such as those associated with syphilis or yaws: (e) leishmaniasis; (f) mydriasis; or (g) cellulites.

(15) Surgical ulcerations, such as those associated with closed incisions or excisions, open incisions or excisions, stab wounds, necrotic incisions or excisions, skin grafts, or donor sites.

(16) Other ulcerations, such as those associated with skin tears (traumatic ulcerations), fistula, peristomal ulcerations, ulcerations associated with aplasia cutis congenita, ulcerations associated with epidermolysis bullosa, ulcerations associated with ectodermal dysplasias, ulcerations associated with congenital protein deficiency, ulcerations associated with

congenital erosive and vesicular dermatosis, ulcerations associated with acrodermatitis enteropathica, and amputation stump ulcerations.

Wound healing is a complex biological process that differs according to the wound type: acute or chronic. The principal elements of wound repair are the immediate events of hemostasis and stimulus for inflammation, then inflammation and cell proliferation and migration, followed by molecular synthesis, collagen polymerization and cross-linking, remodeling, and wound contraction. Inflammation is characterized by vasodilation, increased vascular permeability, leukocyte infiltration, bacterial killing, and macrophage-based stimulation of cellular proliferation and protein synthesis.

In cell proliferation and migration, fibroblasts appear within 2-3 days and dominate wound cell population during the first week. For the initial 2-3 days, their activity is confined to fibroblast replication and migration. At days 4-5, fibroblasts begin to synthesize and secrete extracellular collagen. Fibroblasts produce collagen.

Angiogenesis is regulated by a complex cascade of cellular and molecular events and it is essential to wound repair and scar formation (Aplin *et al.,* 2008). Capillary proliferation is required to support fibroblast migration into wound and fibroblast metabolic requirements. In the absence of angiogenesis, such as in ischemic ulcers or arteriosclerosis obliterans, fibroblast migration arrests and wound healing fails to proceed.

Angiogenesis has the stages of cell attachment, basement membrane degradation and migration, proliferation, and differentiation, and is associated with epithelial cell migration.

Molecular synthesis includes collagen synthesis and proteoglycans synthesis. Collagen synthesis begins with the intracellular phase of monomer synthesis. Secretion into the extracellular space then occurs, followed by polymerization into collagen fibers and cross-linking to increase tensile strength.

Remodeling typically begins 3 weeks after injury. Wound remodeling begins and continues for 2 years. There is a progressive increase in tensile strength as Collagen III is replaced by Collagen I. Epithelialization is the hallmark of successful wound repair and occurs in four phases: mobilization, migration, mitosis, and cellular differentiation.

Granulation tissue contains numerous capillaries and has a support matrix rich in fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and periocytes. If vascular endothelial

growth factor (VEGF) is removed, there is an absence of granulation tissue, and wound angiogenesis and the wound healing process cease.

In chronic wound healing, there is typically an absence of epithelial migration, excessive granulation tissue, and fibrosis, with scarring and impaired function possibly being present.

Although many advances have been made in the understanding of wound healing, the healing of wounds still presents a considerable challenge to the clinician. This is particularly true in patients who are diabetic, who have impaired circulation to the skin, or who are susceptible to infection, such as the result of being in an immunocompromised condition. Additionally, when such wounds do heal, they frequently heal with cosmetically undesirable consequences such as scars or discoloration.

Accordingly, there is a need for an improved method of wound healing that is particularly suitable for application in patients with diabetes, who have poor circulation in the skin, or who are immune compromised. There is a further need for treatments and methods that can reduce or eliminate the consequences that can occur from wound healing, such as scars and discoloration. There is an additional need for factors that are well-tolerated and can be used with other treatments in such patients. (Zaveri, 2007)

The process of healing involves two distinct processes. Regeneration when healing takes place by proliferation of parenchyma cells and usually results in complete restoration of original tissues. Repair, when the healing takes place by proliferation of connective tissue elements resulting in fibrosis and scarring.

6.1.3 Regeneration

Some parenchymal cells are short lived while others have a longer life span. In order to maintain proper structure of tissues, these cells are under the constant regulatory control of their cell cycle. Cell cycle is defined as the period between 2 successive cell divisions and is divided in to 4 unequal phases. The regeneration process include growth factors such as epidermal growth factor, fibroblast growth factor, platelet derived growth factor, endothelial growth factor, transforming growth factor 13.

6.1.4 Repair

Repair is the replacement of injured tissue by fibrous tissue. Two processes are involved in repair (Ali *et al.*, 2005):

1) Granulation tissue formation; and 2) Contraction of wounds.

Repair response takes place by participation of mesenchymal cells (consisting of connective tissue stem cells, fibrocytes and histiocytes), endothelial cells, macrophages, platelets and the parenchymal cells of the injured organ.

6.1.4.1 Granulation Tissue Formation

The term granulation tissue derives its name from slightly granular and pink appearance of the tissue. Each granule corresponds histologically to proliferation of new blood vessels which are slightly lifted on the surface by thin covering of fibroblasts and young collagen. The following three phases are observed in the formation of granulation tissue.

1. **Phase of Inflammation:** Following trauma, blood clots at the site of injury. There is acute inflammatory response with exudation of plasma, neutrophils and some monocytes within 24 hours.

2. **Phase of Clearance:** Combination of photolytic enzymes liberated from Neutrophils, autolytic enzymes from dead tissue cells and phagocytic activity of macrophages clear off the necrotic tissue, debris and red blood cells.

3. **Phase of ingrowth of Granulation Tissue:** This phase consist of two main processes- angiogenesis or neovascularisation and formation of fibrous tissue.

a] Angiogenesis (neovascularisation): Formation of new blood vessels at the site of injury takes place by proliferation of endothelial cells from the margins of severed blood vessels. Initially, the proliferated endothelial cells are solid buds but within a few hours they develop a lumen and start carrying blood. The newly formed blood vessels are leakier accounting for the oedematous appearance of new granulation tissue. Soon, these blood vessels differentiate into muscular arterioles, thin walled venules and true capillaries.
The process of angiogenesis takes place under the influence of the following:

i) Endothelial cell growth factors, which act as positive stimuli and appear in granulation tissue.

ii) Some components of matrix like type IV Collagen, which acts as negative stimuli and appear late in the granulation tissue formation.

b] Fibrous tissue formation: The newly formed blood vessels are present in an amorphous ground substance or matrix. The new fibroblasts originate from fibrocytes as well as by mitotic division of fibroblast. Some of these fibroblasts have morphologic and functional characteristics of smooth muscle cells (myofibroblasts).

Collagen fibrils begin to appear by about 6th day. As maturation proceeds, more and more of the collagen is formed while the number of the active fibroblasts and new blood vessels decrease. The results in formation of inactive looking scar known as *cicatrisation.*

6.1.4.2. Contraction of Wounds

The wound starts contraction after 2-3 days and the process completes by the 14th day. During this period, the wound is reduced by approximately 80% of its original size. Contracted wound results in rapid healing since lesser surface area of the injured tissue has to be replaced.

In order to explain the mechanism of wound contraction following factors have been proposed.

1. Dehydration as a result of removal of fluid by drying of wound was first suggested but without being substantiated.

2. Contraction of collagen was thought to be responsible for contraction but wound contraction proceeds at a stage when the collagen content of granulation tissue is very small.

3. Myofibroblasts appearing in active granulation tissue has resolved the controversy surrounding the mechanism of wound contraction. These cells have features intermediate between fibroblasts and smooth muscle cells. Evidences support that migration of fibroblasts in to the wound area and their active contraction decreases the size of the defect. Morphological as well as functional characteristics of modified fibroblasts or myofibroblasts as follows:

a] Fibrils present in the cytoplasm of these cells resemble those seen in smooth muscle cells.

b] These cells contain actin-myosin similar to that found in non-striated muscle cells.

c] The nuclei of these cells have in folding of nuclear membrane like in smooth muscle cells.

d] These cells have basement membrane and demosomes which are not seen in ordinary fibroblasts.

e] The cytoplasm of these modified cells demonstrates immunofluorescent labeling with anti-smooth muscle antibodies.

f] The drug response of granulation tissue is similar to that of smooth muscle.

6.1.5 Mechanism of wound healing

Healing of skin wounds provides a classical example of combination of regeneration and repair described above. This can be accomplished in one of the two ways.

- Healing by first intention (primary union) and
- Healing by second intention (secondary union).

6.1.5.1 Healing by First Intention (Primary Union)

This is healing of wound with the following characteristics:

1. Clean and uninfected
2. Surgically incised
3. Without much loss of cells and tissue, and
4. Edges of wound are approximated by surgical sutures.

The sequence of events in primary union is as described below.

1. Initial hemorrhage: Immediately after the injury, the space between the approximated surfaces of incised wound is filled with blood which then clots and seals the wound against dehydration and infection.

2. Acute inflammatory response: This occurs within 24 hours with appearance of polymorphs from the margins of incision. By 3rd day, polymorphs are replaced by macrophages.

3. Epithelial changes: The basal of epidermis from both the cut margins starts proliferation and migration towards incision space in the form of epithelial spurs. A well- approximated wound is covered by a layer of epithelium in 48 hours. The migrated epidermal cells separate the underlying viable dermis from the overlying necrotic material and blood clot, forming scab. The basal cells from the margins continue to divide. By 5th day a multilayered new epidermis is formed which differentiates into superficial in deeper layer.

4. Organization: On 3rd day, fibroblasts invade the wound area and on 5th day, new collagen fibrils start forming which dominate till healing completes. Within 4 weeks, the scar tissue along with scanty cellular element, vascular elements, a few inflammatory cells and epithelialised surface is formed.

5. Suture tracks: Each suture is a separate wound and incites the same phenomena as in healing of primary wound i.e. filling the space with hemorrhage, some inflammatory cell reaction, epithelial cell proliferation along the suture track is avulsed and remaining epithelial tissue in the track is absorbed. However, the epithelial cells may persist in the track (implantation or epidermal cysts).

Thus the scars formed in sutured wound are neat due to close apposition of the margins of the wound. The use of adhesive avoids removal of sutures and its complications.

6.1.5.2 Healing by Second Intention (Secondary Union)

This is healing of wound having following characteristics (Harsh, 2000):

1. Open with a large tissue defect.
2. Having extensive loss of cells and tissues and
3. The wound is not approximated by surgical sutures but left open.

The basic events in secondary union are similar but differ in having a larger tissue defect, which has to be bridged. Hence healing takes place from the base upwards as well as from the margins inwards. The healing by second intention is slow and results in large, ugly scar as compared to rapid healing and neat scar of primary union.

The sequence of events in secondary union is illustrated as below:

1. Initial hemorrhage: As a result of injury, the wound space fills with blood and fibrin clot which helps drying.
2. Proliferation epithelial cells do not cover the surface fully until granulation tissue.
3. Inflammatory phase: There is an initial acute inflammatory response followed by appearance of macrophages which clears off the debris as in primary union.
4. Epithelial changes: As in primary healing, the epidermal cells from both the margins of wound proliferate and migrate into the wound in the form of epithelial spurs till they meet in the middle and re-epithelise the gap completely. However, from the base has started healing the wound space. In this way pre-existing viable connective tissue is separated from necrotic material and blood clot, forming scab. Regenerated epidermis may become stratified and keratinized.

5. Granulation tissue: The main bulk of secondary healing is by granulations. Granulation tissue is formed by proliferation of fibroblasts and neovascularisation from the adjoining viable elements. The newly- formed granulation tissue is deep red, granular and very fragile. With time, the scar on maturation becomes pale and white due to increase in collagen and decrease in vascularity. The specialized strictures of skin like hair follicles and sweat glands are not replaced unless their viable residues remain, which may regenerate.

6. Wound contraction: contraction of wound is an important feature of secondary healing, which is not seen in primary healing. Due to the action of myofibroblasts present in granulation tissue, the wound contracts to ⅓rd to ¼th of its original size. Wound contraction occurs at a time when active granulation tissue is being formed.

6.1.6 Complication of wound healing

During the course of healing, following complications may occur:

1. Infection of wound: Due to entry of micro-organisms delay the healing.

2. Implantation: Epidermal cyst formation may occur due to persistence of epithelial cells in the wound after healing.

3. Pigmentation: Healed wounds may have rust – like colour due to staining with haemosiderin. Some colored particulate material left in the wound may persist and impart colour to the healed wound.

4. The deficient scar formation: may occur due to inadequate formation of granulation tissue.

5. Incisional hernia: A weak scar, especially after a laparotomy, may be the sight of bursting open of a wound (wound dehiscence) or an incisional hernia.

6. Hypertrophiea scars and keloid formation: At times the scar formed is excessive, ugly and painful. Excessive formation of collagen in healing may result in keloid (claw like) formation, seen more commonly in blacks. Hypertrophied scars differ from keloid in that they are confined to the borders of the initial wound while keloids have tumor-like projection of connective tissue.

7. Excessive contraction: An exaggeration of wound contraction may result in formation of contractures or cicatrisation e.g. Dupuytren's (palmar) contracture, plantar contracture and Peyronie's disease (contraction of the cavernous tissue of penis)

8. Neoplasia: Rarely scar may be the site for development of carcinoma at later stage e.g. squamous cell carcinoma in scar.

6.1.7 Extracellular matrix (wound strength)

The wound is strengthened by proliferation of fibroblasts and myofibroblasts which gets structural support from the extracellular matrix (ECM). In addition to providing structural support, ECM can direct cell migration, attachment, differentiation and organization.

ECM has five main components:

(1) Collagen (2) Adhesive glycoproteins (3) Basement membrane (4) Elastic fibers and (5) Proteoglycans.

1. Collagen

The collagens are a family of proteins which provide structural support to the multicellular organism. It is the main component of tissue such as fibrous tissue, bone, cartilage, valves of heart, cornea, basement membrane etc.

Collagen is synthesized and secreted by a complex biochemical mechanism of ribosomes. The collagen synthesis is stimulated by various growth factors and degraded by collagenase. Regulation of collagen synthesis and degradation takes place by various local and systemic factors so that the collagen content of normal organs remains constant. On the other hand, defective regulation of collagen synthesis leads to hypertrophied scar, fibrosys and dysfunction.

Depending upon the biochemical composition, 18 types of collagen have been identified called collagen type I to XVIII any of which are unique for specific tissues. Type I, III and V are true fibril collagen which form the main portion of the connective tissue during healing of the wounds in scars. Other types of collagen are non-fibril and amorphous material seen as component of the basement membrane.

Morphologically the smallest units of the collagen are collagen fibrils, which align together into parallel bundles to form collagen fibres and then collagen bundles.

2. Adhesive glycoprotein

Various adhesive glycoproteins acting as glue for the ECM and the cells consist of fibronectin, tenascin (cytotacin) and thrombospondin.

a] Fibronectin (nectere=to bind) is the best characterized glycoprotein in ECM and has binding properties to other cells and ECM. It is of two types: plasma and tissue fibronectin.

b] Tenascin or cytotactin is the glycoprotein associated with fibroblasts and appears in wound about 48 hours after injury.

c] Thrombospondin is mainly synthesized by granules of platelets. It functions as adhesive protein for keratinocytes and platelets but is inhibitory to attachment of fibroblasts and endothelial cells.

3. Basement membrane

Basement membranes are periodic acid Schiff (PAS) positive amorphous structure that lies underneath epithelia of different organs and endothelial cells. They consist of collagen type IV and laminin.

4. Elastic fibres

While the tensile strength in tissue is built up in collagen; the ability is recoil by elastic fibres. Elastic fibres consist of two components; elastic glycoprotein and elastic microfibril. Elastases degrades the elastic tissue e. g. in inflammation, emphysema etc.

5. Proteoglycans:

These are a group of molecules having two components; an essential carbohydrate polymer (called polysaccharide or glycosaminoglycan), and a protein bound to it, hence the name Proteoglycans. Various proteoglycans are distributed in different tissue they are as follows.

1. Chondroitin Sulphate- abundant in cartilage, dermis.
2. Heparin Sulphate- abundant in basement membrane.
3. Dermatan Sulphate- abundant in dermis.
4. Keratan Sulphate- abundant in cartilage.
5. Hyaluronic acid – abundant in cartilage and dermis.

The strength of wound also depends upon the factors like the site of injury, depth of incision and area of wound. After removal of stitches on around 7^{th} day, the wound strength is approximately 10% which reaches 80% in about 3 months.

6.1.8 Factors influencing healing

Two types of factors influence the wound healing, those acting locally and those acting in systemic.

A. Local factors

These include the following factors:

1] Infection is the most important factor acting locally which delays the process of healing.

2] Poor blood supply to wound slows healing e.g. injuries to face heal quickly due to rich blood supply while injury to leg with varicose ulcers having poor blood supply heals slowly.

3] Foreign bodies including sutures interfere with healing and cause intense inflammatory reaction and infection.

4] Movement delays wound healing.

5] Exposure to ionizing radiation delays granulation tissue formation.

6] Exposure to ultraviolet rays facilitates healing.

7] Type, size and location of injury determines healing takes place by resolution or organization.

B. Systemic factors

These include:

1] Age: Wound healing is rapid in young and somewhat slow in aged and debilitated people due to poor blood supply to the injured area in the latter.

2] Nutrition: Deficiency of constituents like protein, vitamin C (scurvy) and zinc delays the wound healing.

3] Systemic infection delays wound healing.

4] Uncontrolled diabetics are more prone to develop infection and hence delay in healing.

5] Haematologic abnormalities like defects of neutrophil functions (chemotoxis and phagocytosis), neutropenia and bleeding disorders slow the process of wound healing.

6.1.9 Screening Models for wound healing activity

1) Resutured incision wound models

These models are employed to asses the skin breaking strength in rats. In these models, animals are divided in to groups. Two para-vertebral straight incision of 6 cm each is made through entire thickness of skin on either side at least 1cm lateral to the vertebral column. Wound is sutured with catgut, sutures are removed on 1st post wounding day and the breaking strength is estimated on 10th post wound day by continuous constant water flow technique (Lee *et al.*, 1968, Ali *et al.*, 2005).

2) Excision wound models

The model is employed to study the rat wound contraction and epithelization. In this model, animals are randomly assigned with groups. A round seal of 2.5cm in diameter is impressed on the dorsal thoracic central region, 5cm away from the ears. The entire thickness of the skin from demarked area is excised to get 500mm^2 wound areas. Animals are subjected to the treatment from 0 day till the wound completely healed or up to 21st post wounding day, whichever is earlier. The observations of percentage wound contraction are made on 4th, 5th, 12th and 16th post wounding days, starting from day 2.

3) Dead space wound models

The models usually employed for assessing the extent of collagenation. In this model, rats are divided in to groups. Wound is created by implanting subcutaneously 2.5 X 0.5cm polypropylene tubes in the lumber region on dorsl side of the animal. Animals receive drugs from 0 day to 9 post wounding day. On the 10^{th} day granulation tissue developed around the tube is harvested. The tubular granulation tissue is further cut into approximately equal pieces. The breaking strength is generally measured by continuous constant water flow technique.

Research on wound healing drugs is a developing area in modern biomedical sciences. Scientists who are trying to develop newer drugs from natural resources are looking towards the Ayurveda, the Indian traditional system of medicine. Several drugs of plant, mineral and animal origin are described in the Ayurveda for their wound healing properties under the term *Vranaropaka.* Most of these drugs are derived from plant origin. Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and patients, but the potential of most remains unexplored. In a few cases, active chemical constituents were identified. Some Ayurvedic medicinal plants, namely, *Ficus bengalensis, Cynodon dactyln, Symplocos racemosa, Rubia cordifolia, Pterocarpus santalinus, Ficus racemosa, Glycyrrhiza glabra, Berberis aristata, Curcuma longa, Centella asiatica, Euphorbia nerifolia, and Aloe vera,* were found to be effective in experimental models (Biswas, 2003). In the present study all the three plants i.e. *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* are selected to establish their ethnomedicinal claims for wound healing activity.

6.2 MATERIALS AND METHODS

6.2.1 Plant material

The fresh leaves of *Urtica parviflora (U. parviflora), Callicarpa arborea (C. arborea)*and root bark of *Morinda citrifolia (M. citrifolia)* were collected at Majhitar, East Sikkim and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131, 132 and 133).

6.2.2 Preparation of extracts

The leaves of *U. parviflora* and *C. arborea,* free from dirt were separated and shade dried for ten days and made to powder by a mechanical grinder. The powdered drugs (500g) were extracted with methanol by continuous hot extraction process (soxhelation). The solvent was recovered and the extracts were concentrated under reduced pressure. In case of *Morinda citrifolia* the clean roots are shade dried for twenty days and then subjected to soxhelation

using methanol as the solvent. The extract yield was found to be 5% for *U. parviflora,* 7.5% for *C. arborea* and 11.0% for *M. citrifolia.*

6.2.3 Animal study

Healthy male albino rats weighing between 160-220gm were used in the study. They were individually housed in aseptic condition and maintained on normal diet and water. They were kept in plastic cages at 23 ± 1^0C in 12:12 hr dark: light cycle. All experiments were carried out between 10:00 and 16:00 hrs. The animal experiments were conducted as per protocol approved by the *Institutional Animal Ethics Committee (IAEC) No.* HPI/07/60/IAEC/0005.

6.2.4 Wound Models

6.2.4.1 Excision wound model

For excision wound study, the male albino rats were divided into eight groups, each comprising six animals. They were starved for 12 hrs prior to wounding. Under light ether anaesthesia, wounding was performed aseptically. A circular wound of about 2.5 cm diameters was made on depilated dorsal thoracic region, washed with normal saline and observed during the study. Wounds were traced on 1mm^2 graph paper on the day of wounding and subsequently on alternative days until healing processes were complete. Changes in wound area were calculated, giving an indication of the rate of wound contraction. The alcoholic extracts (5% w/w) were formulated as an ointment prepared by IP method.

The prepared ointments (500 mg) were applied on the wound, once daily for 18 days, starting from the day of wounding in groups VI, VII and VIII. The extracts were orally fed to the animals in the dose of 300 mg/kg in a form of slurry with the help of oral feeders in group III, IV and V. No medication other than the extracts was given to the test groups. The standard group was treated with Framycetin (1%) ointment (Soframycin skin ointment, Aventis). While the control group only received the vehicle (2% gum acacia) orally. The percentage of wound closure was observed on 2nd to 18th post wounding days. The period of epithelialization was calculated as the number of days required for falling of the dead tissue without any residual raw wound (Manjunath, 2005).

6.2.4.2 Incision wound model

Two paravertebral incisions of 6 cm length were made in the skin on either sides of the vertebral column with the help of a sharp blade. The liner wounds are at least 1cm away from the vertebral column. The wounds were sutured using 4-0 number silk thread using a (No11) bend needle. The sutures are spaced 5 mm apart. On 8th day the sutures were removed and breaking strength was determined on 10th post wounding day. The breaking strength was

measured with a manually operated instrument in terms of weight (Nayak *et al.*, 2006). The animals were treated with drugs as in **6.2.4.1** except that the treatment was given up to 9th day only in case of Incision wound model.

6.2.4.3 Dead space wounds

The wounds were made in the region of axilla and groin under light ether anaesthesia where sterilized grass piths of 2.5 cm length and 0.3 cm diameter were introduced in each side to induce granuloma formation. The wounds were sutured and mopped with a saline swab. The animals were treated with drugs except the control group for 9 days from the day of wounding. Granuloma tissues formed on implanted piths were dissected out on the 10th post wounding day. One of the pith was used to determine the tensile strength by manually operated instrument in terms of weight, while the other pith containing the granuloma tissue was used for estimation of hydroxyproline content by Woessner method (Woessner, 1963).

6.2.4.4 Determination of wound breaking strength

The anesthetized animal was secured to the table, and a line was drawn on either side of the wound 3 mm away from the line. This line was gripped using forceps one at each end opposed to each other. One of the forceps was supported firmly, whereas the other was connected to a freely suspended light weight metal plate. Weight was added slowly and the gradual increase in weight, pulling apart the wound edges. As the wound just opened up, addition of weight was stopped and the weights added was noted as a measure of breaking strength in grams. Three readings ere recorded for a given incision wound, and the procedure was repeated on the contralateral wound. The mean reading for the group was taken as an individual value of breaking strength. The mean value gives the breaking strength for a given group (Nayak *et al.*, 2006).

6.2.5 Histopathology Study

The histopathology study was carried on the section of granuloma tissue to observe the stages of keratinization, fibrosis, collagenation, epithelization and neovascularisation. The tissues were fixed in 10% formalin and dehydrated with 90% ethanol, embedded in paraffin, made in to sections of 7µm thickness, stained with haematoxyline-eosin dye and subjected to microscopy. The results are evaluated by numbering 1 to 5. '1' indicates least and '5' indicates maximum similarity with normal tissue in all the groups.

6.2.6 Statistical Analysis:

Values are expressed as Mean ± SEM. Statistical analysis (Graph Pad Prism Software) was made by using Tukey-Kramer Comparisons ANOVA test at different time intervals. $P<0.001$ was taken as significant compared to control.

6.3 RESULTS OF THE WOUND HEALING ACTIVITY

6.3.1 Excision wound model

The result of the excision wound healing model is presented in **Table 6.2.** From the result it is revealed that all the six groups of animals who received the methanolic extracts and ointments of the three plants as mentioned in the method (**6.2.4.1**) showed increased wound contraction continuously from 2nd day to 18th day. The animals of group VII and VIII who received ointment of MECA and MEMC the healing of wound was found to be completed within 14 days in contrast to group V and VI who received MEMC orally and ointment of MEUP respectively whose healing was completed on day 16 only. The group III and IV who received MEUP and MECA orally have shown complete wound contraction on day 18 whereas the control group I failed to show the healing up to 18 days. The groups VII and VIII showed complete healing within 14 days similar to the group II, which received the standard drug Framycetin ointment. Hence the ointments of the extract of *C. arborea* and *M. citrifolia* are comparable to the standard drug Framycetin in healing of wound. The epithelization period was also found to be less in group V (12.6 days) and in group VII (12.9 days) which is similar to the standard drug (Framycetin) treated group (12.5 days)

6.3.2 Incision wound model

The effect of the test drugs in incision wound model is presented in **Table 6.3.** The breaking strength was found to be maximum in group VIII who received ointment of MEMC (710.00 ± 4.22) and is similar to standard drug Framycetin group (Group II) (712.23 ± 2.84). The other two groups who received ointment of MEUP and MECA (group VI and VII) also showed better breaking strength as compared to orally fed drugs groups i.e. group-III, IV and V.

6.3.3 Dead space wound model

The result of dead space wound model is presented in **Table 6.3.** The three parameters namely dry granuloma weight, breaking strength and estimation of hydroxyproline were examined in this model. The two grass piths collected from each of the animal were air dried for two hours and then subjected to the above mentioned tests. Animals of group VIII treated with ointment of MEMC showed maximum dry granuloma tissue weight (72.01 ± 1.19 mg/100g) which is much higher than that of the standard drug Framycetin treated group (62.12 ± 0.38 mg/100g).The MEUP ointment treated group (Group VI) showed the same dry

granuloma tissue weight with that of the standard drug group i.e. (62.12 ± 0.38 mg/100g).The control group showed the lowest dry granuloma tissue weight (26.32 ± 0.41 mg/100g).

The MEMC ointment treated group (group VIII) also showed maximum breaking strength (600.13 ± 4.36g) amongst all the test groups and is comparable to standard drug treated group (group II) which is found to be 612.13 ± 2.31g) The MEMC ointment treated group also showed maximum amount of hydroxyproline (2397.24 ± 2.01µg/100g) which is comparable to the results found in the standard drug treated group of animals (2439.61 ± 0.87µg/100g) which reveals that the extract of the roots of *M. citrifolia* is found to be the most effective in healing of wound among the three plant drugs used in this study.

6.3.4 Histopathology

The results of the histopathological examinations were recorded in five parameters and are presented in **Table 6.4** and **Figure 6.1** to **6.4.** The MECA and MEMC treated groups (groups VII and VIII) showed similar stage of keratinization (4.1 ± 0.09 and 4.1 ± 0.03 respectively) which is comparable to the effect of the standard drug Framycetin in group II (4.2±0.05). Similarly the MEMC ointment treated group showed maximum epithelization (4.2 ± 0.26) comparable to the standard drug treated group (4.3 ± 0.14).

The stage of fibrosis was also found to be of very high value in case of the two plants i.e. *U. parvifolia* and *C. arborea* (4.0 ± 0.13 and 4.0 ± 0.12) but it was maximum with the ointment of the extract of the plant *M.citrifolia* (4.1± 0.32) which is 4.2 ± 0.15 in case of the standard drug Framycetin.

The collagen formation was maximum in group VII (4.4± 0.16), which received MECA ointment amongst the test groups and is comparable to the standard drug treated group i.e. group II (4.5± 0.17). The stage of neovascularization in MECA ointment treated group was found to be 4.4 0.07 which is similar to the standard drug treated group (4.4 0.09). The value in MEUP ointment treated group is also very appreciable i.e. 4.3±0.08, comparable to the standard drug treated group and is much higher than that of the control group (0.6+0.07). Thus the ointment forms of the extracts of two plants *U. parvifolia* and *C. arborea* were found to have maximum wound healing activity.

Table 6.2 Effect of extracts of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* on excision wound model.

(Group) Treatment	Epithelization Period (days)	Excision wound model (% of wound contraction by day)								
		2	4	6	8	10	12	14	16	18
(Gr I) Control	17.4 ±0.81	15.22 ±0.07	32.14 ±0.28	40.73 ±0.41	59.38 ±1.23	79.11 ±1.86	85.66 ±2.78	91.37 ±2.84	95.23 ±2.92	98.46 ±2.89
(Gr II) Framycetin	12.5 ±0.43	19.28 ±0.06	39.32 ±0.19	79.16 ±0.29	86.44 ±1.01	90.21 ±2.81	98.91 ±2.68	100.00 ±2.55	–	–
(Gr III) MEUP 300 mg/kg p.o	14.1 ±0.69	17.01 ±0.11	34.29 ±0.18	54.16 ±0.37	70.98 ±1.98	83.78 ±2.73	89.23 ±2.91	95.66 ±3.02	99.82 ±2.79	100.00 ±2.13
(Gr IV) MECA 300 mg/kg p.o	13.8 ±0.62	16.56 ±0.08	34.97 ±0.22	56.27 ±0.46	72.14 ±2.14	84.19 ±2.05	91.17 ±2.84	94.92 ±2.93	98.71 ±2.62	100.00 ±1.97
(Gr V) MEMC 300 mg/kg p.o	12.6 ±0.72	18.35 ±0.07	37.00 ±0.25	61.11 ±0.39	78.19 ±2.44	87.09 ±2.21	95.13 ±3.01	98.01 ±3.12	100.00 ±2.81	–
(Gr VI) MEUP 5%w/w	13.2 ±0.48	17.91 ±0.09	36.78 ±0.26	71.23 ±0.49	78.53 ±1.69	86.74 ±1.98	95.34 ±2.32	99.33 ±2.05	100.00 ±1.93	–
(Gr VII) MECA 5%w/w	12.9 ±0.51	18.39 ±0.09	37.81 ±0.28	74.18 ±0.47	80.35 ±1.72	88.35 ±2.00	97.62 ±1.93	100.00 ±1.86	–	–
(Gr VIII) MEMC 5%w/w	13.0 ±0.52	18.99 ±0.77	38.23 ±1.31	75.01 ±0.71	83.24 ±2.99	90.00 ±2.97	98.01 ±3.01	100.00 ±3.69	–	–
One way ANOVA F df P	8.967 7, 40 <0.0001	23.052 7, 40 <0.0001	21.003 7, 40 <0.0001	807.21 7, 40 <0.0001	18.279 7, 40 <0.0001	2.557 7, 40 =850.02	3.050 7, 40 =0.0114	1.26395. 7, 40 =0.2932	0.6671 4, 25 0.5986^{N}	0.8693 2, 15 0.1414^{N}

Superscripted N= Not Significant. Other 'P' values are significant, n = 6.

MEUP = Methanolic Extract of *U. parviflora*
MECA = Methanolic Extract of *C. arborea*
MEMC = Methanolic Extract of *M. citrifolia*

Table 6.3 Effect of extracts of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* on wound healing in incision and dead space wound models.

(Group) Treatment	Incision	Dead space		
	breaking strength(g)	Dry granuloma weight(mg/100g)	Breaking strength(g)	Hydroxyproline (µg/100g)
(Gr I) Control	389.87±3.86	26.32±0.41	380.44±1.12	1401.22±0.98
(Gr II) Standard (Framycetin)	712.23±2.84	62.12±0.38	612.13±2.31	2439.61±0.87
(Gr III) MEUP 300mg/kg p.o	634.47±3.91	44.39±0.53	481.37±3.02	1958.12±1.09
(Gr IV) MECA 300mg/kg p.o	656.81±3.89	49.53±0.49	498.11±3.57	1979.26±1.03
(Gr V) MEMC 3000 mg/kg p.o	677.94±4.68	56.12±1.33	541.34±4.58	1997.42±3.01
(Gr VI) MEUP 5%w/w	700.31±3.67	62.12±0.42	587.19±3.46	2198.78±1.20
(Gr VII) MECA5%w/w	709.19±3.54	68.16±0.44	592.49±3.08	2310.16±1.19
(Gr VIII) MEMC 5%w/w	710.00±4.22	72.01±1.19	600.13±4.36	2397.24±2.01
One way ANOVA				
F	789.11	91.460	563.98	69671
df	7,40;47	7,40;47	7,40;47	7,40;47
P	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Values are mean ±SE of 6 replicant

Tukey-Kramer Multiple Comparisons Test

P values are extremely significant.

Table 6.4 Histopathological examinations of wound treated with the aqueous and methanolic extracts of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* at the end of 10 days.

(Group) Treatment	Parameters				
	Keratinization	Epithelization	Fibrosis	Collagen	Neovascula - risation
(Gr I) Control	0.4±0.09	1.6±0.15	2.5±0.17	2.6±0.17	0.6±0.07
(Gr II) Standard (Framycetin)	4.2±0.05	4.3±0.14	4.2±0.15	4.5±0.17	4.4±0.09
(Gr III) MEUP 300mg/kg p.o	3.6±0.16	3.9±0.17	3.7±0.08	4.0±0.15	3.8±0.10
(Gr IV) MECA 300mg/kg p.o	3.8±0.13	3.9±0.18	3.8±0.09	4.1±0.14	3.9±0.09
(Gr V) MEMC 300mg/kg p.o	4.0±0.47	4.0±1.07	3.9±1.88	4.0±0.86	4.1±1.76
(Gr VI) MEUP 5%w/w	4.0±0.08	4.0±0.09	4.0±0.13	4.3±0.14	4.3±0.08
(Gr VII) MECA 5%w/w	4.1±0.09	4.1±0.08	4.0±0.12	4.4±0.16	4.4±0.07
(Gr VIII) MEMC 5%w/w	4.1±1.03	4.2±0.26	4.1±0.32	4.2±0.54	4.0±0.38
Oneway ANOVA					
F	9.680	4.656	0.6228	2.437	3.914
df	7,40;47	5,30;35	7,40;47	7,40;47	7,40;47
P	< 0.0001^{s}	< 0.0007^{s}	< 0.7339^{s}	< 0.0353^{s}	< 0.0024^{s}

Values are expressed ± SEM, n = 6.
Tukey-Kramer Multiple Comparisons Test
's' indicates P values are considered very significant.

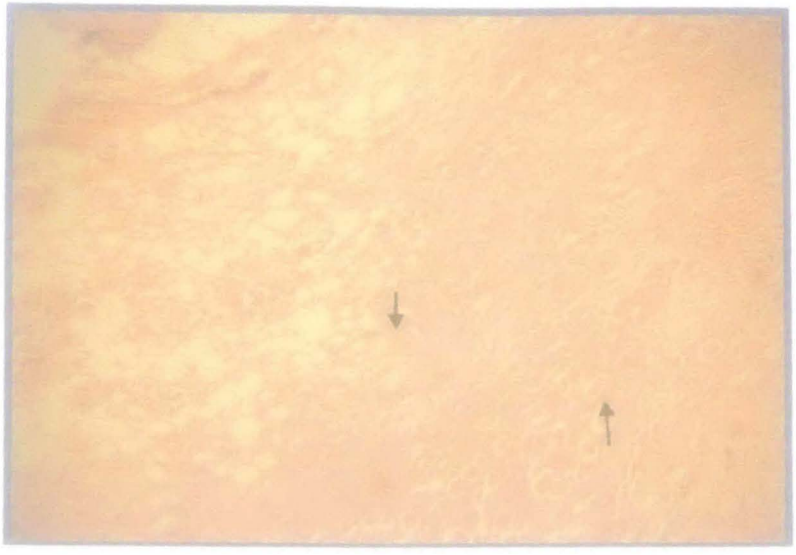

Fig 6.1 Histological section of Granulation tissue of control animal showing more macrophages and less collagen. (M x 400)

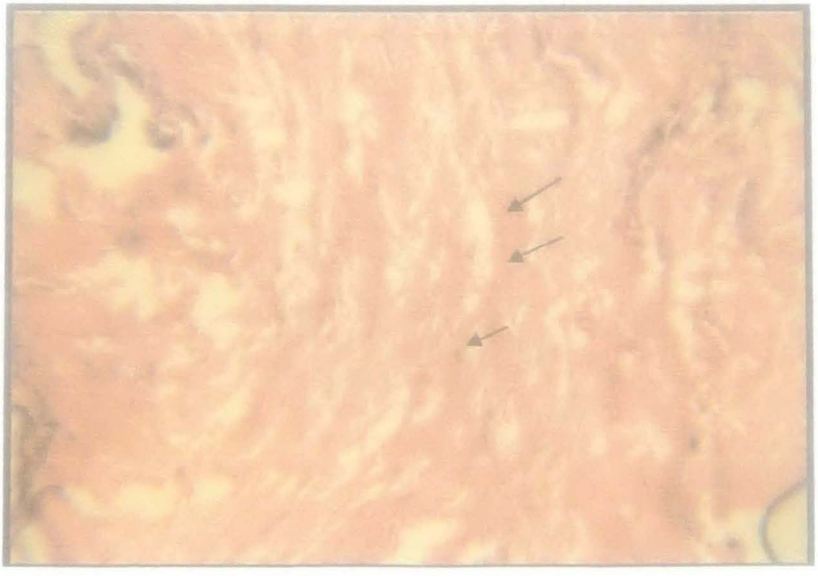

Fig 6.2 Histological section of Granulation tissue of the EEUP ointment treated animal showing very few macrophages and increased collagen deposition. (M x 400)

Fig 6.1 Histological section of Granulation tissue of control animal showing more macrophages and less collagen. (M x 400)

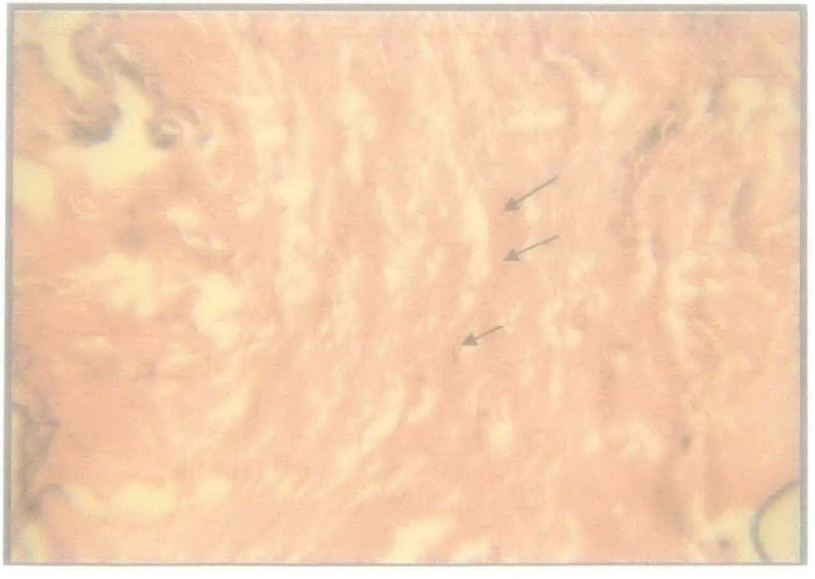

Fig 6.2 Histological section of Granulation tissue of the EEUP ointment treated animal showing very few macrophages and increased collagen deposition. (M x 400)

Fig 6.3 Histological section of Granulation tissue of the EECA ointment treated animal showing collagen fibres well organized into distinct bundles with increased cellularity. (M x 400)

Fig 6.4 Histological section of Granulation tissue of the MEMC ointment treated animal showing collagen fibres well organized into distinct bundles with increased cellularity, joined bundles of collagen fibres with minimal cellularity and increased vascularity. (M x 400)

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CHAPTER 7

ANTIOXIDANT ACTIVITY

7.0 Introduction

7.1 Antioxidants

Antioxidants are the substance that prevent or slow the breakdown of another substance by oxidation. They donate electrons to the free radicals and convert them to harmless molecules. These are substances used by the body to protect itself from damage caused by oxidation. Oxidation is a process that causes damage in our tissues through the work of free radicals and they prevent the oxidation of other chemicals also. In the body, nutrient antioxidants such as beta-carotene (a vitamin A precursor), vitamin C, vitamin E, and selenium have been found (Gamez *et al.*, 1998).

Antioxidants neutralize highly-reactive oxygen free radicals. They act against oxidants to minimize the damage made by them. They are capable of stabilizing or deactivating, free radicals before they attack cells as they are powerful electron donors and react with free radicals before the free radical damage the biomolecules. The formed antioxidant radical is stable and unreactive. The Antioxidants belongs to various classes and act through different mechanism (Nadendla, 2002).

Free radical production is actually a normal part of life. The process of oxidation in the human body damages cell membranes and other structures including cellular proteins, lipids and DNA. When oxygen is metabolized, it creates 'free radicals' which steal electrons from other molecules, causing damage. The body can cope with some free radicals and needs them to function effectively. However, an overload of free radicals has been linked to certain diseases, including heart disease, liver disease and some cancers. Oxidation can be accelerated by stress, cigarette smoking, alcohol, sunlight, pollution and other factors. Antioxidants counteract these cellular byproducts and bind with them before they can cause damage (Corvilain *et al.*, 1991).

7.1.2 Free radicals

Free radicals can be defined as chemical species associated with an odd or unpaired electron. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally to achieve stable configuration (Nadendla, 2002). Harmful free radicals are toxic molecules of oxygen that damage the body. A free radical is an unstable incomplete molecule because it is missing an electron which exists in pair in stable molecules. Free radicals steal an electron from another molecule, thereby create another free radical. This new free radical then duplicates the process, resulting in a chain reaction of events, which can ultimately damage the body. Free radicals are natural by-products of ongoing biochemical reactions in the body, including ordinary metabolic processes and immune system responses. The common free radicals are reactive oxygen species (ROS), namely, superoxide radical, hydroxyl radical and peroxyl radical which can be internally produced by cellular metabolism, inflammation by immune cells and externally by radiation, pharmaceuticals, hydrogen peroxide, toxic chemicals, smoke, alcohol, oxidized polyunsaturated fats

and cooked food. Free radicals can cause damage to parts of cells such as proteins, DNA, and cell membranes by stealing their electrons through a process called oxidation. Reactive oxygen free radicals have been known to produce tissue injury through covalent binding and lipid peroxidation and have been shown to augment fibrosis as seen from increased collagen synthesis (Geesin *et al.*, 1990). Scavenging of free radicals by antioxidants could reduce the fibrosis process in the tissues (Thresiamma *et al.*, 1996). Free radicals may also be a contributory factor in a progressive decline in the function of the immune system (Pike *et al.*, 1995). The defense systems that protect the body from free radical damage include the antioxidant nutrients and enzymes.

Free radicals such as reactive oxygen species (ROS) play important role in the etiology of number of diseases including cardiovascular, ischemic disease, and aging processes (Halliwell *et al.*, 1992; Gutteridge, 1993; Halliwell *et al.*, 1995). When antioxidants are employed in the treatment of such diseases, they participate in body defense mechanism against ROS, which include variety of enzymes such as superoxide dismutase (SOD), which produces hydrogen peroxide from superoxide radicals, catalase (CAT) and glutathione-related enzymes, which decompose hydrogen peroxide (Halliwell, 1990 and Trocino *et al.*, 1995).

7.1.3 The health benefits of antioxidants

Antioxidants work by neutralizing highly reactive, destructive compounds, the free radicals. In biological systems, the normal processes of oxidation produce highly reactive free radicals. Antioxidants work by binding to the free radicals; they transform them into non-damaging compounds or repairs cellular damage. Antioxidants are able to easily donate electrons to molecules in need of an electron, such as free radicals, before they steal one from someplace else, thus stabilize and prevent a damaging chain reaction. The antioxidant molecules interact with the oxygen free radicals and halt the spread of cancer causing cells with damaged DNA before other vital molecules are damaged. Antioxidants help neutralize the production of free radicals which are chemical complexes that cause harm to our cells and play a major role in the disease process. Antioxidants serve as a source of electrons that can be provided to free radicals without damaging the cell components. Antioxidants prevent unstable oxygen molecules (made unstable by loss of one electron) from interacting with other molecules (taking one of their electrons) and consequently causing them to become unstable, a process that starts the free-radical chain reaction. The rationale for the use of antioxidants is well established in prevention and treatment of chronic diseases where oxidative stress plays a major role (Mukherjee *et al.*, 2003 and Rajlakshmi *et al.*, 2003). There are number of lipophilic and hydrophilic low molecular- weight antioxidants, which directly reacts and scavenge the ROS (Sharma *et al.*, 1993).

Antioxidants help alleviate the symptoms and side effects of many of these diseases. According to the free radical theory, radicals damage cells in an organism, causing aging. Antioxidants break the free radical chain reaction by sacrificing electrons, and then humbly existing without stealing more. The body naturally circulates many nutrients for their antioxidant properties, and creates antioxidant enzymes just for the purpose of controlling free radicals and their chain reactions. Antioxidants are thought to thwart heart disease by preventing oxidation. Antioxidants combat chronic inflammation. Vitamin E suppresses platelet stickiness, acting as an anticoagulant to discourage the formation of clots that lead to heart attacks. Vitamin C decreases a blood factor needed to build clots.

Antioxidants fight cancer by neutralizing DNA-damaging free radicals. Antioxidants neutralize free radicals as the natural by-product of normal cell processes. Antioxidants prevent injury to blood vessel membranes, helping to optimize blood flow to the heart and brain, defend against cancer-causing DNA damage, and help lower the risk of cardiovascular disease and dementia, including Alzheimer's disease.

7.1.4 Commonly used antioxidant supplements

Antioxidants are found in the nutrient antioxidants, vitamins A, C and E, and the minerals copper, zinc and selenium. Other dietary food compounds, such as the phytochemicals in plants and zoochemicals from animal products, are believed to have greater antioxidant effects than either vitamins or minerals. These are called the non-nutrient antioxidants and include phytochemicals, such as lycopenes in tomatoes, and anthocyanins found in cranberries. Some antioxidants are made in our cells and include enzymes and the small molecules glutathione, uric acid, coenzyme Q10 and lipoic acid. Antioxidant compounds must be constantly replenished since they are "used up" (converted) in the process of neutralizing free radicals. The repair enzymes that can regenerate some antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase and the other metalloenzymes. Their roles as protective enzymes are well known and have been investigated extensively with *in vivo* models. Several medicinal plants have been screened based on the integrative approaches on drug development from Ayurveda (Mukherjee *et al.*, 2006). Superoxide dismutase is the most important antioxidant enzyme, catalyses the removal of superoxide free radicals in all aerobic and anaerobic organisms, and catalase *in vivo* plays an important role for removal of damaging effects caused by ROS in living systems. Generation of ROS also plays an important role in the etiology of diabetic complications. Under diabetic conditions, ROS are produced via glucose autoxidation (Wolff *et al.*, 1987). Oxidative damage occurs as a consequence of an imbalance between the formation and inactivation of oxygen free radicals. This process leads to the destruction of membrane lipids and production of lipoperoxides and their products. Inactivation and removal of ROS depend on

relations with antioxidative defense mechanisms (Serafini *et al.*, 2004). The free radical nitric oxide (NO) is derived from endothelium and it is a potent vasorelaxant that elicits its effects by activating soluble guanylate cyclase (sGC), thereby stimulating the formation of cyclic guanosine monophosphate (cGMP) (Fukuto *et al.*, 1993). Since the measurement of the NO radical itself is difficult because of being a radical with poor stability with a very short half life, measurement of the end products of NO as nitrite and nitrate (NO^{2-}/NO^{3-}) is often used as a marker for the production of NO radicals (Moshage *et al.*, 1995).

7.1.4.1 Vitamin C (Ascorbic acid)

Vitamin C neutralizes potentially harmful reactions in the watery parts of the body, such as the blood and the fluid inside and surrounding cells. Vitamin C may help decrease total and LDL cholesterol and triglycerides, as well as increased HDL levels. The antioxidant activity of Vitamin C is helpful in the prevention of some types of cancers and cardiovascular diseases. The antioxidant properties of vitamin C are thought to protect smokers, as well as those exposed by passive smoking, from the harmful effects of free radicals. As a powerful antioxidant, vitamin C may help to fight cancer by protecting healthy cells from free-radical damage and inhibiting the proliferation of cancerous cells. The body does not produce vitamin C. Foods containing the highest sources of vitamin C include green peppers, citrus fruits and juices, strawberries, tomatoes, broccoli, turnip greens and other leafy greens, sweet and white potatoes, and cantaloupe.

7.1.4.2 Vitamin E

Vitamin E is the most effective, fat-soluble antioxidant known to occur in the human body. Vitamin E is an antioxidant that prevents free radical damage in biological membranes. Free radicals can cause cell damage that may contribute to the development of cardiovascular disease and cancer. Vitamin E helps protect against heart disease by limiting the oxidation of LDL-cholesterol. Vitamin E helps prevent oxidation of lipoproteins, particularly in smokers, and reduces the stickiness of platelets in the bloodstream. Vitamin E as an antioxidant helps to stabilize cell membranes and protect the tissues of the skin, eyes, liver, breast, and testes, which are more sensitive to oxidation. Vitamin E is found in many common foods, including vegetable oils (such as soybean, corn, cottonseed and safflower) and products made from these oils (such as margarine), wheat germ, nuts and green leafy vegetables, although the researchers evaluated only the pill form of the vitamin.

7.1.4.3 Beta-carotene

Foods rich in beta-carotene protect the body from the damaging molecules (free radicals). The antioxidant actions of Beta-carotene make it valuable in protecting against, and in some cases even reversing, precancerous conditions affecting the breast, mucous membranes, throat, mouth, stomach, prostate, colon, cervix, and bladder. Individuals with highest levels of beta-carotene intake have lower risks of lung cancer, coronary artery heart disease, stroke and age-related eye disease than individuals with lowest levels of beta-carotene intake. The richest sources of beta-carotene are yellow, orange, and green leafy fruits and vegetables (such as carrots, spinach, lettuce, tomatoes, sweet potatoes, broccoli, cantaloupe, and winter squash).

7.1.4.4 Selenium

Selenium is a nonmetallic chemical element. Selenium is used in free radical elimination and other antioxidant enzymes and also plays a role in the functioning of the thyroid gland. Selenium is the central element in glutathione peroxidase (GPx), an antioxidant enzyme that protects cells against the oxidative damage caused by peroxides and free radicals. Selenium forms part of the structure of the important antioxidant enzyme glutathione peroxidase, which in turn recycles glutathione. Dietary selenium comes from cereals, meat, fish, and eggs. Brazil nuts are a particularly rich source of selenium.

Oxygen-dependent deterioration of lipids, known as rancidity is a major problem in the storage of oils (mainly olive oil) but was also considered useful as far back as the 15th century in preparing siccative oil, paints and printing inks. The same oxidation process is also considered important today for natural products used in human consumption such as fats, oils, dressings or margarine but also for chemical and industrial products such as paints, inks, resins, varnishes or lacquers.

The first study of this lipid oxidation problem was those of the Swiss chemist Nicolas-Théodore de Saussure who observed around 1800, using a simple mercury manometer, that a layer of walnut oil exposed to air was able to absorb about 150 times its own volume of oxygen during a one year period. Parallel with these changes, oil became viscous and had a bad smell. Later, Berzelius (who discovered selenium) suggested that this oxidation might be involved in the spontaneous ignition of wool lubrified with linseed oil in textile mills.

Systematic studies of lipid autoxidation may be considered to have begun around the 40s since (Criegee *et al.*, 1939) established that hydroperoxides are the primary products of hydrocarbon oxidation.The major credit for developing the hydroperoxide hypothesis of lipid autoxidation is due to Farmer E J, (Farmer, 1943). Bolland (Q Rev., 1949) established that the primary autoxidation

products of linoleic acid are hydroperoxides (on carbon atom 9 or 13) containing conjugated dienes (Lundberg, *et al.,* 1961). Since the early 1960's, our understanding of the oxidation of unsaturated lipids has advanced considerably as a result of the application of new analytical tools. Detailed studies of the products of polyunsaturated fatty acids were initiated in the 70's by several research groups, revealing more complex mixtures than those previously proposed (Porter, *et al.,* 1980). With the help of HPLC, several hydroperoxide products could be separated after autoxidation of arachidonic acid (Porter, *et al.,* 1980) including products of lipoxygenase action (Porter, *et al.,* 1980). The first demonstration of free radical oxidation of membrane phospholipids was given in 1980, (Porter, *et al.,* 1980) leading to a new fruitful era with a continuous flow of innumerable works devoted to chemistry, biochemistry and medicine.

7.2 Free Oxygen Radicals

Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. Their formation occurs in enzymatic or non-enzymatic reactions involving activated chemical species known as "reactive oxygen species" (ROS) which are responsible for toxic effects in the body via various tissue damages. These ROS include among others hydroxyl radicals, lipid oxyl or peroxyl radicals, singlet oxygen, and peroxinitrite formed from nitrogen oxide (NO), all these groups of atoms behave as a unit and are now named "free radical". These chemical forms are defined as any species capable of independent existence that contains one or more unpaired electrons (those which occupy an atomic or molecular orbital by themselves). They are formed either by the loss of a single electron from a non-radical or by the gain of a single electron by a non-radical. They can easily be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, this mechanism is known as homolytic fission. In water, this process generates the most reactive species, hydroxyl radical OH. Chemists know well that combustion which is able at high temperature to rupture C-C, C- H or C-O bonds is a free-radical process. The opposite of this mechanism is the heterolytic fission in which, after a covalent break, one atom receives both electrons (this gives a negative charge) while the other remains with a positive charge. In eukaryotic organisms, ROS are mainly generated during the normal respiration process involving oxygen, oxidases and electron transports in mitochondria or endoplasmic reticulum.

Oxygen is known since a long time as poisonous as well in plants as in animals or bacteria, mainly when supplied at concentrations greater than those in normal air. Thus, oxygen is able to damage plant tissues in inhibiting chloroplast development, seed viability and root growth. The growth of bacteria (Escherichia coli) was shown to be slowed down by pure oxygen, the toxic effect being enhanced by ionizing radiations. Very early, it appears that the effects of oxygen and those of ionizing radiations on organisms have many similarities. In man, the toxicity of oxygen was studied

in relation with diving, life in submarines and spacecrafts, treatment of some pathologies such as cancer, sclerosis or gangrene. Exposure of man to oxygen at 1 atmosphere pressure causes chest soreness, cough, sore throat, damage to lung alveoli and acute central nervous system toxicity. This toxicity was at the origin of the frequent blindness observed in the early 1940s and solved only around 1954 among infants born prematurely and kept in incubators fed with high oxygen concentrations. Several observations led Gershman R and Gilbert DL to propose in 1954, that most of the damaging effects of oxygen could be attributed to the formation of free oxygen radicals (ROS). Several reactive oxygen species (ROS) are known. Among them, the most frequently studied are given below.

7.2.1 Superoxide radical ($O_2^{\cdot-}$)

This ROS is formed when oxygen takes up one electron and as leaks in the mitochondrial electron transport but its formation is easily increased when exogenous components (redox cycling compounds) are present. Its first production site is the internal mitochondrial membrane (NADH ubiquinone reductase and ubiquinone cytochrome C reductase). This species is reduced and forms hydrogen peroxide (H_2O_2). The production of superoxide radicals at the membrane level (NADPH oxidase) is initiated in specialized cells (oxidative burst) with phagocytic functions (macrophages) and contributes to their bactericid action. The flavin cytosolic enzyme xanthine oxidase found in quite all tissues and in milkfat globules generates superoxide radicals from hypoxanthine and oxygen and is supposed to be at the origin of vascular pathologies.

7.2.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxysomes and mitochondria. Even in normoxia conditions, the hydrogen peroxide production in relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. In plant and animal cells, superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\cdot-}$ thus contributing to the lowering of oxidative reactions. The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. H_2O_2 is also able to diffuse easily through cellular membranes.

7.2.3 Hydroxyl radical ($^{\cdot}OH$)

In the presence of Fe^{2+}, H_2O_2 produces the very active species $^{\cdot}OH$ by the Fenton reaction.

$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + {}^{\cdot}OH + OH^-$

This iron-catalyzed decomposition of oxygen peroxide is considered the most prevalent reaction in biological systems and the source of various deleterious lipid peroxidation products. Another

reaction involving myeloperoxidase and Cl- ions represent an important OH- production process in neutrophils during phagocytosis.

7.2.4 Nitric oxide ($^{\cdot}NO$)

Nitric oxide is produced in various types of cells is well studied in vascular endothelium. While this species is not too reactive (poorly oxidizing function), it reacts rapidly with $O_2^{\cdot-}$ and gives the extremely reactive peroxinitrite (ONOO-). This ROS is naturally formed in activated macrophages and endothelial cells and is considered as an active agent in several pathologies based on inflammation, organ reperfusion and also may play an important role in atherosclerosis.

7.2.5 Singlet oxygen ($^1O_2$)

This chemical form of oxygen is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition (UVA, 320-400 nm). Its toxicity is reinforced when appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen. Several natural sensitizers are known to catalyzed oxidative reactions such as tetrapyrroles (bilirubin), flavins, chlorophyll, hemoproteins and reduced pyridine nucleotides (NADH). Some of these sensitizers are also found in foods and cosmetics. Some others are used for therapeutic purposes (anticancer treatments) and are sensitive to visible light. The presence of metals contributes to increase the production of singlet oxygen, as well as anion superoxide, and thus accelerates the oxidation of unsaturated lipids generating hydroperoxides. It has been suggested that singlet O_2 may be formed during the degradation of lipid peroxides and thus may cause the production of other peroxide molecules. This singlet O_2 formation may account for the chemiluminescence observed during lipid peroxidation.

7.2.6 Ozone (O_3)

This natural compound present in the higher atmosphere and in the lower atmosphere of our polluted cities is a major pollutant formed by photochemical reactions between hydrocarbons and nitrogen oxides. Ozone is not a free radical but, as singlet oxygen, may produce them, stimulates lipid peroxidation and thus induces damages at the lipid and protein levels in vivo mainly in airways. The exact chemistry of ozone-mediated stimulation of peroxidation is not entirely known. Ozone may add on across a double bond and decomposes to form a free radical.

7.2.7 Thiyl radicals ($RS^{\cdot}$)

Thiol compounds (RSH) are frequently oxidized in the presence of iron or copper ions:

$RSH + Cu_2^+ \text{----> } RS^{\cdot} + Cu^+ + H^+$

These thiyl radicals have strong reactivity in combining with O_2:

$RS^{\cdot} + O_2 \text{---> } RSO_2^{\cdot}$

Furthermore, they are able to oxidize NADH into $NAD^{\cdot}$, ascorbic acid and to generate various free radicals ($^{\cdot}OH$ and $O_2^{\cdot-}$). These thiyl radicals may also be formed by homolytic fission of disulfide bonds in proteins.

7.2. 8 Carbon-centered radicals

The formation of these reactive free radical is observed in cells treated with CCl_4. The action of the cytochrome P450 system generates the trichloromethyl radical ($^{\cdot}CCl_3$) which is able to react with oxygen to give several peroxyl radicals (i.e. $^{\cdot}O_2CCl_3$). Reactive oxygen species (ROS) play important role in the etiology of number of diseases including cardiovascular, ischemic disease, and aging processes (Halliwell *et al.*, 1992; Gutteridge, 1993; Halliwell and Gutteridge, 1995). When antioxidants are employed in the treatment of such diseases, they participate in body defense mechanism against ROS, which include variety of enzymes such as superoxide dismutase (SOD), which produces hydrogen peroxide from superoxide radicals, catalase (CAT) and glutathione-related enzymes, which decompose hydrogen peroxide (Halliwell, 1990; Trocino *et al.*, 1995). Excess production of free radicals impacts the pathogenesis and progression of various diseases (Visioli *et al.*, 2000). Lipid peroxides, produced from unsaturated fatty acids via free radicals causes toxic effects and promote the formation of additional free radicals in a chain reaction. Deficient activity of enzymes or scavengers which takes part in neutralizing these free radicals, leads to development of oxidative stress related diseases. The rationale for the use of antioxidants is well established in prevention and treatment of chronic diseases where oxidative stress plays a major role (Mukherjee *et al.*, 2003; Rajlakshmi *et al.*, 2003). There are number of lipophilic and hydrophilic low molecular-weight antioxidants which directly react and scavenge the ROS (Sharma *et al.*, 1993).

7.3 Assay methods

7.3.1 1, 1-Diphenyl-2-picryl hydrazyl radical scavenging activity (DPPH assay)

The antioxidant activity of a substance and the standards can be assessed on the basis of the radical scavenging effect of the DPPH free radical (Gamez *et al.*, 1998; Raja *et al.*, 2005; Sundararajan *et al.*, 2006). The ability of the test substance to scavenge the free radicals can be determined by an *in vitro* assay method using a stable free radical DPPH (1,1-Diphenyl-2-picryl hydrazyl) (Soni *et al.*, 2003). In this method to the ethanolic solution of DPPH (in μM), an equal volume of the test substance is dissolved in ethanol and maintained for at least 20 minutes. The

decrease in absorbance of test mixture due to quenching of DPPH free radical is read at 517nm (Soni *et al.,* 2003).

7.3.2 Superoxide anion scavenging activity assay

The scavenging activity of a substance towards superoxide anion radicals can be measured by this method (Liu *et al.,* 1997). Superoxides are generated in a non enzymatic phenazine methosulphate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It is assayed by the reduction of Nitroblue tetrazolium (NBT). In this method the superoxide anion is generated by Tris-HCl buffer containing NBT solution, NADH solution and different concentration of the test substance. The reaction is initiated by adding PMS to the mixture. After 5 minutes of incubation at room temperature, the absorbance at 560nm is measured by spectrophotometer. The superoxide anion scavenging activity is calculated by the equation:

$$\% \text{ inhibition} = [(A_0 - A_1)/ A_0 \times 100] \quad \text{..... Eqn (1)}$$

Where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance of the test substance.

7.3.3 Nitric oxide radical inhibition assay

Nitric oxide radicals are generated by sodium nitroprusside in aqueous solution at physiological pH, on interaction with oxygen it produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction (Garrat, 1964). Griess Illosvoy reagent can be modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Maracocci *et al.*, 1994; Mukherjee, 2002; Badami *et al.*, 2003).

7.3.4 Lipid peroxidation assay

Lipid peroxidation is evaluated by measuring both thiobarbituric acid reactive substances (TBARS) generation and 4-hydroxynonenal (HNE) concentration. TBARS generation is measured spectrophotometrically at 543nm. Mesangial cells (2·5X106 cells/ml) are incubated for up to 4 h at 37^0C in a shaking bath. For the standard curve, malonildialdehyde bis-(dimethylacetal), followed by acid hydrolysis is used. The HNE concentration can be evaluated by the method described by Esterbauer *et al.* (1991).

7.3.5 Fe (II)-EDTA-H_2O_2 induced oxidative DNA damage assay

In this method the solution of DNA is prepared by dissolving 2 mg of calf thymus DNA in 1 ml of 10 mM tris-HCl pH 7.4, 500 µg DNA and varying concentration of extract (25 - 250 µg) solution, 0.08 mM EDTA, 0.08 mM $FeSO_4$, 0.03% H_2O_2 and 20 mM Na-ascorbate. All solutions are sterilized before use. After incubation at 37°C for 1 hour, DNA nuclease digestion is performed as described by Rahman *et al.*, (1989). The assay determines the acid soluble nucleotides released from DNA

because of enzymatic digestion. Acid soluble nucleotides are determined colorimetrically using the diphenylamine method of Schneider (1957). To a 1.0 ml aliquot, 2.0 ml of diphenylamine reagent (freshly prepared by dissolving 1 g of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4) is added. The tubes are heated in a boiling water bath for 20 min and the intensity of blue color is read at 600 nm.

7.4 Material and methods

7.4.1 Plant materials

Methanol extracts of *Urtica parviflora, Callicarpa arborea* leaves and *Morinda citrifolia* root along with their isolated compounds (described in Chapter 3) were used as test drug in these experiments.

7.4.2 Animals

Wistar albino rats (200–250g) of either sex were maintained under standard environmental conditions and had free access to feed and water. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee.

7.4.3 Instruments, chemicals and drugs

UV-Visible double beam spectrophotometer (Shimadzu Corporation, Japan), cold centrifuge (Remie India Ltd.), mechanical stirrer were used in the experiment. Thiobarbituric acid was obtained from Loba Chemie, India. 1, 1-Diphynyl-2-picryl hydrazyl (DPPH), NADH and nitroblue tetrazolium (NBT) were obtained from Sigma chemicals, St. Louis, USA. Dimethyl sulphoxide, phenazine methosulphate were of analytical grade and were obtained from Ranbaxy fine chemicals.

7.4.4 Antioxidant activity of *Urtica parviflora* leaf

7.4.4.1 1, 1-Diphenyl-2-picryl hydrazyl radical scavenging activity (DPPH assay)

The antioxidant activity of the plant drugs and the standard were assessed on the basis of the radical scavenging effect of the DPPH free radical (Gamez *et al.*, 1998; Raja *et al.*, 2005; Sundararajan *et al.*, 2006). The DPPH scavenging activity of *Urtica parviflora* was measured by spectrophotometric method. To each of the nine glass tubes methanolic solution of DPPH (100 µM, 2.95ml) was taken. Tube No. 1 was treated as control without test compound where 0.05 ml of methanol was added. The second tube contained the standard compound vitamin E. To the tubes numbered from three to seven methanol extract of *Urtica parviflora* (0.05 ml) was added at concentration ranging from 200 to 1000 µg/ml at interval of 200 µg/ml between the tubes. To the tubes numbered eighth and ninth compound I was added in concentration 25 and 50 µg/ml. Absorbance of each tube was recorded at 517nm at regular intervals of 10 min up to 40 min. The

scavenging activity in percentage of inhibition was calculated by comparing the control and test samples applying the **Equation 1** mentioned in **7.3.2.**

7.4.4.2 Lipid peroxidation model

Rat liver homogenate preparation: Anaesthetized adult rats were dissected to take out the liver. The liver lobes are washed with 0.9% sodium chloride solution. Liver lobes are subjected to homogenization using Teflon homogenizer, in ratio 1: 10 of 0.05 M phosphate buffer (pH 7.4). The homogenate is used for the estimation of thiobarbituric acid reactive substances (TBARS). The extent of lipid peroxidation of the homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method (Okhawa *et al.*, 1979; Raja *et al.*, 2005; Sundararajan *et al.*, 2006).with minor modifications (Pandey, 1995). The content was centrifuged at 2000 rpm for 10 min and supernatant was diluted with phosphate buffer saline up to final concentration of protein 0.8-1.5 mg/0.1 ml. Protein concentration was measured by using standard method of Lowery *et al.*, (1951). To study the comparative response, the experiment was performed in nine glass Petri dishes (30 mm ID). Liver homogenate (3ml) was aliquoted to each of the petri dishes. The dish number one and two were treated as control and standard where buffer and vitamin E were added respectively. To the third to seventh dishes, different concentrations of methanol extract (200-1000 µg/ml) and the eighth and ninth dishes compound I (25, 50 µg/ml) were added. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate in nine dishes (Sreejayan, 1997). After 30 min, 100 µl of each reaction mixture was taken into tubes containing 1.5 ml of 10% Trichloro acetic acid. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% thiobarbituric acid. The mixture was heated in a water bath at 85°C for 30 min and in a boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 535nm in a spectrophotometer (Shimadzu model 1601). The TBARS concentration was calculated by using Equation 2 (molar extinction coefficient of TBARS) and expressed as nanomoles (nM)/mg of tissue (Ramazan, 2001). The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the **Equation 1.**

$$\textbf{nM of TBARS/mg of tissue} = \frac{\textbf{OD} \times \textbf{Volume of homogenate} \times 100 \times 10^3}{(1.56 \times 10^5) \times \textbf{Volume of extract taken}} \quad \textbf{--Eqn 2}$$

7.4.4.3 Superoxide scavenging activity

The superoxide scavenging activity of *Urtica parviflora* was determined by the method described by Nishimik *et al.*, (1972) with slight modification. To 1.0 ml of NBT solution containing 156 µM NBT dissolved in 100 mM phosphate buffer, pH 7.4, 1.0 ml of NADH solution containing 468 µM NADH dissolved in 100mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of the methanol

extract (200 to 1000 µg/ml) as well as compound I (25, 50 µg/ml) and standard compound (vitamin E) were added mixed and the reaction was started by adding 100 µl of phenazine methosulfate solution containing 60µM phenazine methosulphate in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25 °C for 5 min and absorbance of the contents at 560 nm was measured against a control sample. Percent inhibition was determined by comparing the results of the test and control samples with the help of **Equation 1** mentioned in **7.3.2**.

7.4.5 Antioxidant activity of *Callicarpa arborea* leaf

The antioxidant activity of methanol extract of *Callicarpa arborea* leaf and its isolated compound II was determined as per the methods described above in **7.4.4** for *Urtica parviflora* leaf.

7.4.6 Antioxidant activity of *Morinda citrifolia* root

The antioxidant activity of methanol extract of *Morinda citrifolia* root and its isolated compound III was determined as per the methods described above in **7.4.4** for *Urtica parviflora* leaf.

7.5 Statistical analysis

All data were expressed as means ± SEM. IC_{50} values were calculated by nonlinear regression analysis after logarithmic transformation of the sample concentrations.

7.6 RESULTS

7.6.1 Antioxidant activity of *Urtica parviflora* leaf

The effect of methanol extract and compound I on scavenging of DPPH radical is presented in Table 7.1. The results showed that the DPPH scavenging capacity of the extract was found to be 75.56±2.64% at 1000 µg/ml. The compound I at 50 µg/ml concentration, on the other hand, exhibited 82.17 ± 2.74 % compared to 85.23 ± 3.23 % for the standard drug vitamin E at 5mM. The IC_{50} value of the extract was found to be 6.9 mg/ml. The percentage of activity was also dependent on time (**Fig 7.1**).

The results presented in **Table 7.2** showed the effect of methanol extract of *Urtica parviflora* leaf and compound I on ferrous sulphate induced lipid peroxidation in rat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition percentage (71.54±3.03) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 82.01±1.08. The same results have also been presented in **Fig 7.2**, which revealed that compound I at 50 µg/ml showed 80.19±2.48% inhibition which is nearly equal to inhibition produced by vitamin E. The IC_{50} value, (concentration of the test substances at which 50 % of inhibition produced) was found to be 859.32±2.79 µg/ml. From the results of quantitative estimation of TBARS

levels it can be said that both the methanol extract at 1000 µg/ml and compound I at 50 µg/ml concentration decreases the levels of TBARS in liver homogenate and is highly comparable to the results of well known antioxidant vitamin E ($p< 0.05$).

The effect of leaf extract and compound I on superoxide scavenging model is presented in **Table 7.3**. It was found that the methanol extract at concentration of 1000 µg/ml caused significant increase of the inhibition up to 60.18 ± 1.74%. The compound I at 50 µg/ml concentration exhibited 69.01 ± 2.74% inhibition of superoxide radicals. IC_{50} was found to be 8.80.03±3.38 µg/ml. Inhibition was found directly proportional to the amount of the extract added.

Table 7.1 Free radical scavenging activity of *Urtica parviflora* leaf extract and Compound I by DPPH reduction.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	-
2	Vitamin E (5 mM)	85.23±3.23	
	Concentration of methanol extract (µg/ml)		
3	200	14.59±3.01	
4	400	39.44±3.18	
5	600	51.87±2.87	698.92±3.43
6	800	69.82±3.01	
7	1000	75.56±2.64	
	Compound I		
8	25	79.81±2.58	
9	50	82.17±2.74	

Values are mean ± SEM of 3 replicates

Table 7.2 Effect of methanol extract of *Urtica parviflora* leaf and Compound I on ferrous sulphate induced lipid peroxidation in rat liver homogenate.

Dish Number	Treatment	Inhibition (%)	IC_{50} value and confidence interval (µg/ml)	TBARS (n moles/mg tissue)
1	Control	-	-	-
2	Vitamin E (5 mM)	82.01±1.08		0.73±0.01*
	Concentration of methanol extract (µg/ml)			
3	200	27.16±2.68		1.09±0.02*
4	400	41.39±2.17		1.06±0.02*
5	600	54.18±2.68	859.32±2.79	1.01±0.01*
6	800	66.34±2.82		0.98±0.01*
7	1000	71.54±3.03		0.81±0.03*
	Compound I			
8	25	69.77±3.11		0.72±0.02*
9	50	80.19±2.48		0.70±0.03*

Values are mean ± SEM of 3 replicates, '*' indicates $p < 0.05$.

Table 7.3 Superoxide radical scavenging activity of *Urtica parviflora* leaf extract and Compound I.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	70.2± 1.65	
	Concentration of methanol extract (µg/ml)		
3	200	15.59 ± 2.13	
4	400	29.01 ± 2.01	880.03±3.87
5	600	36.48 ± 1.93	
6	800	47.38 ± 1.82	
7	1000	60.18 ± 1.74	
	Compound I		
8	25	67.14 ± 2.62	
9	50	69.01 ± 2.74	

Values are mean ± SEM of 3 replicates

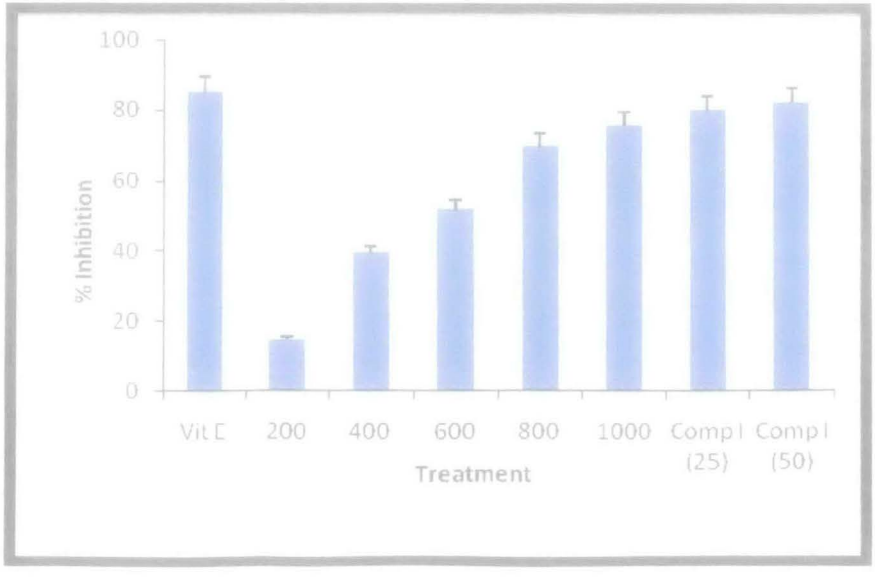

Fig 7.1 Percentage of inhibition of free radical scavenging activity of *Urtica parviflora* leaf extract and Compound I by DPPH reduction. Vitamin E (Vit E) was used as standard.

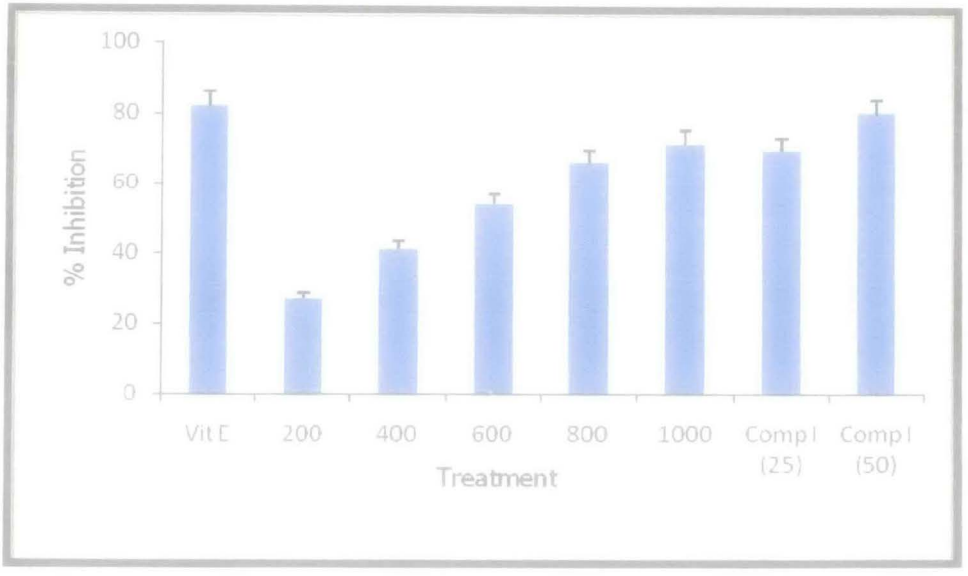

Fig 7.2 Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *Urtica parviflora* leaf and Compound I. Vitamin E (Vit E) was used as standard.

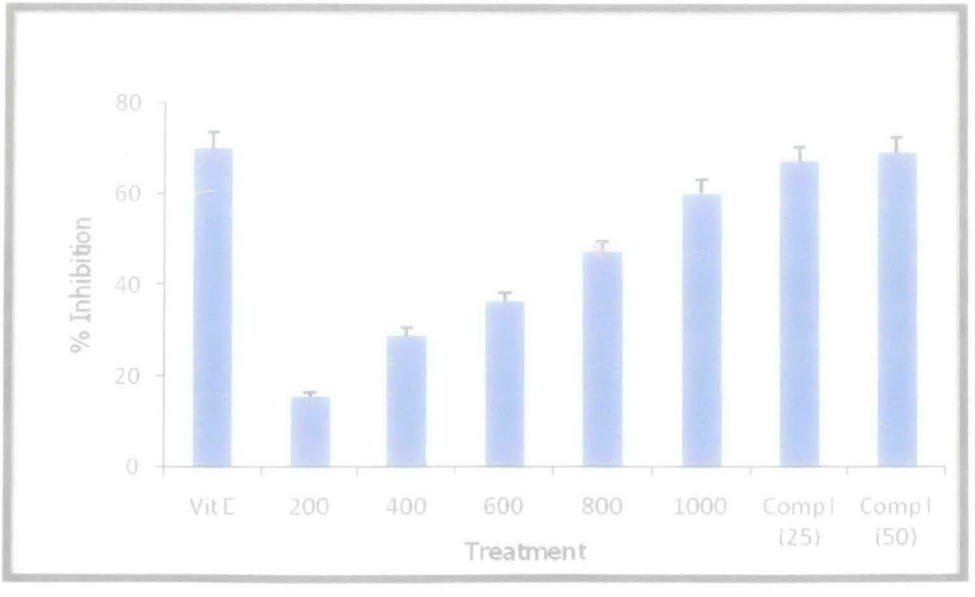

Fig 7.3 Percentage inhibition of Superoxide radical scavenging activity of *Urtica parviflora* leaf methanol extract [illegible] and Compound I. Vitamin E (Vit E) was used as standard.

7.6.2 Antioxidant activity of *Callicarpa arborea* leaf.

The effect of methanol extract and compound II on scavenging of DPPH radical is presented in Table 7.4. The result showed that the DPPH scavenging capacity of the extract was found to be 71.22±3.41% at 1000 µg/ml concentration. The compound II at 50 µg/ml concentration showed 73.65±3.44% inhibition as compared to 83.59±3.12% of the standard drug vitamin E at 5mM. The IC_{50} value of the extract was found to be 593.52 µg/ml. The percentage of activity was also dependent on time as depicted from **Fig 7.3**.

The results presented in **Table 7.5** showed the effect of methanol extract of *Callicarpa arborea* leaf and compound II on ferrous sulphate induced lipid peroxidation in rat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition (61.88±2.73%) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 78.26±3.31. The same result has also been presented in Figure 7.4 which revealed that compound II at 50 µg/ml showed 75.01±4.59% inhibitions. The IC_{50} value was found to be 798.79±4.91µg/ml. From the results of quantitative estimation of TBARS levels it appears that the methanol extract at 1000 µg/ml concentration decreases the levels of TBARS in liver homogenate from 1.08±0.03 to 0.81±0.12 nmoles/mg of tissue. The compound II at 50 µg/ml concentration showed the level of TBARS at (0.72±0.03 nmoles/mg of tissue).

The effect of root extract and compound II on superoxide scavenging model is presented in Table 7.6. It was found that the methanol extract at 1000 µg/ml concentration caused significant increase of the inhibition up to 61.81±2.44%. The compound II at 50 µg/ml exhibited 66.28±2.44% inhibition of superoxide radicals. IC_{50} was found to be 891 µg/ml. Inhibition was found to be directly proportional to the amount of the extract added.

Table 7.4 Free radical scavenging activity of *Callicarpa arborea* leaf extract and Compound II by DPPH reduction.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	83.59±3.12	
	Concentration of methanol extract (µg/ml)		
3	200	10.19±3.67	
4	400	31.23±4.02	593.52±3.87
5	600	[illegible]89±3.41	
6	800	*63.36*±3.68	
7	1000	71.22±3.41	
	Compound II		
8	25	72.37±2.53	
9	50	73.65±3.44	

Values are mean ± SEM of 3 replicates

Table 7.5 Effect of methanol extract of *Callicarpa arborea* leaf and Compound II on ferrous sulphate induced lipid peroxidation in rat liver homogenate.

Dish Number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} value and confidence interval (µg/ml)	TBARS (n moles/mg tissue)
1	Control	-	-	-
2	Vitamin E (5 mM)	78.26±3.31		0.71±0.02*
	Concentration of methanol extract (µg/ml)			
3	200	15.01±2.61		1.08±0.03*
4	400	28.14±3.51		1.04±0.04*
5	600	37.74±3.06	798.79±4.91	1.01±0.04*
6	800	49.62±2.91		0.95±0.03*
7	1000	61.88±2.73		0.81±0.12*
	Compound II			
8	25	71.99±4.56		0.74±0.04*
9	50	75.01±4.59		0.72±0.03*

Values are mean ± SEM of 3 replicates, '*' indicates $p < 0.05$.

Table 7.6 Superoxide radical scavenging activity of *Callicarpa arborea* leaf extract and Compound II.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidenc interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	66.38±2.29	
	Concentration of methanol extract (µg/ml)		
3	200	16.01±2.43	
4	400	31.11±3.08	891.01±2.61
5	600	49.33±2.29	
6	800	57.47±1.48	
7	1000	61.81±2.44	
	Compound II		
8	25	64.45±3.53	
9	50	66.28±2.44	

Values are mean ± SEM of 3 replicates

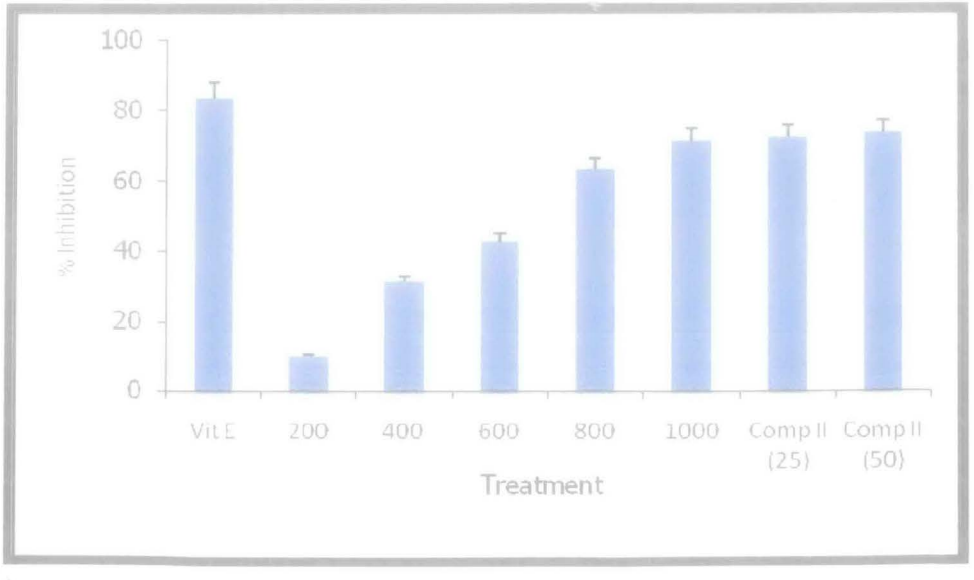

Fig 7.4 Percentage of inhibition of free radical scavenging activity of *Callicarpa arborea* leaf extract and Compound II by DPPH reduction. Vitamin E (Vit E) was used as standard.

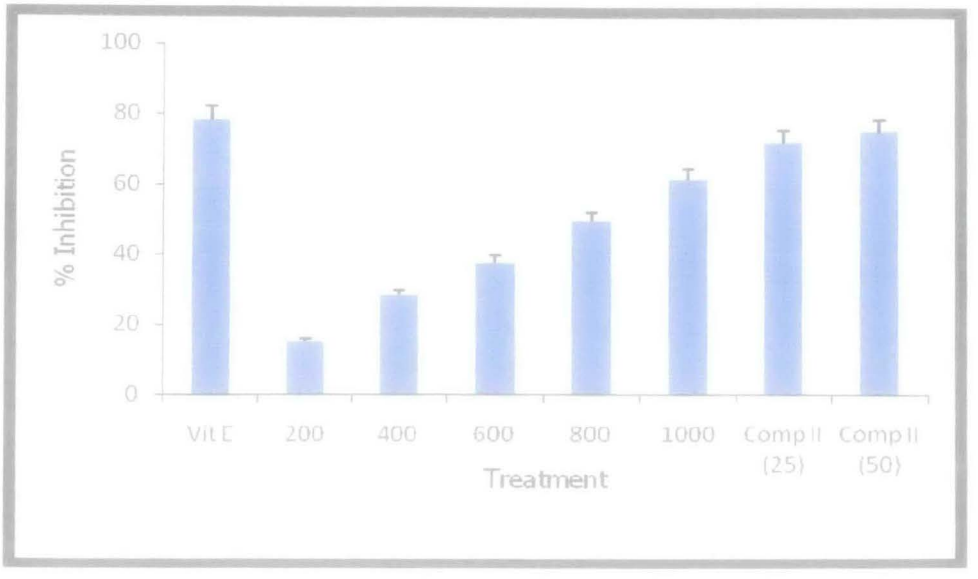

Fig 7.5 **Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *Callicarpa arborea* leaf and Compound II. Vitamin E (Vit E) was used as standard.**

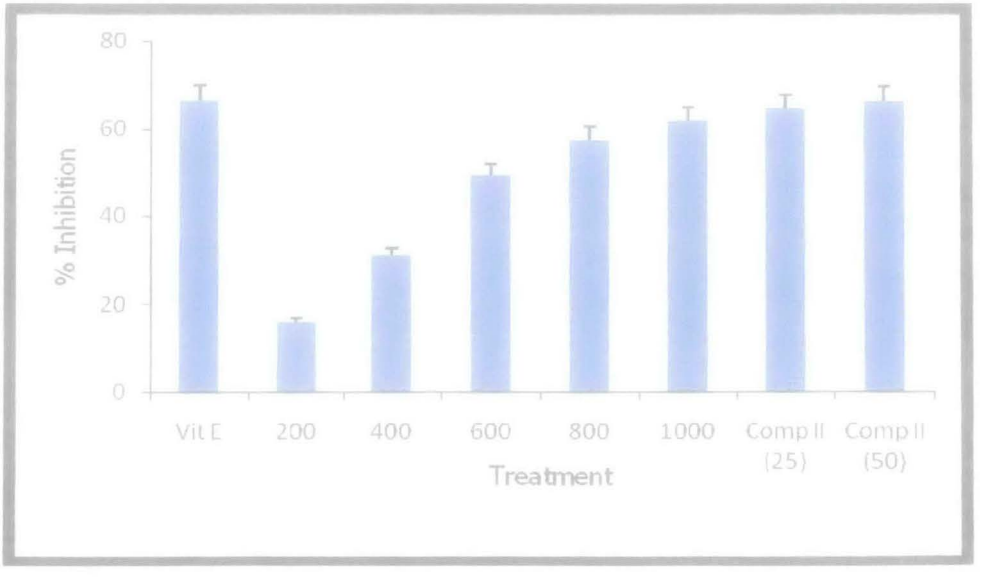

Fig 7.6 **Percentage inhibition of Superoxide radical scavenging activity of *Callicarpa arborea* leaf methanol extract [illegible] and Compound II. Vitamin E (Vit E) was used as standard.**

7.6.3 Antioxidant activity of *Morinda citrifolia* root.

The effect of methanol extract and compound II on scavenging of DPPH radical is presented in **Table 7.7**. The result showed that the DPPH scavenging capacity of the extract was found to be 74.67±3.01% at 1000 µg/ml concentration which is of similar level as compared to compound III at 50 µg/ml concentration, exhibited 74.81±3.14%. The standard drug (vitamin E) at 5mM showed 81.34±4.27 % inhibition. The IC_{50} value of the extract was found to be 591.73 µg/ml. Also the percentage of activity was time dependent **Fig 7.5**.

The results presented in **Table 7.8** showed the effect of methanol extract of *Morinda citrifolia* root and compound III on ferrous sulphate induced lipid peroxidation in rat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition (63.14±2.57%) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 74.61±2.68. The same result have also been presented in **Fig 7.6** which revealed that compound III at 50 µg/ml showed 68.32±3.01% inhibitions, which is almost equal to that of the inhibition produced by vitamin E. The IC_{50} value was found to be 766.3±3.94 µg/ml. From the results of quantitative estimation of TBARS levels, it appears that the methanol extract at 1000 µg/ml concentration decreases the levels of TBARS in liver homogenate from 0.98 ± 0.01 to 0.70 ± 0.12 nmoles/mg of tissue and its compound III at 50 µg/ml concentration showed the level much below TBARS (0.57 ± 0.01 nmoles/mg of tissue), which is much higher rate of decrease than that of the results obtained from vitamin E ($p< 0.05$).

The effect of root extract and compound III on superoxide scavenging model is presented in **Table 7.9**. It was found that the methanol extract at 1000 µg/ml concentration caused significant increase of the inhibition up to 59.63 ± 2063%. The compound III at 50 µg/ml exhibited 68.01 ± 2.01% inhibition of superoxide radicals. IC_{50} was found to be 895.6 µg/ml. Inhibition was found to be directly proportional to the amount of the extract added.

Table 7.7 Free radical scavenging activity of *Morinda citrifolia* root extract and Compound III by DPPH reduction.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	-
2	Vitamin E (5 mM)	81.34±4.27	591.73±3.48
	Concentration of methanol extract (µg/ml)		
3	200	10.17±2.42	
4	400	34.28±2.72	
5	600	44.92±2.85	
6	800	68.49±2.91	
7	1000	74.67±3.01	
	Compound III		
8	25	72.19±3.19	
9	50	74.81±3.14	

Values are mean ± SEM of 3 replicates

Table 7.8 Effect of methanol extract of *Morinda citrifolia* root and Compound III on Ferrous sulphate induced lipid peroxidation in rat liver homogenate.

Tube Number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} value and confidence interval (µg/ml)	TBARS (n moles/mg tissue)
1	Control	-	-	-
2	Vitamin E (5 mM)	74.61±2.68		0.98±0.01*
	Concentration of methanol extract (µg/ml)			
3	200	14.68±3.11		0.97±0.01*
4	400	27.42±3.23		0.96±0.02*
5	600	37.17±3.01	766.3±3.94	0.94±0.01*
6	800	50.51±3.17		0.90±0.01*
7	1000	63.14±2.57		0.70±0.12*
	Compound III			
8	25	66.19±2.21		0.59±0.02*
9	50	68.32±3.01		0.57±0.01*

Values are mean ± SEM of 3 replicates, '*' indicates $p < 0.05$.

Table 7.9 Superoxide radical scavenging activity of *Morinda citrifolia* root extract and Compound III.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidenc interval (µg/ml)
Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	67.33± 2.48	
	Concentration of methanol extract (µg/ml)		
3	200	14.01 ± 2.77	
4	400	24.32 ± 2.37	895.6±5.72
5	600	38.97 ± 2.28	
6	800	51.66 ± 2.50	
7	1000	59.63 ± 2063	
	Compound III		
8	25	67.18 ± 2.17	
9	50	68.01 ± 2.01	

Values are mean ± SEM of 3 replicates

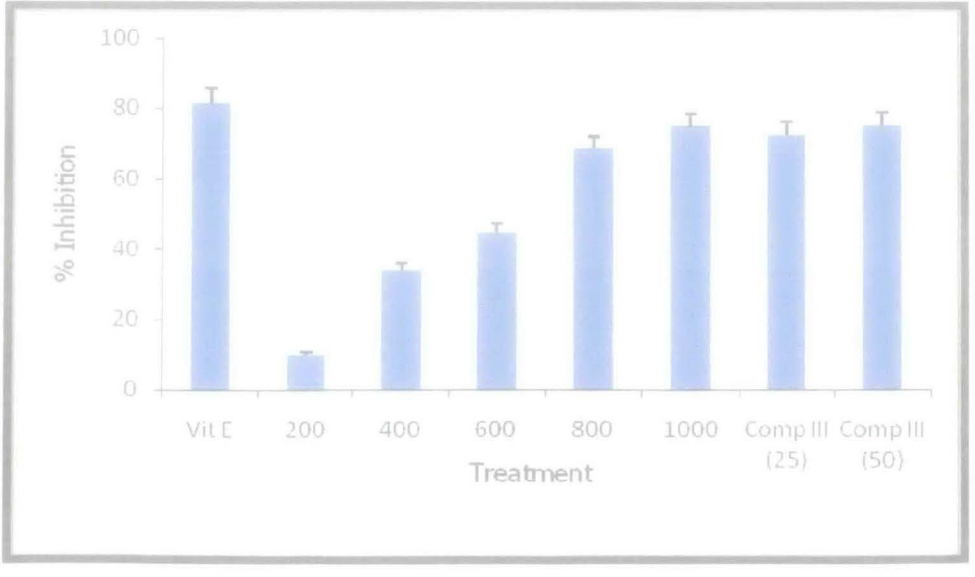

Fig 7.7 Percentage of inhibition of free radical scavenging activity of *Morinda citrifolia* leaf extract and Compound III by DPPH reduction. Vitamin E (Vit E) was used as standard.

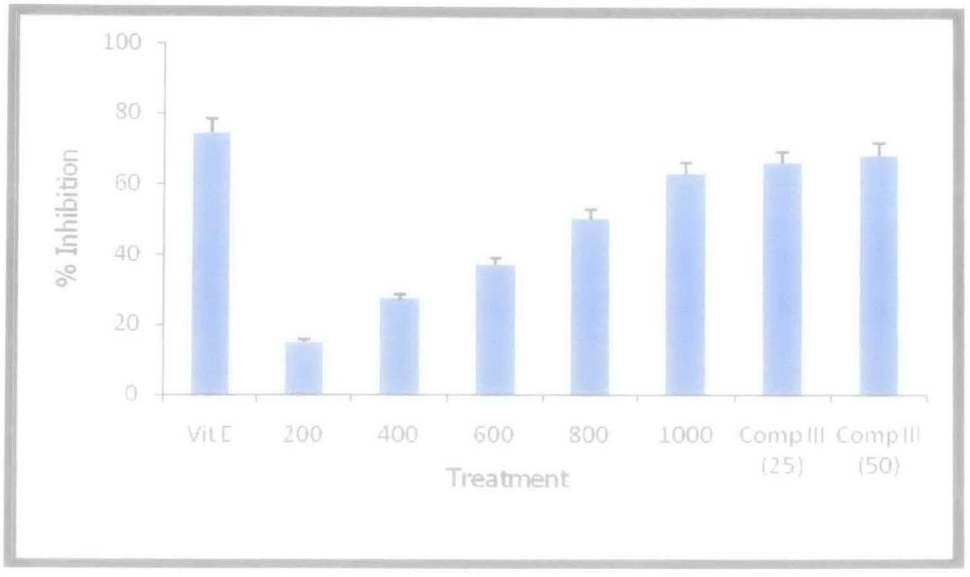

Fig 7.8 Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *Morinda citrifolia* leaf and Compound III. Vitamin E (Vit E) was used as standard.

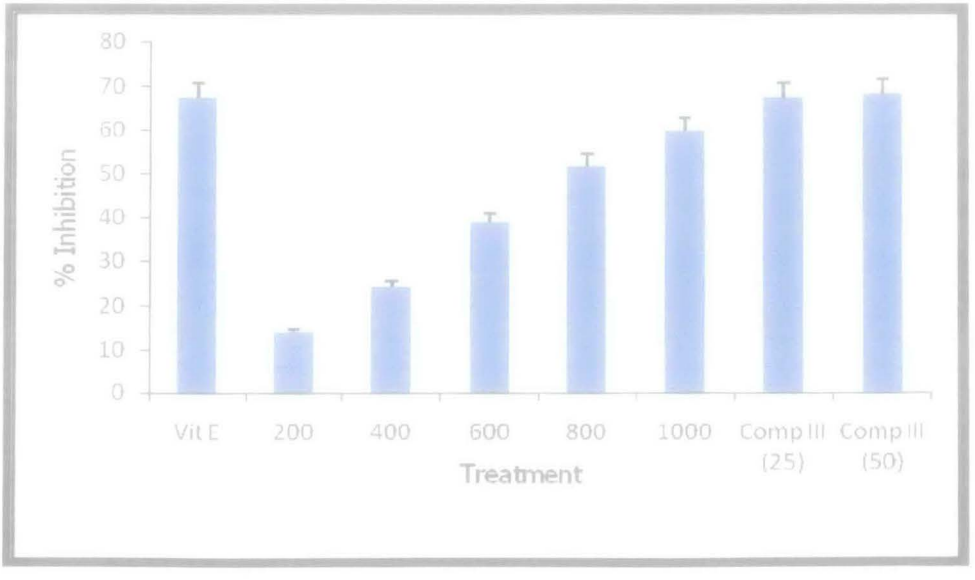

Fig 7.9 Percentage inhibition of Superoxide radical scavenging activity of *Morinda citrifolia* leaf methanol extract and Compound III. Vitamin E (Vit E) was used as standard.

CHAPTER 8

HYPOGLYCEMIC ACTIVITY

8.0 INTRODUCTION

8.1 Diabetes

Diabetes is a disorder of the carbohydrate, fat and protein metabolism. It is attributed to the diminished production of insulin or mounting resistance to its action. Chronic hyperglycemia during diabetes causes glycation of body proteins that in turn leads to secondary complications affecting the eyes, kidneys, nerves and arteries (Sharma *et al.*, 1993).

Diabetes can be classified into two types: (a) type 1 diabetes (insulin-dependent Diabetes mellitus or IDDM) and (b) type 2 diabetes (non-insulin dependent Diabetes mellitus or NIDDM). Exercise and having a controlled diet are recommended for the treatment of both types of diabetes. In addition, insulin is used to treat cases of type 1 diabetes. Oral hypoglycemic agents (such as sulfonylureas, biguanidines, thiazolidinediones and α-glucosidase inhibitors) are often used to treat cases of type 2 diabetes. When therapy with oral hypoglycemic agents is ineffective, insulin also can be used to treat type-2 diabetes (Liu *et al.*, 1996; Zhao, 1999).

In exception to western medicine, diabetes has been treated orally with various medicinal plants or their extracts based upon 'folklore' medicine. Diabetes comprised of a group of metabolic disorders characterized by an alteration in the metabolism of carbohydrates, proteins and fatty substances. This disorder causes a complete or relative insufficiency in insulin secretion and or its action. An investigation of hypoglycemic agents originating from plants used in traditional medicine would be of major public health importance (Almeida *et al.*, 2006).

8.1.1 Prevalence of Diabetes and role of plant derived drugs

Diabetes mellitus is the metabolic disorder with the highest rate of prevalence and mortality world-wide (Harris *et al.*, 1998). The incidence of type 2 diabetes is increasing worldwide. Although genetic factors may play a role, life-style changes such as the consumption of a Western diet which are high in fat, leads to obesity which can be a factor also contributing to the increase of this disease. Life-style factors, such as increased fat intake and reduced exercise, have been shown to be associated with obesity and insulin resistance (Lipman *et al.*, 1972; Lovejoy *et al.*, 1992). It is observed in rats that, high fat feeding induces a state of insulin resistance associated with diminished insulin-stimulated glycolysis and glycogen synthesis. This disease is a result of the peripheral insulin-responsive tissues, such as muscle and adipose tissue, displaying a significant decrease in response to insulin resulting in an increase in circulating glucose and fatty acids in the blood. The low response to insulin results in a decrease in glycolysis which in turn initiates gluconeogenesis and glycogenolysis in the liver, both of which are 'switched off' by insulin under normal conditions. Pancreatic β cells are able to cope with the initial insulin resistant phase by producing an excess of insulin and

increasing the amount of insulin secreted (Pirol *et al.*, 2004). The resulting hyperinsulinaemia to maintain normoglycaemia eventually brings about β cell dysfunction (Khan, 2003) leading to full blown diabetes. It is evident that type 2 diabetes is dependent on insults occurring both at peripheral as well as the β cell level (Khan *et al.*, 2000).

Biguanides, such as metformin, became available for treatment of type 2 diabetes in the late 1950s, and have been effective hypoglycaemic agents ever since (Vigneri *et al.*, 1987). Little is known about the exact molecular mechanism of these agents. As an insulin sensitizer, metformin acts predominantly on the liver, where it suppresses glucose release (Goldfine, 2001). Metformin has also been shown to inhibit the enzymatic activity of complex I of the respiratory chain and thereby impairs both mitochondrial function and cell respiration, and in so doing decreasing the ATP/ADP ratio which activates AMP-activated protein kinases causing catabolic responses on the short term and insulin sensitization on the long term (Brunmair *et al.*, 2004; Tiikkainen *et al.*, 2004). This drug has been proven effective in both monotherapy and in combination with sulfonylureas or insulin (Davidson *et al.*, 1997). However, their cost is very high and the development of more affordable alternative therapies would be an advantage. It is for this reason that scientists are investigating the efficacy of indigenous plant extracts in their own country (Chadwick *et al.*, 2007). Plants such as *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* in my experimental work may be the answer to this problem.

Diabetes mellitus and its complications constitute a major health problem in modern societies (Li *et al.*, 2007). The prevalence of diabetes has increased over the past 20 years, adding urgency to the need of treatment with oral medications (Chehade *et al.*, 2000). Targeting postprandial hyperglycemia may be difficult with conventional diabetes therapy and in this regard, the availability of a-glucosidase inhibitors is helpful (Van de Laar *et al.*, 2005; Scheen, 2003). a-Glucosidase inhibitors are nontoxic and well tolerated, and with mild antihyperglycemic activity, either used as monotherapy or adjuncts to any other oral diabetic agents (Charpentier, 2002). It has long been recognized that many naturally occurring substances have inhibitory effect of aglucosidase, in plant materials such as fruits, leaves, seeds etc. (Shim *et al.*, 2003; Ye *et al.*, 2002). So the study of those bioactive constituents represents a promising approach to the discovery of new diabetes drugs. Teas, rooibos and honeybush are reported to have multiple biochemical and pharmacological activities, such as anticarcinogenic, antioxidant, antiangiogenic, antiviral and antidiabetic effects (Gomes *et al.*, 1995; Shukla *et al.*, 2002 and Skrzydlewska *et al.*, 2002).

Diabetes affects nearly 10% of population of the world (Yanardag *et al.*, 2003). Overall prevalence among Indians is about 1.73% (Park, 1997). Diabetes mellitus is an independent

risk factor for the development of coronary artery diseases, myocardial infarcation, hypertension and dyslipidemia. Clinically diabetic patients are characterized by marked increase in blood glucose level followed by normal or mild hyperlipidemia. It is a chronic disease characterized by high blood glucose levels due to absolute or relative deficiency of circulating insulin levels. Though different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, there is an increase demand by patients to use the natural products with antidiabetic activity. Insulin cannot be used orally and continuous use of synthetic drugs causes side effects and toxicity. At present, second and third generation sulfonylurea are the oral pharmacological agents used to counteract insulin secretion deficiency in diabetes (Puri et al, 2002). Plants have long been a source of traditional antidiabetic medicines (Blumenthal, 2000 & Kirtikar, 1988). Herbal drugs are prescribed widely even when their biologically active compounds are unknown, because of their effectiveness, less side effects and relatively low cost (Venkatesh, 2003). Herbal preparations alone or in combination with oral hypoglycemic agents sometimes produce a good therapeutic response in some resistant cases where modern medicines alone fail (Anturlikar, 1995).

In recent years there has been an upsurge in the clinical use of indigenous drugs. Indian medicinal plants and their derivatives have been an invaluable source of therapeutic agents to treat various disorders including diabetes (Koehn and Carter, 2005). Polyherbal preparations, originally used in the traditional systems of medicine, are now being investigated and effectively tried in a variety of path physiological states (Shah *et al.*, 1997). Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of diabetes (Ajgaonkar, 1979; Alarcon-Aguilara *et al.*, 1998). The hypoglycemic activity of a large number of these plants been evaluated and confirmed in different animal models (Karaway *et al.*, 1984; Bopanna *et al.*, 1997; Bhandari *et al.*, 1998; Jouad *et al.*, 2000; Sugihara *et al.*, 2000; Takako *et al.*, 2005). Side effects and expenses associated with allopathic drugs have provoked the need for research into drugs, which are without the side effects, especially those belonging to the traditional systems of medicine like Ayurveda, Homoeopathy, and Unani etc (Sharma *et al.*, 2007).

Diabetes mellitus, both of the insulin-dependent diabetes mellitus (IDDM) and non-insulindependent diabetes mellitus (NIDDM) type, is a common and serious disorder throughout the world (Keen, 1986; Harris *et al.*, 1987). This metabolic disorder often leads to physical disability arising from the vascular complications of coronary artery disease and cerebrovascular disease, resulting in renal failure, blindness, and limb amputation in addition to neurological complications and premature death (Goldstein and Massry, 1978; Weidmann *et al.*, 1993; Strippoli *et al.*, 2003; He and King, 2004). Treatment of diabetes mellitus by insulin

and oral hypoglycemic drugs fails to prevent these complications in many patients, indicating that additional alternative treatment could be helpful (Cherng *et al.*, 2005).
Type 2 diabetes (DM2) is one of the primary threats to human health due to its increasing prevalence, chronic course and disabling complications. According to the World Health Organization (WHO, 2005) there were 150 million people over 20 years of age living with diabetes in 2000 and they project that by 2025 there will be 300 million people living with this condition. The increase is expected to be 42% in developed countries and 70% in developing countries (WHO, 2005; King, *et al.*, 1998).

One of the principal objectives when treating patients with DM2 is to control glucose levels. Presently, there is an arsenal of synthetic hypoglycemic drugs available; however, these drugs normally cause side effects prompting the patients to stop taking the medication and DM2 progresses with further acute and chronic complications and even death. For this reason, a phytomedicine capable of treating the disease at early stages, but with fewer side effects and less expensive, will be of great help to the diabetic patients specially due to the extended belief that natural treatments cause less harm to the organism (Revilla-Monsalve *et al.*, 2007).

Metabolic syndrome (MS) is also known as syndrome X or insulin resistance syndrome (Boehm and Claudi-Boehm, 2005). In accordance with World Health Organization (WHO), the MS components are: visceral fat obesity, hypertension, insulin resistance and dyslipidemia (Isomaa *et al.*, 2001). Therefore, International Diabetes Federation (IDF) mentions that MS is a cluster of the most dangerous heart attack risk factors such as diabetes and prediabetes, abdominal obesity, high cholesterol and high blood pressure (IDF on-line definition, 2005). Some authors included liver steatosis as an important factor to diagnosing this complex metabolic disease (Masuzaki *et al.*, 2004; Den Boer *et al.*, 2004). Although the pathogenesis of metabolic syndrome is still not fully clear, a large body of evidence indicates that insulin resistance could be the central abnormality (Wajchenberg *et al.*, 1994). These diseases occur due to several factors such as genetic background alterations, lifestyle and other related factors (Fowler *et al.*, 2005).

8.1.2 Hypoglycemic models

Hypoglycemic activity of drugs, including the products derived from medicinal plants, is conventionally assessed in diabetic animal models by observing drug-induced fall in fasting blood glucose (FBG) or suppression of glucose tolerance curve. Diabetes is induced experimentally by partial or total pancreatecctomy, exposure to antislet cell antibodies, but most commonly by injecting chemical agents, such as alloxan or streptozotocin, that cause widespread destruction of insulin-secreting pancreatic beta cells (Kaneto *et al.*, 1995).

However, beta cells being extensively eliminated/ or destroyed in the diabetic animals, several drugs that require functional pancreas cannot be satisfactorily tested in them (Puri, 2006).

Moreover, the drug-induced fall in FBG as a criterion for hypoglycemic effect has been reported to be a relatively intensive method of assessment and sometimes gives inconsistent results. Post-treatment suppression of glucose tolerance curve, though a sensitive method of assessment, suffers from a major drawback in the test is associated with high mortality in diabetic animals (Puri *et al.*, 2002).

Hyperglycemia is common and is due to multiple factors, including decreased insulin release, increased glucagons release and increased output of adrenal glucocorticoids and catecholamines and hypoglycemia is most commonly the result of taking drugs used to treat diabetes mellitus or other drugs, including alcohol. However a number of other disorders, including end-stage organ failure and sepsis, endocrine deficiencies, large mesenchymal tumors, insulinoma, and inherited metabolic disorders are also associated with hypoglycemia. Hypoglycemia defined as a plasma glucose level <2.5 to 2.8 mmol/L (45 to 50 mg/dL).

Experimental animal models in which diabetes is induced by administration of alloxan, streptozotocin or other agents have been used effectively to study etiologies, complications, treatment and prevention of disease (Park, 1997).

8.1.3 Ethnomedicinal antidiabetic of Sikkim and Darjeeling Himalayas

Sikkim and Darjeeling Himalayan region is characterized by a rich floral diversity and an equally rich ethnomedicinal tradition. Herbal medicine is the dominant system of medicine practiced by the local tribes of this region for the treatment of diabetes. It is reported that 37 species of plants belonging to 28 families are used as antidiabetic agents in the folk medicinal practices in the region and 81% of these plants are hitherto unreported as hypoglycemic agents. This finding may lead to serious research towards developing new and efficient drugs for diabetes (Chhetri *et al.*, 2005).

The efficacy of these ethnomedicinal plants needs to be subjected to pharmacological validation. Some antidiabetic plants may exert their action by stimulating the function or number of cells and thus increasing insulin release (Persaud *et al.*, 1999). In some other plants, the effect is due to decreased blood glucose synthesis due to the decrease of the activity of enzymes like glucose-6-phosphatase, fructose1,6-bisphosphatase, etc. In still other plants, the activity is due to slow absorption of carbohydrate and inhibition of glucose transport (Madar, 1984). However, these products may interact with the conventional diabetes medicines (Shane-McWhorter, 2001). Therefore, a cautious approach should be adopted before

administering these drugs. Of course, this primary information is important in view that it may lead to serious pharmacological research and can provide great value in selecting plant material for drug discovery (Lewis *et al.*, 2004).

Worldwide, over 1200 species of plants have been recorded as traditional medicine for diabetes (Marles and Farnsworth, 1995). Some of these plants have been evaluated in laboratories and in a number of cases their efficacy has been confirmed, for instance, *Panax ginseng (ginseng), Opuntia cactus (cactus), Tecoma stans* (trompeta), *Syzygium cumini* (jambol˜ao) (Li *et al.*, 2004). Specific chemical constituents of these plants, such as polysaccharides, alkaloids, triterpenoids and xanthones, are believed to be responsible for the hypoglycemic effects and they can be related to actions including increased insulin release and increased glucose metabolism in the body periphery, among others (Wang and Ng, 1999; Tatiana *et al.*, 2006). Many plants like *Allium cepa, Alium sativum, Ficus bengalensis, Gymnema sylvestre, Pterocarpus marsupium, Trignella foenumgraecum, Engenia jambolana* etc. have ben shown to possess potent antidiabetic activity (Melander, 1988).

The literature survey indicates plants such as *Cyamopsis tetragonoloba* Linn.(Mukhtar *et al.*, 2004), *Polygala arvensis, Polyalthia longifolia, Pterocarpus marsupium, Azadirachta indica, Abroma angusta, Ocimum sanctum, Murraya koenigii* (Vinuthan *et al.*, 2004), *Lantana camara* Linn. are tested for their antihyperglycemic potential against alloxan induced hyperglycemia in laboratory animals. Whereas plants like *Memordica charantia* Linn (Prasanna *et al.*, 2005), *Acacia catechu* Willd., *Ficus hispida* (Ghosh *et al.*, 2004) were tested against glibenclamide induced hypoglycemia and plants like *Cassia kleinii, Petroselinum crispum* (Yanardag *et al.*, 2003), *Brassica nigra* Linn. are tested against streptozotocin induced hyperglycemia in animals respectively. Many herbal agents have been described for the treatment of diabetis mellitus in ancient literature. They have been shown to have hypoglycemic action in both animals as well as humans.

The Ethnomedicinal plants which are practiced by the tribes of Sikkim and Darjeeling Himalayas are given in **Table 8.1.**

Table 8.1 Antidiabetic medicinal plants from Sikkim and Darjeeling Himalayas (Chhetri *et al.*, 2005).

Botanical name and family	Habit	Local name: N-Nepali; L-Lepcha; T-Tibetan	Method of use and administration
Abroma augusta (L.) L.f., Sterculiaceae	Shrub	Ulatkamal (N)	Stem bark and leaf decoction (10–20 ml) taken one time each alternate day in empty stomach for 4–6 week.
Abutilum indicum (L.) Sw., Malvaceae	Shrub	Ghantiphool (N)	Decoction of stem bark (25–50 ml) given two times daily (after principal meals) for 3–4 weeks.
Aconitum palmatum D. Don., Ranunculaceae	Herb	Seto bikhumma (N); Nyini (L); Bhongnanukpo (T)	Root decoction (10–15 ml) taken with a cup of milk one time daily (after lunch) for 7–10 days
Aloe barbadensis Mill, Liliaceae	Herb	Ghew kumari (N); Kumari (T)	Fresh leaf pulp (40–50 g) taken once a day in empty stomach for 10–12 weeks.
Asparagus racemosus Willd., Liliaceae	Climbing shrub	Kurilo (N); Neusiri (T)	Decoction of tender shoots (25 ml) taken once a day for 6–8 weeks.
Berberis aristata DC., Berberidaceae	Shrub	Sano Chutro (N); Sutangkung (L); Skyerba (T)	Root bark extract (5–10 ml) taken twice daily after breakfast and dinner) for 1–2 weeks
Boenninghausenia albiflora (Hook. f.) Reich ex Meissn., Rutaceae	Herb	Chirbirpatay (N)	The whole plant is crushed without water and the juice (5–10 ml) taken one to two times daily for 3–4 weeks.
Calamus rotanga (L.), Arecaceae	Climbing shrub	Bet (N)	Raw fruit (1–2) taken as masticatory two times daily (after breakfast and lunch) for 6–8 weeks.
Campylandra aurantiaca Baker, Liliaceae	Herb	Nakima (N)	Flowers are made into curry and taken with staple food two times per week for 4–6 weeks
Cannabis sativa (L.), Cannabaceae	Under shrub	Bhang (N)	Leaf extract (5–10 ml) taken two times daily for 3–4 weeks.
Catharanthus roseus (L.) G. Don., Apocynaceae,	Herb	Sada bahar (N)	Raw leaf (1–2) chewed daily for 2 weeks.
Cinnamomum tamala (Buch.-Ham.) Nees and Eberm., Lauraceae,	Tree	Sinkauli (N); Napsor (L); Mensing (T)	Decoction of stem bark taken three times daily for 3–4 weeks
Cissampelos pareira (L.), var.hirsuta (Buch.-Ham ex DC) Forman, Menisper-maceae	Climber	Batulpatay (N)	Root bark extract (5–10 ml) tken one to two times daily for 2–3 weeks
Coccinea grandis (L.) Voigt., Cucurbitaceae	Climber	Tilkor (N)	Fresh root extract (5–10 ml.) taken two times daily (before principal meals) for 3–4 weeks
Costus speciosus (Koening) Sm., Costaceae	Herb	Betlouri (N); Ruyang (L)	Decoction of rhizome (10–20 ml) taken two to three times daily for 2–4 weeks
Ficus racemosa (L.), Moraceae	Tree	Dumri (N)	Fruit juice (20–25 ml) taken two times daily (before meals) for 4–8 weeks
Girardiana heterophylla Decne., Urticaceae	Shrub	Bhangre sisnu (N)	Root decoction (25–50 ml) taken two times daily for 4–8 weeks
Gynocardia odorata R. Br., Flacourtiaceae	Tree	Gantay (N); Tukkung (L)	Fruit juice (10–15 ml) taken one time daily for 2 weeks

Botanical name and family	Habit	Local name: N-Nepali; L-Lepcha; T-Tibetan	Method of use and administration
Ipomoea batatus (L.) Lamk., Convolvulaceae	Herb	Sagarkhanda (N)	The juice of the aerial part of the plant (25–30 ml) taken two times daily for 3–4 weeks
Litsea cubeba Pers., Lauraceae	Tree	Siltimmur (N)	One raw fruit chewed as masticatory two times daily for 4–6 weeks
Momordica chrantia (L.), Cucurbitaceae	Climber	Karela (N)	Fruit extract (25 ml) taken two times daily for 12–14 weeks
Nardostachys jatamansi DC., Valerianaceae	Herb	Jatamansi (N), Spanpos (T)	Decoction of rootstock (30–50 ml) taken once daily for 2–3 weeks.
Oroxylum indicum (L.) Vent. Bignoniaceae	Tree	Totola (N), Phagorip (L), Sonaka (T)	Stem bark decoction (15–20 ml) or juice (5–10 ml) taken two to three times daily
Paederia foetida (L.), Rubiaceae	Climber	Birilahara (N), Takpoedrik (L)	Leaf infusion (50–60 ml) taken one time in the morning for 2–3 weeks
Panax pseudoginseng Wall., Araliaceae	Herb	Panch patay (N)	Dried rhizome powder (0.5–1 g) taken one time daily with warm milk
Picrorhiza kurrooa Royle ex Benth. Scrophulariaceae	Herb	Kutki (N), Putse sel (T)	Dry rhizome powder (0.5 g) taken with two tablespoon of curd and a pinch of pepper power one time daily for 1–2 weeks
Potentilla fulgens Wall., Rosaceae	Herb	Banmula (N)	Decoction of root (20–25 ml) taken two times daily for 4–8 weeks
Quercus lanata Sm., Fagaceae	Tree	Banj (N)	Decoction of stem bark (20–25 ml) taken one or two times daily for 2–3 weeks
Saraca asoca (Roxb.) De Wilde, Caesalpiniaceae	Tree	Asok (N)	Infusion of the dry flower (50–100 ml) taken two times daily (before principal meals) for 4–5 weeks
Stephania glabra (Roxb.) Miers, Menispermaceae	Climber	Tamarkay (N), Kanthey (L)	Root decoction (20–25 ml) taken with milk two to three times daily for 1–2 weeks
Swertia angustifolia Buch.-Ham.ex D. Don., Gentianaceae	Herb	Patlay Chireto (N)	Infusion of whole plant (40–50 ml) taken two times daily (before principal meals for 3–4 weeks
Swertia chirayita (Roxb. ex Flem.) Karst., Gentianaceae	Herb	Chireto (N), Rungkyon (L), Tagota (T)	Infusion of the whole plant (50–60 ml) taken one time daily in empty stomach for 2 weeks
Syzygium cuminii (L.) Skeels, Myrtaceae,	Tree	Kyamuna (N), Dzambu (T)	Decoction of stem bark (25–30 ml) taken three times daily for 2–3 weeks
Trigonella foenum-graecum (L.), Fabaceae,	Herb	Methi (N)	Sprouted seeds mixed with chilly, salt and garlic and ground into a paste. 5–10 g of the paste taken with two principal meals daily
Urtica dioica (L.), Urticaceae	Herb	Sisnu (N), Sarong (L)	Decoction of young leaves and shoots (50–100 ml) taken as curry one or two times daily with meals for 4–8 weeks
Zingiber officinale Rosc., Zingiberaceae	Herb	Adua (N), Heng (L), Beasga (T)	Decoction of rhizome (25–50 ml) taken as herbal tea with a pinch of salt two to three times daily for 8–12 weeks

8.2 MATERIAL AND METHODS

8.2.1 Plant materials

The fresh leaves of *Urtica parviflora (U. parviflora), Callicarpa arborea (C. arborea)* and root bark of *Morinda citrifolia (M. citrifolia)* were collected at Majhitar, East Sikkim and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131, 132 and 133). The concentrated methanolic extract of the leaves of *Urtica parviflora, Callicarpa arborea* was used for the study whereas the concentrated chloroform extract of the roots of *Morinda citrifolia* was used. Further the compounds I,II, and III isolated from the leaves of *Urtica parviflora, Callicarpa arborea* and roots of *Morinda citrifolia* respectively were used in this study.

8.2.2 Animals

Male Wistar rats (6 weeks old) weighing 150-200g obtained from Ghosh Enterprises, Kolkata were used. The animals were kept in well ventilated and clean animal house of Himalayan Pharmacy Institute, Sikkim, at 23^0-27^0C, 55% humidity and allowed to acclimatized with free access to food (Local animal feed) and water *ad libitum* for one week under 12 hr each of light and dark cycles. The animal studies were approved by Institutional Animal Ethics Committee.

8.2.3 Induction of experimental diabetes

Diabetes was induced by a single intra peritoneal injection of freshly prepared Streptozotocin (STZ) in a dose of 50mg/kg body weight dissolved in citrate buffer pH 4.5 to overnight fasted rats. The hypoglycemic activity on these animals was carried out after one week of STZ injection when the stabilization of diabetes was ensured (Murali *et al.*, 2004). The animals with fasting serum glucose (FSG) of 240 mg/dl and above were used (Anand *et al.*, 2007).

8.2.4 Effect of different extracts and isolated compounds of the respective extracts on glucose tolerance test (GTT)

Different extracts and the isolated compounds were assessed by oral glucose tolerance test (GTT) based antidiabetic activity in STZ induced diabetic rats (Anand *et al.*, 2007). In this method, the advantage is that the same group of animals served as their self control. Six groups (Group I-VI) of six rats each (one group for each extract or compounds) were used in this experiment. The animals were fasted overnight and fasting blood samples were drawn from the tail vein. To get the GTT pattern in untreated animals, empty gelatin capsules were fed to the animals after withdrawal of fasting blood samples. Again after 90 minutes blood samples were withdrawn. This sample was taken as '0' hour value for GTT. The animals were given immediately aqueous glucose solution 2 g/kg of body weight orally and blood samples were withdrawn at 1, 2 and 3 hr after glucose administration to get the GTT pattern of the untreated diabetic animals (diabetic control). After a week, same animals were again fasted

overnight to carry out GTT with drug. Fasting blood samples were drawn. The extracts and isolated compounds were administered orally to rats in gelatin capsules. Animals of group I-III received methanolic extract of the leaves of *Urtica parviflora* (MEUP), methanolic extract of the leaves of *Callicarpa arborea* (MECA), and chloroform extract of the roots of *Morinda citrifolia* (CEMC). The animals of group IV to VI received the isolated compound I,II, and III at the dose of 50mg/kg body weight **(Table 8.2).** After 90 min (this time was allowed for their effect to take place in the body), blood samples were drawn again. This served as '0' hr blood sample of the treated diabetic rats in GTT. Then the GTT was carried on as described above to get the glucose tolerance pattern of the same diabetic animals but after treatment with the extracts. The GTT pattern of untreated diabetic animals and the GTT pattern of treated animals after one week showed no significant difference. So no separate group of untreated control animals for each drug was required.

8.2.5 Assessment of antidiabetic effect in rats (serum glucose level)

The effect of the extracts and their isolated compound on fasting serum glucose (FSG) in experimental diabetes induced by STZ (section 8.2.3) was assessed as follows. Nine groups (I-IX) each of six rats were used. Group I served as normal healthy controls and group II as untreated diabetic animals and was given water during the experiment period as the vehicle. Group III to VIII diabetic rats were given effective dose of MEUP, MECA, CEMC, compound I, II and III (dose in **Table 8.2**). Group IX received the standard drug glibenclamide (Sigma Chemicals, USA) in a dose of 0.20 g/kg body weight orally. The drugs were given once daily in the morning for seven days. The animals had free access to food and water. The fasting blood was collected in the beginning and on the 8^{th} day. Serum glucose (Fasting serum glucose, FSG) and urine glucose levels were also estimated at the same time. FSG was estimated using Kits of Ranbaxy Laboratories, New Delhi, India and the urine glucose level was estimated using Uristix of Bayer Diagnostics Pvt. Ltd, Mumbai, India.

8.2.6 Assessment of hypoglycemic activity of Plant drugs in normal healthy rat

It is essential to estimate the glucose tolerance of normal healthy animals. The animals were divided into six groups. Each group consists of six animals. The animals were caged for eight days. On the first day they were kept fasted for overnight and had free access to water. On the first day "fasting" blood glucose level was tested in the blood samples collected from the tip of the tails. The animals were allowed the normal feed and water.

On day eight, to the overnight fasted animals drugs were administered (as per the doses given in the **Table 8.2**). After 90 minutes of drug administration blood samples were withdrawn which served as '0' hr blood sample. After collection of blood samples, the animals were administered glucose solution 2g/kg of body weight orally. Again blood samples were collection at 1,2 and 3 hr after glucose administration to get the GTT pattern in normal healthy animals.

8.2.7 Statistical analysis

The result were expressed as mean ± S.D.. The statistical analysis involved in two groups was performed by means of paired *t* test, whereas analysis of variance (ANOVA) followed by Dunette's multiple comparison test were used in order to compare more than two groups. All the data were processed with Graph Pad Prism, version 4.01 software.

$P < 0.05$ was considered significant and $P < 0.01$ was considered more significant.

8.3 RESULTS OF HYPOGLYCEMIC ACTIVITY

8.3.1 Effect of extracts and isolated compounds on glucose tolerance test

The results of the antidiabetic activity on glucose tolerance in streptozotocin induced diabetic rat is presented in the **Table 8.3**. From the result it has been revealed that MEUP (methanolic extract of *Urtica parviflora*) succeeded to control the rise of serum glucose level (70.6%) within 1st hour of GTT in streptozotocin induced diabetic rats, followed by Compound I treated group i.e. Group IV (60.0%) and Compound II treated group i.e. Group V (55.2%). The serum glucose levels between '0' hour and 1 hour are generally compared in these type of studies because there is sudden rise of serum glucose level at the 1st hour after glucose loading in '0' hour. The immediate rise of serum glucose level challenges the efficacy of the test drugs to control it. The CEMC treated (24.24%) and compound III (isolated from *Morinda citrifolia*) treated group (17.8%) failed to normalize the rise of serum glucose level. Thus out of the three plants studied, the plant *Urtica parviflora*, was found to half remarkable hypoglycemic activity.

8.3.2 Assessment of antidiabetic effect (serum glucose level) in rats

The result of the antidiabetic effect of test drugs as well as of standard drug (Glibenclamide) is presented in the **Table 8.4**. The percentage reduction of serum glucose level by the MEUP treated group is maximum (27.2), which is much higher than the standard drug, Glibenclamide treated group (18.5). Even the percentage reduction of serum glucose level by MECA treated group is slightly higher (19.2) than the standard drug treated group. Compound II and Compound I treated groups showed this value at 16 and 15.2 respectively. The percentage reduction values are obtained when compared to diabetic control value. The result indicates high efficacy of MEUP and MECA in reducing serum glucose level in diabetic rats.

8.3.3 Assesment of hypoglycemic activity of the plants drug in normal healthy rats

The result of this activity is presented in the **Table 8.5**. The antidiabetic activity of the extracts and their respective isolated compounds was evaluated by considering the percentage reduction when compared to the value of the control group. Compoud I and Compound II exhibited same percentage reduction i.e. 14.1%, followed by MEUP and MECA respectively. Compound III and CEMC showed low values (3.5% and 2.3% respectively).

It is fairly evident from the studies performed in all the three models that maximum percentage reduction of serum glucose level was found with the MEUP and Compound I, isolated from the same plant *Urtica parviflora*. Hence the plant *Urtica parviflora* is having highest hypoglycemic activity, followed by *Calicarpa arborea* and *Morinda citrifolia*.

Table 8.2 The solvent extracts, their respective isolated compounds with doses and rout of administration used for this experiment.

Gr. No.	Plant Drug	Dose	Rout of administration
I	Methanolic Extract of *Urtica parviflora* (MEUP)	200 mg/kg body weight	Oral
II	Methanolic Extract of *Callicarpa arborea* (MECA)	200 mg/kg body weight	Oral
III	Chloroform Extract of *Morinda citrifolia* (CEMC)	200 mg/kg body weight	Oral
IV	Compound I	50 mg/kg body weight	Oral
V	Compound II	50 mg/kg body weight	Oral
VI	Compound III	50 mg/kg body weight	Oral
VII	Glibenclamide	0.20g/kg body weight	Oral
VIII	Streptozotocin	50 mg/kg body weight	Intra peritoneal
IX	Aqueous Glucose Solution	2g/kg body weight	Oral

Table 8.3 Antidiabetic activity on glucose tolerance in streptozotocin induced diabetic rats during GTT (Anand *et al.*, 2007).

(Group) Treatment	GROUP	Serum Glucose (mg/dl)					Increase in serum glucose (mg/dl) between 0 and 1 hr
		Fasting	0 hr	1 hr	2 hr	3 hr	
Group-I MEUP	C	278±7.0	287±6.4	362±6.7	351±5.8	332±6.0	75
	T	281±6.3	289±5.5	311 ±4.7**	300±4.8	287±5.1	22 (70.6%)#
Group-II MECA	C	287±6.1	280±5.8	358±4.4	330±4.7	309±4.8	78
	T	309±5.2	319±5.0	355 ±6.3**	333±6.2	296±6.7	36 (53.8%)#
Group-III CEMC	C	301±4.8	315±6.2	381±5.4	342±5.5	333±5.3	66
	T	313±5.7	318±4.8	368 ±6.1**	342±5.6	317±6.0	50 (24.24%)#
Group-IV Compound I	C	300±5.7	306±5.9	376±6.0	326±4.8	306±5.2	70
	T	289±5.1	290±5.3	318 ±5.6**	271±5.5	249±6.1	28 (60.0%)#
Group-V Compound II	C	311±4.4	317±4.6	384±5.2	361±5.2	336±5.4	67
	T	303±4.7	311±4.8	341 ±4.9**	312±4.7	292±6.1	30 (55.2%)#
Group-VI Compound III	C	307±5.2	316±5.3	389±6.2	364±4.8	343±4.5	73
	T	299±6.2	317±5.8	377 ±6.1**	350±6.8	326±6.7	60 (17.8%) #

[Values are mean ± SD from 6 animals in each group]

P values: *<0.05; **<0.01 as compared with control values at the same time
C = Control, T = Treated
# Percent of the untreated control of the same group.

Table 8.4 Effect of treatment of diabetic rats with glibenclamide, extracts and their respective isolated compouds for one week on glucose levels (Sharma *et al.*, 2006).

Groups of animals	Glucose levels (mg/dl) before and after one week of treatment			
	Before		After one week	
	Serum	Urine	Serum	Urine
(Gr I) Healthy Control	85±6.9	-	87±7.2	-
(Gr II) Diabetic Control	262±6.8	++	275±6.5	++
(Gr III) MEUP treated	255±5.5	++	200±7.0** 27.2#	+-
(Gr IV) MECA treated	260±5.8	++	222±6.1** 19.2#	+
(Gr V) CEMC treated	249±6.6	++	256±6.7** 6.9#	++
(Gr VI) Compound I treated	252±5.7	++	233±5.9** 15.2#	+
(Gr VII) Compound II treated	257±5.2	++	231±5.9** 16#	+
(Gr VIII) Compound III treated	251±5.6	++	263±6.2** 4.3#	++
(Gr IX) Glibenclamide treated	261±6.5	++	224±7.0** 18.5#	+

[Values are mean ± SD from 6 animals in each group]

P<0.01 when compared with the diabetic control values.

The percentage reduction is of one week value when compared with corresponding diabetic control value.

'+-' indicates presence of urine sugar in trace quantity, '++' indicates presence of urine sugar in more quantity, '-' indicates absence of urine sugar, '+' indicates presence of urine sugar in minimum quantity.

Table 8.5 Antidiabetic activity of the extract and isolated compounds on Serum glucose level during GTT in normal healthy rats (Anand *et al.*, 2007).

(Group) Treatment	Serum Glucose (mg/dl)					Increase in serum glucose (mg/dl) between 0 and 1 hr
	Fasting	0 hr	1 hr	2 hr	3 hr	
(Gr I) CONTROL	84 ±5.6	79 ±7.8	164 ±7.9	114 ±5.7	103 ±4.3	85
(Gr II) EEUP	81 ±6.1	75 ±6.6	150 ±5.1	97 ±4.5	76 ±3.3	75 (11.7%*)
(Gr III) EECA	82 ±6.3	76 ±6.2	154 ±5.3	104 ±5.0	86 ±4.1	78 (8.2%*)
(Gr IV) CEMC	80 ±5.9	76 ±6.5	159 ±6.0	116 ±5.7	104 ±5.1	83 (2.3%*)
(Gr V) Compound I	83 ±5.8	74 ±6.4	147 ±5.5	98 ±4.7	78 ±3.3	73 (14.1%*)
(Gr VI) Compound II	84 ±6.2	76 ±6.8	149 ±5.4	99 ±4.8	81 ±3.8	73 (14.1%*)
(Gr VII) Compound III	82 ±5.8	77 ±7.1	159 ±6.0	115 ±5.7	104 ±5.1	82 (3.5%*)

*Percent of the untreated control of the same group.

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CHAPTER 9

ANTIMICROBIAL ACTIVITY

9.0 INTRODUCTION

9.1.1 Infection and diseases

Infectious diseases are the leading cause of death worldwide, especially in developing countries. In recent times, epidemics of infections due to drug resistant and unknown microbial organisms have posed enormous health concern. Also they are the underlying cause of death in approximately 80% of cases even in the United States of America (Pinner *et al.*, 1996). This situation has called for renewed strategies on treatment and prevention, of which the development of new antimicrobial agents is one of the strategies (Fauci, 1998).

In recent time, it is observed that there is rapid increase of pathogenic bacteria become multidrug resistant. This alarming situation has its origin in the excessive and often inappropriate use of antibiotics in human and animal health care for the treatment and prevention of bacterial infections. Since the development of the first commercially available antibiotic penicillin in the 1940s, the high expectations by man in the healing power of these 'wonder drugs' has not been fulfilled, as the drug resistance posed problems in it. To control this situation, newer drugs armed with competent mechanism should be developed. The plant derived antimicrobial agents may be the solution to these problems.

The continuous use of antibiotics has resulted in multi-resistant bacterial strains all over the world and as expected, hospitals have become breeding grounds for human associated microorganisms (Mainous *et al.*, 2001) and spreading the nosocomial infections. As an example, it is now estimated that about half of all *Staphylococcus aureus* strains found in many medical institutions are resistant to antibiotics such as methicillin (Roder *et al.*, 1999). The emergence among enterococci of resistance to another useful and widely effective antibiotic, vancomycin (Novak *et al.*, 1999), might accelerate the spread of vancomycin-resistant genes, via plasmids, throughout other species, eventually limiting the efficacy of this drug.

The discovery of two classes of antimicrobial peptides, non-ribosomally synthesized (Hancock *et al.*, 1999) present in bacteria, lower eukaryotes and plants; secondly ribosomally synthesized peptides, of wider distribution (Boman, 1995; Broekaert *et al.*, 1997; Hancock *et al.*, 1998; Hoffmann *et al.*, 1999; Thevissen *et al.*, 1999; Zasloff, 2002; Ezekowitz *et al.*, 2003), provided a new therapeutic strategy to fight microorganisms. The knowledge acquired in the past two decades and the discovery of new groups of antimicrobial peptides makes natural antibiotics the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections (De Lucca, 2000; Hancock, 2000; Welling *et al.*, 2000; Selitrennikoff, 2001). In plants, a similar picture is slowly emerging *e.g.* A new family of antimicrobial peptides has been described from *Macadamia integrifolia* of which the first purified member has been termed MiAMP2c (Marcus *et al.*, 1997).

Plants have provided western medicine with an abundance of drugs and treatments for a variety of health problems (Lewis, 1977; Bruneton, 1999). While species used in traditional medicines continue to be the most reliable sources for the discovery of useful compounds, the screening of plants growing under various stresses (Ben *et al.*, 1992; Hanawa *et al.*, 1992; Kruger *et al.*, 1994; Broekaert *et al.*, 1997; Mohamed *et al.*, 1997; Dubery *et al.*, 1999; Pernas *et al.*, 2000) has provided yet another source for compounds with useful activities against microbes.

Recently, medicinal plants have become the focus of intense study regarding their conservation and potential pharmacological effects. Indeed, the search for new pharmacologically active agents, through the screening of natural sources such as microbial fermentations and plant extracts, has led to the discovery of many clinically useful drugs that now play major roles in the treatment of human diseases (Yue-Zhong, 1998; Leitao *et al.*, 2006; Funke, 2006).

Plants normally grow on different nature of soils which are extremely rich in microorganisms and infection remains a rare event. To keep out potential invaders, plants produce a wide range of selective antibacterial compounds either in a constitutive or an inducible manner (Cammue *et al.*, 1992). Among these compounds several low molecular weight proteins or peptides with antibacterial or antifungal activity have been isolated in recent years from various plants (Terras *et al.*, 1992; Hejgaard *et al.*, 1992; Roberts *et al.*, 1986) and are believed to be involved in a defence mechanism against phytopathogenic fungi by inhibiting microorganisms growth through diverse molecular modes, such as binding to chitin or increasing the permeability of the fungal membranes or cell wall. Also plants prevent the entry of invaders by localized production of antimicrobial low molecular weight secondary metabolites known as phytoalexins (Van *et al.*, 1989; Maher *et al.*, 1994). Moreover, the synthesis of many presumed defence related proteins are induced when plants are confronted with pathogens (Linthorst, 1991).

A large group of low molecular weight natural compounds that exhibit antimicrobial activity has been isolated from animals and plants during the past two decades. Among them, cationic peptides are the most widespread (Sergio *et al.*, 2003).

9.1.2 Historic use of plants as antimicrobials

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being. Their role is two fold in the development of new drugs: (1) they may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or; (2) a phytomedicine to be used for the treatment of diseases. There are numerous illustrations of plant derived

drugs. Some selected examples, including those classified as antiinfectives are presented below.

The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha* and related species, has been used for many years as amoebicidal drug as well as for the treatment of abscesses due to *Escherichia histolytica* infections (Cowan, 1999). Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs naturally in the bark of *Cinchona* tree. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malarial parasites have become resistant to the quinines, therefore antimalarial drugs with novel mode of action are required (Cowan, 1999).

The higher plants have made important contributions in the areas beyond antiinfectives, such as in cancer therapies, the antileukaemic alkaloids, vinblastine and vincristine are used. They are obtained from the Madagascan periwinkle (*Catharanthus roseus* Syn. *Vinca roseus*) (Nelson, 1982). Other cancer therapeutic agents include taxol, homoharringtonine and several derivatives of camptothein are plant originated. A well-known benzylisoquinoline alkaliod, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including cytomegalovirus, measles and HIV (Turano *et al.*, 1989). Most recently, three new atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B, and C were isolated from a newly described species tropical liana *Ancistrocladus korupensis* from the rainforest of Cameroon. The three compounds showed potential anti-HIV with michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effects of HIV-1 and HIV-2 on human lymphoblastoid target cell *in vitro* (Boyd *et al.*, 1994).

9.1.3 Antimicrobial compounds from plants

It is estimated that today, plant materials are present in, or have provided the models for 50% Western drugs (Robbers, 1996). Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most are secondary metabolites, of which at least 12,000 have been isolated, a number estimated to be less than 10% of the total. In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odors; others (quinones and tannins) are responsible for plant pigment. Many compounds are

responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds. Useful antimicrobial phytochemicals can be divided into several Categories. Laboratories of the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro*. More of these compounds should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies as well as an examination of their effects on beneficial normal micro biota. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would be facilitated. Also, alternative mechanisms of infection prevention and treatment should be included in initial activity screenings. Disruption of adhesion is one example of an anti-infection activity not commonly screened for currently. Attention to these issues could usher in a badly needed new era of chemotherapeutic treatment of infection by using plant-derived principles (Cowan, 1999).

9.1.4 Therapeutic benefit of natural antimicrobials

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin (Trease, 1972). Though most of the clinically used antibiotics are produced by soil micro-organisms or fungi, higher plants have also been a source of antibiotics (Trease, 1972). Examples of these are the bacteriostatic and antifugicidal properties of Lichens, the antibiotic action of allinine in *Allium sativum* (Garlic), or the antimicrobial action berberines in goldenseal (*Hydrastis canadensis*) (Trease, 1972). Plant based antimicrobials represent a vast untapped source for medicines. There is a great need of continued and further exploration of plant antimicrobials. Plants based antimicrobials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Many plants have tropisms to specific organs or systems in the body. Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis*. *Hydrastis* not only has antimicrobial activity, but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds (Murray, 1995).

9.1.5 Economic benefit

Worldwide, there has been a renewed interest in natural products due to the factors such as: consumer's belief that natural products are superior; their dissatisfaction with conventional medicines; changes in laws allowing structure-function claims which results in more liberal advertising and national concerns for health care cost (Cowan, 1999). Sales of products in this

market have increased dramatically in the last decade. Sales of botanical products in the United States have reached $3.1 billion of the $10.4 billion dollar dietary supplement industry 1996 (NBJ Sept, 1998). The industry anticipates growth in the order of 15–20% into the new millenium (Johnston, 1997). This growth rate will be maintained in an industry that is still considered to be in its infancy. Many plants which are previously collected from wild sources will need to be cultivated to meet the demands of the consumer. This represents many opportunities for the cultivation of medicinal crops for industries.

A market study show the exponential growth in the sale of plant based antimicrobials. In reviewing the top botanicals used as antiinfectives, the primary botanical used as an antimicrobial is *Hydrastis* with sales of 4.7% in 1995 (Gruenwald, 1997). While antiinfectives agents make up 24 % of the pharmaceutical market (1992 Census of Manufactures, 1994).

9.1.6 Plants with promising antiinfective activity

Many scientists emphasize on drug discovery from ethnomedicinal information using the 'Third Generation Approach'. This method differs from other methods as in this case clinical evaluation in humans takes place before the precise active constituents are known but the chemical composition and safety of the extracts are determined before formulation into dosage forms.

Plants containing protoberberines and related biflavones show antimicrobial activity. Some of these plants which are used in traditional African system of medicine and other plants are discussed below.

9.1.6.1 *Garcinia kola*, Bitter Kola (Guttiferae)

Garcinia kola is found in moist forest and grows as a medium size tree, up to 12 m high. It is cultivated and distributed throughout west and central Africa. Medicinal uses include purgative, antiparasitic and antimicrobial agents. The seeds are used in the treatment of bronchitis and throat infections. They are also used to prevent and relieve colic, cure head or chest colds and relieve cough. Also the plant is used for the treatment of liver disorders and as a chewing stick (Iwu, 1999).

The constituents include—biflavonoids, xanthones and benzophenones. The antimicrobial properties of this plant are attributed to the benzophenone, flavanones. This plant has shown anti-inflammatory, antimicrobial and antiviral properties. Studies show very good antimicrobial and antiviral properties. In addition, the plant possesses antidiabetic and antihepatotoxic activities (Iwu, 1999).

9.1.6.2 *Aframomum melegueta* (Zingiberaceae), Grains of Paradise

This is a spicy edible fruit that is cultivated and occurs throughout the tropics. It is a perennial herb. The medicinal uses of *Aframomum* include aphrodisiac, measles, and leprosy, taken for excessive lactation and post partum hemorrhage, purgative, galactogogue, anthelmintic and hemostatic agent. The constituents are essential oils—such as gingerol, shagaol and paradol. Studies show antimicrobial and antifungal activity and effective against schistosomes (Iwu, 1999).

9.1.6.3 *Xylopia aethiopica*, (Abbibacceae), Ethiopian Pepper

It is an evergreen, aromatic tree growing up to 20 m high with peppery fruit. It is native to the lowland rainforest and moist fringe forest in the savanna zones of in Africa. Medicinal uses of the plant are, as a carminative, as a cough remedy, and as a post partum tonic and lactation aid. Other uses are stomachache, bronchitis, biliousness and dysentery. It is also used externally as a poultice for headache and neuralgia. It is used with lemon grass for female hygiene. It is high in copper, manganese, and zinc (Smith, 1996).

Key constituents are diterpenic and xylopic acid. In studies, the fruit as an extracts has been shown to be active as an antimicrobial against gram positive and negative bacteria. Though, it has not been shown to be effective against *E. coli* (Iwu, 1999). Xylopic acid has also demonstrated activity against *Candida albicans* (Boakye-Yiadom, 1977).

9.1.6.4 *Cryptolepis sanguinolenta* Lindl. Schltr. (Periplocaceae)

A shrub that grows in the rainforest and the deciduous belt forest, found in the west coast of Africa. Related species appear in the east and southern regions of the continent. Its main medicinal use is for the treatment of fevers. It is used for urinary tract infections, especially *Candida*. Other uses are in inflammatory conditions, malaria, hypertension, microbial infections and inflammatory conditions of the stomach (Iwu, 1999).

9.1.6.5 *Amomum cannicarpum* (*Zingiberaceae*)

Zingiberaceae is one of the essential oil bearing plant families. These plants are mostly terrestrial, rhizomatous herbs. *Amomum* seeds are used as spices and their plant parts are used in traditional medicine for curing toothache, dysentery, diarrhoea, rheumatism, vomiting, dyspepsia, and lung diseases. *Amomum subulatum* or 'large cardamom' distributed in the eastern Himalayas is the most investigated *Amomum* species. Thirty-three constituents out of forty-one, containing 91.48% of the essential oil from the fruits of *A. cannicarpum*, are identified by GC-MS. The percentage of oxygenated sesquiterpenes in the oil is 47.97%, followed by monoterpene hydrocarbons (21.29%) and oxygenated monoterpenes (20.43%). The percentage of sesquiterpene hydrocarbons in the oil is relatively low (1.78%). The major

constituents of the oil are pinene (14.00%), elemol (10.45%) and cadinol (8.50%). The oil at a 33.3% (*V/V*) concentration in dimethyl sulfoxide showed good activity against the Gram-negative bacteria *Salmonella typhi*, *Pseudomonas aeruginosa* and *Proteus vulgaris* in comparison with streptomycin at 2 g per disc and against the fungi *Candida albicans* and *C. glabrata*, in comparison with the antifungal control, fluconazole at 2g per disc. They show no activity against *Bacillus subtilis*, *B. cereus*, *Klebsiella pneumoniae* and *Escherichia coli* (Sabulal *et al.*, 2006).

9.1.6.6 *Moringa oleifera*

Three fractions from the leaves of *Moringa oleifera* have antibacterial action against *Escherichia coli*, *Klebsiella aerogenes*, *Klebsiella pneumoniae*, *S. aureus*, and *B. subtilis*. They show strong inhibitory activity against *E. coli*, *S. aureus* and *B. subtilis* but clear zone of inhibition is also noted against *Klebsiella aerogenes* and inhibition against *Aspergillus niger*. Aqueous extract of *M. oleifera* leaves posseses significant antimicrobial activity against Gram positive and negative fungal species. The fractions are effective against the growth of pneumoniae, *A. fumigates*, *A. flavus* and *P. expansum*. The small proteins/peptides play an important role in plants of antimicrobial defense system and the plant resources can be utilized for isolation of antimicrobial peptides or small proteins (Dahot, 1998).

9.1.6.7 *Lantana camara* (Verbenaceae)

This plant has antimicrobial properties against three Gram-positive and two Gram negative bacteria, a non-acid fast bacterium, and the yeast, *Candida albicans* (Rajakaruna *et al.*, 2002).

9.1.6.8 Brazilian medicinal plants

The antibiotic activities of the ethanol extracts from 16 species of plants used in Brazilian folk medicine have been determined against *Staphylococcus aureus*, *Micrococcus flavus*, *Bacillus cereus*, *B. subtilis*, *Salmonella enteretidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Mycobacterium phlei*, *M. smegmatis* and *M. fortuitism*, and the yeasts *Candida albicans* and *C. krusei*. *Lafoensia pacari* and *Pterodon polygalaeflorus* show activity against the bacterial strains, and none amongst them are active against yeasts. The ethanolic extract from the leaves of *L. pacari* has minimum inhibitory concentration (MIC) values of 312.5 to 2500, 250, 625 and 1250 µg/mL, respectively, against eight different Gram-positive strains of *Staphylococcus aureus*, the Gram-negative *Proteus mirabilis* and the acid-fast bacilli *Mycobacterium phlei*, *M. fortuitum* and *M. smegmatis*. The ethanolic extract from the stem of *L. pacari* has the MIC value of 625 µg/mL against *S. aureus*. The crude extracts contain tannins, steroids, phenols, flavonoids, triterpenes and saponins. The activities are sufficiently high to present the possibility of future identification of the active components by bioassay-guided fractionation and purification (Maria *et al.*, 2006).

9.1.6.9 *Oxylipins* in plants have antimicrobial activity (Oxylipins)

Plant oxylipins are a large family of metabolites derived from polyunsaturated fatty acids and their biosynthesis depends upon the characterization of mutants or transgenic plants. Oxylipins have direct influence on the antimicrobial effect, stimulation of plant defense gene expression, and regulation of plant cell death. The precise contribution of individual oxylipins to plant defense remains essentially unknown. Most oxylipins are able to impair growth of some plant microbial pathogens and many oxylipins show inhibitory activity toward at least three different microbes. They strongly inhibit mycelial growth and spore germination of eukaryotic microbes. Oxylipins contribute to plant defense through their effects both on the plant and on pathogens, possibly through related mechanisms (Isabelle *et al.*, 2005).

9.1.6.10 *Aloe excelsa* (Aloe)

The fleshy leaves and roots of most species within the *Aloe* family are used in many traditional treatments (Mabberley, 1990). Traditional healers and indigenous people utilize mainly the leaf sap of this genus for the treatment of wounds, burns, rashes, itches, cracked lips and cracked skin (Cera *et al.*, 1980). *Aloe exclesa* leaf sap shows promising evidence of antibacterial and antifungal effect. A killing effect is seen even at the 10% dilution, indicating that the candidicidal compound is relatively potent. This evidence has shown that *A. excelsa* holds excellent potential as an antifungal agent against dermatophyte species *C. albicans, C. tropicalis* and *T. mentagrophytes* and other fungal species isolated from human superficial mycosis. As the number of organisms increases, the results become more credible. Further, these findings could be used to develop suitable dosage forms such as cream, ointment, and lotion as per the requirement of the treatment (Coopoosamy *et al.*, 2007).

9.1.6.11 *Sagittaria pygmaea* (Alismaceae)

Several plants of the genus *Sagittaria* has been used in traditional Chinese medicine for the treatment of various skin diseases. Phytochemical investigation reveals the presence of, ent-pimarane, ent-labdane and ent-kaurane direpinoides. *S.sagittifolia*, and several plant of this genus have series of new anti bacterial ent-rosane diterpinoides (Xue–Ting *et al.*, 2007).

9.1.6.12 Brazilian *Drosera*

The antimicrobial activity of three different extracts (hexane, ethyl acetate, methanol) obtained from Brazilian *Drosera* species (*D. communes, D. montana, D. brevifolia, D. villosa* var. graomogolensis, *D. villosa* var. villosa, Drosera sp. 1, and Drosera sp. 2) are tested against *Staphylococcus aureus, Enterococcus faecium, Pseudomonas aeruginosa, Escherichia coli, Salmonella choleraesuis, Klebsiella* pneumonia, and *Candida albicans* (a human isolate)

showed antimicrobial activity. Better activity can be observed with *D. communism* and *D. Montana* ethyl acetate extracts. Photochemical analyses from *D. communism, D. Montana* and *D. brevifolia* yields 5-hydroxy-2-methyl-1, 4- naphthoquinone (plumbagin); long chain aliphatic hydrocarbons are isolated from *D. communis* and from *D. villosa*, a mixture of long chain aliphatic alcohols and carboxylic acids, are isolated from *D. communism* and 3-O-acetylaleuritolic acid from *D. villosa* (Ferreira *et al.*, 2004).

9.1.6.13 Tannins in many perennial plants has antibacterial-promoting effects

Many tree leaves have antimicrobial factors, like tannins, essential oils, or other aromatic compounds (Kumar *et al.*, 1984). Nutritional and toxic effects of tannins present in various foodstuffs feed and fodder (Kumar *et al.*, 1984; Mehanso *et al.*, 1987). Antibacterial-promoting effects have been reported for plant tannins and flavornoids (Haslam, 1989; Scalbert, 1991; Chung *et al.*, 1998). Tannins and flavonoids present in plant leaves show antimicrobial activity against bacterial pathogens in ruminants, especially organisms such as *Staphylococcus aureus, Streptococcus* sp., coagulate-negative *Staphylococci,* Gram-negative rods, *Klebsiella* sp., *Escherichia coli* and *Enterobacter* sp. in *in vitro* disc diffusion method (Min *et al.*, 2008).

9.1.6.14 Antibacterial essential oils obtained from *Helichrysum* Species (Asteraceae)

The genus *Helichrysum* (Asteraceae) consists of about 500 species. There are 245 *Helichrysum taxa* found in Southern Africa which are divided in 30 groups (Hilliard, 1983). *Helichrysum cymosum* and *H. fulgidum* are aromatic perennial herbs with yellow flowers and characteristic odors, which are widespread in southern tropical Africa. Of all *Helichrysum* species occurring in Southern Africa, *H. cymosum, H. odoratissimum, H. petiolare* and *H. nudifolium* are among the best known and commonly used plants. The smoke of many *Helichrysum* species is used as ritual incense, called 'inphepho'. There are several different ways of administering these traditional medicines. To relieve cough and cold, a tea of leaves or the leaves boiled in milk are taken; for pain relief, leaves are burned and the smoke is inhaled. Leaves are widely used on wounds to prevent infection. Proven antimicrobial activity of these plants will provide scientific evidence for traditional use in wound dressing. Recent studies on essential oils from African *Helichrysum* species include those on oils of *H. bracteiferum, H. cordifolium, H. faradifani, H. gymnocephalum, H. hypnoides, H. kraussii, H. odoratissimum, H. rugulosum, H. rusillonii, H. se- laginifolium, and H. splendidum* (De Medici *et al.*, 1992; Lwande *et al.*, 1993; Theron *et al.*, 1994; Cavalli *et al.*, 2001; Bougatsos *et al.*, 2004). A systematic research on the chemical composition of *Helichrysum* species (Chinou *et al.*, 1996; Roussis *et al.*, 2002) has been reported, consisting of the study of the chemical constituents and antimicrobial activity of the essential oils obtained from the aerial parts of *Helichrysum cymosum* and *H. fulgidum* (Chinou *et al.*, 1996).

9.1.6.15 Antibacterial activity of plant essential oils

Majority of the essential oils show antibacterial activity. However Cinnamon, clove and lime oils are found to be inhibiting both Gram-positive and Gram-negative bacteria. Cinnamon oil can be a good source of antibacterial agents. Cinnamon oil has the most potential bactericidal properties. It can be used as an antibacterial supplement in the developing countries towards the development of new therapeutic agents. Additional *in vivo* studies and clinical trials are needed to test the potential of this oil as an antibacterial agent in topical or oral applications (Ignacimuthu *et al.*, 2006).

9.1.6.16 Antimicrobial activity of some ethno medicinal plants

Ethnomedicinal plants used in folkloric medicine such as *Acalypha fruticosa, Peltophorum pterocarpum, Toddalia asiatica, Cassia auriculata, Punica granatum* and *Syzygium lineare* exhibit antimicrobial activity against one or more microorganisms at three different concentrations of 1.25, 2.5 and 5 mg/disc. High antifungal activity is seen in the methanol extract of *Peltophorum pterocarpum* and *Punica granatum* against *Candida albicans*. Plants like *Toddalia asiatica, Syzygium lineare, Acalypha fruticosa* and *Peltophorum pterocarpum* could be potential sources of new antimicrobial agents.

9.2 Materials and Methods

9.2.1 Plant materials

Methanol extracts of *Urtica parviflora, Callicarpa arborea* leaves; *Morinda citrifolia* root and their respective isolated compounds (described in **Chapter 3**) were used as test drugs in this study.

9.2.2 Microorganisms

A total of 257 bacterial strains belonging to different genera were tested in this study. The test organisms were obtained from Department of Bacteriology, Calcutta School of Tropical Medicine, Kolkata, India and Institute of Microbial Technology (IMTECH), Chandigarh, India. All the strains are of human origin and were isolated in Himalayan Pharmacy Institute, Sikkim. Some *Staphylococcus* strains were kindly provided by Prof. (Mrs.) Sujata Ghosh Dastidar, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

9.2.3 Chemicals

Dimethyl sulphoxide (DMSO) and Mueller Hinton Agar were obtained from Ranbaxy Fine Chemicals.

9.2.4 Media

9.2.4.1 Liquid media

9.2.4.1.1 Peptone water

Peptone water having the following composition was used for the cultivation of bacterial strains as well as for spot inoculation.

Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%

The pH was adjusted to 7.2 to 7.4 and the volume was made up with distilled water.

9.2.4.1.2 Alkaline peptone water

The alkaline medium was prepared for the cultivation of *Vibrio cholerae*, as follows:

Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 8.5 to 9.0

9.2.4.1.3 Nutrient broth

Bacteriological peptone (Oxoid)	- 1.0%
Beef extract (Oxoid)	- 0.5%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 7.2 to 7.4

9.2.4.1.4 Solid media

9.2.4.1.4.1 Nutrient agar

This medium was used to isolate pure cultures of Gram-positive bacteria. It contained the following ingredients:

Agar (Oxoid)	- 3.0%
Beef extract (Oxoid)	- 0.5%
Bacteriological peptone (Oxoid)	- 1.0%
Sodium chloride (Analar)	- 0.5%
pH adjusted to	- 7.2 to 7.4

9.2.4.1.4.2 Bromothymol blue lactose agar

This medium consisted of the following ingredients:

Agar (Oxoid)	- 3.0%
Bacteriological peptone (Oxoid)	-1.0%
Beef extract (Oxoid)	- 0.5%
Sodium chloride (Analar)	- 0.5%

The pH was adjusted to 7.2 to 7.4 and 1.25 ml of bromothymol blue was added per 100 ml of the medium. After sterilization, 1.0% lactose was added, steamed for 30 minutes and poured in sterile petri dishes. This medium was used to isolate pure cultures of Gram- negative bacteria.

9.2.5 Preservation of bacterial cultures

All the strains of Staphylococci, Streptococci, Bacilli, *E. coli*, Klebsiellae, Salmonellae, Shigellae, Citrobacter, *Pseudomonas* spp. and Vibrios were preserved as stab slant cultures at a temperature of 4ºC and in freeze dried state after checking of purity and identification where necessary. Routine subculturing of the Gram-positive bacteria was carried out on nutrient agar and Gram-negative strains on bromoothymol blue lactose agar (Barrow *et al.,* 1993).

9.2.6 Standard antibiotics

The standard antibiotics used in the studies were amoxycillin (Lyka Labs, India) and gentamycin (Hindustan Antibiotics, Pimpri, Pune) obtained from the respective manufacturers.

9.2.7 Preparation of impregnated discs of extract and standard antibiotics

The discs of 7.25 mm diameter were prepared by punching of Whatman No.1 filter paper and were sterilized by dry heat at 160ºC for an hour in batches of 100 in screw capped Bijou bottles. The dried extract (semisolid) of *Urtica parviflora* leaf, *Callicarpa arborea* leaf and *Morinda citrifolia* root were weighed and dissolved in 0.5 ml of dimethyl sulphoxide, as the extracts are not fully soluble in water, and then diluted in sterile distilled water to make the required stock solutions. For each extract three stock solutions were prepared. Similarly the stock solutions of the control antibiotics were prepared by dissolving the required amount of amoxycillin or diluting required amount of gentamicin in 10 ml of sterile distilled water separately to prepare two fold serially dilutions of the antibiotics (0–1000 µg/ml concentrations). All the stock solutions were then kept at 4ºC and used for three months. For preparation of antibiotic impregnated discs 1.0 ml of the stock solutions of the antibiotic were added separately to each bottle of 100 discs. Each disc adsorbed 0.01 ml of the solution, so the entire 1.0 ml volume was adsorbed by 100 discs, each giving the required two fold concentrations of 0–1000 µg/ml. The procedure was repeated for preparation of impregnated

discs of the plant extracts and their isolated compounds. The discs were used in wet condition and for further use they were stored at 4°C, as the discs can retain their moisture and potency for at least 3 months in the screw capped bottles.

9.2.8 Antimicrobial activity of *Urtica parviflora* leaf

Microbial sensitivity tests were performed by disc diffusion method (Dash, 1977). The nutrient agar plates, containing an inoculum size of 10^5-10^6 cfu/ml of bacteria were used. Previously prepared crude methanol extract (Concentration 128-2000 µg/ml) and isolated compound (Concentration 0-1000 µg/ml) discs were placed aseptically on sensitivity plates. The discs containing no test compound and standard antibiotics (Amoxycillin and Gentamicin) served as negative and positive controls respectively. All the plates were then incubated at 37°C±2°C for 18 hr. The sensitivity was recorded by measuring the clear zone of inhibition on agar plate around the discs.

The MICs were determined by the standard agar dilution method (Dastidar, 1995). The crude methanol extract was dissolved in 0.5 ml of dimethyl sulphoxide, as they are not fully soluble in water, and then diluted by sterile distilled water to make solution. The drug solution was then added to the molten nutrient agar in different tubes to give final concentrations of 0–128 µg/ml and subsequently increasing it by two fold concentration up to 2000 µg/ml. The concentrations of the tubes were mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. Bacterial cell suspensions were spot inoculated on the plates using a bacterial planter (10 µl). The final number of cfu inoculated onto the agar plates was 10^5 for all strains. The inoculated plates were then incubated at 37°C±2°C for 18 h. The lowest concentration of the plate, which did not show any visible growth after incubation, was considered as MIC. The same method was followed for the isolated Compound I also. The agar plate containing only sterile distilled water and Amoxycillin was served as negative and positive control respectively.

9.2.9 Antimicrobial activity of *Callicarpa arborea* leaf

The antibacterial activity of ethanol extract of *Callicarpa arborea* leaf and its isolated compound II was determined as per the methods described above in **9.2.8** for *Urtica parviflora* leaf.

9.2.10 Antimicrobial activity of *Morinda citrifolia* root

The antibacterial activity of methanol extract of *Morinda citrifolia* root and its isolated compound III was determined as per the methods described above in **9.2.8** for *Urtica parviflora* leaf.

9.3 RESULTS

9.3.1 Antimicrobial activity of *Urtica parviflora* leaf

The methanol extract of *Urtica parviflora* leaf exhibited a significant *in vitro* antimicrobial activity against 257 stains of Gram-positive and Gram-negative bacteria including MRSC. All the three reference MRSC strains of bacteria were found to be sensitive between 256 and 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the leaf extract presented in **Table 9.1** showed that out of 257 bacteria, the growth of 168 isolates were inhibited at a concentration of 128 – 512 μg/ml. 76 isolates were resistant at <1000μg/ml, while remaining 13 isolates where resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 58 out of 63 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 92 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed both against Gram-positive and Gram-negative bacteria. The isolated compound I was also tested for antimicrobial activity. The result is presented in **Table 9.2**, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the compound I except the *Vibrio cholera* 14033. It was interesting to note that all the MRSC strains were susceptible to Compound I at concentration of 128-256 μg/ml, while they are resistant to both the standard antibiotics used.

9.3.2 Antimicrobial activity of *Callicarpa arborea* leaf

The methanol extract of *Callicarpa arborea* leaf exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria. The results of the antimicrobial spectrum of the leaf extract presented in **Table 9.3** showed that out of 257 bacteria, the growth of 221 isolates were inhibited by the extract at a concentration of 128 – 512 μg/ml. 35 isolates were resistant at <1000μg/ml, while remaining 1 isolate was resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 51 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 128-256 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed both against Gram-positive and Gram-negative bacteria but more sensitive to Gram-negative strains. The isolated compound II was also tested for antimicrobial activity. The result is presented in **Table 9.4**, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the Compound II. It was noted that all the MRSC strains were resistant to compound II at concentration of 128 μg/ml, while they are resistant to both the standard antibiotics used.

9.3.3 Antimicrobial activity of *Morinda citrifolia* root

The methanol extract of *Morinda citrifolia* root exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multiresistant *Staphylococcus* (MRSC) strains. All the three reference MRSC strains of bacteria were found to be sensitive within 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the root extract presented in **Table 9.5** showed that out of 257 bacteria, the growth of 217 isolates were inhibited by the extract at a concentration of 128– 512 μg/ml, 37 isolates were inhibited at a concentration of 1000μg/ml, while the remaining 03 isolates where inhibited at concentration >2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 64 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the ethanol extract was directed both against Gram-positive and Gram-negative bacteria. The isolated compound III was also tested for antimicrobial activity. The result is presented in **Table 9.6**, which revealed that all the isolates were sensitive at 128-512 μg/ml concentration of the compound III except the *P. aeruginosa*. It was interesting to note that all the MRSC strains were susceptible to Compound III at a concentration of 128 μg/ml, while they were resistant to the two standard antibiotics used.

Table 9.1 *In vitro* antimicrobial spectrum of methanol extract of *Urtica parviflora*.

Bacterial Species	No. of strn.	MIC of leaf extract (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	> 2000	0.25	0.5	8	64	128	256	> 1000
E.Coli	70	03	21	21	24	01	-	07	03	03	05	15	37
Klebsiella spp.	12	-	-	03	09	-	-	-	-	-	-	02	10
Salmonella Spp.	18	-	07	06	05	-	-	-	-	-	01	04	13
Shigella spp.	34	02	02	10	14	06	-	-	-	-	02	10	22
Vibrio cholerae	15	-	03	04	06	02	-	-	-	-	-	03	12
Citrobacter spp.	15	-	02	03	08	02	-	-	-	-	-	01	12
Pseudomonas aeruginosa	15	-	01	09	03	02	-	-	-	-	-	02	14
Bacillus subtilis	06	1	03	02	-	-	-	-	04	01	-	01	13
Staphylococcus aureus	62	21	24	10	07	-	01	27	12	13	-	09	-
Streptococcus faecalis	10	05	04	01	-	-	01	05	04	-	-	-	-
Total	257	32	67	69	76	13	02	39	23	17	08	47	121

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 9.2 The MIC of 15 sensitive bacteria against methanol extract of *Urtica parviflora* and Compound I.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	MEUP	CM I	Amoxycillin	Gentamycine	
E.Coli 832	512	512	0.50	0.25	+
E.Coli TG_1	512	512	0.50	>256	+
E.Coli 871	256	512	0.50	0.50	+
E.Coli HD_{10}	512	512	0.25	0.50	+
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	256	256	8.0	1.0	+
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	++
Bacillus lichenfermis 10341	512	256	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	256	512	8.0	>256	++
V.Cholerae 14033	1000	1000	8.0	>256	+
Klebsiella pneumoniae.	256	256	256	0.50	++
Pseudomonas aeruginosa	256	256	2.0	0.50	++

MEUP: Methanol extract of *Urtica parviflora*; CM I= Compound I; '+' = ≤ 10mm; '++'= ≥12mm; inoculum size used 10^5 cfu per spot for all organisms except *S.aureus*, where 10^6 cfu where used. The results are expressed as Mean ± S.E.M (n=3).

Table 9.3 *In vitro* antimicrobial spectrum of *Callicarpa arborea* leaf extract.

Bacterial Species	No. of strn.	MIC of leaf extract (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	> 2000	0.25	0.5	8	64	128	256	> 1000
E.Coli	70	11	22	27	08	01	-	07	03	03	05	15	37
Klebsiella spp.	12	01	02	04	05	-	-	-	-	-	-	02	10
Salmonella Spp.	18	02	04	06	06	-	-	-	-	-	01	04	13
Shigella spp.	34	07	09	12	06	-	-	-	-	-	02	10	22
Vibro cholera	15	01	06	08	-	-	-	-	-	-	-	03	12
Citrobacter spp.	15	02	03	06	04	-	-	-	-	-	-	01	12
Pseudomonas aeruginosa	15	01	03	08	03	-	-	-	-	-	-	02	14
Bacillus subtilis	06	03	02	01	-	-	-	-	04	01	-	01	13
Staphylococcus aureus	62	13	22	24	03	-	01	27	12	13	-	09	-
Streptococcus faecalis	10	04	07	-	-	-	01	05	04	-	-	-	-
Total	257	45	80	96	35	01	02	39	23	17	08	47	121

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus*, where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 9.4 The MIC of 15 sensitive bacteria against methanol extract of *Callicarpa arborea* leaf and Compound II.

Name of the Organisms	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	MECA	CM II	Amoxycillin	Gentamicin	
E.Coli 832	256	128	0.50	0.25	++
E.Coli TG_1	256	128	0.50	>256	++
E.Coli 871	256	128	0.50	0.50	++
E.Coli HD_{10}	256	256	0.25	0.50	++
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	128	128	8.0	1.0	+
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	++
Bacillus lichenfermis 10341	512	512	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	+
S.typhimurium NCTC 74	256	256	8.0	>256	+
V.Cholerae 14033	128	128	8.0	>256	+
Klebsiella pneumoniae.	256	256	256	0.50	+
Pseudomonas aeruginosa	512	512	2.0	0.50	+

MECA: Methanol extract of *Callicarpa arborea* leaf; CM II: Isolated compound of *Callicarpa arborea*; '+'= ≤ 10mm; '++'= ≥12mm; innoculum size used 10^5 cfu per spot for all organisms except S.*aureus*, where 10^6 cfu where used. The results are means ± S.E.M (n=3).

Table 9.5 *In vitro* antimicrobial spectrum of *Morinda citrifolia* root extract.

Bacterial Species	No of strn	MIC of root extract (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	> 2000	0.25	0.5	8	64	128	256	> 1000
E.Coli	70	08	12	35	14	01	-	07	03	03	05	15	37
Klebsiella spp.	12	01	02	04	05	-	-	-	-	-	-	02	10
Salmonella Spp.	18	01	08	09	-	-	-	-	-	-	01	04	13
Shigella spp.	34	07	09	12	06	-	-	-	-	-	02	10	22
Vibro cholerae	15	02	02	08	02	01	-	-	-	-	-	03	12
Citrobacter Spp.	15	02	02	06	04	01	-	-	-	-	-	01	12
Pseudomonas aeruginosa	15	01	06	05	03	-	-	-	-	-	-	02	14
Bacillus subtilis	06	03	02	01	-	-	-	-	04	01	-	01	13
Staphylococcus aureus	62	29	20	10	03	-	01	27	12	13	-	09	-
Streptococcus faecalis	10	04	06	-	-	-	01	05	04	-	-	-	-
Total	257	58	69	90	37	03	02	39	23	17	08	47	121

Inoculum size used 10^5 cfu per spot for all the organisms except *S.aureus,* where the inoculum size per spot was 10^6 cfu. The results are the mean value of triplicate tests.

Table 9.6 The MIC of 15 sensitive bacteria against methanol extract of *Morinda citrifolia* root and Compound III.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	MEMC	CM III	Amoxycillin	Gentamicin	
E.Coli 832	256	256	0.50	0.25	++
E.Coli TG_1	512	512	0.50	>256	++
E.Coli 871	256	512	0.50	0.50	++
E.Coli HD_{10}	256	256	0.25	0.50	++
S. aureus NCTC 6571	128	128	0.50	1.0	++
S. aureus NCTC 8530	128	128	0.50	0.50	++
S. aureus Bang 44	128	128	8.0	1.0	++
S. aureus ML 275	128	128	0.50	1.0	++
S. epidermidis 865	128	128	0.50	0.50	+
Bacillus lichenfermis 10341	512	512	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	128	128	8.0	>256	++
V.Cholerae 14033	512	512	8.0	>256	+
Klebsiella pneumoniae.	512	512	256	0.50	+
Pseudomonas aeruginosa	512	1000	2.0	0.50	+

MEMC: methanol extract of *Morinda citrifolia* root; CM III: Isolated compound of *Morinda citrifolia*; '+'= ≤ 10mm; '++'= ≥12mm; innoculum size used 10^5 cfu per spot for all organisms except S.*aureus*, where 10^6 cfu where used. The results are means ± S.E.M (n=3).

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CHAPTER 10

ANTIINFLAMMATORY & ANTIPYRETIC ACTIVITY

(A) ANTIINFLAMMATORY ACTIVITY

10.1 Inflammation

Based on visual observation, an inflammation is characterized by five cardinal signs, namely redness (rubor), swelling (tumour), heat (calor), pain (dolor) and loss of function. The first four of these signs were named by Celsus in ancient Rome (30–38 B.C.) and the last by Galen (A.D 130–200) (Hurley, 1972). Inflammation is described as "the succession of changes which occurs in a living tissue when it is injured provided that the injury is not of such a degree as to at once destroys its structure and vitality" (Sanderson, 1871) or "the reaction to injury of the living microcirculation and related tissues (Spector *et al.*, 1963). Although, in ancient times, inflammation was recognized as being part of the healing process, up to the end of the 19th century, inflammation was viewed as being an undesirable response that is harmful to the host. However, beginning with the work of Metchnikoff and others in the 19th century, the contribution of inflammation to the body's defensive and healing process was recognized (Hurley, 1972). The classical description of inflammation accounts for the visual changes seen. Thus, the sensation of heat is caused by the increased movement of blood through dilated vessels into the environmentally cooled extremities, also resulting on the increased redness (due to the additional number of erythrocytes passing through the area). The swelling (edema) is the result of increased passage of fluid from dilated and permeable blood vessels into the surrounding tissues, infiltration of cells into the damaged area and in prolonged inflammatory responses deposition of connective tissue. Pain is due to the direct effects of mediators, either from initial damage or that resulting from the inflammatory response itself, and the stretching of sensory nerves due to oedema. The loss of function refers to either simple loss of mobility in a joint, due to the oedema and pain, or to the replacement of functional cells with scar tissue. Today it is recognized that inflammation is far more complex than might first appear from the simple description given above and is a major response of the immune system to tissue damage and infection; although not all infection gives rise to inflammation. Inflammation is also diverse, ranging from the acute inflammation associated with *S. aureus* infection of the skin, through to chronic inflammatory processes resulting in remodeling of the artery wall in atherosclerosis; the bronchial wall in asthma and chronic bronchitis and the debilitating destruction of the joints associated with rheumatoid arthritis. These processes involve the major cells of the immune system, including neutrophils, basophils, mast cells, T-cells, B-cells, etc. However, examination of a range of inflammatory lesions demonstrates the presence of specific leukocytes in any given lesion. That is, the inflammatory process is regulated in such a way that ensures the appropriate leukocytes are recruited for their respective functions. These events are controlled by a host of extracellular mediators and regulators, including cytokines, growth factors, eicosanoids (prostaglandins, leukotrines, etc) and peptides. The role of inflammation as a healing, restorative process, as well as its aggressive role, is also more

widely recognized today. Inflammation is now considered as the full circle of events, from initiation of a response, through the development of the five cardinal signs stated before, to healing and restoration of normal appearance and function of the tissue or organ. However, in certain conditions it appears to be no resolution and a chronic state of inflammation develops that may last the life of the individual. Such conditions include the inflammatory disorders rheumatoid arthritis, osteoarthritis, inflammatory bowel diseases, retinitis, multiple sclerosis, psoriasis and atherosclerosis. In order to study inflammation, a multidisciplinary approach is necessary and knowledge of the immune system is required, in order to understand the events involved in initiation and maintenance of the inflammatory conditions. Recently it is recognized that the underlying genetics and molecular biology basis to cellular responses are also important in order to identify genetic predisposition to inflammatory diseases, while pharmacological studies are necessary to identify targets and for development of novel treatments to bring relief from chronic life-threatening inflammatory conditions. Thus research into inflammation includes not only the study of immunological and cellular responses involved but also the pharmacological processes involved in drug development to treat it (Rios *et al.*, 2005).

Many of the drugs used in the treatment of inflammatory conditions, predate our current understanding of the biochemical processes involved in the disease. Traditionally, the standard treatments for rheumatoid arthritis has been to use a non-steroidal anti-inflammatory drug (NSAID), such as aspirin, for pain relief and to use corticosteroids or even disease-modifying anti-rheumatic drugs in an attempt to reduce other symptoms of the disease. For many years the pharmaceutical industry attempted to develop NSAIDs which shared the therapeutic action of aspirin but which did not cause the main adverse event, namely gastric ulceration. However, while all these drugs had clinical utility they also eroded the gastric mucosa. The fact that a large number of patients with severe chronic inflammatory disease fail to respond to conventional systemic or topical therapy resulting in a huge clinical and socio-economic burden urgently needs the development of novel therapies.

10.2 Nonsteroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are the most commonly used drugs in inflammatory diseases, since they are effective in management of pain, fever, redness, edema arising as a consequence of inflammatory mediator release (Ferreira, 2002, Mitchell *et al.*, 1999). The drugs are named NSAIDs because they are structurally different from steroidal anti-inflammatory drugs (Vane *et al.*, 1990). As NSAIDs possess analgesic, antipyretic and anti-inflammatory effects, they have certain advantages in the treatment of diseases with pain, fever and inflammation, when compared to

steroidal anti-inflammatory and narcotic analgesic drugs (Amadio *et al.*, 1993). Although NSAIDs have distinct chemical structures, they have similar therapeutic and side effects (Rainsford, 1999). Studies have shown that both therapeutic and side effects of NSAIDs are dependent on cyclooxygenase (COX) ('constitutive' COX-1 and 'inducible' COX-2) inhibition (Warner *et al.*, 1999). It has been suggested that COX-2 inhibition is responsible for the therapeutic effects of NSAIDs, while COX-1 inhibition causes the gastrointestinal and renal side effects (Patrignani, 2000, Xie *et al.*, 1991, Zhao *et al.*, 2001). Although both isoforms had similar amino acid sequence and catalytic activity, they were demonstrated to have different functions (Maricic *et al.*, 1999, Xie *et al.*, 1991). Induction of COX-2 by various growth factors, proinflammatory agents, endotoxins, mitogens, tumor agents (Ferraz *et al.*, 1997, Michauluart *et al.*, 1999, Mitchell *et al.*, 1994) indicates that this isoform may have a role in formation of pathological processes, such as inflammation (Siegle *et al.*, 1998, Vane *et al.*, 1994), COX-1 products and prostaglandins (PGI_2 and PGE_2). **Fig 10.1** gives a summary of the biosynthesis of prostaglandins (PGs).

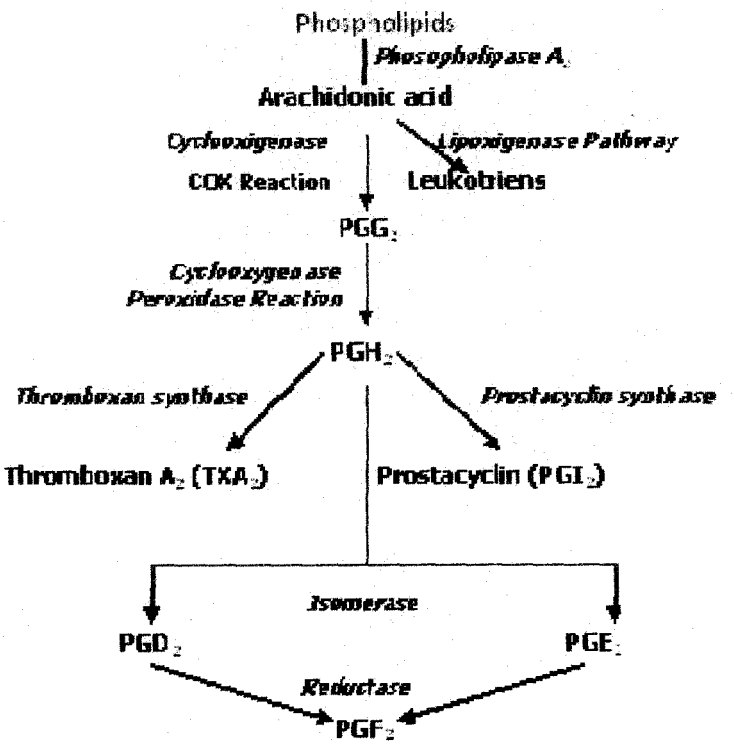

Fig 10.1 Summary of the biosynthesis of PGs.

10.2.1 Classification of NSAIDs

NSAIDs can be grouped in four categories -

1. Selective COX-1 inhibitors, such as aspirin.
2. Non-selective COX inhibitors: a number of the NSAIDs examined exhibit COX-1/COX-2 IC_{50} ratios between 0.5 and 3.0.
3. Relatively selective COX-2 inhibitors, such as meloxicam, nimesulide, diclofenac with COX-1/COX-2 IC_{50} ratios of 10 to 20.
4. Highly selective COX-2 inhibitors comprising three experimental compounds (NS-398, L-745, 337 and SC-58125, the prototype of celecoxib) with COX-1/ COX-2 IC_{50} ratios from 140 to 250 and rofecoxib with the ratio > 400 (Patrignani, 2000).

10.2.2 Mechanisms of action of NSAIDs

These mechanisms comprise inhibition of synthesis of COX and leukotriene (LO) products, prevention of release of toxic oxygen radicals and lysosomal enzymes, prevention of neutrophil aggregation, adhesion and chemo taxis, and uncoupling of oxidative phosphorylation (Bednarek D et al., 1999, Brooks P M et al., 1991, Celotti F et al., 2001, Higgs G A et al., 1980, Jacobs J W G et al., 1996, Mahmud T et al., 1996). Recently, it has been suggested that stimulation of peroxisome proliferators-activated receptors (PPARs) and inhibition of nuclear factor kappa B (NF-κB) and other transcription factors play an important role in the mechanism of actions of NSAIDs. PPARs are members of the nuclear receptor family. Three genes encode the peroxisome proliferators activated receptor (PPAR) family members, PPAR-α, PPAR-β/δ and PPAR-γ. All three PPARs are widely expressed in different tissues. Influence of PPARs on gene expression may occur directly through promoter-binding and transcriptional modulation of target genes or through indirect interference with other transcription factor pathways leading in most cases to transcription inhibition. PPARs regulate transcription of target genes involved in lipid and lipoprotein metabolism, glucose homeostasis and cell differentiation (particularly adipogenesis). PPARs inhibit the activation of certain inflammatory response genes acting as transrepressors.

10.2.3 Methods of study of antiinflammatory activity

a) Carrageenan induced paw edema method

This is a commonly employed method based on inhibition of the paw edema, induced by the injection of phlogistic agents (Inflammation causing agents). Carrageenan is a marine derived polysaccharide which is injected (0.1ml of 0.2% soln) in the subplantar region of hind paw of rats and the swelling or edema caused, is measured. The swellings can be measured with the help of plethysmograph (Volume) or by slide caliper (Thickness).

Other commonly used phlogistic agents (Vogel, 2002) are:

0.05ml undiluted fresh egg white
0.1ml of 1% ova albumin solution
0.1ml of 1% formalin
0.1ml of 1-3% dextran solution
0.1ml of 0.1% trypsin solution
0.1ml of 0.1% collagenase solution etc.

b) Cotton pellets method

The subcutaneous implantation of sterile cotton pellets into experimental animals is carried out to asses the effects of drugs on the formation of granulation tissue. The initial phase of the response occurs within the first 3 hr and is described as transudative phase, which is characterized by the accumulation of low protein exudates. The second phase of the response involves increase in vascular permeability and the subsequent appearance of exudates. The third phase of the response is described as the proliferative phase and is characterized by the appearance of collagen in the granuloma on day 4 after pellet implantation. This model has also been used to determine the contribution of several inflammatory mediators to the cellular response seen in implanted sponges (Meier *et al.*, 1950).

Rheumatoid arthritis is a chronic inflammatory disorder of unknown etiology characterized by joint pain and swelling with multiple extra-articular manifestations. The adjuvant related diseases which manifestation is synovitis, similar to that of rheumatoid arthritis can be produced in rats injecting mineral oil containing cell protein from mycobacterium. In addition similar conditions in joint tissue can be produced in rabbits with streptococcal infections and in mice with mycobacterial infections (Nigel, 1987). The biochemical characteristics in Rheumatoid arthritis are due to chronic inflammatory parameters such as perturbations in serum proteins, low plasma level of histidine, increased fluid volume, color change of serum from pale yellow to white or dark yellow viscosity falls and poor clotting.
The level of inhibition of edema can be calculated for each drug using the equation (Perez, 1986):

$$\textbf{Inhibition (\%)} = 100\{1 - (a-x/b-y)\}$$

Where,
a= mean paw volume of treated animals after carrageenan injection
x = mean paw volume of treated animals before carrageenan injection
b = mean paw volume of control animals after carrageenan injection
y = mean paw volume of control animals before carrageenan injection

or by using the following formula:

% Inhibition of Paw Edema = (Vc-Vt) 100/ Vc

Where, Vc =Volume of paw edema of control animals in ml, and
Vt = Volume of paw edema of drug treated animals in ml.

10.3 Anti-inflammatory activity of *Urtica parviflora* leaf, *Callicarpa arborea* leaf and *Morinda citrifolia* root.

10.3.1 Materials and Methods

10.3.1.1 Plant materials

The fresh leaves of *Urtica parviflora (U. parviflora), Callicarpa arborea (C. arborea)* and root bark of *Morinda citrifolia (M. citrifolia)* were collected at Majhitar, East Sikkim and were authenticated by Botanical Survey of India (BSI), Gangtok, Sikkim and the herbaria were preserved in the institutional museum (HPI / PK/ No. 131, 132 and 133).

10.3.1.2 Preparation of extracts and their respective isolated compounds

The leaves of *U. parviflora* and *C. arborea,* free from dirt were separated and shade dried for ten days and made to powder by a mechanical grinder. The powdered drugs (500g) were extracted with ethanol by continuous hot extraction process (soxhelation). The solvent was recovered and the extracts were concentrated under reduced pressure. In case of *M. citrifolia* the clean roots are shade dried for twenty days and then subjected to soxhelation using ethanol as the solvent. The extract yield was found to be 5% for *U. parviflora* (MEUP), 7.5% for *C. arborea* (MECA) and 11.0% for *M. citrifolia* (MEMC) and their Isolated compounds as compound I, II, and III were obtained as described in **Chapter 3,** used in the study.

10.3.1.3 Instruments and Chemicals

Plethysmometer (Model-520-R, IITC Life sciences, USA); Ibuprofen, phenidone, carrageenan and arachidonic acid were purchased from Sigma chemicals. All other chemicals were of analytical grade and procured locally.

10.3.1.4 Test animals

Healthy male albino rats weighing between 160-220gm were used in the study. They were individually housed in aseptic condition and maintained on normal diet and water. They were kept in plastic cages at 23 ± 1^{0}C in 12:12 hr dark: light cycle. All the experiments were carried out between 10:00 and 16:00 hrs. The animal experiments were conducted as per protocol approved by the *Institutional Animal Ethics Committee (IAEC) No.* HPI/07/60/IAEC/0005.

10.3.1.5 Carrageenan induced rat paw edema test

The rat paw edema method of Winter *et al.*, (1962) was used. The extracts, MEUP, MECA and MEMC at the dose of 200mg/kg b.w. and the isolated compounds I, II, and III at the dose of 30mg/kg b.w. were administered orally to the animals of group III to VIII respectively. Control animals of group I received (2%w/v CMC 1ml/kg b.w.p.o) and the animals of group II(standard) received Ibuprofen at the dose of 100mg/kg b.w. Thirty minutes after administration of the test drugs inflammation was induced by subplantar injection of 0.1 ml of 1% w/v carrageenan solution. Edema was assessed in terms of volume of distilled water displaced by the paw before and at 0, 1, 2, 3, and 4 hours after induction of inflammation. The percentage inhibitions of paw edema in the animals were determined using the following equation.

% Inhibition of Paw Edema = (Vc-Vt) 100/ Vc

Where, Vc = Volume of paw edema of control animals in ml and
Vt = Volume of paw edema of drug treated animals in ml.

10.3.1.6 Cotton pellet induced granuloma test

Cotton pellet granuloma was induced according to the method of D' Arcy *et al.*, Sterilised cotton pellets each weighing 10mg were implanted in both axilla and groin of each rat under light ether anaesthesia. Forty eight rats were divided into 8 (eight) groups, six animals in each group. The group I was treated with 2% CMC (1ml/kg b.w.p.o) and group II was treated with Indomethacin at a dose of 10mg/kg b.w.p.o (Mujumdar *et al.*, 2000). Animals of group III to V received MEUP, MECA and MEMC at the dose of 200mg/kg and the groups VI to VIII received the compound I, II, and III at the dose of 30mg/kg b.w.p.o. After 7 (seven) days the animals were sacrificed by cervical dislocation and the cotton pellets along with the granuloma tissues were collected and dried in an oven at 60^0C, weighed and resulted weights were compared with control. The percentage inhibition of granuloma by the test drugs was determined.

10.3.1.7 Arachidonic acid induced paw edema test

Paw edema was induced by single injection of 0.1ml of 0.5% w/v arachidonic acid in 0.2M carbonate buffer (pH 8.4) into the right hind paw of each rat. The test drugs, MEUP, MECA and MEMC were administered at the dose of 200mg/kgb.w.p.o to animals of group III to V. The control group I received 2.0% CMC solution (1ml/kg b.w.) and the standard group II received the Standard drug, Phenidone at the dose of 200mg/kg b.w.p.o. Animals of group VI to VIII received the compound I, II, and III at a dose of 30mg/kg b.w.p.o. respectively. All the treatments were given before 2 (two) hours of administration of arachidonic acid and the paw

volumes were measured after one hour of administration of the same with the help of a Plethysmometer. The paw volumes of different groups of animals were compared with the control.

10.4 Results of the Antiinflammatory activity of *Urtica parviflora* leaf, *Callicarpaarborea* leaf and *Morinda citrifolia* root

10.4.1 Carrageenan-induced edema test

The results of the carrageenan induced paw edema study are presented in **Table 10.1**.From the result it is evident that the test drugs inhibited the paw edema in dose and time dependent manner. From the table, it is seen that Compound II in Group VII showed percentage inhibition of 17.39 at the 1st hour, where as the standard drug Ibuprofen had percentage inhibition of 18.26 followed by MECA (16.52). In the 2nd hour maximum inhibition percentage amongst the test drugs treated groups was observed incase of Compound II treated group which was continued for 3rd (48.76) and 4th hour (61.69). It indicates the high efficacy of Compound II as compared to the standard drug Ibuprofen. In other words it can be said that Compound II at the dose of 30mg/kg b.w.p.o. has anti-inflammatory activity in carrageenan induced paw edema in rats similar to the standard drug at the dose of 100mg/kg b.w.p.o. In conclusion out of the three plants the plant *C.arboria* (contains compound II) has got maximum anti-inflammatory activity.

10.4.2. Cotton pellet induced granuloma test

There was dose dependant reduction in granuloma tissue formation in extract, isolated compouds and Ibuprofen treated rats as shown in **Table 10.2**. The activity was found to be statistically significant for the dose ranges used. In this test the standard drug Indomethacin at the dose of 10 mg/kg b.w.p.o. showed maximum (51.94%) inhibition of granuloma formation followed by Compound II (38.20%) at the dose of 30 mg/kg b.w.p.o. and MECA (36.01%) at the dose of 200 mg/kg b.w.p.o. In conclusion, out of three plants the plant *C. arborea* (contains Compound II) has got maximum anti-inflammatory activity.

10.4.3. Arachidonic acid induced paw edema test

The effect of methanolic extracts of *U. parviflora, C. arborea, M. citrifolia* and their respective compounds on arachidonic acid induced inflammation in rats is presented in **Table 10.3.** The activity was found to be statistically significant for the dose ranges used. The anti-inflammatory activity of Compound II (64.06%) at the dose of 30 mg/kg b.w.p.o. is comparable to that of standard drug Phenidone (71.86%) at the dose of 200 mg/kg b.w.i.p. followed by Compound III (63.20%) at the dose of 30 mg/kg b.w.p.o and MEMC (56.70%) at the dose of 200 mg/kg b.w.p.o..

(B) ANTIPYRETIC ACTIVITY

10.5 Pyrexia (Fever)

The oral human body temperature is 98.6^{0}F or 36.8±0.4^{0}C. The axillary temperature is 36.5^{0}C and the rectal body temperature is 37.5^{0}C. The body temperature is affected by food or liquid ingestion, physical activities, presence of infection, malignancy, collagen vascular diseases and undiagnosed causes.

Fever is the elevation of body temperature above the normal circadian range i.e. above 100.4^{0}F. "Fever is nature's engine which she brings into the field to remove her enemy", except children with febrile seizures, pregnant women; patients with impaired cardiac, pulmonary and cerebral function. Very high grade fever is termed as hyperpyrexia or hyperthermia (Guyton, 1998).

10.5.1 Mechanism of Fever Production

Normally the infected or damaged tissue or exogenous pyrogens release from virus, bacteria, fungi, endotoxins, Ag-Ab reaction, drug etc. initiates the enhanced formation of proinflammatory mediators or the endogenous pyrogens (cytokines, such as interleukin 1β, α, β, and TNF-α), which increase the synthesis of prostaglandin E_2 (PgE_2) near hypothalamic area and thereby trigger the hypothalamus to elevate the body temperature (Spacer *et al.*, 1994).

The history of analgesic, antipyretic and anti-inflammatory substances started with the use of decocted salicylate-containing plants by ancient Greek and Roman physicians. Willow bark was already mentioned in the **Corpus Hippocraticum** (a collection of medical scripts compiled by Alexandrian scholars in approximately 300 BC) as a substance for treating fever and pain conditions. Over the past 140 years other substances have been introduced for therapy, collectively termed as nonsteroidal anti-inflammatory drugs (NSAIDs), after P. S. Hench discovered the anti-inflammatory properties of glucocorticoids in 1949. NSAIDs, which possess analgesic, anti-inflammatory and antipyretic properties, are a heterogeneous group of substances without any uniform chemical properties (although most are organic acids), but nevertheless share the same therapeutic and side effects. In the past few years there have been significant advances in explaining the mechanism of action of NSAIDs (Jürgen, 2000).

10.6 Mechanism of action of NSAIDs

In the 1930s, Goldblatt and Von Euler showed that human seminal fluid contained a component that reduced blood pressure, the effects of which could not be classified among the

tissue hormones known at the time. Von Euler termed these new, unknown substances 'prostaglandins' because he presumed that these mediators were produced in the prostate (Goldblatt, 1933, Von Euler, 1935). After Bergström and Sjövall achieved the first chemical identification of a prostaglandin at the beginning of the 1960s, the era of prostaglandin research began (Bergström, 1962). It turned out that these hormones could be synthesized by many mammalian cells and that they participate in the regulation of numerous physiological functions. Another milestone was the discovery by Vane and co-workers that the analgesic, antipyretic and anti-inflammatory properties of acetylsalicylate were based on the inhibition of prostaglandin synthesis (Vane, 1971). Vane showed that the acidic anti-inflammatory analgesics decreased pro-inflammatory prostaglandin concentrations by inhibiting cyclo-oxygenase. This finding made sense because the prostaglandins characterized in the 1960s were found to be substantially involved in bringing about and maintaining inflammatory processes by increasing vascular permeability and amplifying the effects of other inflammatory mediators such as kinins, serotonin and histamine. Prostaglandin E_2 (PGE_2) is also involved in the induction of fever. As it is known that, prostaglandins are not themselves significant mediators of pain; instead, they increase the sensitivity of nociceptors to other stimuli in traumatized tissue. They switch normally non-excitable polymodal receptors (Silent nociceptors) into a state in which they are easily excitable (**Fig 10.2**).

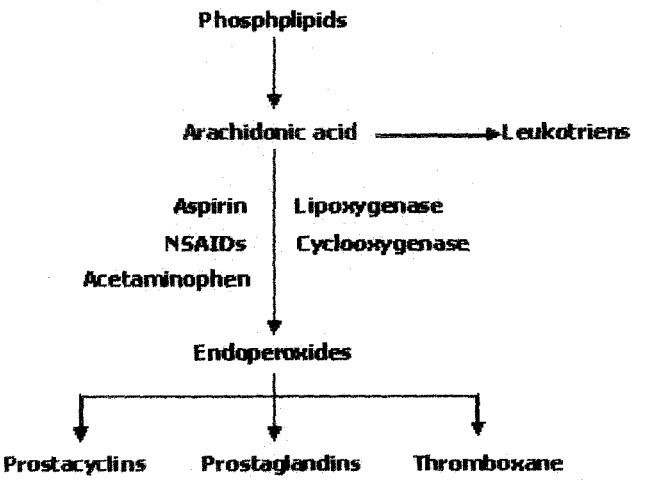

Fig 10.2 Mechanism of action of antipyretics.

10.6.1 NSAIDs as inhibitors of cyclo-oxygenase-1 and cyclooxygenase-2

In 1990, the first evidence for the existence of an inducible isoform of cyclo-oxygenase (COX) was published by P. Needleman (Fu *et al.*, 1990; Masferrer *et al.*, 1990). Structural analysis showed that the isoenzymes COX-1 and COX-2 had an amino acid sequence homology of approximately 60%. However, the isoforms encoded by different genes differ in their tissue

distributions and regulation of expression. COX-1 is expressed constitutively in almost all cell types, including thrombocytes and those present in kidney, stomach and vascular endothelium, and is synthesized and regulated as a so-called 'housekeeping enzyme' involved in physiological adaptation. COX-2 in contrast is inducible; induction can occur during tissue damage or inflammation in response to cytokines (tumor necrosis factor- α, interleukin-1), mitogens and growth factors. The induction of COX-2 has been observed in macrophages and monocytes, endothelial cells, chondrocytes and osteoblasts. An increased COX-2 level has also been registered in the synovial tissue of patients with rheumatoid arthritis and osteoarthritis (Crofford, 1996). These first findings led to the hypothesis that a selective blockade of the COX-2 isoform should lead to the inhibition of inflammation and pain without impeding the COX-1-dependent effects in the GI tissue and kidney, and in blood coagulation (Vane, 1994). This hypothesis led to an intensive worldwide search for selective COX-2 inhibitors. Most of the antipyretic drugs inhibit COX-2 expression to reduce the elevated body temperature by inhibiting PgE_2 biosynthesis (Cheng *et al.*, 2005). These synthetic agents irreversibly inhibit COX-2 with a high selectivity and are toxic to the hepatic cells, glomeruli, cortex of brain, and heart muscles. Natural COX-2 inhibitors have lower selectivity with fewer side effects (Cheng *et al.*, 2005).

10.6.2 Salicylates

Also Salicylates are the class of compounds, that are widely valued for their pain killing, antipyretic and anti-inflammatory properties (Moncada *et al.*, 1979 and Insel, 1991). The most commonly known and used salicylates are salicylic acid (also called 2-hydroxybenzoic acid), aspirin (acetylsalicylic acid or ASA) and sodium salicyclates. They are used extensively for the relief of headache, inflammation, arthritis pain, and some are employed in the treatment of heart attacks and strokes in the elderly (Rainsford, 1984). Their mode of action is the inhibition of the synthesis of Prostaglandin and its derivatives that cause inflammation, pain, rise in temperature and related diseases (Moncada *et al.*, 1979; Meade *et al.*, 1993).

Salicylate toxicity and poisoning are rare in recommended doses, however, salicylate poisoning and its side effects are prominent problem in developing countries where they are used as antipyretic in the management of infectious malaria, both in children and in elderly people. Meanwhile, there are development and introduction of new analgesic, antipyretic and anti-inflammatory agents that compete with aspirin (Fadeyi *et al.*, 2004).

10.7 Antipyretic activity of *U.parviflora* leaf, *C.arborea* leaf and *M. citrifolia* root

10.7.1 Material and Methods

10.7.1.1 Animals

Albino rats (Wistar strain) of either sex weighing 180-200 g were used. The animals were maintained under suitable nutritional and environmental conditions throughout the experiment. All the experimental protocols were approved by the Institutional animal ethics committee vide its no. HPI/07/60/IAEC/0005.

10.7.1.2 Induction of fever by Brewer's yeast (Pyrexia)

Yeast induced pyrexia (Burn *et al.,* 1997) was used to evaluate the antipyretic activity of the extracts of the plants with their isolated compounds. Male rats of Wistar strain (11-12 weeks) were procured from Ghosh Enterprises, Kolkata. They were housed in polypropylene cages with paddy house bedding under standard laboratory condition. The animals had access to laboratory chow and water *ad libitum*. The animals were kept fasting for 24 hours prior to the experiment and were divided into eight groups of six animals each. The body temperature of each rat was recorded by measuring rectal temperature at predetermined time intervals with the help of tele-thermometer. Fever was induced by injecting 15% suspension of Brewer's yeast (*Saccharomyces cerevisiae*), following a standard method (Murugesan *et al.*, 2000). The rats were allowed to remain quiet in the cage for sometimes. A thermister probe was inserted 3-4 cm deep into the rectum, after fastened the tail, to record the basal rectal temperature. The animals were then given a subcutaneous injection of 10 ml/kg of 15% w/v Brewer's yeast suspended in 0.5% w/v methylcellulose solution (Debprasad *et al.*, 2005) and the animals were returned to their housing cages. After the 19hrs of yeast injection, the rats were again restrained in individual cages to record their rectal temperature. Immediately the MEUP, MECA and MEMC extracts were administered orally at dose of 200 mg/kg to the Group III to V animals; the last three groups received 30 mg/kg of isolated compounds Compound I, Compound II and Compound III respectively, the Group I received 5 ml/kg of methylcellulose solution as vehicle control and the Group II animals were administered with 150 mg/kg of Paracetamol (for each rat 27 mg of Paracetamol was dissolved in 0.5 ml methylcellulose solution and diluted with 4.5 ml distilled water) as standard drug. Rectal temperatures of all the rats were recorded at immediately before extract or vehicle or Paracetamol administration, 19hrs and again at 1hour interval up to 23 hrs, after yeast injection (Chattopadhyay *et al.*, 2002).

10.7.1.3 Statistical analysis

The values are expressed as Mean ± SEM; where number of observation in a group is 6. The data of all the groups are compared with the mean value of respective control group and level of significance are cited.

10.8 Results of the antipyretic activity of *U.parviflora* leaf, *C. arborea* leaf and *M. citrifolia* root.

The effect of MECA, MEMC, MEUP and their isolated compounds on yeast-induced pyrexia is presented in **Table 10.4.** The data revealed that the rectal temperature of 38.19^{0}C at 19 hr was markedly elevated to 40.52^{0}C at the 21 hr and then slowed down for vehicle control. The results showed that the MECA at doses of 200 mg/kg b.w.p.o. caused significant lowering of the body temperature up to 4 hr following administration, as the normal mean temperature 39.20^{0}C at 19 hr was reduced to 38.00^{0}C at 23 hr. While maximum lowering of body temperature was noticed in case of its isolated compound treated group i.e. Compound II treated group, which received 30 mg/kg b.w.p.o. In this group the body temperatures slightly increased at 19hr (37.59^{0}C) from the basal temperature (37.55^{0}C) and slightly go on increasing up to 21 hr (38.46^{0}C) and then decreased to 37.79^{0}C. The compound succeeded to keep the body temperature near normal. The pattern of body temperature was different from other groups. In the paracetamol treated group the body temperature was 38.46^{0}C at 19 hr after the subcutaneous injection of yeast suspension which rose to 38.31^{0}C in the next hour and then slowed down up to 23 hr. It was found that the antipyretic effect of Compound II at 30 mg/kg b.w.p.o. is similar to the paracetamol group. The study revealed that MECA and compound II i.e. the plant *C. arborea* possess the maximum antipyretic activity out of the three plants studied. The statistical analysis also revealed that the temperature difference was significant in this case.

Table 10.1 Acute anti-inflammatory activity of *U. parviflora, C. arborea, M. citrifolia* and their respective compounds on carrageenan induced rat paw edema.

GROUP	TREATMENT	Paw volume in ml and inhibition %			
		1hr	2hr	3hr	4hr
I	CONTROL	1.15±0.09	1.29±0.07	1.62±0.05	2.01±0.01
II	IBUPROFEN 100 mg/kg	0.94±0.02* (18.26)	0.90±0.04* (30.23)	0.82±0.01* (49.38)	0.74±0.02 (63.18)
III	MEUP 200 mg/kg	1.01±0.05* (12.17)	0.97±0.04* (24.80)	0.94±0.03* (41.97)	0.90±0.03* (55.22)
IV	MECA 200 mg/kg	0.96±0.01* (16.52)	0.92±0.02* (28.68)	0.85±0.05* (47.53)	0.79±0.07* (60.69)
V	MEMC 200 mg/kg	0.97±0.05* (15.65)	0.92±0.03* (28.68)	0.84±0.02* (48.14)	0.79±0.02* (60.69)
VI	Compound I 30 mg/kg	1.08±0.03* (6.08)	1.00±0.04* (22.48)	0.95±0.02* (41.35)	0.91±0.03* (54.72)
VII	Compound II 30 mg/kg	0.95±0.06* (17.39)	0.91±0.03* (29.45)	0.83±0.03* (48.76)	0.77±0.06* (61.69)
VIII	Compound III 30 mg/kg	0.98±0.09* (14.78)	0.94±0.05* (27.13)	0.90±0.06* (44.44)	0.85±0.02* (57.71)

Values are expressed as Mean ± SEM, (n=6). '*' indicates $P<0.01$

Table 10.2 Effect of methanol extracts of leaves of *U. parviflora, C. arborea, M. citrifolia* and their respective isolated compounds on cotton pellet induced granuloma in rats.

Group	Treatment (mg/kg b.w.p.o)	Weight of dry cotton pellet granuloma (mg)	% Inhibition of granuloma
I	Control (2% CMC)	36.23±1.37	_
II	Indomethacin 10 mg/kg	17.41±1.44***	51.94
III	MEUP 200 mg/kg	27.01±0.78**	25.44
IV	MECA 200 mg/kg	23.18±0.97**	36.01
V	MEMC 200 mg/kg	26.65± 0.99*	26.44
VI	Compound I 30 mg/kg	25.01±1.68**	30.96
VII	Compound II 30 mg/kg	22.39±0.82***	38.20
VIII	Compound III 30 mg/kg	29.41±0.64*	23.18

Values are expressed as Mean ± SEM (n=6). '*' indicates $P<0.05$, '**' indicates $P<0.01$ and '***' indicates $P<0.001$.

Table 10.3 Effect of methanolic extracts of *U. parviflora, C. arborea, M. citrifolia* and their respective compounds on arachidonic acid induced inflammation in rats.

GROUP	Treatment	Paw volume in (ml) at 1 hr after arachidonic acid injection	% Reduction in Paw volume
I	Control (2%CMC)	2.31±0.03	–
II	Phenidone 200 mg/kg i.p	0.65±0.02**	71.86
III	MEUP 200 mg/kg p.o	1.94±0.04 NS	16.01
IV	MECA 200 mg/kg p.o	1.31±0.02**	43.29
V	MEMC 200 mg/kg p.o	1.00±0.02**	56.70
VI	Compound I 30 mg/kg p.o	1.58±0.01*	31.60
VII	Compound II 30 mg/kg p.o	0.83±0.03**	64.06
VIII	Compound III 30 mg/kg p.o	0.85±0.21**	63.20

Values are expressed as Mean± SEM, (n=6). '*' indicates $P \leq 0.05$; '**' indicates $P \leq 0.001$ and NS=Not Significant

Table 10.4 Antipyretic effect of MEUP, MECA, MEMC and their respective compounds on albino rats.

(Group) Treatment (Oral)	Basal temperature in ^0C	RECTAL TEMPERATURE °C IN HOURS ± SEM				
		19 Hour	20 Hour	21 Hour	22 Hour	23 Hour
(Gr I) CONTROL	37.22	38.19 ±0.31	39.11 ±0.28*	40.52 ±0.14	40.00 ±0.20*	39.51 ±0.15
(Gr II) Paracetamol	37.78	38.46 ±0.11*	38.31 ±12	38.05 ±32**	37.81 ±0.01***	37.82 ±0.02**
(Gr III) MEUP 200 mg/kg	37.05	38.20 ±0.13	39.00 ±0.22*	38.92 ±.0.30	38.85 ±0.10	38.77 ±0.13*
(Gr IV) MECA 200 mg/kg	37.42	39.20 ±0.60	38.55 ±0.28*	38.35 ±0.31** *	38.13 ±0.09	38.00 ±0.06*
(Gr V) MEMC 200 mg/kg	37.61	39.50 ±0.19	40.50 ±0.98	38.90 ±0.68*	38.70 ±0.49	38.37 ±0.33
(Gr VI) Compound I 30 mg/kg	37.51	38.80 ±0.13	39.71 ±0.13	38.40 ±0.13	38.20 ±0.13	38.15 ±0.13
(Gr VII) Compound II 30 mg/kg	37.55	37.59 ±0.02***	38.30 ±0.01***	38.46 ±0.61	38.22 ±0.81*	37.79 ±1.21***
(Gr VIII) Compound III 30 mg/kg	37.40	38.71 ±0.74	39.9 ±0.37**	38.42 ±0.62	38.01** ±0.12	37.89 ±0.22*

Values are expressed as Mean ± SEM ; '*' indicates $P \leq 0.05$. '**' indicates $P \leq 0.01$ and '***' indicates $P \leq 0.001$

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CHAPTER 11

DISCUSSION

The use of plant and animal parts for medicine has long being in existence and is widely documented in records in ancient China, India and Egypt. These ancient indigenous practices were discovered by a series of 'trial and error', which then could not be substantiated by proven scientific theories. However, these practices have produced results of proven efficacies compared to conventional modern medicine (Chopra *et al.*, 1956). In recent times, herbal medicines have become indispensable and are forming an integral part of the primary health care system of many nations. A recent survey in the United States of America (USA) indicate an expected 20% annual growth in herbal medicine in the next 5 years (Saxena, 2001) with an estimated 80% of the world population living in the developing countries still relying on plants for health care. In the USA, the total number of visits to unconventional healers in 1988 was 425 million compared with 388 million visits to primary health care physicians, accounting for an estimated $13.7 billion in the unconventional market (Eisenberg *et al.*, 1993). In view of this large dependence on traditional health practices, the World Health Organisation (WHO) recognized the implicit role of herbal medicine in the Alma Mata declaration of Health for All by the Year 2000 A.D. In 1978, WHO approved the use of these natural products (Schillhorn, 1997). More than 60% of approved and pre-new drug application (NDA) candidates are either natural products or related to them, not including biologicals such as vaccines and monoclonal antibodies (Cragg *et al.*, 1997).

Nature apparently optimizes certain compounds through many centuries of evolution. Secondary metabolism has evolved in nature in response to needs and challenges of the natural environment. Nature has been continually carrying out its own version of combinatorial chemistry (Verdine, 1996) over 3 billion years during which bacteria have inhabited the earth (Holland, 1998). Combinatorial chemistry practiced by nature is much more sophisticated than that in the laboratory, yielding exotic structures rich in stereochemistry, concatenated rings, and reactive functional groups (Verdine, 1996). As a result, an amazing variety and number of products have been found in nature. The total number of natural products produced by plants has been estimated to be over 500,000 (Mendelson *et al.*, 1995). One-hundred sixty thousand natural products have been identified, a value growing by 10,000 per year (Henkel *et al.*, 1999). About 100,000 secondary metabolites of molecular weight less than 2500 have been characterized, half from microbes and the other half from plants (Fenical *et al.*, 1993; Berdy, 1995; Roessner *et al.*, 1996). More natural product research is needed to face the challenges of: unmet medical needs, remarkable diversity of structures and activities, utility as biochemical probes, novel and sensitive assay methods; improvements in isolation, purification, and characterization; and new production methods (Clark, 1996).

The traditional medicine is still the mainstay of health care and most drugs come from plants. Although many plants have long been recognized and widely used in Nepalese traditional medicine, some are relatively unexplored and not arrived to mainstream medicine (Bhattarai *et*

al., 2006). Therefore, the search on new drugs must be continued and natural products from plants, microorganisms, fungi and animals can be the source of innovative and powerful therapeutic agents for newer, safer and affordable medicines (Cooper, 2001; Lindequist *et al.,* 2005).

There are many ethno-pharmacological tradition exists in developing countries like India. Many recent entrepreneurs of botanicals have created a vast, frequent, and indiscriminate use of plants and plant extracts by millions of people, generating not only health dangers but also permanent destruction of large areas of the precious and irreplaceable primary rainforest. Proliferation of small, unregulated firms that promote and market the use of hundreds of poorly studied natural remedies. In fact, the majority of plants used in folk medicine, and phytomedicines in general, are traditionally sold over the counter, and lack adequate pharmacological, toxicological, and clinical evaluation (Liebstein, 1927). Thus it makes an urgent need for determination of the efficacy of medicinal plants and their therapeutic usefulness as well as for safety.

It is true that, in contrast to wealthy communities, the use of herbal products in developing regions is not an alternative to other effective medicines, but indeed the only option for primary health care. Therefore, implementation of good practices for production, development, and use of these compounds, as well as their scientific evaluation and separation of toxic products, are of primary importance to public health. In response to these circumstances, the World Health Organization (WHO), in the context of its Resolution WHA 31.33, has recognized the importance of medicinal plants for primary healthcare and has recommended to its Member States the use of a comprehensive approach to medicinal plants (WHA, 1978).

Three plants were selected for this research work. They are *Urtica parviflora* Roxb., family-Urticaceae, *Callicarpa arborea* Roxb., family-Verbenaceae and *Morinda citrifolia* Linn., family-Rubiaceae. These plants were subjected: (i) to evaluation for their bioactivity both *in vitro* and *in vivo*, to justify their use in ethnomedicine; (ii) to isolation and identification of the bioactive principle(s) in pure form. Standard methods were followed for the collection and processing of the plants and their useful parts. The extractions of the plant parts and prescreening of the extracts were done by standard protocols and the universally accepted methodologies, as described in Materials and Methods.

11.1 Phytochemical studies and ethno-medicinal importance

This research work includes the study of the phytochemistry, toxicity, antimicrobial potency and pharmacology of the three above said medicinal plants. These plants are in use in the traditional medicines, which is practiced by the local hill people of Sikkim Himalayan region.

Authentication of the plants was made with the help of qualified scientists of the Botanical Survey of India, Gangtok region, Sikkim, India.

The powdered plant materials were subjected to methanol extraction (70%) in a Soxhlet extractor fitted with a waterbath. The methanol extracts were concentrated, suspended in hot distilled water, cooled and the blast precipitate was filtered off. The water soluble component was fractionated by extracting it successively with petroleum ether, chloroform and acetone. The chloroform soluble fraction was subjected separately to chromatographic analysis in case of *U. parviflora* and *M. citrifolia.* Similarly, the acetone soluble fraction was taken for chromatographic analysis in *C. arborea.* The aqueous, and petroleum ether fraction did not show any positive pharmacological activities under purview of this investigation and was discarded. Using multistep column chromatography technique with various developing phases Compound I, compound II and Compound III were isolated from the above stated fractions of *U. parviflora* leaf, *C. arborea* leaf and *M. citrifolia* root respectively.

11.1.1 *Urtica parviflora* Roxb.

The word 'Urtica' came from the Latin word 'urtic' referring the pain caused in the hand or any part of the body by touching the aerial part having stings (hair). It produces an urticarial inflammatory nettle rash, accompanied by a considerable burning and itching sensation. The rashes may come out in large or small patches, remains for few minutes or several hours and may disappear quite abruptly. Urtication was practiced for the treatment of certain diseases and involved of beating the skin with a bunch of nettles. The result is erythema and whealing but after the third or fourth successive application, the skin ceases to react under fresh contact (BoDD, 2007). The stem fibers are used to make ropes, the leaves are used as fodder, and the young shoots are used as a seasoning substitute for sorrel. This plant is in use in the traditional medicine practiced by local Bungthings (Medicine Practitioners) to treat dislocation and fracture of bones, fever, cold and cough, and liver diseases (Gurung 1, 2002). About 50 species are found in northern temperate regions, a few in tropical and south temperate regions. Amongst them *U. ardens, U. parviflora, U. thunbergiana, U. fissa, U. mairei, U. dioica, U. angustifolia, U. urens, U. atrichocaulis, U. taiwaniana, U. laetevirens, U. hyperborean, U. cannabina, U. triangularis* are important species. *Urtica parviflora* is confused with *U. ardens*, and *U. dioica* but that species have denser, setulose indumentum and ovate leaf blade with the surface conspicuously wrinkled and the margin sharply doubly serrulate (Jiarui *et al.*, 2003). Cooked tender leaves of stinging nettles are eaten not only in India but in several countries (Hadjichambis *et al.*, 2007).

11.1.2 *Callicarpa arborea* Roxb.

This plant is called 'Sunga' in Lepcha and 'Guenlo' in Nepali belongs to family *Verbenaceae.* Ethnomedicinally the plant (bark) is used as tonic and carminative, applied in cutaneous diseases, rheumatism and gonorrhoea (Gurung 2, 2002), paste of the bark and leaf applied on scorpion sting area of skin (Anonymous, 1976). The literature review reveals that almost no work on pharmacological activity had been performed on this plant previously.

11.1.3 *Morinda citrifolia* Linn.

The common name of this plant is called 'Indian Mulberry' or 'Noni' and in Nepali it's called as 'Hardikath', belongs to family *Rubiaceae.* The root is cathartic. The leaf is used as febrifuge and tonic; heals wounds and ulcers. The baked fruit is given in asthma and dysentery. The leaf juice is applied to gout externally (Gurung 3, 2002). Traditionally the tender leaves and fruits are used as food. Apart from the traditional use of this plant in Sikkim, it has numerous uses in many countries (Etkin *et al.*, 2003) and it has several pharmacological activities (Wang *et al.*, 2002). People are crazy about this plant. They use it for diabetes, high blood pressure, cancer, and many other illnesses (Abbott, 1985). Noni is a traditional remedy to treat broken bones, deep cuts, bruises, sores, and wounds (Bushnell *et al.*, 1950). Morton gave numerous references for medicinal uses of Noni (Morton, 1992). In addition, Polynesians are reported to have successfully used Noni to treat breast cancer and eye problems.

11.2 Isolation and identification of compounds derived from plant extracts

The preliminary phytochemical group tests indicated the presence of amino acids, proteins, steroids and triterpenoids in *Urtica parviflora.* The column chromatography and TLC studies confirmed the chemical nature of the Compound I. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (IR, GC-MS, ^{13}C and ^{1}H NMR) data (Faizi *et al.*, 2001; Peirs *et al.*, 2006).

The white shining, needle shaped crystals isolated from *Urtica parviflora* Roxb. yielded (β-sitosterol (Compound I). The UV absorption spectrum showed strong absorption at 492 nm, which implied the presence of steroidal ring in its structure and the UV spectrum of Compound I is similar to the standard spectrum of β-sitosterol with the presence of phenolic aromatic rings. The Melting Point range is within 128.5^{0}-$129.2^{0}C$. The IR spectrum confirmed the presence of hydroxyl group and aromatic ring in the compound. The ^{13}C NMR spectrum showed C-5 and C-6 double bond carbons at δ 122.09 and 138.29 suggesting the sitosterol structure. The fragmentation ion at m/z 414 in its mass spectrum, inferred the compound is corresponding to the molecular formula $C_{29}H_{50}O$ and the compound should be + (–) β-

sitosterol. The structure of the compound was further confirmed by elemental analysis of the available literature of β-sitosterol. All the recorded spectral and elemental analysis and evidence discussed in **Chapter 3** conclusively prove the identity of the isolated compound as **β-sitosterol**. This confirmed that β-sitosterol is one of the major bioconstituent of the plant *Urtica parviflora* Roxb. Presence of this compound is not reported in the available literature.

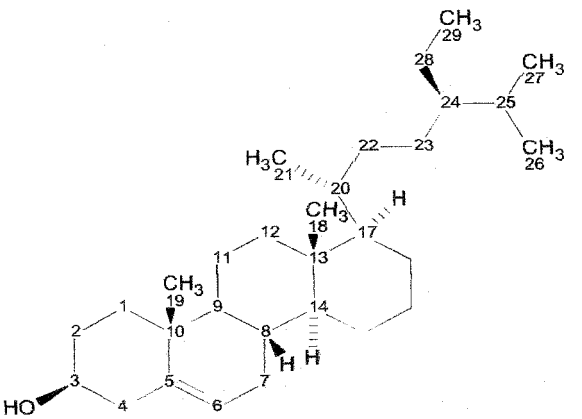

Fig 11.1 Compound I (β-sitosterol) isolated from methanol extract of U. parviflora.

In the second plant, *Callicarpa arborea* Roxb., the preliminary phytochemical group tests indicated the presence of steroids, flavonoids, proteins. It was further subjected to chemical tests, column chromatography and TLC to confirm the chemical nature of the isolated compound, which yielded a triterpenoid glycoside. The chemical nature of the isolated compound was further characterized by comparison of its physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data with that of the reported values of triterpenoid glycoside (Alvarez *et al.,* 2003; Abe *et al.,* 2002 and Yoshida *et al.,* 2005).

The colourless amorphorphus powder material was isolated from *Callicarpa arborea.* The compound was melted at 139^{0}–143^{0}C.The UV absorption spectrum showed a significant absorption at 242 nm indicates that the compound is an isoprene derivative (Yu *et al.,* 2003). The IR spectrum confirmed the functional groups of OH (2928 cm^{-1}), COO (1691 cm^{-1}) of COOH, C=C of steroidal moiety; matched with the reference spectrum of Oleanolic acid. The ^{1}H and ^{13}C NMR spectra of Compound II showed that most of the signals of the aglycone were in good agreement with literature data of oleanolic acid (Kubota *et al.,* 1968). The ^{13}C NMR showed the presence of carbonyls and hydroxyl groups and the fragmentation ion at 248 (m/z) in its mass spectrum inferred the compound is having the molecular formula of $C_{30}H_{48}O_3$ and ascertained the Oleanolic acid moiety. From these data, it is concluded that the structure of the isolated triterpenoid is of **Oleanolic acid**.

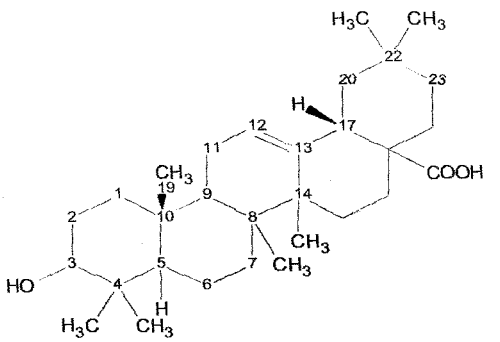

Fig 11.2 Compound II (Oleanolic acid) isolated from methanol extract of *C. arborea.*

The preliminary phytochemical group tests in the extract obtained from the plant *Morinda citrifolia* Linn. indicated the presence of alkaloids, amino acids, reducing sugars, steroids, triterpenoids and anthraquinones which was further subjected to thin layer chromatographic study and coloumn chromatographic separation of the chloroform fraction to yield an anthraquinone derivative (Compound III). The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, MS, ^{13}C and ^{1}H NMR) data (Rajendran *et al.,* 2007; Ling *et al.,* 2002). The UV spectrum of Compound III is identical with that of anthraquinone glycoside. The IR spectrum shows the presence of absorption bands at 1633, 1614, 1035, 3572, 3636 and 2984 cm^{-1}. The IR spectrum confirmed the presence of OH, OCH_3, C=C, C=O groups in compound III. The proton and carbon NMR of Compound III matched with reference spectra as follows: C-3 at δ 26.69, C-6 δ 16.27, C-8 at δ 113.39 to 161.07. The fragmentation ion at 291 m/z inferred the compound is anthraquinone derivative i.e **1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone** (Kamiya, *et al.,* 2005; Chan *et al.,* 2005 and Wab *et al.,* 2007).

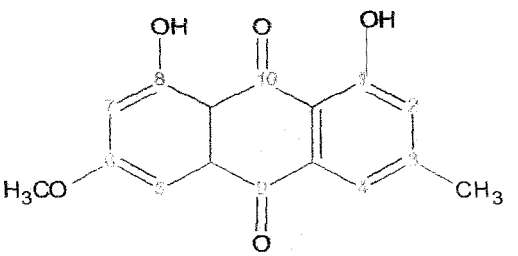

Fig 11.3 Compound III (1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone) isolated from methanol extract of *Morinda citrifolia* Linn.

11.3 Toxicity Study

The Arithmetical method of Karber, was used to evaluate the acute toxicity in adult albino rats (**Chapter 4**). The animal studies were approved by Institutional Animal Ethics Committee. In this study, ethanolic extracts were used instead of methanol extracts to avoid the toxicity of methanol itself. The LD_{50} (Median Lethal Dose) (Ghosh, 1984) of orally administered ethanolic extracts of leaves of *U.parviflora*, *C. arborea* and root bark of *M. citrifolia* was found to be 3500 mg/kg, 1666.67 mg/kg and 950 mg/kg body weight per oral respectively. *Urtica parviflora* is a commonly used plant in Sikkim which is consumed as cooked food and has low toxicity.

11.4 Hepatoprotective study

Chapter 5 of this thesis deals with the hepatoprotective activity. Liver is the principal organ of metabolism and excretion and is subject to a number of diseases which may be classed as liver cirrhosis (cell destruction and increase in fibrous tissue), acute chronic hepatitis (inflammatory disease) and hepatitis (non-inflammatory condition). The terminal events in the attack on the liver by carbon tetrachloride, which is commonly used as a liver toxicant, involved in the production of a highly reactive radical leading to lipid oxidation and the inhibition of the calcium pump of the microsome, giving rise to liver lesions. A number of plant drugs (cholagogues) are in use to treat biliary disorders and other liver disorders (Evans, 2006). In this study only *Urtica parviflora* was selected for the study. Other two plants i.e. *Callicarpa arborea* and *Morinda citrifolia* were not included because they did not showed significant anti-hepatotoxic activity. Also the ethanolic extract of *Urtica parviflora* was selected as because methanol and any other organic solvent may induce hepatotoxicity.

The results of hepatoprotective effect reveals that the administration of ethanol extract at 250 mg/kg body weight protected the liver partially in CCl_4 induced liver damage in albino rats. The histopathology showed, that the liver necrosis was controlled in rats treated with ethanol extract at 500 mg/kg body weight along with CCl_4. The histology of the livers was found normal when treated with ethanol extract and standard drug Silymarin at 750 mg/kg p.o. and 100 mg/kg body p.o. weight respectively. In these groups, the distribution of Kupffer cells and sinusoidal cells was normal with clear bile canaliculi. To elucidate the biochemical mechanism of hepatoprotective activity of the *U.parviflora* extract, the levels of ALT, AST, ALP, total protein and bilirubin were estimated. Carbon tetrachloride (CCl_4) is the extensively studied liver toxicant and its metabolite, trichloromethyl peroxy radical ($CCl_3O_2^{\cdot}$) is involved in the liver damage. The toxic chemical causes oxidative degradation in the adipose tissue resulting in infiltration of fat into the hepatocytes. The increased level of serum bilirubin reflected the depth of jaundice and the increment in the transaminases and alkaline phosphatase were clear indication of cellular leakage and the loss of cellular integrity of the cell membrane (Sarawat

et.al 1993) as because in hepatocellular injury (e.g. hepatitis), the damaged liver cells develop leaky membranes, allowing for escape of intracellular enzymes into the bloodstream (Green *et al.*, 2002; Pratt *et al.*, 2000). The major intracellular enzymes are aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

The results showed that the administration of the test formulation to the animals at a dose of 750 mg/kg body weight returned the elevated enzymes level, total protein and bilirubin near to normal, than that of the animals that received only CCl_4 and found to be closer to the Silymarin (standard drug) treated animals. Oral administration of *U.parviflora* ethanol leave extract revealed significant protection against the hepatic cell damage, in a dose dependent manner up to the dose of 750 mg/kg body weight p.o. The findings of the study provide some scientific basis to the traditional use of *U.parviflora* leaves in the management of hepatic disorders. The data obtained are consistent with the literature on hepatoprotective activity of *U. parviflora* leaves through the studies of enzyme assays and histopathological examination of liver in rats (Gurung, 1999; Kar *et al.*, 2007).

11.5 Wound healing study

Wound care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1–3% of modern drugs (Mantle *et al.*, 2001). Reports about medicinal plants affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelization and wound contraction are abundant in the scientific literature (Ulubelen *et al.*, 1995; Choi *et al.*, 2001; Bairy, 2002).

In the wound healing study (**Chapter 6**), three wound models were selected. They are Excision wound model, Incision wound model, and Dead space wound model. The result of excision wound model revealed that the ointments prepared from the extract of *C. arborea* and *M. citrifolia* are comparable to the standard drug Framycetin (ointment) in healing of wound. The epithelization period was also found to be less in Group V, which received MEMC orally (12.6 days) and in Group VII, which received MECA ointment (12.9 days) is similar to the standard drug (Framycetin) treated group (12.5 days). The effect of the test drugs in incision wound model showed that The breaking strength was found to be maximum in Group VIII which received ointment of MEMC (710.00 ± 4.22) and is similar to standard drug Framycetin group (Group II) (712.23 ± 2.84). The other two groups who received ointment of MEUP and MECA (Group VI and VII) also showed better breaking strength as compared to orally fed drugs groups.

Three parameters were studied in dead space wound model, namely dry granuloma weight; breaking strength and estimation of hydroxyproline. Animals of group VIII treated with ointment of MEMC showed maximum dry granuloma tissue weight (72.01 ± 1.19 mg/100g) which is much higher than that of the standard drug Framycetin treated group (62.12 ± 0.38 mg/100g).The MEUP ointment treated group (Group VI) showed the same dry granuloma tissue weight with that of the standard drug group i.e. (62.12 ± 0.38 mg/100g) as compared to the control group, which showed the lowest dry granuloma tissue weight (26.32 ± 0.41 mg/100g). The MEMC ointment treated group (Group VIII) also showed maximum breaking strength (600.13 ± 4.36g) amongst all the test groups and is comparable to standard drug treated group (Group II) which is found to be 612.13 ± 2.31g). The MEMC ointment treated group also showed maximum formation of hydroxyproline (2397.24 ± 2.01µg/100g) which is comparable to the results found in the standard drug treated group of animals (2439.61 ± 0.87µg/100g) as hydroxyproline and collagen are primarily responsible for the strength of tissues (Harkness, 1961). It reveals that the extract of the roots of *M. citrifolia* is found to be the most effective in healing of wound among the three plant drugs in this model.

The results of the histopathological examinations were recorded in five parameters i.e. Keratinization, Epithelization, Fibrosis, Collagen, and Neovascularisation. Wounds of all ages create an impression of individuality on a microscopic level as specific as a finger print. The microscopy showed that the MECA and MEMC treated groups (Groups VII and VIII) had similar stage of keratinization (4.1 ± 0.09 and 4.1 ± 0.03 respectively) which is comparable to the effect of the standard drug Framycetin in Group II (4.2±0.05). Similarly the MEMC ointment treated group showed maximum epithelization (4.2 ± 0.26) comparable to the standard drug treated group (4.3 ± 0.14). The stage of fibrosis was highest in case of *Morinda citrifolia* ointment, compared to *U. parvifolia* and *C. arborea* (4.0 ± 0.13 and 4.0 ± 0.12). The collagen formation was maximum in Group VII (4.4± 0.16), which received MECA ointment amongst the test groups and is comparable to the standard drug treated group i.e. Group II (4.5 ± 0.17). The stage of neovascularization in MECA ointment treated group was found to be 4.4±0.07 which is similar to the standard drug treated group (4.4± 0.09). The value in MEUP ointment treated group is also very appreciable i.e. 4.3±0.08, and is comparable to the standard drug treated group and is much higher than that of the control group (0.6+0.07). Thus the ointment forms of the extracts of two plants *U. parvifolia* and *C. arborea* were found to have potent wound healing activity.

It can be concluded from this study that, the three plants have significant wound healing activity. The test drugs used in this study showed variability in different parameters. The MECA 5%w/w and MEMC 5%w/w ointments have high incision breaking strength. The incision breaking strength depends upon the formation of collagen in the skin. The great bulk of

collagen is formed and gain in tensile strength of healing skin is achieved within 18 days, and the wound reached its maximal strength at the end of this time (Howes *et al.,* 1929). During fixation of wounds with formalin, it was taken care of the factor that, formalin has the potency to increase the breaking strength of wounds (Levenson *et al.,* 1965). Also there were variations in the phase of the skin cycle as well as in skin thickness observed in the study (Butcher, 1934; Chase *et al.,* 1953; Randall *et al.,* 1954; Strauss *et al.,* 1953). The highest wound healing activity showed by the ointment prepared from the methanolic root bark extract of *Morinda citrifolia* may be due to the presence of the anthraquinone moiety i.e. **1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone.**

11.6 Antioxidant study

The **Chapter 7** deals with the antioxidant study. DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable dimagnetic molecule. Due to its odd electron, the methanolic solution of DPPH shows a strong absorption at 517 nm. DPPH radical reacts with suitable reducing agents and then by accepting an electron becomes paired off and the solution loses colour stoichometrically with the number of electrons taken up (Nagai *et al.,* 2003). Such reactivity has been widely used to test the ability of compound to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. The effect of the methanol extract of *U. parviflora* and Compound I in DPPH scavenging model revealed that the extract of *U. parviflora* at 1000 µg/ml concentration showed an inhibition to DPPH reduction by 75.56 % as compared to the inhibition produced by Vitamin E (85.23 %). Compound I at 50 µg/ml showed higher inhibition of DPPH radical, 82.17% compared to the standard drug Vitamin E i.e. 85.23 % at 5 mM concentration. The percentage of activity was found to be time dependent.

The results of ferrous sulphate induced lipid peroxidation, showed that methanol extract of *U. parviflora* leaf at 1000 µg/ml concentration produced maximum percentage inhibition (71.54 %) of lipid peroxidation as compared with the standard antioxidant Vitamin E, while compound I at 50 µg/ml showed an inhibition of 80.19 % similar to the inhibition produced by Vitamin E i.e. 82.01%. The inhibition could be attributed to the prevention of ferryl-perferryl complex or by change of the Fe^{3+} / Fe^{2+} ratio or by reduction of the rate of conversion of ferrous to ferric or by chelation of iron itself or combination thereof (Braugghler *et al.,* 1986).

Superoxide radical O_2^- is highly toxic species, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyse the breakdown of superoxide radical (Govindarajan *et al.,* 2003). Reduced phenazine methosulfate assay was used to measure the superoxide dismutase activity of methanol extract of *U. parviflora* and Compound I. The methanol extract showed significant

scavenging capacity of superoxide free radical at a concentration of 1000 µg/ml by 60.18 %. The Compound I at 50 µg/ml concentration showed maximum inhibition of superoxide radicals (69.01 %) as compared with standard drug Vitamin E (70.2 %) at 5mM concentration.

The results of antioxidant activity of methanol leaf extract of *C. arborea* on ferrous sulphate induced lipid peroxidation showed that the extract inhibited the lipid peroxidation in a dose dependent manner. Compound II at 50 µg/ml concentration exhibited 75.01% inhibition, as compared to the inhibition produced by vitamin E (78.26%) at 5 mM concentration. The DPPH scavenging capacity of the extract was found to be 71.22% at the maximum tested concentration (1000 µg/ml). While the Compound II at 50 µg/ml exhibited 73.65% inhibition compared with 83.59% for the standard drug vitamin E at 5mM. In the superoxide radical scavenging activity, results indicated that the scavenging capacity of the extract was 61.81% at 1000 µg/ml concentration as compared with standard drug Vitamin E (66.38 %) at 5 mM concentration. The Compound II, at 50 µg/ml exhibited similar inhibition of superoxide radicals, as compared with the standard drug (66.28%). IC_{50} was found to be 891.01µg/ml. The inhibition was proportional to the amount of the extract added.

The results of antioxidant activity of methanol root extract of *M. citrifolia* on ferrous sulphate induced lipid peroxidation showed that the extract inhibited the lipid peroxidation in a dose dependent manner. Compound III at 50 µg/ml concentration exhibited 68.38 % inhibition, as compared to the inhibition produced by vitamin E (74.61%) at 5 mM concentration. The DPPH scavenging capacity of the extract was found to be 74.67% at the maximum tested concentration (1000 µg/ml). While the Compound III at 50 µg/ml exhibited 74.81% inhibition compared with 81.34 % for the standard drug vitamin E at 5mM. In the superoxide radical scavenging activity, results indicated that the scavenging capacity of the extract was 56.63% at 1000 µg/ml concentration as compared with standard drug Vitamin E (67.33 %) at 5 mM concentration. The Compound III, at 25 µg/ml exhibited similar inhibition of superoxide radicals, as compared with the standard drug (67.18%). IC_{50} was found to be 895.6µg/ml. The inhibition was proportional to the amount of the extract added.

The methanol extracts of *U. parviflora* leaf *C. arborea* leaf and *M. citrifolia* root as well as Compound I, Compound II and Compound III showed significant antioxidant effects in concentration dependent manner in all the models tested.

11.7 Hypoglycemic study

Diabetes mellitus has a high prevalence, morbidity and mortality rate worldwide and is regarded as an incurable but controllable disease. Many synthetic drugs, plant remedies and dietary traditions are in use to minimize the suffering that it causes. The potential role of medicinal plants as hypoglycemic agents has been reviewed by several authors and is

supported by ethno botanical surveys, and the use in traditional medicines in numerous cultures (Afifi *et al.*, 2005; Grover *et al.*, 2002; Ivorra *et al.*, 1988; Jouad *et al.*, 2001; Li et al., 2004; Yeh *et al.*, 2003; Chhetri *et al.*, 2005).

The results of the antidiabetic activity (**Chapter 8**) on glucose tolerance in streptozotocin induced diabetic rat reveals that, MEUP (methanolic extract of *Urtica parviflora*) succeeded to control the rise of serum glucose level (70.6%) within 1st hour of GTT in streptozotocin induced diabetic rats, followed Compound I treated group (60.0%) and Compound II (isolated from *Callicarpa arborea*) treated group (55.2%). The serum glucose levels between '0' hour and 1 hour are generally compared in these type of studies because there is sudden rise of serum glucose level at the 1st hour after glucose loading in '0' hour. The immediate rise of serum glucose level challenges the efficacy of the test drugs to control it. The CEMC and compound III (isolated from *Morinda citrifolia*) treated groups failed to normalize the rise of serum glucose level. Thus out of the three plants studied, the plant *Urtica parviflora*, was found to have remarkable hypoglycemic activity.

The antidiabetic effect of test drugs as compared to the standard drug, Glibenclamide is very much significant. The percentage reduction of serum glucose level by the MEUP treated group (27.2) is much higher than the standard drug, Glibenclamide treated group (18.5). Also MECA treated group is slightly higher (19.2) than the standard drug treated group. The percentage reduction values are obtained when compared to diabetic control value. The result indicates high efficacy of MEUP and MECA in reducing serum glucose level in diabetic rats.

In the study of hypoglycemic activity of the plant drugs, in normal healthy rats by considering the percentage reduction when compared to the value of the control group, it is revealed that Compoud I and Compound II exhibited same percentage reduction i.e. 14.1%, followed by MEUP and MECA respectively.

It is fairly evident from the studies performed in all the three models that maximum percentage reduction of serum glucose level was found with the MEUP and Compound I, isolated from the same plant *Urtica parviflora*. Hence the plant *Urtica parviflora* is having highest hypoglycemic activity, followed by *Calicarpa arborea* and *Morinda citrifolia* in streptozotocin induced diabetes in rat.

Streptozotocin induced hyperglycemia has been described as an important experimental model to study activity of hypoglycemic agents (Szkudelski, 2001). It selectively destroys the pancreatic insulin secreting β-cells, leaving less active cell resulting in a diabetic state (Szkudelski, 2001; Kamchouing *et al.*, 1998). From this study, it can be inferred that the plant, *Urtica parviflora* and its isolated compound i.e. β-Sitosterol may have restorative activity on

insulin secreting β-cells in pancreas like the standard drug Glibenclamide, which is a sulphonylurea hypoglycemic drug, known to stimulate insulin secretion from the pancreas.

11.8 Antimicrobial study

Now a days, mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective and as new, particularly viral, diseases remain intractable to this type of drug (Cowan, 1999). Synthetic antibiotics used to control infection produces adverse toxicity to host organs, tissues and cells. The toxicity produced by the antimicrobial agents can be prevented or antagonize with herbs (Lin *et al.*, 1989). Herbal molecules are safe, and will overcome the resistance produced by the pathogens since they are in combined form or in pooled form of more than one molecule in the protoplasm of the plant cell. Some herbs have antibacterial and antifungal properties which will be useful to clinical use (Kalemba *et al.*, 2003). Some *in vitro* studies have been conducted and proved that herbal oral liquids can be used clinically to overcome drug resistant strains and different serotype strains of infection (Lu *et al.*, 2002). Antimicrobial drugs have received immense importance in last few decades. The plant derived antimicrobials are structurally different from those, which are isolated from microbes. The antimicrobials of plant origin include flavonoids, essential oils, alkaloids, anthraquinones, triterpenoids etc. One of the main approaches for the discovery of antimicrobials from higher plants is the evaluation of the medicinal plant extracts on pathogenic microbes (Verpoorte *et al.*, 1982).

In this study, the methanol extract of *Urtica parviflora* leaf exhibited a significant antimicrobial activity against 257 stains of Gram-positive and Gram-negative bacteria including MRSC strain. All the three reference MRSC strains of bacteria were found to be sensitive between 256 and 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the leaf extract described in **Chapter 9** showed that, out of 257 bacteria, the growth of 168 isolates were inhibited (65.36%) at a concentration of 128–512 μg/ml. 76 isolates (29.57%) were resistant at <1000μg/ml, while remaining 13 isolates (5.05%) were resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 58 out of 63 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 92 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed both against Gram-positive and Gram-negative bacteria. The isolated Compound I was also tested for antimicrobial activity. The result reveals that all the isolates were sensitive at 128-256 μg/ml concentration of the Compound I except the *Vibrio cholerae* 14033. It was interesting to note that all the MRSC

strains were susceptible to Compound I at concentration of 128-256 μg/ml, while they are resistant to both the standard antibiotics used.

The methanol extract of *Callicarpa arborea* leaf exhibited a significant antimicrobial activity against 257 Gram-positive and Gram-negative bacteria. The results showed that out of 257 bacteria, the growth of 221 isolates (85.99%) were inhibited by the extract at a concentration of 128 – 512 μg/ml. 35 isolates (13.61%) were resistant at <1000μg/ml, while remaining 1 isolate (0.38%) was resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 51 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 128-256 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the extracts was directed against both Gram-positive and Gram-negative bacteria but more sensitive to Gram-negative strains. The isolated Compound II was also tested for antimicrobial activity. The result is reveals that all the isolates were sensitive at 128-256 μg/ml concentration of the Compound II. It was noted that all the MRSC strains were resistant to compound II at concentration of 128 μg/ml, while they are resistant to both the standard antibiotics used.

The methanol extract of *Morinda citrifolia* root exhibited a significant antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multiresistant *Staphylococcus* (MRSC) strains. All the three reference MRSC strains of bacteria were found to be sensitive within 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the root extract showed that out of 257 bacteria, the growth of 217 isolates (85.99%) were inhibited by the extract at a concentration of 128– 512 μg/ml, 37 isolates (14.39%) were inhibited at a concentration of 1000μg/ml, while the remaining 03 isolates (1.16%) where inhibited at concentration >2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 64 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it can be inferred that the antimicrobial activity of the methanol extract was directed both against Gram-positive and Gram-negative bacteria. The isolated compound III was also tested for antimicrobial activity. The result revealed that all the isolates were sensitive at 128-512 μg/ml concentration of the Compound III except the *P. auruginosa*. It was observed that all the MRSC strains were susceptible to Compound III at a concentration of 128 μg/ml, while they were resistant to the two standard antibiotics used.

The present investigation therefore reveals that the methanol extracts of *Urtica parviflora* leaf, *Calicarpa arborea* leaf and *Morinda citrifolia* root have a significant degree of antimicrobial activity, which may be due to the presence of Compound I, Compound II and Compound III as evident by the tests. This can explain the rationale for the use of the plants in treating infections in traditional medicine.

11.9 Antiinflammatory and antipyretic study

Inflammation is a homeostatic response to pathogens and tissue injury. Inhibiting such processes may do more harm than good and may be associated with some degree of cellular and organ system toxicity. The use of anti-inflammatory agents should therefore be carefully considered, restricted to a limited time, and followed up with more appropriate therapies to address the underlying cause of inflammation (Mujumdar *et al.,* 2000).

From the result of the carrageenan induced paw edema study, presented in **Chapter 10**; it is evident that the test drugs inhibited the paw edema in dose and time dependent manner. Compound II showed percentage inhibition of 17.39 at the 1st hour, where as the standard drug Ibuprofen had percentage inhibition of 18.26 followed by MECA (16.52). In the 2nd hour maximum inhibition percentage amongst the test drugs treated groups was observed incase of Compound II treated group which was continued for 3rd (48.76) and 4th hour (61.69). It indicates the high efficacy of Compound II as compared to the standard drug Ibuprofen. In other words it can be said that Compound II at the dose of 30mg/kg b.w.p.o. has anti-inflammatory activity in carrageenan induced paw edema in rats similar to the standard drug at the dose of 100mg/kg b.w.p.o. In conclusion out of the three plants the plant *C. arborea* (contains Compound II) has got maximum anti-inflammatory activity.

There was dose dependant reduction in granuloma tissue formation in extract, isolated compouds and Indomethacin treated rats. The activity was found to be statistically significant for the dose ranges used. In this test the standard drug Indomethacin at the dose of 10 mg/kg b.w.p.o. showed maximum (51.94%) inhibition of granuloma formation followed by Compound II (38.20%) at the dose of 30 mg/kg b.w.p.o. and MECA (36.01%) at the dose of 200 mg/kg b.w.p.o. In conclusion, out of three plants the plant *C. arborea* (contains Compound II) has got maximum anti-inflammatory activity in cotton pellet induced granuloma model.

The effect of methanolic extracts of *U. parviflora, C. arborea, M. citrifolia* and their respective compounds on arachidonic acid induced inflammation in rats was found to be statistically significant for the dose ranges used. The anti-inflammatory activity of Compound II (64.06%) at the dose of 30 mg/kg b.w.p.o. is comparable to that of standard drug Phenidone (71.86%)

at the dose of 200 mg/kg b.w.i.p. followed by Compound III (63.20%) at the dose of 30 mg/kg b.w.p.o and MEMC (56.70%) at the dose of 200 mg/kg b.w.p.o..

The effect of MECA, MEMC, MEUP and their isolated compounds on yeast-induced pyrexia revealed that the rectal temperature of 38.19^{0}C at 19 hr was markedly elevated to 40.52^{0}C at the 21 hr and then slowed down for vehicle control. The results showed that the MECA at doses of 200 mg/kg b.w.p.o. caused significant lowering of the body temperature up to 4 hr following administration, as the normal mean temperature 39.20^{0}C at 19 hr was reduced to 38.00^{0}C at 23 hr. While maximum lowering of body temperature was noticed in case of its isolated compound treated group i.e. Compound II treated group, which received 30 mg/kg b.w.p.o. In this group the body temperatures slightly increased at 19hr (37.59^{0}C) from the basal temperature (37.55^{0}C) and slightly go on increasing up to 21 hr (38.46^{0}C) and then decreased to 37.79^{0}C. The compound succeeded to keep the body temperature near normal. The pattern of body temperature was different from other groups. In the paracetamol treated group the body temperature was 38.46^{0}C at 19 hr after the subcutaneous injection of yeast suspension which rose to 38.31^{0}C in the next hour and then slowed down up to 23 hr. It was found that the antipyretic effect of Compound II which is Oleanolic acid (Tang *et al.*, 2000; Jeong *et al.*, 1999) at 30 mg/kg b.w.p.o. is similar to the paracetamol group. The study revealed that MECA and compound II i.e. the plant *C. arborea* possess the maximum antipyretic activity out of the three plants studied. The statistical analysis also revealed that the body temperature differences were significant in this case.

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CHAPTER 12

SUMMARY & CONCLUSION

CONCLUSION

The objective of the investigation was to explore and prove the ethnomedicinal claims of three medicinal plants practiced by traditional healers of Sikkim in their health care system. Numerous medicinal plants used by the ethnic communities of Sikkim are neither systematically documented nor scientifically tested. The scientific studies of these ethnomedicines can lead to the discovery of promising biomolecules (drugs) and may be the answer to many illnesses in a cost effective way. In purview of this, it was thought to be much worthy to take up the present research project i.e. *"Chemical and Pharmacological Evaluation of Ethnomedicinal Plants of Sikkim"* so as to explore the pharmacology and development of some novel molecules from the indigenous plants.

The Chapter 1 of this thesis deals with the importance and development of herbal and traditional medicine in the present scenario. Herbal medicines include herbs, herbal preparations, natural products of plant origin and finished herbal products that contain active ingredients, parts of plant, or other materials, or their combination. Interest in natural sources as curatives for a variety of maladies or recreational use reaches back to the earliest points of history. Natural products now and in future will continue to exist and grow to become even more valuable as sources of new drug leads. This is because the degree of chemical diversity found in natural products is broader than that from any other source, and the degree of novelty of molecular structure found in natural products is greater than from any other source. Drug development over the years has relied only on a small number of molecular prototypes to produce new medicines. So, research into the use of plant-derived natural products should be emphasized as they possess broad spectrum of activities. To fully capitalize on the extensive biodiversity available to us in natural products, high efficient screening processes need to be improved. This chapter deals with such approaches which are used to develop drug from herbal sources.

The Chapter 2, deals with the reports on phytochemical and pharmacological analysis of *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn. The morphology, ethno-medicinal uses and advance research on these plants consisting of various pharmacological activities have been elaborated in the form of review of literature. Though some reports on phytochemical and pharmacological analysis of *Urtica parviflora, Callicarpa arborea* and *Morinda citrifolia* exist in the literature, the present study revealed the presence of additional constituents with significant pharmacological activities.

In Chapter 3, the modern methodologies for extraction and isolation of bioactive compounds from *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn. along with phytochemical group tests have been presented. The isolated bioactive compounds were analysed by using physical spectroscopic methods like UV, IR, ^{1}H NMR, ^{13}C NMR, Mass and melting point determination etc. The evidence presented conclusively proved that the isolated bioactive compounds isolated are β-Sitosterol from *Urtica parviflora* Roxb. Leaf, Oleanolic acid from *Callicarpa arborea* Roxb. leaf and 1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone from *Morinda citrifolia* Linn. root. The isolation of phytoconstituents bear much significance because the presence of β-Sitosterol in the leaf of *Urtica parviflora* Roxb. And 1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone are not reported previously.

In Chapter 4, the oral acute toxicity study of ethanol extracts of *Urtica parviflora* Roxb. leaf, *Callicarpa arborea* Roxb. leaf and *Morinda citrifolia* Linn. root are described. The protocol design and methodology was approved by the Institutional Animal Ethics Committee. The LD_{50} (Median Lethal Dose) of orally administered ethanolic extracts of leaves of *U. parviflora*, *C. arborea* and root bark of *M. citrifolia* were found to be 3500 mg/kg, 1666.67 mg/kg and 950 mg/kg body weight per oral respectively in albino rats.

Chapter 5 deals with the hepatoprotective activity of ethanol extract of *Urtica parviflora* Roxb. leaf only. Other two plant drugs namely methanol extracts of, *Callicarpa arborea* leaf and root bark of *Morinda citrifolia* did not showed any significant hepatoprotective activity in the pilot study, thus excluded from the purview of this study. Also in this study the ethanolic fraction of *Urtica parviflora* was chosen because of it showed higher hepatoprotective activity as compared to other fractions. Oral administration of *U.parviflora* leaf extract restored the hepatic cell damage in a dose dependent manner providing significant protection at a dose of 750 mg/kg body weight as compared to the standard drug Silymarin. The findings provide the scientific basis for traditional use of *U. parviflora* leaves for managing hepatic disorders. The data obtained are consistent with literature report on ethnopharmacological usage of the plant as a hepatoprotective agent. This was further evident from the enzyme assays such as ALT, AST, ALP, Total protein and bilirubin along with the histopathological examination of liver in rats treated with the leaf extract. The mechanism by which *U. parviflora* exerts its protective action against carbon tetrachloride induced alterations in the liver may be due to the antioxidative effect of the plant extract.

Chapter 6 deals with the wound healing activity of three plant extracts from *Urtica parviflora* Roxb., *Callicarpa arborea* Roxb. and *Morinda citrifolia* Linn. respectively. Three wound models such as Excision, Incision, and Dead space were selected to evaluate the wound healing

potential. Separate group of animals were treated with ointments prepared from the extracts and with oral feeding of the extracts. Three parameters were studied in dead space wound model, namely dry granuloma weight, breaking strength and estimation of hydroxyproline. The histopathological examinations were recorded in five parameters i.e. Keratinization, Epithelization, Fibrosis, Collagen, and Neovascularisation.

Wounds of all ages create an impression of individuality on a microscopic level as specific as a finger print. Wounds are disruption of the normal anatomic structure and function and the body restore this abnormality with the process of wound healing, which is a very complex, multifactor sequence of events involving several cellular and biochemical changes to regenerate and reconstruct the disrupted anatomical continuity with the maintenance of the functional status of the skin.

The results in this study are in support that the wound healing and repair is accelerated by applying extract ointments as well as oral feeding. It can be concluded from this study that, the three plants have significant wound healing activity which could be credited to the presence of their active principles and showed variability in different parameters. The enhanced capacity of wound healing with the *Callicarpa arborea* could be explained on the basis of the anti-inflammatory effect of the plant and presence of Oleanolic acid moiety, that are well documented in other chapters. The highest wound healing activity showed by the ointment prepared from the methanolic root bark extract of *Morinda citrifolia* may be due to the presence of the anthraquinone moiety i.e. 1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone, which has antimicrobial property. This ability was especially obvious when the data were compared with the other plants. All the three plants are used by the local communities to treat fracture and dislocation and other skin injuries. exhibited wound healing potential in one or more parameters.

In Chapter 7, antioxidant activities of methanol extracts of *Urtica parviflora* Roxb. leaf, *Callicarpa arborea* Roxb. leaf and *Morinda citrifolia* Linn. root as well as their respective isolated compounds, Compound I, II and III has been presented. The antioxidant activity was studied by *in vitro* ferrous sulphate induced lipid peroxidation, DPPH (1,1-diphenyl –2 picryl hydrazyl) free radical, superoxide radical scavenging assay using vitamin E (5mM) as standard drug. The percentages of inhibitions were calculated as compared with standard drugs. It was observed that the methanol extracts of all the plants exhibited a considerable inhibition of lipid peroxidation and possessed DPPH radical, and superoxide radical scavenging activity. The percentage of inhibition was on a concentration dependent manner in all the models. Further study with compound I and compound II at 25 and 50 µg/ml concentrations showed significant antioxidant effects in concentration dependent manner in all the models tested. In conclusion,

the presence of β-sitosterol, Oleanolic acid and anthraquinone glycoside isolated from the plants could be responsible for the observed antioxidant activity.

Chapter 8 deals with the hypoglycemic activity of the plant drugs. Hypoglycemic activity of drugs is conventionally assessed in diabetic animal models by observing drug-induced fall in fasting blood glucose (FBG) or suppression of glucose tolerance curve. Diabetes is induced experimentally by partial or total pancreatecctomy, exposure to antislet cell antibodies, or by injecting chemical agents, such as alloxan or streptozotocin, that cause widespread destruction of insulin-secreting pancreatic beta cells. All the plant extracts were subjected to hypoglycemic activity in albino rats. Diabetes was induced in this study by a single intra peritoneal injection of freshly prepared Streptozotocin (STZ) in a dose of 50mg/kg body weight dissolved in citrate buffer pH 4.5 to overnight fasted rats. The hypoglycemic activity on these animals was carried out after one week of STZ injection when the stabilization of diabetes was ensured. The animals with fasting serum glucose (FSG) of 240 mg/dl and above were used for this activity along with the normal rats.

The result reveals that methanolic extract of *Urtica parviflora* succeeded to control the rise of serum glucose level (70.6%) within 1st hour of Glucose Tolerance Test in streptozotocin induced diabetic rats, followed by its isolated compound β-sitosterol (60.0%) and Compound II, which is Oleanolic acid, isolated from *Callicarpa arborea* (55.2%). *Morinda citrifolia* and its isolated compound 1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone failed to normalize the rise of serum glucose level. Thus out of the three plants studied, the plant *Urtica parviflora*, was found to have remarkable hypoglycemic activity.

The antidiabetic effect of test drugs as compared to the standard drug, Glibenclamide is very much significant. The percentage reduction of serum glucose level by the methanol extracts of *Urtica parviflora* (27.2) and *Callicarpa arborea* (19.2) are much higher than the standard drug, Glibenclamide (18.5); which indicates high efficacy of them, in reducing serum glucose level in diabetic rats.

In the study of hypoglycemic activity of the plant drugs, in normal healthy rats by considering the percentage reduction when compared to the value of the control group, it is revealed that Compoud I and Compound II exhibited same percentage reduction.

In the present study, it is fairly evident from the studies performed in all the three models that maximum percentage reduction of serum glucose level was found with the methanolic extract of *Urtica parviflora* and Compound I, isolated from the same plant. Hence the plant *Urtica parviflora* is having highest hypoglycemic activity, followed by *Calicarpa arborea* and *Morinda citrifolia* in streptozotocin induced diabetes in rat. This is further confirms the strong glucose

lowering effect of nettles of other species as reported earlier. This effect may be caused in part by the reduction of intestinal glucose absorption.

In conclusion, the present study seems to support the potential of a methanol extract of *Urtica parviflora* as an effective hypoglycaemic agent when compared with Metformin, a drug which has long since been used for the treatment of Type 2 diabetes. This study shows that *Urtica parviflora* can restore the normal blood levels of glucose.

Chapter 9, which deals with the antimicrobial activity of methanol extracts of *Urtica parviflora* leaf, *Callicarpa arborea* leaf, *Morinda citrifolia* root; Compound I, Compound II and Compound III has been studied *in vitro*. The *in vitro* test was carried out by agar dilution and disc diffusion method.

The methanol extract of *Urtica parviflora* leaf exhibited a significant antimicrobial activity against 257 stains of Gram-positive and Gram-negative bacteria including MRSC, sensitive between 256 and 1000 μg/ml concentration of the extract. The percentage of inhibition was 65.36 at a concentration of 128–512 μg/ml. 29.57% bacteria were resistant at 1000μg/ml, while remaining 5.05% were resistant up to 2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 58 out of 63 Gram-positive bacteria were sensitive between 128 and 256 μg/ml, while out of 179 Gram-negative isolates, 92 were sensitive between 256-512 μg/ml concentration of the extract. Hence, the antimicrobial activity of the extracts was directed against both Gram-positive and Gram-negative bacteria. In case of Compound I, which is β-sitosterol, all the isolates were sensitive at 128-256 μg/ml concentration except the *Vibrio cholerae* 14033 and all the MRSC strains were susceptible to Compound I at the minimum concentration, while they are resistant to both the standard antibiotics amoxycillin and gentamycin.

The methanol extract of *Callicarpa arborea* leaf exhibited a significant antimicrobial activity against 257 Gram-positive and Gram-negative bacteria. The results showed that 85.99% isolates were inhibited by the extract at a concentration of 128 – 512 μg/ml and 13.61% were resistant at 1000μg/ml, while remaining 0.38% was resistant up to 2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 51 out of 78 Gram-positive bacteria were sensitive in the minimum concentration, while out of 179 Gram-negative isolates, 73 were sensitive between 128-256 μg/ml concentration of the extract. The antimicrobial activity of the extracts was directed against both Gram-positive and Gram-negative bacteria but more sensitive to Gram-negative strains. The isolated Compound II, which is Oleanolic acid, is sensitive to all strains at the minimum concentration but MRSC strains were resistant to

compound II at concentration of 128 μg/ml, while they are resistant to both the standard antibiotics used.

The methanol extract of *Morinda citrifolia* root also exhibited a significant antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multi-resistant *Staphylococcus* (MRSC) strains. All the three reference MRSC strains of bacteria were found to be sensitive within 1000 μg/ml concentration of the extract. The results of the antimicrobial spectrum of the root extract showed that it is sensitive to 85.99% isolates, at a concentration of 128– 512 μg/ml, 14.39% of the isolates were inhibited at a concentration of 1000μg/ml, while the remaining 1.16% where inhibited at concentration 2000 μg/ml, the highest concentration of the extract tested. The MIC tests revealed that 64 out of 78 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 73 were sensitive between 256-512 μg/ml concentration of the extract (zone diameter 10-14 mm). Hence, it can be inferred that the antimicrobial activity of the methanol extract was directed both against Gram-positive and Gram-negative bacteria. The isolated Compound III, which is 1-8 dihydroxy, 3 methyl, 6 methoxy anthraquinone, which was tested for antimicrobial activity revealed that all the isolates were sensitive at 128-512 μg/ml concentration of the Compound III except the *P. auruginosa*. It was observed that all the MRSC strains were susceptible to Compound III at a concentration of 128 μg/ml, while they were resistant to the two standard antibiotics used.

The present investigation therefore reveals that the methanol extracts of *Urtica parviflora* leaf, *Calicarpa arborea* leaf and *Morinda citrifolia* root have a significant degree of antimicrobial activity, which may be due to the presence of Compound I, Compound II and Compound III as evident by the tests. This can explain the rationale for the use of the plants in treating infections in traditional medicine.

Chapter 10 is devoted to the anti-inflammatory and antipyretic activity of plant drugs. The results of the antiinflammatory activity of *Urtica parviflora* leaf, *Callicarpa arborea* leaf and *Morinda citrifolia* root, which was carried out in three models i.e. Carrageenan-induced edema test, cotton pellet induced granuloma test, and arachidonic acid induced paw edema test. It reveals that, Compound II, which is Oleanolic acid, at the dose of 30mg/kg b.w.p.o. has anti-inflammatory activity in carrageenan induced paw edema in rats similar to the standard drug at the dose of 100mg/kg b.w.p.o.

In conclusion out of the three plants the plant *C. arborea* (contains Compound II) has got maximum anti-inflammatory activity. There was dose dependant reduction in granuloma tissue formation in extract, isolated compouds and Indomethacin treated rats. Again in cotton pellet induced granuloma model, methanolic leaf extract of *Callicarpa arborea* and its isolated compound, Oleanolic acid proved their efficacy in inhibiting granuloma formation (36.01% and 38.20%) at the dose of 30 mg/kg b.w.p.o. and 200 mg/kg b.w.p.o respectively. In conclusion, out of three plants the plant *C. arborea* (contains Oleanolic acid) has got maximum anti-inflammatory activity.

The anti-inflammatory activity of Compound II (64.06%) at the dose of 30 mg/kg b.w.p.o. is comparable to that of standard drug Phenidone (71.86%) at the dose of 200 mg/kg b.w.i.p. followed by Compound III (63.20%) at the dose of 30 mg/kg b.w.p.o and MEMC (Methanol extract of *Morinda* citrifolia, 56.70%) at the dose of 200 mg/kg b.w.p.o. in arachidonic acid induced inflammation in rats.

It was found that the antipyretic effect of Compound II, which is Oleanolic acid, at 30 mg/kg b.w.p.o. is similar to the antipyretic activity of paracetamol in Brewers' yeast induced pyrexia model in rats. The study revealed that MECA and compound II i.e. the plant *C. arborea* possess the maximum antipyretic and anti-inflammatory activity out of the three plants studied. The statistical analysis also revealed the significance.

All the experiments which were performed both by using *in vitro* and *in vivo* models to establish and authenticate the ethnomedicinal claims on plants of the Sikkim Himalayan region have yielded valuable information. It proves the right importance of use of these plants by the people of this region as aids in their health care system in form of indigenous ethnomedicines since time immemorial. In the context of combinatorial chemistry and phytomedicine and herbal preparations (wherein large numbers of compounds are present as a mixture) may lead to the development of novel drugs. Many drug discoveries produce the key components and lead molecules, which are identified as fingerprints obtained from the studies of herbal medicines as well as ethnomedicines.

The studies undertaken in this thesis was also an attempt towards the search of such lead molecules from the plant drugs, which may contribute a little but impotant step in the development of newer molecules of high therapeutic value. Most of the work presented in this thesis has been authenticated with concomitant publications in national and international journals.

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Hepatoprotective Effect of the Ethanolic Extract of *Urtica parviflora* Roxb. in CCl_4 Treated Rats

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Abstract: The ethanolic extract of leaves of *Urtica parviflora* (EEUP) was evaluated for the hepato protective effect in carbon tetrachloride (CCl_4) induced hepatotoxicity in rats to prove its ethnomedicinal claim by the hill people of Sikkim. Hepatotoxicity was induced in Swiss Albino male rats of Sprague Dawley strain by subcutaneous injection of carbon tetrachloride at the dose of 1 mL kg^{-1} body weight. The hepatoprotective activity was evaluated by the assay of liver function biochemical parameters such as aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), total bilirubin, serum protein and by study of histopathology of the livers. The toxic effect of carbon tetrachloride was controlled significantly by the EEUP at 250, 500 and 700 mg kg^{-1} p.o. ($p<0.05$) as compared to the CCl_4 treated animals by restoration of the levels of serum bilirubin, proteins and hepato protective enzymes. Histopathological studies revealed that the centrolobular necrosis induced by CCl_4 was recovered to normal state by EEUP in a dose dependent manner. The study confirms the possible hepatoprotective potentiality of the ethanolic extract of leaves of *Urtica parviflora* which had been collected from Sikkim. Studies are under process to isolate and characterize the bioactive component present in the plant as well as to establish the mechanism of action underlying for its hepatoprotective potentiality.

Key words: Hepatoprotective activity, *Urtica parviflora*, carbon tetrachloride

INTRODUCTION

Urtica parviflora Roxb. (Urticaceae) is a perennial shrub used in traditional medicine in Sikkim, Darjeeling and in North Bengal (Gurung, 1999). The roots are employed for the treatment of fractures of bone and dislocations of joints (Ramachandran, 1992). The leaves are used in dysentery, joint pain and liver disorders (Gurung, 1999). The inflorescences are used as cleansing agent after parturition and in the treatment of dermatitis in the alpine region of central and eastern Himalayas (Ramachandran, 1992). Urtication was practiced for the treatment of certain diseases and consisted of beating the skin with a bunch of nettles (White, 1887). The result was erythema and whealing but after the third or fourth successive application, the skin ceased to react under fresh contact (White, 1887). Acetylcholine, histamine and 5-hydroxytryptamine have been implicated in itching from the stinging hairs (Emmelin and Feldberg, 1947; Saxena *et al*., 1965). The hairs are described by Thurston and Lersten (1969) and Uphof and Hummeh (1962). Recently, Oxalic acid and tartaric acid were isolated from other species of *Urtica* (*U. thunbergiana*) as major long-lasting pain-inducing toxins (Fu *et al*., 2006). The roots of stinging nettle (*Urtica dioica*) had been studied for the treatment of Benign Prostatic Hyperplasia (BPH) and associated Lower Urinary Tract Symptoms (LUTS) (Egon, 2001). Liver is an important organ actively involved in many metabolic functions and is the frequent target of number of toxicants (Meyer and Kulkarni, 2001). Therefore, the disorders associated with this organ are numerous and varied (Wolf, 1999). Liver disorders have long been recognized as one of the most important health problems in the developing countries. Hepatitis is one of the most common diseases in the Eastern Himalayan region of Sikkim (Roy Burman, 2003). In absence of a reliable liver protective drug in the modern medicine, there are number of medicinal preparations in Ayurveda, recommended for the treatment of liver disorders (Chatterjee, 2000). In view of this, the present study has been undertaken to investigate the hepatoprotective activity of *U. parviflora* Roxb. leaves against the CCl_4 induced hepatotoxicity in albino rats.

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MATERIALS AND METHODS

Plant materials: The leaves of *Urtica parviflora* Roxb was collected from Majhitar, East Sikkim, India in March 2006. The plant was identified by the Botanical Survey of India (BSI), Gangtok, Sikkim. The voucher specimen (HP-124) has been retained in our laboratory for future reference. The collected leaves were air-dried and pulverized in a mechanical grinder.

Preparation of extracts and phytochemical study: The leaves (500 g) were coarsely powered and subjected to successive solvent extraction with petroleum ether (60-80°C), benzene, chloroform, ethanol and water. In the preliminary hepatoprotective studies, ethanolic extract shown to have better protection than the other extracts. Therefore the ethanolic extract was further used for pharmacological screening. The extract was suspended in aqueous Tween 80 solution (0.5%). The chemical constituents of the extracts was identified by qualitative chemical tests and further confirmed by thin layer chromatography for the presence of alkaloids, sterols, tannins, reducing sugars and flavonoids (Trease and Evans, 1996).

Animals: Swiss Albino male rats of Sprague Dawley strain, weighing 150-175 g each, were used. They were housed under standard conditions of temperature (23±1°C) and relative humidity (55±10%); 12/12 h light/dark cycle and fed with standard pellet feed and water *ad libitum*. The Institutional Animal Ethics Committee reviewed the entire animal protocols prior to the experiment (No. HPI/IAEC/PK/08/2006).

Experimental induction of liver damage: Liver damage was induced in rat by administering CCl_4 subcutaneously in the lower abdomen at the dose of 1 mL kg^{-1} body weight except the animals of first group. CCl_4 was administered on every first and fourth day of the week up to 13 weeks. The rats were divided into 5 groups, 8 animals in each. Group 1 served as control, receiving Tween80 solution (0.5%) orally. Group 2 received only CCl_4. In a pilot study it was observed that the ethanolic extract showed prominent liver protection against CCl_4 induced liver damage. The ethanol extract of *U. parviflora* Roxb leaves (EEUP) was administered orally to groups 3, 4 and 5 at a dose o f 250, 500 and 750 mg kg^{-1} p.o. body weight, respectively. Reference drug Silymarin 100 mg kg^{-1} p.o. was administered to Group 6 animals in Tween80 solution (0.5%). Every day at 9.00 am, a known quantity of food was replenished. The animals were kept starved over night one day before the last day of the experiment. On the next day they were sacrificed and blood was collected making an incision on jugular vein.

Enzyme assay: The serum was separated from the blood for biochemical estimation by centrifugation at 2500-3000 rpm. Different parameters like serum alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP) activity were measured according to the method of Reitmen and Franckel (Reitman and Frankel, 1957).

Estimation of total protein and bilirubin: The level of total protein (TP) was estimated in the serum of the animals by Biuret method (Kingsley and Frankel, 1964). The level of bilirubin was estimated by the method of Mallory with slight modification (Mallory and Evelyn, 1939).

Histopathological examination: The liver lobes of the animals were removed and washed with normal saline (Fig. 1a). Small pieces of liver tissue were preserved in 10% formalin solution for histological analysis. The pieces were dehydrated with 90% ethanol, embedded in paraffin, cut into thin sliced sections (7 μm thick), stained with haematoxylin-eosin dye and observed under a light microscope, for cell necrosis, vascular degenerative changes, inflammation and fibrosis.

Statistical analysis: The data were analysed statistically using one-way analysis of variance followed by Dunnett t-test. The data are expressed as mean±SEM p-values less than 0.05 indicate significance.

RESULTS AND DISCUSSION

The phytochemical studies (chemical test and TLC) revealed that presence of alkaloids, sterols, tannins, flavonoids and reducing sugar (Table 1). The biochemical mechanism of hepatoprotective activity of the EEUP, the levels of ALT, AST, ALP, total protein and bilirubin were shown in Table 2. EEUP has shown dose dependent and significant ($p<0.05$ compared to CCl_4 group) hepatoprotective effect at the dose levels 250, 500 and 750 mg kg^{-1} p.o. The increase in the level of hepatic enzymes and serum bilirubin reflected the depth of jaundice, cellular leakage and the loss of cellular integrity of the cell membrane (Sarawat *et al*., 1993). The ethanol extract at the dose level of 750 mg kg^{-1} p.o decreased the elevated levels of ALT, AST, ALP and Bilirubin at the levels of 47.9, 58.3, 63.7 and 0.74 IU L^{-1} (Table 2) after CCl_4 induction. Single dose of CCl_4 caused centrolobular necrosis, extending to midzone with neutrophilic collection inside the lobule (Fig. 1b). The decrease in

Table 1: Thin layer chromatography study of the ethanol leave extract of *U. parviflora*

Chemical group	Solvent system	Spray reagent	hRf values
Alkaloids	Chloroform: Methanol (9:1)	Dragendroff's	28
Sterols	Chloroform: Methanol (1:1)	Libermann Burchard	65-75
Tannins	Ethyl acetate: Benzene (9:11)	Folin	0-5
Reducing sugars	n-Butanol: Acetic acid: Water (4:1:5)	Aniline hydrogen phthalate	15-22
Flavonoids	Toluene: Ethyl acetate: Acetone (2:4:4)	5% alcoholic $AlCl_3$	35

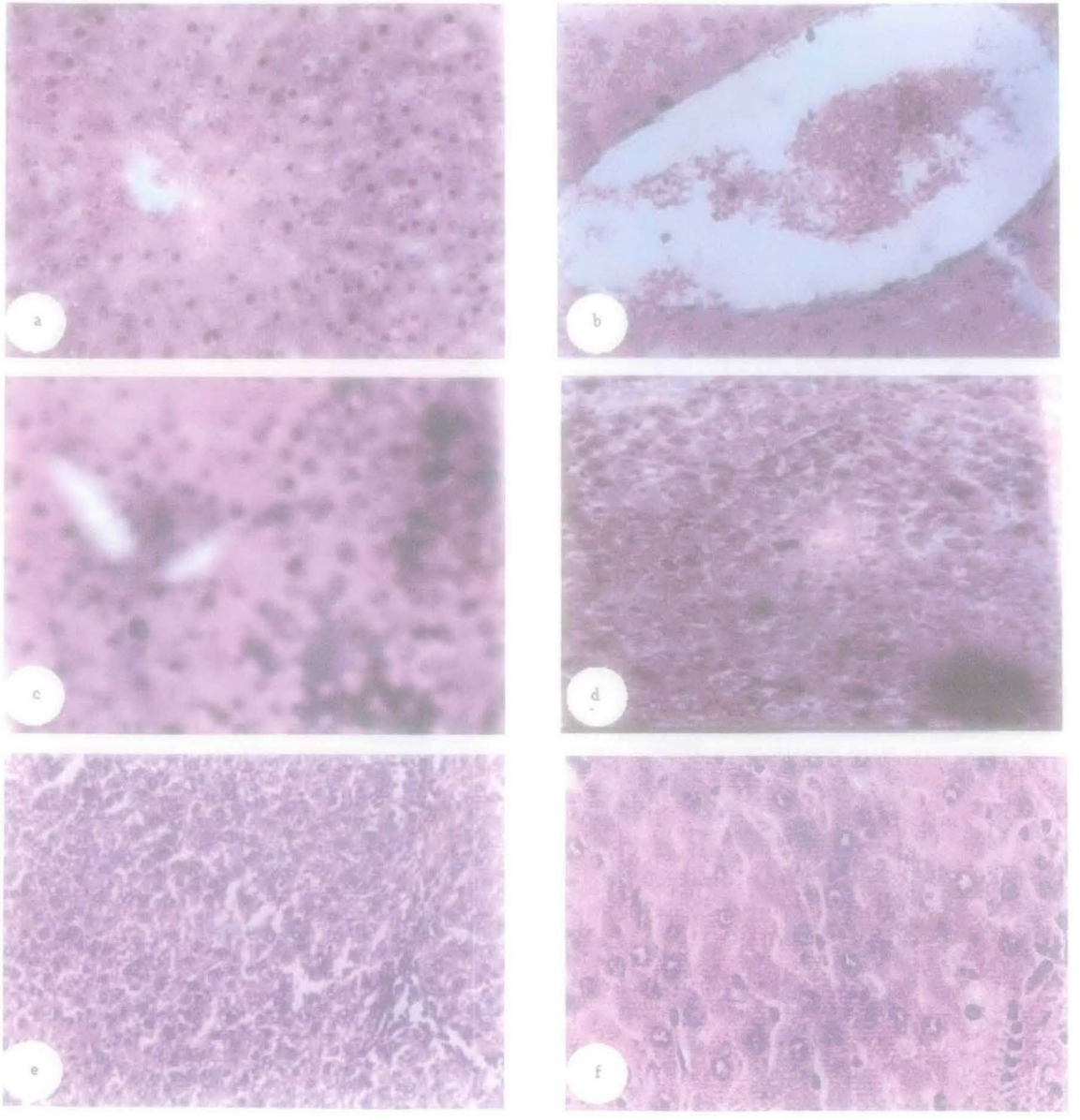

Fig. 1: Photomicrographs of liver sections of rat stained with haematoxylin and eosin (x100). (a) Liver section from normal rat showing normal liver architecture with normal hepatocyte morphology, (b) Liver section from CCl_4-treated rat showing centrolobular necrosis extending to midzone with neutrophillic collection, (c) Liver section recovering from CCl_4 induced toxicity in EEUP 250 mg kg^{-1} treated rat (d) Liver section depicting the clear bile canaliculi; normal distribution of kupffer cells and sinusoidal cells in EEUP 500 mg kg^{-1} treated rat and (e, f) Liver architecture almost normal in EEUP 750 mg kg^{-1} and Silymarin 100 mg kg^{-1} treated rats

Table 2: Effect of ethanol leaves extract of *U. Parviflora* on CCl_4 induced hepatotoxicity in rats

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
ALT (IU L^{-1})	30.63±0.32	120.00±8.81[a]	118.30±6.44[b]	96.70±6.43[b]	47.90±4.98[b]	30.23±1.82[b]
AST (IU L^{-1})	60.12±0.05	65.00±6.40[a]	148.90±7.76[b]	81.20±5.18[b]	58.30±7.41[b]	58.10±4.56[b]
ALP (IU L^{-1})	58.29±0.12	108.20±3.39[a]	89.70±1.73[b]	71.40±4.81[b]	63.70±3.67[b]	57.90±1.89[b]
Bilirubin (IU L^{-1})	0.49±0.05	2.67±0.13[a]	1.15±0.12[b]	0.86±0.09[b]	0.74±0.04[b]	0.56±0.02[b]
Total protein (g dL^{-1})	8.15±0.27	5.21±0.13[a]	6.17±0.27[b]	7.56±3.32[b]	9.54±2.13[b]	9.23±1.13[b]

Values are expressed as mean±SEM from 6 rats in each observation, [a]: $p<0.05$ compared to control group, [b]: $p<0.05$ compared to CCl_4 treated group

hepatic transminase enzymes after the EEUP treatment is an indication of the stabilization of plasma membranes as well as repair of hepatic tissue damage caused by CCl_4. This effect is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (Raja *et al.*, 2007). In CCl_4 treated rats liver section, the cells of centrolobular region showed vacuolated cytoplasm, the vacuolar size showed variations from spherical to large droplet structures. In most of the necrotic cells centrally placed nuclei were suspended in small amount of cytoplasm, which continued by cytoplasmic strands that traverse through the vacuoles connected to the periphery of cytoplasm. Kupffer cells and sinusoidal cells showed arrest in distribution. The administration of ethanol extract at 250 mg kg^{-1} p.o. body weight protected the liver partially (Fig. 1c). The extent of the necrotic region was reduced significantly. Numbers of necrotic cells located in this region were considerably reduced and were retained in immediate vicinity of the vein. The centrolobular region of rat liver treated with EEUP at 500 mg kg^{-1} p.o. body weight along with CCl_4 showed normal cellular architecture without any necrotic cells (Fig. 1d). The histology of the livers was found normal when treated with EEUP and standard drug Silymarin at 750 mg kg^{-1} and 100 mg kg^{-1} p.o. body weight, respectively (Fig. 1e, f). Histopathological studies revealed the significant protection of liver by EEUP after CCl_4 induction, the study reports are similar to the literature (Shyamal, 2006), but only the dose was higher (750 mg kg^{-1}) in this study and revealing at higher dose of EEUP can be employed as hepatoprotective medicine. The sub-chronic and chronic toxicity studies to be performed on this plant to assure the safety of *Urtica parviflora*. Further detailed investigations by the researchers are in progress to isolate the phytoconstituents of the plants responsible for the hepatoprotective activity.

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