Chemical And Pharmacological Evaluation Of *Colebrookea oppositifolia* Smith And *Heracleum nepalense* D. Don. Dc.

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2006

DEDICATED TO

MY

GRAND PARENTS

AND

THE ALMIGHTY GOD

HIMALAYAN PHARMACY INSTITUTE

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CERTIFICATE

This is to certify that the thesis entitled "Chemical and Pharmacological Evaluation of *Colebrookea oppositifolia* Smith and *Heracleum nepalense* D. Don. DC" submitted by Sri Suvakanta Dash, Sr. Lecturer, Himalayan Pharmacy Institute, Majhitar, Rangpo, E. Sikkim-737132 for the award of Ph.D. (Pharmacy) degree of University of North Bengal, is absolutely based upon his own research work under the supervision of Prof. Lila Kanta Nath, Professor and Principal, Himalayan Pharmacy Institute, Majhitar, Rangpo, E. Sikkim-737132, and that neither his thesis nor any part of the thesis has been submitted for any degree or any other academic award anywhere before.

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Suvakanta Dash

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PREFACE

From the very primitive age people have relied on nature for their basic needs, be it for food, shelter, or medicine. Plants have formed the basis of sophisticated traditional systems of medicines that have been in existence for thousands of years in the countries like India. The newly implemented Intellectual Property Regimens (IPR) has got tremendous impact on the molecular medicines discovered in recent era. It is expected that developing countries may face a problem of higher prices for drugs in near future on implementation of the IPR as compared to the existing system. To meet the need for protecting the health of common and poor people, herbal and traditional medicine will play an important role in the new IPR regimen. India has rich heritage in herbal medicine, unfortunately we have not added our scientific strength to enrich our knowledge in this domain, rather ignored them by respecting only modern allopathic system of health support. However time has now more than ripen to initiate scientific investigations on our traditional herbal medicines. Initial field survey has led to the conclusion that different plants are being used by the traditional healers of Sikkim to combact various diseases. The work embodied in this thesis is a humble and sincere effort to explore and screen two ethnomedicine *Colebrookea oppositifolia* Smith and *Heracleum nepalense* D.Don DC used by traditional healer of Sikkim in their health care since time immemorial. The finding of this investigation revealed that the crude extracts of the above two plants are very useful in enhancement of bioavailability of antibiotics, stimulation of immune response and possessing antioxidant and antimicrobial activities. 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 09 well-defined chapters and finally a summary and finally the conclusion has been drawn along with the summary of the whole work. At the end of each chapter the necessary references have been list, which are superscripted in the text. Thus the investigated works deals with a scientific approach to explore the pharmacological activities of the compounds isolated from *C.oppositifolia* and *H.nepalense*, two popular ethnomedicine from Himalayan Sikkim region, for the benefit of the entire mankind.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1. Herbal medicines: definition and developments.

The use of substances of plant origin for food as well as medicine can be said as old as mankind itself. Primitive man depended on plants and animals for food and medicine and learnt to identify those, which were useful to them, poisonous and harmful. As our primitive ancestors evolved in to human, the diseases they brought with them, and these acquired along the evolutionary way, became social and cultural facts as well as pathological states. Since evolution, diseases have threatened not only well being sufferers and their fellows, but also integrity of the community. Therefore it is of primary importance to the members of every group to try and maintain their health of those who fall ill.

Every human community responded to this challenge by developing a medical system. Therefore, the medical system is a *"pattern of social and cultural system that evolves to deliberate behavior to enhance health"* [(1)]. Ever since the most ancient times, human beings have found remedies within their habitat, and have adopted different therapeutic strategies depending both upon climatic, pedagogical, phytogeographic and faunal characteristics, as well as peculiar cultural and sociostructural typologies [(2)]. Traditional systems of medicines contain beliefs and practices in order to cure illness. Such acts and avoidance that constitute preventive medicines in traditional system are often quite different from those of modern medicine.

Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients, parts of plant, or other materials, or their combination. Traditional use of herbal medicines refers to the long historical use of these medicines. Their use is well established and widely acknowledged to the safe and effective, and accepted by national authorities.

World Health Organization (WHO) has defined herbal medicines as " finished labeled medicinal products that contain active ingredients, aerial or underground parts of the plant or other plant materials or combinations thereof, whether in the crude state or as plant preparations". The same WHO document adds, " medicines containing plant

material combined with chemically defined active substances, including chemically defined isolated constituents of plants are not considered to be herbal medicines".

Herbal medicines, as defined by WHO, can therefore be classified into three categories as follows:

- Phytomedicines or phytopharmaceuticals sold as over- the- counter (OTC) products in modern dosage forms such as tablets, capsules and liquids for oral use.
- Dietary supplements containing herbal products, also called nutraceuticals, available in modern dosage forms.

  Consumers in developed countries and these in urban areas of developing countries use these two types of herbal medicines. These herbal medicines are gradually occupying increasing shelf-shape in modern pharmacies.
- Herbal medicines consisting of crude, semiprocessed or processed medicinal plants. These have a vital place in primary healthcare in developing countries.

Early human recognized their dependence on nature in both health and illness. Led by instinct, taste, and experience, primitive men and women treated illness by using plants, animal parts, and minerals that were not part of their usual diet. Physical evidence of use of herbal remedies goes back some 60,000 years to a burial site of a Neanderthal man uncovered in 1960 [3]. In a cave in northern Iraq, scientists found what appeared to be ordinary human bones. An analysis of the soil around the bones revealed extraordinary quantities of plant pollen that could not have been introduced accidentally at the burial site. Someone in the small cave community had consciously gathered eight species of plants to surround the dead man. Seven of these are medicinal plants still used throughout the herbal world [4]. All cultures have long folk medicine histories that include the use of plants. Even in ancient cultures, people methodically and scientifically collected information on herbs and developed well-defined herbal Pharmacopoeias. Indeed, well into the 20th century much of the Pharmacopoeia of scientific medicine was derived from the herbal lore of native peoples. Many drugs, including strychnine, vincristine, taxol, curare, and ergot, are of herbal origin. About one-quarter of the prescription drugs dispensed by community pharmacies in the United States contain at least one active ingredient derived from plant material [5].

Middle East medicine. The invention of writing was a focus around which herbal knowledge could accumulate and grow. The first written records detailing the use of herbs in the treatment of illness are the Mesopotamian clay tablet writings and the Egyptian papyrus. About 2000 B.C., King Assurbanipal of Sumeria ordered the compilation of the first known materia medica--an ancient form of today's United States Pharmacopoeia containing 250 herbal drugs (including garlic, still a favorite of herbal doctors). The Ebers Papyrus, the most important of the preserved Egyptian manuscripts, was written around 1500 B.C. and includes much earlier information. It contains 876 prescriptions made up of more than 500 different substances, including many herbs [6].

Greece and Rome. One of the earliest materia medica was the Rhizotomikon, written by Diocles of Caryotos, a pupil of Aristotle. Unfortunately, the book is now lost. Other Greek and Roman compilations followed, but none was as important or influential as that written by Dioscorides in the 1st century A.D., better known by its Latin name De Materia Medica. This text contains 950 curative substances, of which 600 are plant products and the rest are of animal or mineral origin [6]. Each entry includes a drawing, a description of the plant, an account of its medicinal qualities and method of preparation, and warnings about undesirable effects.

Muslim world. The Arabs preserved and built on the body of knowledge of the Greco-Roman period as they learned of new remedies from remote places. They even introduced to the West the Chinese technique of chemically preparing minerals. The principal storehouse of the Muslim materia medica is the text of Jami of Ibn Baiar (died 1248 A.D.), which lists more than 2,000 substances, including many plant products. Eventually Christian doctors traveling with the Crusaders reintroduced this entire body of knowledge to Europe. Indeed, during the Middle Ages, trade in herbs became a vast international commerce.

China and Japan. The earliest written evidence of the medicinal use of herbs in China consists of a corpus of 11 medical works recovered from a burial site in Hunan province. The burial itself is dated 168 B.C., and the texts (written on silk) appear to have been

composed before the end of the 3rd century B.C. Some of the texts discuss exercise, diet, and channel therapy. The largest, clearest, and most important of these manuscripts, called by its discoverers Prescriptions for Fifty-Two Ailments, is predominantly a Pharmacological work. More than 250 medicinal substances are named. Most of the substances are derived from herbs and wood, grains, legumes, fruits, vegetables, and animal parts. Underlying this entire text is the view that disease is the manifestation of evil spirits, ghosts, and demons that must be repelled by incantation, rituals, and spells in addition to herbal remedies.

By the Later Han Dynasty (25-220 A.D.), medicine had changed dramatically in China. People grew more confident of their ability to observe and understand the natural world and believed that health and disease were subject to the principles of natural order. However, herbs still played an important part in successive systems of medicine. The Classic of the Materia Medica, compiled no earlier than the 1st century A.D. by unknown authors, was the first Chinese book to focus on the description of individual herbs. It includes 252 botanical substances, 45 mineral substances, and 67 animal-derived substances. For each herb there is a description of its medicinal effect, usually in terms of symptoms. Reference is made to the proper method of preparation, and toxicities are noted [4].

Since the writing of the Classic of the Materia Medica almost 2,000 years ago, the traditional Chinese Materia Medica has been steadily increasing in number. This increase has resulted from the integration into the official tradition of substances from China's folk medicine as well as from other parts of the world. Many substances now used in traditional Chinese medicine originate in places such as Southeast Asia, India, the Middle East, and the Americas. The most recent compilation of Chinese materia medica was published in 1977. The Encyclopedia of Traditional Chinese Medicine Substances (Zhong yao da ci dian), the culmination of a 25-year research project conducted by the Jiangsu College of New Medicine, contains 5,767 entries and is the most definitive compilation of China's herbal tradition to date.

Traditional Chinese medicine was brought to Japan via Korea, and Chinese-influenced Korean medicine was adapted by the Japanese during the reign of Emperor Ingyo (411-453 A.D.). Medical envoys continued to arrive from Korea throughout the next century, and by the time of the Empress Suiko (592-628 A.D.), Japanese envoys were being sent directly to China to study medicine. Towards the end of the Muromachi period (1333-1573 A.D.) the Japanese began to develop their own form of traditional oriental medicine, called kampo medicine. As traditional Chinese medicine was modified and integrated into kampo medicine, herbal medicine was markedly simplified.

In North America, early explorers traded knowledge with the Native American Indians. The tribes taught them which herbs to be used to sharpen their senses for hunting, to build endurance, and to bait their traps. In 1716, French explorer Lafitau found a species of ginseng, *Panax quinquefolius* L., growing in Iroquois territory in the New World. This American ginseng soon became an important item in world herb commerce [7]. The Jesuits dug up the plentiful American ginseng, sold it to the Chinese, and used the money to build schools and churches.

As medicine evolved in the United States, plants continued as a mainstay of country medicine. Approaches to plant healing passed from physician to physician, family to family. Even in America's recent past, most families used home herbal remedies to control small medical emergencies and to keep minor ailments from turning into chronic problems. During this period there was a partnership between home folk medicine and the family doctor [8]. Physicians often used plants and herbal preparations to treat common illness. Until the 1940s, textbooks of Pharmacognosy that characterize plants as proven-by-use prescription medicines contained hundreds of medically useful components on barks, roots, berries, leaves, resins, twigs, and flowers.

As 20th-century technology advanced and created a growing admiration for technology and technologists, simple plant-and-water remedies were gradually discarded. Today, many Americans have lost touch with their herbal heritage. Few Americans realize that many over-the-counter (OTC) and prescription drugs have their origins in medicinal

herbs. Cough drops that contain menthol, mint, horehound, or lemon are herbal preparations; chamomile and mint teas taken for digestion or stomach disorder are time-honored herbal remedies; and many simple but effective OTC ache-and pain-relieving preparations on every druggist's and grocer's shelf contain oils of camphor, menthol, or eucalyptus. Millions of Americans greet the morning with their favorite herbal stimulant--coffee.

Despite the importance of plant discoveries in the evolution of medicine, some regulatory bodies such as the U.S. Food and Drug Administration (FDA), the main U.S. regulatory agency for food and drugs consider herbal remedies to be worthless or potentially dangerous [9]. Indeed, today in the United States, herbal products can be marketed only as food supplements. If a manufacturer or distributor makes specific health claims about a herbal product (i.e., indicates on the label the ailment or ailments for which the product might be used) without FDA approval, the product can be pulled from store shelves.

Despite FDA's skepticism about herbal remedies, a growing number of Americans are again becoming interested in herbal preparations. This surge in interest is fueled by factors that include the following:

- ❖ Traditional European and North American herbs are sold in most U.S. health food stores. The same is true for Chinese and, to a lesser extent, Japanese herbal medicinals. Ayurvedic herbals are available in most large U.S. cities, as are culinary and medicinal herb shops called botanicals that sell herbs from Central and South America and Mexico. The reemergence of Native American Indian cultural influences has increased interest in Native American Indian herbal medicines.
- ❖ Pharmaceutical drugs are seen increasingly as over prescribed, expensive, even dangerous. Herbal remedies are seen as less expensive and less toxic.
- ❖ Exposure to exotic foreign foods prepared with non-European culinary herbs has led many Euroethnic Americans to examine and often consider using medicinal herbs that were brought to the United States along with ethnic culinary herbs.
- ❖ People increasingly are willing to "self-doctor" their medical needs by investigating and using herbs and herbal preparations. Many Americans--especially those with chronic

illnesses such as arthritis, diabetes, cancer, and AIDS are turning to herbs as adjuncts to other treatments.

India underwent a similar process in the development of its medicine. The healing that took place before India's Ayurvedic medical corpus was similar to that of ancient Egypt or China (i.e., sickness was viewed as a punishment from the gods for a particular sin). The earliest mention of the Rig Veda dates back to as early as 3500 BC. Ayurveda, considered as an Upaveda (or the supplementary Hymns designed for more detailed instruction of the mankind) has very strong foundations in the ancient medical science of India. World Health Organization estimates nearly 3200 million (80%) inhabitants of the World rely chiefly on traditional medicines for their primary healthcare needs, as a major part of traditional therapy involves the use of plant extracts or their active principles [5]. Materia medica of India provides lots of information on the folklore practices and traditional aspects of therapeutically important natural products. Indian traditional medicine is based on various systems including Ayurveda, Siddha, Unani and Yoga. It is generally believed that around one fourth of the active ingredients used in the modern medicines were derived from plant and/or plant products. Hence it will be worthwhile and beneficial to explore the plant kingdom for suitable and effective remedies and bring them into a framework of rational scientific use [10].

Ayurveda

Ayurveda is the science of life. Scattered references to health and diseases are quoted in the Vedas, especially in the Rig Veda and Atharva Veda got about 114 *hymns*, which describe the treatment of diseases, and Ayurveda has originated from those *hymns*. It is based on fundamental doctrines known as *darshanas*, which encompass physical, chemical, biological and spiritual sciences.

Ayurveda born out of intuition and revealation, developed in due course into eight well-defined specialized branches and two major schools.

- The school of physician (*Atreya sampradaya*)
- The school of surgeons (*Dhanvantari sampradaya*)

The basic theories of Ayurveda arose from the concepts of *panchamahabhutas* and *Trodosha*, which embrace the process of creation and evolution of the universe and all laws of life therein. According to Ayurveda the human body and all matter in the universe are composed of *panchamahabhutas*. Treatment of disease involves avoiding the causative factor, intake of suitable diets, adoption of appropriate activities and regimens, which would restore the balanced state of the body, or use of surgical procedures. So far as the function of the body concerned this system considers the body, mind and soul as complementary to one another. Thus Ayurveda is not merely medical science but is in fact a way of life [11].

Siddha

The Siddha system of medicine owes its origin to the Dravidian culture, which is of prevedic period. The ancient literature revealed that the Vedic Aryas owed allegiance to the cult of Shiva and worship of the phallus (*linga*), which was later, incorporated into the Vedic culture. The Shiva cult is associated with its medical counterpart, the Siddha system of medicine, which is mainly therapeutic. Mercury, sulfur, iron, copper, gold, bitumen, white, yellow and other minerals as well as vegetable poisons are extensively used in the Pharmacopoeia of the Siddha tradition. The Siddha system of medicine is prevalent in the southern states of India as well as in Sri Lanka, Malaysia and Singapore where the Dravidian civilization was dominant. The principles and doctrines of this system are both fundamental and applied and have a close similarity to Ayurveda [11].

Unani

Unani Tibb or Graeco-Arab medicine traced back to the system of Greek medicine, which was developed during Arab civilization. The basic framework of this system consists of the four humor theory of Hippocrates, which presupposes the presence of four humors; blood: phlegm, yellow bile and black bile. The basic philosophy of Tibb advocates that the body is composed of matter and spirit. These were taken, as a whole because harmonious life is possible only when there is a proper balance between bodily (physical) and spiritual (mental) function. Unani Tibb seeks the restoration of the body as a whole to its original state [12].

Yoga

Yoga is a traditional science based on techniques. It helps us to coordinate body and mind more effectively and enables a person to maintain tranquility of mind with greater calmness in the conscious state, and is perhaps the easiest and safest method to promote mental health. It can also be used as a preventive and curative technique for the management of various psychic and psychosomatic disorders. Although yoga had been described in the *Vedas* about 4000 years ago, it was presented by sage Patanjali in an abridged form about 2500 years ago [13].

His methods of integrated yoga are the most important and these are:

- *Yama* or improvement in our social behaviour
- *Niyama* or improvement in our personal behaviour
- Physical postures
- Breath holding practices
- Contemplation
- Meditation
- Attainment of super consciousness.

Regular practice of the integrated yoga can prevent the development of various psychosomatic disorders and also improve individual's resistance and ability to endure stressful situation more effectively [14,15]. Studies on normal individuals showed that a regular practice of yogic postures leads to psychological improvement in the intelligence and memory quotient and a decrease in the pulse rate, blood pressure, respiration and body weight. The integrated type of yoga has been used for the treatment of many stress disorders like hypertension, anxiety, neurosis, mucous colitis, bronchial asthma, diabetes mellitus, migraine and rheumatic disorders of the spine. In addition to the integrated practice of yoga, many other methods of yoga are available that could be used for the promotion of mental health, among them, the practice of *Kundalini* yoga [16] is the most important one.

1.2. Herbal medicines: a valuable therapy for different diseases [17, 18]

Herbal medicines remain the major source of health care for the world population. WHO has recognized herbal medicine as an essential building block for primary health care of vast countries like India and China. They are prepared from a variety of plant material like leaf, stem, root, bark and so on. They usually contain many biologically active ingredients and are used primarily for treating mild or chronic ailments. Herbs can be prepared at home in many ways, either fresh or dried ingredients. Herbal teas and infusions can be steeped to varying strengths. Roots, bark or other plant parts can be boiled into strong solutions called decoctions. Honey or sugar can be added to infusions and decoctions to make sweet. Herbal remedies can also be purchased in the form of pills, capsules or powders, or in more concentrated liquid forms called extracts and tinctures. They can be applied topically in form of creams or ointments, soaked into cloths and used as compresses, or applied directly to the skin as poultices. According to World Health Organization 80 percent of global population still depends on traditional or alternative medicine as the preferred form of healthcare, even with the spectacular advances in molecular biology, hospital medicine and physiological chemistry that have greatly enhanced our understanding and treatment of diseases.

Herb based therapies of traditional medical system is now recommended for the treatment of several degenerative and chronic diseases where modern pharmaceutical agents have proved inadequate. The acceptance of these techniques as standard healthcare options has posed serious conceptual problems in the development of public health programs that are responsible for the real needs of the population, with a tremendous impact in the cost of healthcare inventions, preventive medicine and self healing. The major characteristics of modern and traditional medicines are given in Table 1.1. Plants are considered to be medicinal if they possess pharmacological activities of possible therapeutic use. In spite of advances in modern system of medicine, there are various areas like tropical diseases, herpes, AIDS, cancer, and bronchial asthma etc., which will remain a formidable challenge to present day drug therapy. There is a possibility of finding a cure for them from the drugs of herbal origin. Almost half of the useful drugs today are derived from natural sources. Herbal medicines have yielded many useful compounds and plant

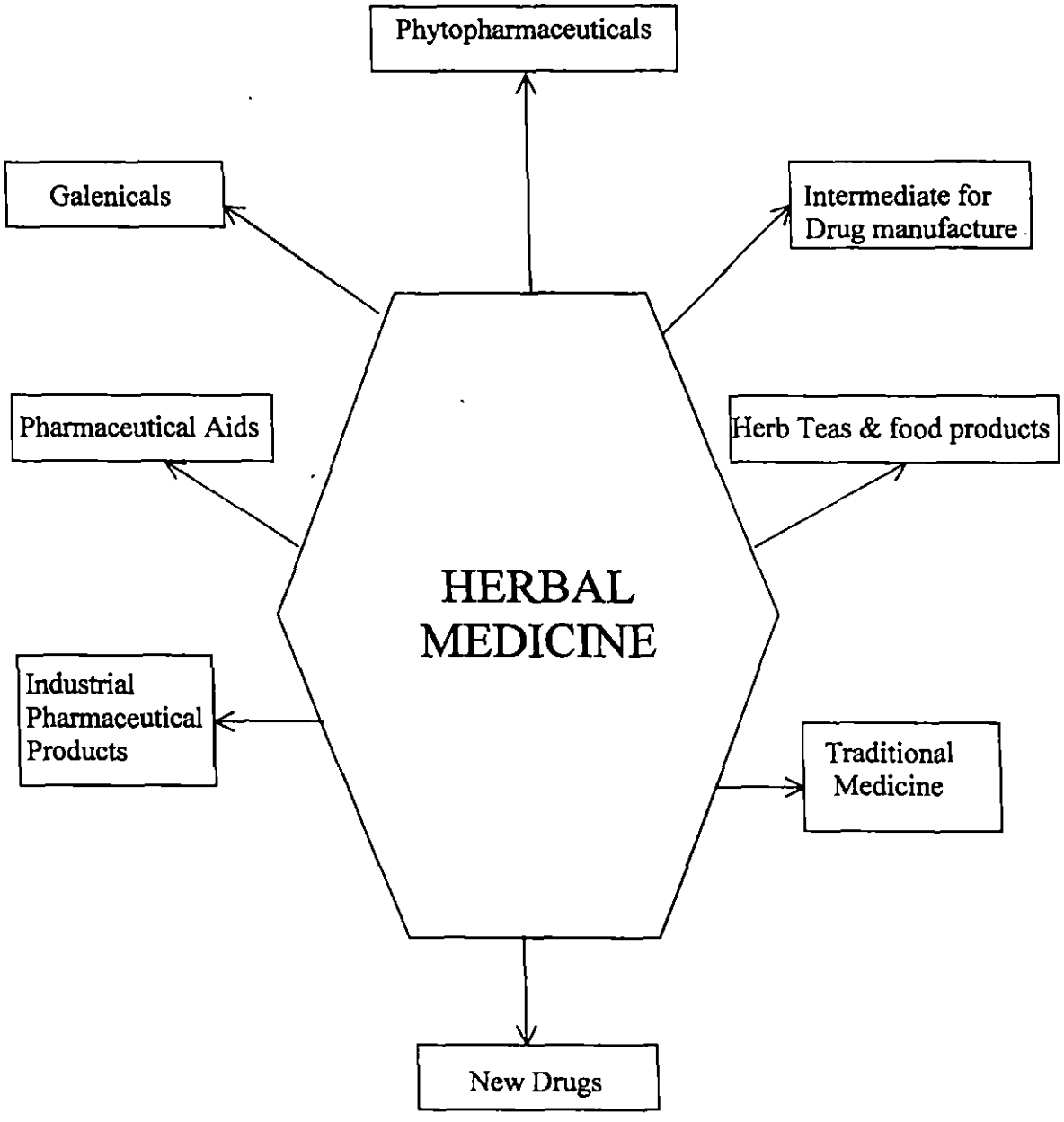

Figure 1.1 Role of Herbal Medicine.

derived ingredients, which are important components of modern phytopharmaceuticals. Today, the global market is floated with herbal preparations. A number of companies, including some multinational are entering into the area of herbal medicines. These medicines are available for each and every disorder including diabetes, ulcer, cancer, inflammation, hepatitis and asthma.

1.3. Drug discovery from natural products.

Natural products, including plants, animals and minerals have been the basis of treatment of human diseases. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists.

However, the basis of its development remains rooted in traditional medicine and therapies. The history of medicine includes many ludicrous therapies. Nevertheless, ancient wisdom has been the basis of modern medicine and will remain as important source of future medicine and therapeutics. The future of drug discovery from natural products will be more holistic, personalized and involve wide use of ancient and modern therapeutic skills in a complementary manner so that maximum benefits the patients and the community [17]. The approaches for drug development from natural sources generally consist of the steps as shown in Figure 1.2. The Greek physician Galen (AD 129–200) devised the first Pharmacopoeia describing the appearance, properties and use of many plants of his time. The foundations of the modern Pharmaceutical industry were laid when techniques were developed to produce synthetic replacements for many of the medicines that had been derived from the forest. Natural products chemistry actually began with the work of Serturner, who first isolated morphine from opium. This, in turn, was obtained from opium poppy (*Papaver somniferum*) by processes that have been used for over 5000 years. Many such similar developments followed. Quinine from cinchona tree had its origin in the royal households of the South American Incas. Before the first European explorers arrived, the native people of the America had developed complex medical systems replete with diagnosis and treatment of physical as well as mental illnesses.

Table 1.1 Salient features of modern and traditional medicines.

Modern medicine	Traditional medicine
• Universally accepted and globally spread	• Based on culture. Practice is regionalized without absolute uniformity.
• Training is intense standardized and confirmed by certification and registration	• Training varies; generally trained by a single mentor, confirmed with jewelry or dressings.
• Based on the disease	• Believes in holistic approach.
• Practice is institutionalized and developed through scientific studies	• Home serves as clinic or patients are called home for consultation and treatment
• Categorized on specialities i.e. doctor, nurse, physiotherapist etc.	• Healers may be called as specialist
• It is expensive and not easily available in remote areas	• Being culture based, healers are accessible and affordable.
• Treatment provided by doctors to physically and mentally illed patients.	• Traditional healers are highly respected, relatively old people and are vary renowned in primary health care system.

Indigenous people derived medicines and poisons from thousands of plants. A review of some plants that originated from Central and South America indicates that most of them either had potentially toxic characters or components of food sources. The following are few examples [18]: In the early 1500s, Indian fever bark was one of the first medicinal plants to find appreciative consumers in Europe. Taken from the cinchona tree (*Cinchona officinalis*), the bark was used as an infusion by native people of the Andes and Amazon highlands to treat fevers. Jesuit missionaries brought the bark back to Europe. By the early sixteenth century, this medicine was known as 'Jesuit fever bark', quite a transformation. The name coca (*Erythroxylum coca*) comes from an Aymara word meaning 'tree'. In Andean cultures, the leaves of the coca tree have been primarily chewed to obtain perceived benefits. From ancient times, indigenous people have added alkaline materials such as crushed seashells or burnt plant ashes to the leaves in order to accentuate the pharmacologically active moiety of coca. In 1860, a German chemist Carl Koler isolated cocaine, the chemical responsible for the biological activity. He found that cocaine could act as a local anesthetic in eye surgery. As the years passed, scientists observed that cocaine paralyzed nerve endings responsible for transmitting pain. As a local anesthetic, it revolutionized several surgical and dental procedures. Pot curare arrowhead poison used in East Amazon is predominately obtained from the species *Strychnos guianensis*. Tube curare in the West Amazon is from *Chrondrodendron omentosum*; curare in modern medicine is made from this species and named as tubocurarine. The jaborandi tree (*Pilocarpus jaborandi*) secretes alkaloid rich oil. Several substances are extracted from this aromatic oil, including the alkaloid pilocarpine, a weapon against the eye disease, glaucoma. American Indians on the island of Guadeloupe used pineapple (*Ananas comosos*) poultices to reduce inflammation in wounds and other skin injuries, to aid digestion and to cure stomach ache. In 1891, an enzyme that broke down proteins (bromelain) was isolated from the fresh juice of pineapple and was found to break down blood clots. Other pharmaceuticals that have their origin in botanicals include atropine, hyoscine, digoxin, colchicine and emetine. Reserpine, an anti-hypertensive alkaloid (*Rauwolfia serpentina*) became available as a result of work carried out by Ciba-Geigy in India. It is pertinent to note that most of these early discoveries are mainly based on traditional medicines; many products could act as poisons in toxic doses.

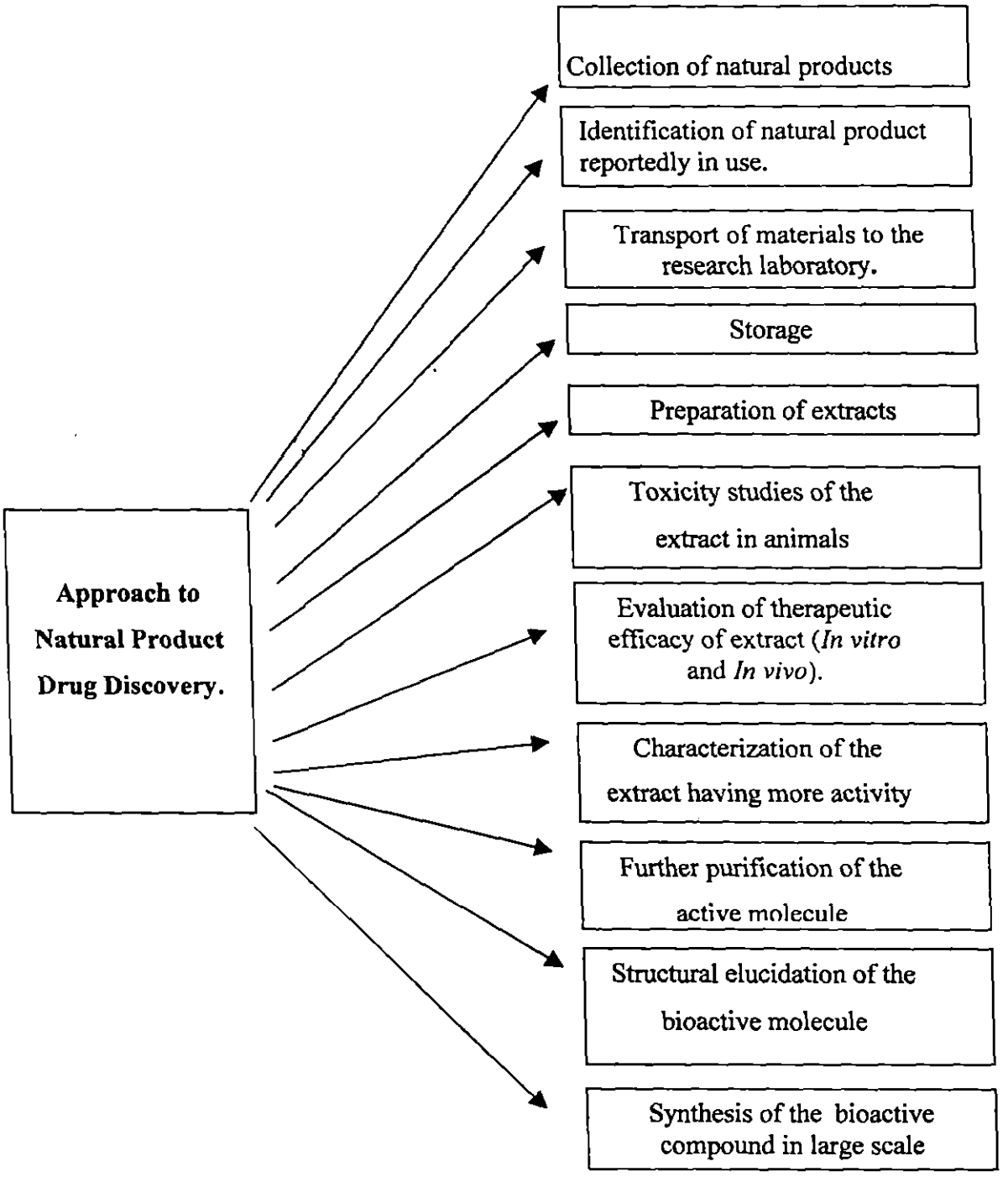

Figure 1.2. Approach for the Drug Development from Natural sources.

1.3.1. Discovering medicines or poisons?

A major problem with traditional, indigenous medicines is discovering a reliable 'living tradition' rather than relying upon second-hand accounts of their value and use. In many parts of the world the indigenous systems of medicine have almost completely broken down and disappeared. This includes mostly developed countries and some developing countries where the indigenous population has been marginalized. In others, the system is fragmented with the use of indigenous materials being limited to small tribal and geographical areas, as in many parts of Africa. In anthropological terms these are 'little traditions', while the Ayurvedic Indian and traditional Chinese systems are living 'great traditions'. Although the little traditions are an excellent repository of knowledge about medicinal and poisonous properties of botanicals, researchers have mainly exploited poisonous sources. This may be primarily because of many reasons. First, it is relatively easy to present and demonstrate poisonous characteristics of botanicals. Second, there may not be a written documentation and poisonous characters get predominance by word of mouth. Third, for an outsider, poisonous characteristics differentiate between ordinary and extraordinary material for pharmaceutical development. Fourth, a considerable time period is required to demonstrate true medicinal activities with proven safety profile. Great traditions have relatively organized database, and more exhaustive description of botanical material is available that can be tested using modern scientific methods. Ayurveda and Chinese medical systems thus have an important role in bio-prospecting of new medicines [17].

1.3.2. Serendipity and synthetic dominance

Pharmaceutical research took a major leap when alongside natural products chemistry, pharmacologists, microbiologists and biochemists began to unravel the chemistry of natural processes in human, animals, plants and microorganisms. Advances in synthetic organic chemistry led to the identification of many key chemical molecules that offered more opportunities to develop novel compounds. Many new drugs emerged by this route, particularly those now being used to treat infections, infestations, cancers, ulcers, heart and blood pressure conditions. Many drugs were developed through random screening of thousands of chemicals synthesized as dye-stuffs and the like; many others resulted from

serendipity (happy chance) arising from sharp-eyed observations of physicians and scientists. Examples of such drugs include sulfonamides, isoniazid, anti-psychotics, anti-histamines and penicillin. Emergence of the modern pharmaceutical industry is an outcome of all these different activities that developed potent single molecules with highly selective activity for a wide variety of ailments. The drugs produced in many cases improved with nature, viz. a new range of local anaesthetics from cocaine avoided its dangerous effects on blood pressure; chloroquine is much less toxic than quinine. These successes and many more like them resulted in reduced interest in natural products drug discovery and many major drug companies almost neglected such divisions. Work on developing new drugs for the treatment of the world's major diseases, malaria, trypanosomiasis, filariasis, tuberculosis, schistosomiasis, leshmaniasis and amoebiasis came almost to a standstill. In addition, although botanical medications continued to be produced in every country, the clinical efficacy of these was usually not evaluated and the composition of these complex mixtures was only crudely analysed. Thus, herbal medicines became the domain of 'old wives' tales' and quack medicine, exploitation of the sick, the desperate and the gullible. Sadly, herbal medicines continued to reflect poor quality control both for materials and clinical efficacy [17, 18].

1.3.3. Back to traditional wisdom

Lag phase for botanical medicines is now rapidly changing for a number of reasons. Problems with drug-resistant microorganisms, side effects of modern drugs, and emerging diseases where no medicines are available, have stimulated renewed interest in plants as a significant source of new medicines. Pharmaceutical scientists are experiencing difficulty in identifying new lead structures, templates and scaffolds in the finite world of chemical diversity. A number of synthetic drugs have adverse and unacceptable side effects. There have been impressive successes with botanical medicines, most notably quinghaosu, artemisinin from Chinese medicine. Considerable research on pharmacognosy, chemistry, pharmacology and clinical therapeutics has been carried out on Ayurvedic medicinal plants [19]. Numerous molecules have come out of Ayurvedic experiential base, examples include rauwolfia alkaloids for hypertension, psoralens in vitiligo, holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic

agents, mucuna pruriens for Parkinson's disease, piperidines as bioavailability enhancers, baccosides in mental retention, picrosides in hepatic protection, phyllanthins as antivirals, curcumine in inflammation, withanolides, and many other steroidal lactones and glycosides as immunomodulators [20]. A whole range of chronic and difficult-to-treat diseases such as cancers, cardiovascular disease, diabetes, rheumatism and AIDS, all require new effective drugs. Most developing countries have relied and will continue to rely on traditional natural medicines due to the deterrence of high costs of modern allopathic medicines. Current estimates indicate that about 80% of people in developing countries still rely on traditional medicine based largely on various species of plants and animals for their primary healthcare. The uses of alternative medicine therapy in US have been increased by almost 50% from 1990 to 1997 [21].

1.3.4. Botanical medicine: research, development and markets

Thirty per cent of the worldwide sales of drugs are based on natural products. Though recombinant proteins and peptides account for increasing sales rates, the superiority of low-molecular mass compounds in human disease therapy remains undisputed mainly due to more favorable compliance and bioavailability properties. Approaches to improve and accelerate the joint drug discovery and development process are expected to take place mainly from innovation in drug target elucidation and lead structure discovery. Therefore, Grabley and Thiericke [21] have correctly emphasized the need for new concepts to generate collection of large compounds with improved structural diversity.

There are number of problems connected with the search for new prototype drugs of biological origin. Investigations of plants used in traditional and modern medicine in China serve as a source of inspiration and as models for the synthesis of new drugs with better therapeutic, chemical or physical properties than the original compounds [22]. The World Health Organization also has recognized the importance of traditional medicine and has been active in creating strategies, guidelines and standards for botanical medicines.

Commercially, these plant-derived medicines are worth about US$ 14 billion a year in the United States and US$ 40 billion worldwide. Americans paid an estimated US$ 21.2 billion for services provided by alternative medicine practitioners [23]. A 1997 survey estimated that over 12% of adults had used herbal medicine during 1996 compared with 2.5% in 1990, resulting in a increase in business of US$ 5.1 billion [24]. Lilly Research Laboratories markets several million dollars worth of vincristine and vinblastine – the periwinkle derivatives used to treat childhood leukaemia and Hodgkin's disease. The US National Cancer Institute regularly earmarks large appropriations to screen 50,000 natural substances for activity against cancer cell lines and the AIDS virus. China, Germany, India and Japan, among others, are also screening wild species for new drugs.

Proven agro-industrial technologies need to be applied to the cultivation and processing of medicinal plants and the manufacture of herbal medicines [25]. The mass screening of plants in the search for new drugs is vastly expensive and inefficient. It would be cheaper and perhaps more productive to re-examine plant remedies described in ancient and medieval texts [26]. Many higher plants produce economically important organic compounds such as oils, resins, tannins, natural rubber, gums, waxes, dyes, flavours, fragrances, pharmaceuticals and pesticides. Advances in biotechnology, particularly methods for culturing plant cells and tissues, should provide new means for the commercial processing of even rare plants and the chemicals that they produce. These new technologies will extend and enhance the usefulness of plants as renewable resources of valuable chemicals. In future, biologically active, plant-derived chemicals can be expected to play an increasingly significant role in the commercial development of new products for regulating plant growth and for insect and weed control [27].

Natural products research continues to explore a variety of lead structures, which may be used as templates for the development of new drugs by the pharmaceutical industries. While microbial products have been the mainstay of industrial natural products discovery, in recent years phytochemistry has again become a field of active interest. Drug discovery programmes based on microbial products and phytochemicals have been discussed and contrasted [28]. Glaxo PLC, embarked a programme wherein extracts and

fermentation broths were screened in order to detect bioactive principles [29]. Many other multinationals and academic institutions have created joint programmes for plant medicine research, for example, Virginia Polytechnic Institute, Bedrijf Geneesmiddelen Voorziening Suriname, Conservation International–Suriname, and Bristol–Myers Squibb Pharmaceutical Research Institute. Several such projects were sponsored by the Federal Agencies of USA. University of Chicago at Illinois, University of Mississippi, Xeenova, Ayur-Core, Inc. and Bio-Ved Pharmaceuticals represent additional examples. Indian pharmaceutical companies have launched new projects: Dabur, Zandu, Arya Vaidya, Nicholas Piramal, Lupin and Ranbaxy are few prominent examples. The Pharmaceutical Research and Development Committee (PRDC), under the Ministry of Chemicals, Government of India also underlines the importance of traditional medicines [30].

Opportunities for multidisciplinary research that joins the forces of natural products chemistry, molecular and cellular biology, synthetic and analytical chemistry, biochemistry and pharmacology to exploit the vast diversity of chemical structures and biological activities of natural products are best discussed by Clark [31].

The exploration of structural chemical databases comprising a wide variety of chemotypes, in conjunction with databases on target genes and proteins, will facilitate the creation of new chemical entities through computational molecular modeling for pharmacological evaluation [32, 33]. In natural drug discovery it is important to follow systems-theory and systems-biology applications to facilitate the processes [34]. Routine random efforts are not likely to increase the desired success rate of discovery, while experience indicates that a modified collection policy offered better chances for the discovery and development of agents for treatment of AIDS and cancer [35, 36]. Numerous drugs have entered the International Pharmacopoeia through ethnobotany and traditional medicines [37, 38]. There are many similarities in traditional systems of medicine as well as ethnomedicines being connected to each other as 'great traditions and little traditions'. All botanical drugs will have to fulfill the international requirements on quality, safety and efficacy [39].

1.3.5. Drug discovery: intentional not coincidental

In the sequence of their appearance, the scientific disciplines involved in drug discovery were chemistry, pharmacology, physiology, microbiology, biochemistry and molecular biology. It can be shown that new therapeutic classes of drugs like muscle relaxants, diuretics, antibiotics, recombinant proteins, monoclonal antibodies and others were generated on the basis of scientific opportunities rather than therapeutic need. All these drugs were created within the confines of a chemical paradigm of medicine and drug therapy. We are now witnessing the entry of a new informational paradigm into medicine that is most prominently represented by genomic sciences. This paradigm will bring two important changes in the therapy of diseases. First, molecular biology has matured to such a degree that it can now study complex genomes and their functionality in complex organisms such as humans. Therefore, results from these studies no longer have to be translated into the context of medicine: they are already within this context. Secondly, drug therapy that used to be largely symptomatic, will now aim at targets that are closer to the causes of diseases. Therapeutic progress, which used to be indirect, conjectural and coincidental, is about to become more directed, definitive and intentional. The future drug discovery will be more often based on intent rather than coincidence. Proper bioprospecting of medicinal sources will be an important factor [40].

1.4. Ethnopharmacology in drug evaluation

Interest in traditional drugs is thus not new but has been spurred in recent years by methodological advances in phytochemistry, a growing number of ethnobotanical studies, and a upsurge of interest in renewable resources in traditional medicine.

The observation, identification, description and experimental investigation of the ingredients and the effects of indigenous drugs are a truly interdisciplinary field of research. The term Ethnopharmacology has been used loosely to describe this field [44]. Ethnopharmacologic research is based on botany, pharmacology and chemistry, but other disciplines have made vital contributions. Based on these considerations, Ethnopharmacology is defined as " the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man" [45]. This study of

traditional drugs is not meant to advocate a return to the use of these remedies in their aboriginal form, or to exploit traditional medicines. The objectives of ethnopharmacology are to rescue and document an important cultural heritage before it is lost, and to investigate and evaluate the agents employed. Thus, it plays an immense role in evaluation of natural products and more particularly the herbal drugs from traditional and folklore resources.

1.5. Phytochemical analysis of ethnomedicine

Phytochemical studies should be tailored to match the biological activity. The chemical studies should provide information that will be used in the standardization and quality control of the finished product. Even when the product is used as whole herbs, it is imperative that the chemistry of the plant material be thoroughly studied so that storage conditions, stability and ingredient integrity can be determined. For example, it will be important to determine the stability if active constituent in a crude drug is a glycoside, which will likely hydrolyze when stored with high moisture content. Isolation of phytoconstituents using Coloumn chromatography monitor by Thin layer chromatography and finally confirmation of structure by using various spectral methods like IR spectroscopy, ^{1}HNMR Spectroscopy, ^{13}CNMR Spectroscopy and Mass Spectroscopy are involved in phytochemical investigation [41].

1.6. Toxicity studies of ethnomedicine

Herbal products used in traditional medicines are generally non-toxic due to dilution with inert herbal ingredients. The traditional system of medicines advocates a liquid dosage form, which encourages the use of extremely low concentration of active ingredients in the finished products. The problem of toxicity of synthetic drugs arises mainly due to modification of the original structure to increase the biological activity. In the traditional formulations, therapeutically active substances in plants exist in conjunction, and bound with other substances like tannins, carbohydrates, amino acids, proteins, vitamins, trace metals, etc. and consumed as decoctions. Moreover major part of the formulations remain as food materials and consumed by the human body along with the therapeutically active substances. When used in the right form, these herbs do not upset or cause toxicity in the

body, rather they maintain the physiological balance of the body. This explains why herbal extracts produce little or no toxicity, even when large amount is consumed. Over processing food is not beneficial in promotion of condition of health.

Among so many co-existed compounds in the plants one may exist in the state of highly reactive. It has been suggested that the reactivity of the single compound with the physiological mediators of the human systems leads to the manifestation of the phenomenon described as drug toxicity. Phytomedicines, with a little amount of processing, can be considered as health foods. They promote the health since they contain, apart from the active drug molecule, other substances required to maintain the overall physiological functions of the body. This is why "bitter leaf" (*Vernonia amygdalina*) can be used as a food and as a drug in the treatment of diabetes, without any manifestation of toxicity. However, when purified extracts or isolated compounds are used as phytomedicines, they should be subjected to same rigorous testing procedures used to standardize other medicinal agents.

1.7. Pharmacological evaluation of ethnomedicine

The single major factor, on which the decision to select one or more medicinal plant candidates for development into phytomedicines on commercial scale is the wealth of ethnomedicinal information on its efficacious use in the long history of traditional medicines. The scientific literature is now replete with reproducible experimental results on the pharmacological properties of herbal extracts. These results were obtained using the same laboratory animal models and techniques that have been accepted scientifically for testing the pharmacological activity of the pharmaceutical products [42,43]. Modern chemical methods have led to a dramatic increase in the number of natural or synthetic molecules available for pharmaceutical research. At the same time, recent developments in cellular and molecular pharmacology provide an increasing number of selective tests able to identify the activity and the mechanisms of action of biologically active molecules. Paradoxically, however, the availability of numerous sophisticated techniques makes the pharmacological research much easier. A major problem encountered in the pharmacological evaluation of plant drugs is that of solubility. The active constituents

occur naturally as soluble salts or organic complexes with solubility enhancing matrices, but the extraction process sometimes leads to the dissociation of the organic compounds from the water-soluble components with the resultant production of insoluble extractives. Problem arises from attempts to resolubilize the material in aqueous media for pharmacological evaluation, and the use of organic solvents is often precluded because of probable interference with bioassay methods.

1.8. Clinical evaluation of ethnomedicine

Prior to detailed laboratory studies of any plant used in traditional medicine, it is important to establish whether the remedy does in fact posses the claimed therapeutic properties. It is important to emphasize the fact that a given plant may not show pharmacological activity in laboratory animals but it does not necessarily mean that the plant devoid of therapeutic application. A carefully designed clinical evaluation will help to determine if the remedy is active within measurable parameters. Such studies are not the same with a clinical trial of a new drug entity. This form of evaluation merely evaluates the traditional use of the drug in a clinical setting. The usual ethical considerations are followed, and the dosage form is standardized in terms of posology. It is not supposed to address any of the issues meant for a controlled clinical study. It is neither randomized nor double blinded. Good reliable results can obtain for most diseases with a small study population of 8 to12. It should, however, provide clear go/no go decision points [45]. Conducting a clinical evaluation at the beginning of the study will give the candidate drugs a better chance of making it as a reliable indicator of substances that can be used in the production of effective and safe phytomedicines and natural personal care products.

Perhaps clinical evaluation of traditional medicine is even more useful not as a tool for pharmaceutical drug discovery but as a method to provide a better understanding of the spectrum of activity or limitations of ethnomedical remedies and practices. In this case establishment of clear and unambiguous parameters for measuring expected outcomes is required. Three fundamental features determine the scientific rigor and validity of the experimental approach to assessing herbal efficacy: Randomization is the hallmark of the randomized controlled trial (RCT), ensures that the various treatment groups are indeed

comparable and that the only significant difference is the treatment they receive. Blindness minimizes bias by both patient and investigator, and could be considered crucial in a system of medicine that is relatively more subjective than modern medicine. The third leg of the RCT tripod is the measurement of predetermined outcomes that should complete, appropriate, and accurate. As has been noted by Keravitz, measurement of predetermined outcomes is a property that is recommended not only for RCT but also for good observational studies used in the evaluation of herbal medicines. The overriding consideration in determining the outcomes should be the interest of the patient, since ultimately the objective is to improve their health status. Both generic and disease specific outcomes are now combined in the determination of the usefulness of therapeutic interventions in complex clinical situations. In certain situations, measurement of various aspects of functional status and well-being and other quality of life assessments are considered more relevant to the patient than disease-specific outcome measurements [46]. It has been observed that although RCT is a valuable method for the evaluation of ethnomedical remedies and the so-called complementary and alternative medicines, the method may have some important drawbacks that could limit its application in ethnomedical evaluation [47].

Some of the problems with the RCT method include generalizability of the results produced because the study populations are often limited to a fairly narrow spectrum of patients that may not be representative of either the population or the situation in actual practice in order to enhance internal validity and to keep sample sizes reasonable [47]. The results obtained from RCT's are also rendered less relevant to real life situations by the mere fact that the experiments are usually conducted by experienced clinical investigators, and the careful monitoring of the patients in a clinical trial setting may produce results that are better than real situations. In order to overcome the limitation of the RCT approach for clinical evaluation of ethnomedical remedies, alternative approaches have been suggested that attempt to narrow the difference between efficacy and effectiveness.

These alternative approaches include:

• *Quasi-experimentals*: Patients are assigned to treatment condition not as individuals but as members of a group. This method dispenses with randomization and blindness but solves the problem of selection bias that is common with evaluation of traditional medicines.

• *Regression-discontinuity*: The designed is not randomized but provides for full experimental control without randomization. It is based on the assumption that there are predictable relationships between pre-test and post-test scores for all subjects. By using the same patients as controls, it is possible to determine the effect of a given therapy by assessing the observed difference between the regression linear obtained by plotting the pre-test scores and the post treatment effects.

• *Cohort studies*: This involves following a group of patients in time. Both prospective (forward in time) and retrospective cohort studies are valid approaches to assess the effect of a given therapy.

• *N=1 trials*: These are conducted with just one individual patient, with all the rigor and a true experimental approach. The experiment can be blinded, and definitive decisions can be made regarding the efficacy of a given treatment on the individual patient.

• *Case control studies*: patients are selected based on the presence or absence of the diseases or outcome of interest t time t=0. Assessment is then made on the exposure status of both cases at an earlier time (t=1). This time is particularly useful in epidemiological research.

1.9. New drug molecules from nature

Throughout the ages humans are relied on nature for their basic needs, be it for food, shelter or medicine. Plants formed the basis of sophisticated traditional system that has been in existence for thousands of years in countries such as India and China.

The use of plants in the traditional medicinal system of many other cultures has been extensively documented. These plant-based systems continue to play an essential role in health care, and as discussed earlier, it has been estimated by the World Health Organization that approximately 80 percent of the world's inhabitants rely mainly on traditional medicines for the primary health care. Plant products also play an important role in the healthcare systems of the remaining 20 percent of the population, mainly residing in the developed countries. Table 2 represents the model lists of medicinal plants that were recommended by WHO for different therapeutic activity.

Today, pharmaceutical companies have to spend millions of dollars to develop a new drug molecule. Currently, approaches to improve and accelerate the joint drug discovery and development process are expected to arise mainly from innovation in drug target elucidation and lead finding. Therefore, new concepts to generate compound libraries with improved structural diversity are desirable and it can be provided by natural products due to wide chemical diversity. It is necessary to discover and develop new drugs in such a way as to maximize the return on the investment. Natural products can stand as a good alternative in finding drug molecules at low cost and in time frame with newer developments in the field.

A recent survey has found that 477 out of 868 drug molecules discovered from various sources during the period of 1981 to 2002 were natural products or derived from the natural products i.e. almost 55 percent of the drugs discovered till date [48]. In 2000 alone, seven out of twenty best-selling non-protein drugs were either derived from natural products, or developed as a result of lead compounds generated from natural product sources. These included several statins, the largest selling class of drugs, enalaprils and augmentin, with combined annual sales in excess of US $ 20 billion. Although natural products constitute only one percent of all published chemical structures, yet natural products and substances derived naturally account for about 35 percent of the total pharmaceutical market. The key advantage of natural products over synthetic compounds is their greater chemical diversity. Although, it has been estimated that less than 10 percent of biodiversity has been tested for biological activity, a comparison of the

structure of compounds in published data base revealed that 40 percent of the chemical scaffolds found in natural products are absent in synthetic chemistry.

A number of natural products and natural product-derived compounds are in the market as drug molecules in various therapeutic categories [48]. The recent examples are Ertapenam (Antibacterial, 2002), Micafungin (Antifungal, 2002), Abacavir (Antiviral, 1999), Frovatriptan (Antimigraine, 2002), Zofenapril (Antihypertensive, 2000), Arglabin (Anticancer, 1999), Gefitinib (Anticancer, 2002) and many more. So, there is an urgent need to identify novel, active chemotypes as lead compounds for effective drug development in many therapeutic areas. Drug discovery from nature was de-emphasised at a time when there has been rapid progress in technology that could have enhanced the process of drug discovery. There is a need to re-emphasise and enhance research in natural products, because only a small fraction of plant species has been investigated so far.

1.10. Scope and aim of phytomedicine in the near future

The scope and aim of the phytomedicine in near future will be its extensive research, regardless of whether phytopharmaceuticals are classified as conventional or traditional drugs. As we enter into a new millennium, it may be urged that interest in herbal medicines and natural products in general is at an all-time high. The last decade has witnessed a greater use of botanical products among members of the general public through self-medication than never before. This phenomenon has been mirrored by an increasing attention to phytomedicines as form of alterative therapy by the health professions inclusive of pharmacy and medicine. The search for new pharmacologically active compounds for drug development is an important issue but not the only one, as the trend toward using standardized plant extracts of high quality, safety, and efficacy will continue. Therefore, all efforts have to be targeted to reveal the chemical-pharmacological profiles of extracts and fixed combinations and to rationalize their therapeutic applications. Whether in the future the highly active, safe, and causally acting phyto-preparations will be able to replace some synthetic drugs, or in other cases are potent enough to be applied in combination with synthetic drugs, depends on the level of

evidence-based therapeutic efficacy achieved. In this context, it will also be necessary to find scientific explanations and rationales for the fact that many phyto-preparations, usually applied in low doses relative to the amount of defined bioactive compounds contained in an extract preparation, exhibit no immediate pharmacological or therapeutic effect and achieve their optimal efficacy only after long-term (three to four weeks) treatment. The greatest challenge for phytomedicine research, however, will be the shift of paradigms, which is occurring in chemotherapy. This change can be described as a withdrawal from monosubstance therapy and a transition to treatment of patients with drug combinations consisting of two, three, or more single drugs. This multi-chemotherapy has been introduced, for example, in the treatment of AIDS, hypertension, and many other diseases. The second paradigm shift can be defined as a change in the strategy of medication, characterizable as a multitarget therapy. Taking tumor therapy as an example, this new strategy aims to destroy tumor cells not via direct interaction of the drug with the tumor cell cycle but via various other mechanisms, which do not damage healthy cells. This medication could be directed, e.g., to induce apoptosis of tumor cells, to inhibit angiogenesis, to stimulate specific and nonspecific immune defense mechanisms, to induce the expression of antioncogenes, and to activate the production of cell-protecting proteins (heat shock proteins). This new, very ambitious therapeutic strategy is still in its infancy, but it is a challenge for phytomedicine research because the attempt to treat diseases according to this strategy is an actually old phytotherapeutic concept.

Table 1.2. Model list of medicinal plants recommended by WHO

Gastro intestinal tract remedies

Anti-diarrheals

Acacia arabica
Acacia catechu
Berberis aristata
Commiphora mukul
Punica granatum

Laxatives

Aloe ferox
Cassia acutifolia
Chicorium intybus
Glycyrrhiza glabra
Plantago ovata, P.psyllium
Rhamnus frangula
Ricinus communis

Carminatives

Cinnamomum zeylanicum
Elettaria cardamomum
Matricaria chamomilla
Origauni spp.
Tyhmus vulgare, Ocimum sanctum,
Umbelliferous fruits. *Pimpinella anisum*
Zingiber officinalis

Spasmolytics

Atropa belladona
Datura spp.
Hyoscyamus spp.
Solenostemma spp.

Stomachics

Rheum officinalis

Anti-emetics

Atropa belladona
Hyoscyamus spp.
Mentha spp.
Zingiber officinalis
Santalum album

Anti-helmintics

Alziba anthelmintics
Artemesia cina

Analgesics and anti-inflammatory agents

Lactuca sativa
Matricaria chamomilla
Peganun harmala

Remedies for arthritic condition

Capsicum minimum, C.annum
Commifora mukul
Withania somnifera

Remedies for eye disease

Berberis aristata
Rosa damascena

Treatment for snakebites and scorpion and insect stings

Aloe spp.
Azadirachta indica
Heliotropium strigosum

Anti-allergics

Cydonia oblonga

Remedies for upper respiratory diseases

Adhatoda vasica
Allium cepa
Althaea officinalis
Ammi visnaga
Cassia fistula
Hibiscus sabdarriffa
Linum usitatissimum
Mentha spp.
Nigella sativum
Ocimum sanctum
Prunus domestica
Psidium guajuava
Tilla sometosa, T.ulmifolia

Plants reported to induce aphrodisiac effect

Aleurites moluccana
Costus speciosus
Celba pentandra
Artocarbus heterophyllus
Piper betle
Pandanus amarylifolius
Lamulus symphomonia
Areca catechu
Erythroxylum coca
Magnolifa fuscata
Ipomeoea mauritania
Semecarpus anacardium
Asparagus racemosus
Mucuna prurita
Boerhaavia diffusa
Maranta arundinacea
Canna edulis
Butea superba
Dioscorea triphylla
Piper longum

Remedies for skin diseases

Aloe vera, A. barbadenses, A. ferox
Ammi majus
Azadirachta indica
Ficus carica
Fumaria officinalis, Lasoria alba
Lupinus termis
Martricaria chamomilla
Nymphaea alba
Urginea maritima
Zingiber officinalis

Remedies for urinary infection

Ammi visnaga
Balanites aegyptiaca
Cucumis sativus
Cymbopogon prodmus
Nymphaea alba
Raphanus sativus

Treatment for burn, scals, wounds, abscesses and swellings

Aloe vera, A.barbadense, A. ferox
Lawsonia alba
Linum usitatissimum

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CHAPTER 2

REVIEW OF LITERATURE

2.1. *Colebrookea* species.

Colebrookea belongs to family Labiatae (sub- Lamiaceae), which comprises of 100 genera and about 3000 species having considerable economic and medicinal importance. Most of the species found in India are distributed in the hill regions. The family yields many useful steroids, flavonoids and glycosides [1]. Many members of the family are used as culinary or medicinal herbs, as sources of volatile oils and in some cases for the preparation of constituents of the volatile oils such as menthol and thymol. In addition to the volatile oils, constituents of the family include diterpenoids and triterpenoids, saponins, a few pyridine and pyrollidine alkaloids, insect moulting hormones, polyphenols, tannins, coumarins and furanoids. Some of the flavonoid present in the species has been demonstrated to have CNS stimulant activity [2].

2.1.1 *Colebrookea oppositifolia* Smith [3].

The synonyms of the plant are:

Beng. – *Pansara*; Hindi – *Binda, pansara*; Kan. – *Falia, tuggigidda*; Mar. – *Bhamini, dusarika jhar*; Oriya – *Bosiki, darigopi*; Tel. – *Jolidi*, *Nep.* – Dhusure, Lepcha – Kumfyjemkung.

2.1.1.1. Distribution

The plant is found to be distributed throughout India in the hilly regions, at elevation ranging from 3000 to 5000 ft, especially in valleys and ravines, often gregarious near watercourses [3].

2.1.1.2. Features of different parts of the plant

Colebrookea oppositifolia is a densely tomentose, hoary, much branched shrub or small tree. The plant is 1.2 to 3.6 m tall, bearing oblong-elliptic leaves with smooth grey bark. Trunk stout, stems irregularly indented, bark corky, and leaves 10 – 20 cm × 3.8 – 5.0 cm. Flowers small, numerous, white, red or pale chocolate, in crowded spikes; fruits 1.2 cm, nuts, usually one, hairy [3]. The leaves are used as fodder. The wood is grayish white, moderately hard (736-738 kg/m^3) and closed grained. It is converted into charcoal for gunpowder. (Fig. 2.1)

Figure 2.1. Photograph showing the twig of *Colebrookea oppositifolia* Smith obtained from the Rangpo region of Sikkim.

2.1.1.3. Pharmaceutical and Phytochemical aspects

In folk medicine, the leaves as well as root of *Colebrookea oppositifolia* is being used to treat various ailments. Ethnobotanical studies indicate that the decoctions of *Colebrookea oppositifolia* leaves and stem bark are widely used among the tribal populations of Sikkim to treat skin infections [2], indigestion [2], diarrhoea [5], wounds and cuts [4]. Alcoholic extracts of leaves is reported to be useful in asthma [6], epilepsy [7] and helminthes [8]. It is administered either as decoction, infusion or tincture. The leaves pastes are applied to wounds and bruises, and are also used in eye diseases. The roots are used in epilepsy by the tribals of western Himalaya. The defatted alcoholic extract of the roots showed CNS excitation with increased rate of respiration and induced motor incoordination in mice [3]. Leaf decoction is also used as aphrodisiac and relief from tension (personal experience of the authors).

Phytochemical analysis of *C.oppositifolia* revealed the presence of number of flavonoids: Baicalein, tri-O-methyl [9], Chrysin [10], Colebrookia flavonoid [11], 2'–5–7– trihydroxy flavone [10], 2–hydroxy –2– 3 –7–8– tetramethoxy flavonol [12], 4'–5–6–7– tetramethoxy flavone [13], Ladanein [10], Negletin [10], Quercetin [14] and Scutellarein [9]. The bark also contains steroid, lipid, Triacontane and triacontene [8]. The alkanol and alkene (C_5 or more) isolated from plant showed remarkable Anthelminetic avtivity. Methanol: water (1:1) extract of dried aerial part of *C.oppositifolia* showed significant cytotoxic and antitumor activity [15].

2.2. *Heracleum Species* [2]

The Genus belongs to the family Umbelliferae (sub Apiaceae). The family contains about 275 genera and 2850 species, out of which *Heracleum* contains 70 species. The constituents of the family, other than volatile oil and resins, include coumarins, furocoumarins, chromonocoumarins, flavonoids, terpenes and sesquiterpenes, triterponoid saponins and acetylenic compounds. Alkaloids remain as rare constituent in the species.

2.2.1. *Heracleum nepalense* D. Don. DC [2]

The synonyms of the plant are: Nepali – *Chimphing*, Lepcha – *Sanben*.

Figure 2.2. Photograph showing the plant *Heracleum nepalense* D.Don. DC obtained from Pstangu region of Sikkim.

2.2.1.1. Distribution

This species is a forest dweller and is found in North- Eastern part of Sikkim Himalayan range at an altitude of 2000 – 3000 m. This plant is found in several parts of the Himalayan range of Sikkim, including spring banks, freshly burnt area, and association with Rhododendron. (Fig. 2.2)

2.2.1.2. Features of different parts of the plant

It is a small shrub 0.9 to 1.2m high glabrescent stem. Leaves 25 cm long uni or bipinnate; leaflets sessile oblong or ovate, irregularly toothed; flowers white, in umbels. Flowering in July and fruits are obovate. Fruiting August to.September. The dried herb is used as a fodder in winter. The seeds are being used for preparation of chatni [1].

2.2.1.3. Pharmaceutical and Phytochemical aspects

Ethnomedicinal studies indicate that the root juice is being used to treat various ailments [2]. The root juice is said to posses antidiarrhoeal, aphrodisiac and analgesic property. The plant is used in veterinary medicine. It exhibits stimulant property and increases the rate of respiration and blood pressure in goats. The essential oil from fruits showed moderately good antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Vibrio cholerae* [16]. Phytochemical analysis of the root of the plant revealed the presence of a number of Coumarin: Bergapten [17, 18, 19], Pimpinellin [17], Saphondin [17].

2.3. Effect of herbs on bioavailability and pharmacokinetics of drugs

The therapeutic effectiveness of a drug depends upon the ability of the dosage form to deliver the medicament to its site of action at a rate and amount sufficient to elicit the desired pharmacologic response. This attribute of the dosage forms referred to as physiologic availability, biologic availability or simply bioavailability. For most drugs, the pharmacologic response can be related directly to the plasma levels. Thus the term **bioavailability** is defined as the rate and extent (amount) of absorption of unchanged drug from its dosage form. It is an absolute term. The concentration of drug in plasma and hence the onset of action, and the intensity and duration of response depend upon the bioavailability of drug from its dosage form. Other processes that play a role in the

therapeutic activity of a drug are distribution and elimination. In order to administer drugs optimally, knowledge is needed not only of the mechanisms of drug absorption, distribution, metabolism and excretion (*ADME*) but also the rates (kinetics) at which the processes occur. So **pharmacokinetics** is defined as the study of time course of drug *ADME* and their relationship with its therapeutic and toxic effects of the drug [20].

There is a growing awareness that herbal remedies and other phytochemicals could severely affect the disposition of drugs and chemicals. During the past five years, a few herbs that have been widely popularized, such as ginkgo, ginseng, and St. John's wort, were specifically cited as causing, or suspected of causing, interactions with drugs (Table 2.1). A possible interaction refers to the possibility that herbs may alter the bioavailability or the clinical effectiveness of drug when both are given concurrently. The net result may be an increase or a decrease in effect of one or both substances. The main causative factors in the effect of herbs on bioavailability of drugs were identified as the phytoconstituents that modulated a major of drug metabolizing enzyme system, Cytochrome P450 (CYP) known as acromyn [21]. Additionally, molecules that are substrate of P-glycoprotein efflux transporter pumps may also get affected by the interaction of herbs [22].

The cytochrome P450 (CYP) enzyme system consists of a superfamily of hemoproteins that catalyse the oxidative metabolism of a wide variety of exogeneous chemicals including drugs, carcinogens, toxins and endogenous compounds such as steroids, fatty acids and prostaglandins [22]. The CYP enzyme family plays an important role in phase-I metabolism of many drugs. The broad ranges of drugs that undergo CYP mediated oxidative biotransformation are responsible for the large number of clinically significant drug interactions during multiple drug therapy. The enzyme cytochrome P450 is derived from the proteins that have a heme group and unusual spectrum. The name cytochrome P450 is appropriated from the fact that these enzymes are characterized by a maximum absorption wavelength of 450 nm in reduced state in the presence of carbon monoxide. Naming a cytorome P450 gene included root symbol "CYP" for humans ("CYP" for mouse and Drosophilla), an Arabic numeral denoting the CYP family (e.g. CYP1, CYP2), letters A, B, C indicating subfamily (e.g. CYP3A, CYP3C) and another Arabic numeral representing the

Table 2.1. Herbs reported to have potential for drug interactions.

Herb	Source	Interactions reported or suspected
St. John's wort	*Hypericum perforatum* (Whole plant)	Warfarin (to cause bleeding); serotonin-uptake inhibitors (to cause mild serotonin syndrome); indinavir (increased bioavailability); digitoxin, theophylline, cyclosporin, phenprocoumon, and oral contraceptives (all with reduced bioavailability)
Ginseng	*Panax ginseng* (Root)	Antidepressants such as phenelzine sulfate (to cause manic episodes, headache); warfarin (to cause bleeding or to decrease effectiveness); corticosteroids (potentiation); estrogens (potentiation)
Ginkgo	*Ginkgo biloba* (Leaf)	Warfarin (to cause bleeding)
Ginger	*Zingiber officinale* (Rhizome)	Sulfaguanidine (enhance absorption)
Garlic	*Allium sativum* (Bulb)	Warfarin (to cause bleeding)
Rhubarb	*Rheum officinale* (Root)	Cardiac glycosides and antiarrhythmic agents (potentiating by reducing potassium via laxative effect)
Aloe	*Aloe ferox* (Leaf sap)	Cardiac glycosides and antiarrhythmic agents (potentiating by reducing potassium via laxative effect)
Astragalus	*Astragalus membranaceus* (Root)	Cyclosporine, azathioprine, methotrexate (to impair intended immuno-suppressive effects).
Bupleurum	*Bupleurum falcatum* (Root)	Sedatives (potentiation)
Liquorice	*Glycyrrhiza uralensis* (Root)	Corticosteroids and thiazide diuretics (potentiation); digitalis or other cardiac glycosides (increased sensitivity)

individual gene/isoenzyme/isoform (e.g. CYP3A4, CYP3A5) [23]. CYP3A4 isoenzyme is the most predominant isoenzyme in the liver and is involved in the metabolism of approximately 30-40% of drugs [24, 25].

Literature and anecdotal reports suggest that concomitant oral administration of some natural products and pharmaceuticals may affect metabolism in human and significantly increase the plasma-drug concentration [26-76].

Bailey *et.al* reported a significant pharmacokinetic and pharmacodynamic interaction of felodipine (a calcium antagonist) when the drug was given with concomitant intake of grape fruit juice; this investigation was carried out in a cohort of subjects who had a borderline hypertension [26]. As a result of the pharmacokinetic interaction, parameters such as peak plasma concentration (C_{max}) and area under plasma concentration-curve (AUC) increased approximately by 2.2 fold and 2.5 fold respectively of control treatment (water alone). In a similar fashion, the pharmacodynamic interaction resulted in a significant 2-fold decrease in diastolic blood pressure and a 2-fold increase in heart rate measures compared to the treatment. Interestingly, the concomitant administration of felodipine with orange juice to the same subjects did not alter the pharmacokinetics or pharmacodynamics of felodipine. Another contemporary work showed that other dihydropyridine substrates such as nitredipine and nisoldipine when administered with grape fruit juice exhibited increased rate and extent of absorption when compared to the respective control treatment[27].

Proppe *et.al* observed a stable creatine clearance in all but one patient, in spite of significant alternation in the pharmacokinetics of cyclosporine following intake of grape juice [28]. In a group of women patients, it has been shown that the intake of grape juice significantly inhibited the metabolism of 17 β-estradiol (hormone supplement) and as result significantly increased the bioavailable amounts of 17 β-estradiol and its metabolite, estrone. However, it appeared that this pharmacokinetic interaction between grape juice and 17 β-estradiol might not be clinically important [29].

Anderson *et.al* have reported, a 2-fold increase in the exposure of midazolam following grapefruit juice administration in patients with liver cirrhosis. In the same report, authors concluded that those liver cirrhotic patients are more dependent on the intestinal metabolism of midazolam than the normal subjects who have the normal liver function [30].

Nagy *et.al* have reported an approximately 3.2 fold increase in the availability of albendazole, when grape juice was ingested with the drug [31]. In another study, the bioavailability of sildenafil was enhanced, while the absorption was delayed when grape juice was administered with sildenafil [32]. Both C_{max} and AUC values of cisapride were tremendously increased by concomitant grape juice ingestion, however, neither T_{max} nor elimination half-life values were altered [33]. In case of saquinavir, a HIV protease inhibitor, concomitant intake of grape juice increased the bioavailability of saquinavir by approximately 1.7 fold [34].

One of the main causative factors in the grape juice effect on drug availability was identified as a group of furanocoumarins that inhibited a major drug metabolizing enzyme system, cytochrome P450. In particular, CYP3A4 appears to be the enzyme most affected, though the entire CYP system is inhibited to some extent [35, 36]. CYP is mainly found in the small intestine and liver; it is believed that the major effect of grape juice is to inhibit the small intestine CYP, thus preventing the drug from being metabolized before it enters the blood stream. In fact, the process has been described as more than simple inhibition: to a certain extent the enzyme is bound to the chemical compounds in the juice and washed away from the small intestine. Repeated administration of the juice increases its effect, rather than causing a rebound of enzyme levels [37].

Interaction between grape juice and clinically used drugs has been reported in recent years. These drugs include cyclosporine, midazolam and trizolam. All of these drugs are substrates for CYP3A4 and undergo extensive metabolism by intestinal CYP3A4 [38, 39].

Different other studies have been conducted to know the compounds in which grape juice involved in the drug interaction. Analysis of ethyl acetate extracts from grape juice

revealed the presence of several furanocoumarins of which bergamottin, the parent compound of 6', 7'-dihydroxybergamottin, is the major one and was found to be a mechanism based inactivator of CYP3A4 in human liver microsomes [40]. The other non-flavonoid components found in grape juice such as limolin and obacunone, a triterpene-derived product, also reduced microsomal testosterone 6β-hydroxylation in human liver [41].

Ping. Cheun Ho *et al.* studied the effect of flavonoids and furanocoumarin compounds found in grape juice, on activity of human liver CYP3A4. The study has reported that besides the flavonoids, other compounds found in grapefruit including furanocoumarins can produce strong inhibition of CYP3A4. The grape juice drug interactions could involve CYP3A4 inhibition by more than one component present in grape juice [42]. Other *in vivo* and *in vitro* studies have shown that flavonoids can enhance or inhibit the activities of certain P450 isozymes [43, 44, 45, 46, 47].

When predicting potential drug-herbs interactions, the role of the membrane transporter P-glycoprotein (Pgp) must also be considered [48]. Pgp, a member of the superfamily of proteins known as the ATP-binding cassette, is capable of pumping a wide range of structurally diverse, lipophilic drugs out of cells, potentially acting as a defense mechanism. This glycoprotein is well recognized because of its contribution to multiple-drug resistance during cancer chemotherapy. Pgp has been discovered in the liver, kidney (where it pumps drug out of the body through the urine), bloodbrain and bloodtestis barrier, lymphocytes, and the placenta. Pgp has also been located in the enterocytes of the small and large intestine, where its role is to carry lipophilic molecules from the enterocyte back into the intestinal lumen for elimination. Many hydrophobic drugs are either metabolized by CYP3A or pumped back into the lumen by Pgp after intestinal absorption and enterocyte uptake. Therefore, CYP3A and Pgp, acting in tandem, may decrease the oral absorption and delivery of different pharmaceuticals [49].

Abernethy *et.al* have shown that grape juice can inhibit the P-glycoprotein related efflux transport of talinolol both *in vitro* and *in vivo* situations [50]. In another study, it was

reported a decrease in the concentrations of celiprolol following grape juice administration. The authors have attributed a physical interaction of grape juice such that it interferes with phase of celiprolol. The data is well supported by the fact that itracanozole's treatment increases the plasma concentrations of celiprolol due to itracanozole's known inhibitory on CYP3A4 and P-glycoprotein [51]. Oral bioavailability of some protease inhibitor was unexpectedly increased by about 25% when the patients were preadministered with grape juice [52].

Piperine (1-piperoyl piperidine) a major component of the Piper species *Piper nigrum* L. (Piperaceae) is a potent inhibitor of drug metabolism and inhibited hepatic monooxygenase and UDP-glucuronyltransferase [53, 54].

Hiwale *et.al* studied the effect of piperine on bioavailability and pharmacokinetics of β-lactam antibiotics in rats. It has been observed that an earlier t_{max}, higher C_{max} and AUC were obtained in the mice that received both the drugs concurrently [55]. In addition piperine has also been shown to enhance the bioavailability of drugs like vascicine, spartenin, barbiturate and oxyphenbutazone, zoxazolamine, propranalol and theophylline in animal experiments [56-59].

Bano *et.al* studied the effect of piperine on pharmacokinetics of phenytoin in the healthy human volunteers and reported the significant increase in the bioavailability of the phenytoin in volunteers treated with piperine [60]. In another study, piperine was reported of the increase in bioavailability of indomethacin in rabbits, including t_{max}, C_{max} and AUC [61].

St. John's Wort (SJW), an herbal product derived from the perennial plant *Hypericum perforatum* and has gained immense popularity as an antidepressant. In recent years, there have been numerous case reports of interactions between SJW and prescription drugs metabolized by CYPs such as indinavir, cyclosprorin A, warfarin, digoxin, and theophylline [62]. Several *in vitro* and *in vivo* studies have evaluated the effects of SJW extract on the expression and activity of hepatic CYP3A4 and the drug efflux protein P-glycoprotein (Pgp) [63-67]. These data suggest that SJW induces CYP3A4 activity, which

could explain many of these drug interactions. Additionally, SJW may also induce intestinal Pgp expression and activity and influence pharmacokinetics of drugs such as indinavir and digoxin [63, 64]. A pharmacokinetic interaction between SJW and theophylline was reported, where plasma concentrations following SJW therapy were decreased [68]. Since theophylline is metabolized mainly by CYP1A2, it is possible that induction of CYP1A2 by SJW may explain the reduced theophylline concentrations and loss of therapeutic efficacy. The use of medicinal herbs has particularly increased over the past few years among specific patient populations including HIV-infected patients. St. John's wort altered pharmacokinetics of the HIV protease inhibitor, indinavir in individuals on retroviral therapy [69]. According to the Card L, St. John's wort induces CYP3A4, which metabolizes most protease inhibitors. The concomitant administration of St. John's wort with protease inhibitors could result in the induction of CYP3A4, increased metabolism, and sub therapeutic levels of the protease inhibitor.

Rosemary, *Rosmarinus officinalis* (Labiatae) is used externally to improve circulation in hypotonic circulatory disorders, rheumatic conditions, eczema, and as a poultice for poorly healing wounds. Rosemary is taken orally for dyspeptic disorders, loss of appetite, liver and gallbladder complaints, and blood pressure problems [70]. It inhibits the binding of doxorubicin and vincristine to Pgp, thereby increasing the intracellular accumulation of these chemotherapeutic agents [71]. Rosemary may thus also increase cyclosporine plasma concentrations by increasing its oral bioavailability, through inhibition of Pgp activity.

Strandell *et. al* studied the interactions of herbals and other natural remedies on cytochrome P450. They confirmed that extracts of herbal preparation were potent inhibitor of all tested metabolic enzymes [72]. In another study, it was reported that garlic inhibited the metabolic enzymes and increases the bioavailability of different pharmaceuticals [73]. Traditional Chinese medicines (TCM) are believed by many to be safe and used for self-medication without supervision. Although the risk appears to be low, certain TCM have been associated with a number of serious adverse reactions. In different studies it was reported that many Chinese medicinal herbs were inhibitors or inducers of hepatic CYP450 [74-76].

2.4. Immunostimulatory agents from herbal source

Immunostimulants or immunopotentiators are drugs leading predominantly to a non-specific stimulation of immunological defense mechanisms [77,78]. Change in the immunity leads to incurable life threatening diseases like cancer and AIDS. So, stimulation of the host immune response presents an interesting supplementary approach for conventional anti-AIDS therapy. The stimulation of immune response by using herbal products, as a possible therapeutic measure has become a subject of scientific investigations. The basic concept has, however, existed in the ancient Vedic scripture, the Ayurveda, and has been practiced in Indian traditional medicine for many centuries. One of the therapeutic strategies in Ayurveda medicine is to increase body's natural resistance to the disease causing agent rather than directly neutralizing the agent itself; in practice, this is achieved by using extracts of various plant materials called *rasayanas* (Charak Samhita, 1000 BC)[79]. This concept in modern scientific understanding would mean enhancement of immune responsiveness of an organism against a pathogen by non-specifically activating the immune system using immunostimulatory agents of plant origin. It is now being recognized that immunostimulation could provide an alternative to conventional chemotherapy for a variety of diseased conditions, especially when host's defense mechanisms have to be activated under the conditions of impaired immune response [80].

The basic function of the immune system is to protect against foreign pathogens and infectious agents. This is achieved either through innate or natural immunological mechanisms which essentially serve as a short term first line of defense or through elaborate adaptive mechanisms which are highly specific, complex and are marked by diversity and memory. In both types of immunity, cells and molecules play important roles. While in natural or innate responses the cellular players are monocytes, macrophages, polymorphonuclear phagocytes and natural killer cells, in the adaptive immunity the pivotal role is played by two classes of lymphocytes, viz., T (thymus derived) and B (Bursa- or bone marrow derived) cells and these are assisted by accessory cells such as antigen presenting cells. Further, the cellular dichotomy in adaptive immune response is also reflected in functional division of labour as the T cells serve as effectors of cell mediated immune responses such as delayed type hypersensitivity and killing of virus

infected cells and also as helpers for the production of highly specific proteins, called antibodies, by the B lymphocytes. These antibodies possess binding sites complementary to the antigen and are responsible for their removal from the system. The molecular constituents of the natural immune system are the complement proteins some of which help in opsonisation of foreign bacteria, lysozyme, defensin peptides, certain cytokines, etc [81-83]

Although extensive work has been carried out in the field of medicinal chemistry during this century, it is only in the last two decades that a number of compounds with immunostimulating activity have been identified from the plant materials. These include: alkaloids, terpenoids, quinines in one category and polysaccharides, peptides, glycoproteins and nucleotides in the other.

Upadhaya S.N. has highlighted the therapeutic potential of immunomodulatory agents from plant products. They have evaluated Indian medicinal plants for immunostimulatory activity. The author had also reviewed the Ayurvedic preventive healthcare medicines. A list of Ayurvedic medicinal plants showing immunostimulatory activity has been provided which includes agents like *Withania somnifera, Allium sativum, Azadirachta indica, Piper longum, Asparagus racemosus, Glycyrrhiza glabra, Aloe vera, Gmelina arborea* and *Tinospora cordifolia* [80].

Thatte and Dahanukar, have described how the description of ancient writings can lead to the development of new immunostimulatory agents. The experiments carried out to prove the *rasayana* concept of Ayurveda have demonstrated that *Asparagus racemosus, Tinospora cordifolia* and *Withania somnifera* protected animals against infections in normal and immunosupressed states induced by hemisplenectomy or surgery [85]. These plants also produced leucocytosis with predominant neutrophilia and prevented, to varying degrees, the leucopenia induced by cyclophosphamide. They were found to activate the polymorphonuclear and monocyte-macrophage systems. Only these *rasayanas* which produced sweet (madhur) vipaka (*Tinospora cordifolia, Asparagus racemosus, Emblica officinalis, Terminalia chebula* and *Withania somnifera*) were found to stimulate the

reticulo-endothelial system, but not these like *Acorus calamus*, *Commiphora mukul*, and *Picorrhiza kurroa*, which produced bitter (katu) vipaka [84].

Among the immunostimulant *rasayanas*, *Tinospora codiofolia* has been extensively studied by Dahanukar *et al* [85]. It has been found to activate the mononuclear cells to release cytokines like GMCSF [86] and IL-1 in a dose dependent manner [84]. Whole aqueous extract of *Tinospora codiofolia*, standardized using HPTLC, has been evaluated as an adjuvant in clinical conditions like obstructive jaundice, tuberculosis and cancer chemotherapy and has been found to increase the efficacy of conventional therapy. Active principle of *Tinospora codiofolia* were found to possess anticomplementary and immunomodulatory activities. Syringin (TC-4) and cordial (TC-7) inhibited the *in vitro* immunohaemolysis of antibody coated sheep erythrocytes by guinea pig serum by inhibiting the C-3 convertase of the classical complement pathway. The compounds also gave rise to significant increases in IgG antibodies in serum. Both humoral and cell-mediated immunity were dose dependently enhanced. Macrophage activation was reported by cordioside (TC-2), cordiofolioside A (TC-5) and cordiol (TC-7) and this activation was more pronounced with increasing incubation time [87].

The effect of *Asparagus racemosus, Tinospora cordifolia, Withania somnifera* and *Piccorrhiza kurroa* on macrophage function obtained from mice treated with the carcinogen, ochratoxin (OTA) was evaluated by Dhuley J.N. [88]. Treatment with these plants significantly attenuated the OTA induced suppression of chemostatic activity as well as IL-1 and TNF-α production by macrophages. Moreover, *Withania somnifera* potentiated macrophage chemotaxis and *Asparagus racemosus* induced excessive production of TNF-α as compared to controls.

Ray *et al* demonstrated that ovalbumin immunized mice treated with *Azadirachta indica* leaf extract had higher IgG and IgM levels and anti-ovalbumin antibody titers as compared to control (humoralresponse). *Azadirachta indica* also induced cell-mediated response as seen from the enhancement of macrophage migration inhibition and footpad thickness [89]. These finding were supported by Ansari et al. They found that *Azadirachta indica*

potentiated the antibody titres following typhoid H antigen immunization and induced delayed hypersensitivity following administration of tuberculin and DNBC to animals. In human volunteers, it stimulated humoral immunity by increasing total lymphocyte and T-cell count in 21 days [90].

Oral treatment with leaf extract of *Azadirachta indica* reversed the inhibitory of restraint stress on formation of anti-sheep RBC antibody titres in rats immunized with sheep RBC and also the increase in footpad thickness. It reversed the DDT induced suppression of antibody response and leucocyte migration inhibition in tetanous toxoid immunized rats. Restraint stress along with administration of DDT in subthreshold doses resulted in an inhibition of the immune response. *Azadirachta indica* attenuated the immunotoxicity of environmental and xenobiotic stressors [91]. In another study, the animals were treated intraperitoneally with the neem oil. Peritoneal macrophages exhibited enhanced phagocytic activity and expression of MHC class-II antigens. Neem oil treatment also induced the production of gamma interferon. Spleen cells showed a significantly higher proliferative response of Con A and tetanus toxoid TT *in vitro* compared to that of controls. Pretreatment with neem oil, however, did not augment the anti TT antibody response. These data suggested that neem oil acts as a non-specific immunostimulant for cell mediated immune mechanisms [92].

Root suspension of *Janakia arayalpathra* was found to have immunostimulatory properties in mice. It stimulated an increase in humoral antibody titres and also of antibody secreting spleen cells in the plaque forming cells assay following immunization with sheep erythrocytes. It also increased the number of peritoneal macrophages and produced an increase in delayed hypersensitivity reaction in mice [93].

The alkaloidal fraction of *Boerrhiva diffusa* significantly restored the suppressed humoral response in stressed rats as observed by Mungantiwar et. al., wherein *Boerrhiva diffusa* increased the suppressed antibody titres following immunization by sheep RBCs in rats subjected to restraints stress. It also significantly reversed the depleted adrenal cortisol

level and elevated plasma cortisol level in the stressed rats, thus appearing to have a corticosteroid sparing effect in experimental stress [94].

Immune-21, a polyherbal natural product has been shown to exhibit significant immunopotentiating and immunoprophylactic activity, both *in vitro* and *in vivo* [95]. In another study, one hundred and seventy-eight ethanolic plant extracts from the pharmacopoeia of the Tacana, an ethnic group from Bolivia, were screened for immunomodulatory activity using complement cascade inhibition and ADP-induced platelet aggregation inhibition assays. Six plants impaired both complement pathways (classical and alternative): stem bark from *Astronium urundeuvea* (Anacardiaceae), *Cochlospermum vitifolium* (Cochlospermaceae), *Terminalia amazonica* (Combretaceae), *Triplaris americana* (Polygonaceae), *Uncaria tomentosa* (Rubiaceae) and *Euterpe precatoria* (Arecaceae) roots. Inhibition of complement cascade was independent of essential ion complexation, and was not due to direct hemolytic activity on target red blood cells. For *A. urundeuvea*, *C. vitifolium*, and *T. amazonica*, anti-inflammatory activity relied on cyclo-oxygenase inhibition. Four of these species (*A. urundeuva, T. americana, U. tomentosa* and *E. precatoria*) are used traditionally to treat inflammatory conditions [96].

Jayathirtha *et. al.* made an attempt to assess the immunomodulatory activity of methanol extracts of whole plant of *Eclipta alba* and *Centella asiatica* at five dose levels, ranging from 100 to 500 mg/kg body weight., using carbon clearance, antibody titer and cyclophosphamide immunosupression parameters. In the case of *E. alba,* the phagocytic index and antibody titer increased significantly and the F ratios of the phagocytic index and WBC count were also significant. Regression analysis showed linearity in patterns of the dose response relationship, greatest in the case of the phagocytic index, moderate in the WBC count and lowest in the antibody titer. For *C. asiatica*, significant increases in the phagocytic index and total WBC count were observed and the F ratio of the phagocytic index was also significant. Regressed values revealed maximum linearity in the case of the phagocytic index, moderate linearity in the total WBC count and lowest linearity in the antibody response [97].

Joharapurkar *et al* developed a new model to screen the immunomodulatory activity of *Rubia cordifolia*. He concluded that, the minimum dose of pyrogallol, which can induce significant immunosupression, was 50 mg/kg bodyweight. Thus, pyrogallol can be used as an experimental tool to induce immunosupression while screening the immunomodulatory activity [98].

2.5. Antioxidants from herbal source

During normal biochemical reactions in our body there is a generation of reactive oxygen and nitrogen species (ROS and RNS). These are enhanced during patho-physiological conditions creating 'oxidative stress'. During this phenomenon cellular constituents get altered resulting in various diseased states. This may be effectively neutralized, by enhancing the cellular defense mechanisms, in the form of antioxidants. So, an antioxidant may be defined as any substance that when present in low concentrations compared to that of an oxidizable substrate significantly delays or inhibits oxidation of the substrate [99,100].
The generation of ROS begins with the rapid uptake of oxygen and activation of NADPH oxidase and the production of the superoxide free radicals (O_2^-) Superoxide is then rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD).

These ROS can act by either of the two oxygen dependent mechanisms resulting in the destruction of the microorganism or other foreign matter. The reactive species can also be generated by the myeloperoxidase-halide-H_2O_2 system. The neutrophil cytoplasmic granules contain the enzyme myeloperoxidase (MPO). In presence of chloride ion, which is ubiquitous, hydrogen peroxide is converted to hypochlorous acid (HOCl), a potent oxidant andantimicrobial agent [101].

The MPO dependent mechanism, though not as important as the previous one, is still essential. ROS are generated from superoxide and H_2O_2 produced via respiratory burst by Fenton (A) and/or Haber-Weiss (B) reactions.

(A) $H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$

(B) $O_2^- + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2$

Reactive nitrogen species are also important. The free radical nitric oxide (NO), first described as endothelium derived relaxation factor (EDRF), is produced from arginine by nitric oxide synthatase (NOS). An inducible nitric oxide synthatase is capable of continuously producing large amount of NO. In activated cells, it acts as a killer molecules[102].

There is increasing evidence to support the involvement of free radical reactions in several human diseases. Free radical reaction is an important pathway in a wide range of unrelated biological systems. Amongst the very many ways to chemically injure and kill cells, an important class of reactions is that producing free radical intermediates which trigger a network of multifarious disturbances because of ubiquity of molecular oxygen in aerobic organisms and its ability to accept electron [103].

Oxygen derived free radicals are often mediators and/or products of normal, pathological or toxic free radical reactions. A vast amount of circumstantial evidence implicates oxygen-derived free radicals (superoxide and hydroxyl radical) and high-energy oxidants (peroxynitrile) as mediators of shock, inflammation and ischemia reperfusion injury. Active oxygen species and other free radicals have long been known to be mutagenic. Further, these agents have more recently emerged as mediators of the other phenotypic and genotypic changes that lead from mutations to neoplasia. Therefore, free radicals may contribute widely to cancer development in humans. In last decade, evidences have been accumulated that the free radical process known as lipid peroxidation plays a crucial and causative role in the pathogenesis of atherosclerosis, cancer, myocardial infraction, and aging. Participation of free radical oxidative interactions in promoting tissue injury in conditions like brain trauma, ischaemia, toxicity and also in neuro-degenerative diseases such as Parkinson's disease, Alzheimer's dementia, multiple sclerosis and lipofuscinosis are now well documented [104].

The involvement of reactive oxygen species (ROS) in the pathogenesis of several lung diseases has also been suggested. The pioneer studies on the role of free radical reactions in the genesis and the expression of cellular and tissue damage has been carried out mainly in the liver, in particular, during the last 25 years by using acute rat poisoning with carbon

tetrachloride (CCl_4) as a model system. Several scientists have demonstrated the different mechanisms by which carbon tetrachloride activation to free metabolites lead to liver degeneration and necrosis [105].

Studies in experimental models have incriminated reactive oxygen species as primary mediators in the pathogenesis of ischaemic, toxic and immunologically mediated renal injury. Diabetes mellitus is also associated with oxidative reactions, particularly those that are catalyzed by decompartmentalized transition metals, but their causative significance in diabetic tissue damage remains to be established [105].

More interestingly, free oxygen radicals are increasingly discussed as important factors involved in the phenomenon of biological aging. Higher formation rates of free radicals from senescent animals observed in isolated biological material (mainly mitochondria), accumulation of the free radical damage and changes of antioxidants capacities appear to prove correctness of this assumption. The basic idea behind this assumption is that aging results from random deleterious effects of tissue brought about by free radicals. A conclusion from the data available so far was the free radicals are very likely to contribute considerable to the development of stochastic disorders observed during the progress of aging [106].

Plants and other organisms have evolved a wide range of mechanisms to contend with this problem, with a variety of antioxidant molecules and enzymes. In traditional South-East Asian medicine the therapeutic value of the parenchymatous leaf-gel of *Aloe vera* for inflammation-based diseases was reported. Extracts from leaf-gel contain glutathione peroxidase activity. The low molecular weight constituents of this extract inhibit the release of reactive oxygen species (ROS) by phorobol myristic acetate (PMA)- stimulated human polymorphonuclear leucocytes (PMN) [107].

The antioxidant effects of oils isolated from onion and garlic on nicotine-induced lipid peroxidation in rat tissues were studied. Lipid peroxidation was significantly increased in the tissues of nicotine treated rats. Both the garlic oil and onion oil supplementation to

nicotine treated rats increased resistance to lipid peroxidation. With garlic oil or onion oil supplementation, nicotine treated rats showed increased activities of antioxidant enzymes and increased concentrations of glutathione [108].

Kamat *et al.* have examined the antioxidant effects of crude extract as well as purified polysaccharide fraction of *Asparagus racemosus* against membrane damage induced in rat mitochondria and liver by free radicals generated during γ-irradiation. They found that these materials had potent antioxidant properties *in vitro* [109].

Selvam *et.al.* have isolated turmeric antioxidant protein (TAP) from the aquous extract of turmeric. The protein showed a concentration-dependent inhibitory effect on lipid peroxidation. Ca^{2+}-ATPase of rat brain homogenate was protected to nearly 50% of the initial activity from the lipid peroxidant induced inactivation by this protein. This protection of Ca^{2+}-ATPase activities was found to be associated with the prevention of loss of –SH groups [110]. Turmeric contains several small molecular weight components with antioxidant, medicinal and immunomodulatory activities. Natural curcuminoids, isolated from turmeric, were compared for their potential use as anti-promoters [111]. The curcuminoids inhibited lipid peroxidation besides the production of superoxides and hydroxyl radical.

The antioxidant activity of tannoid an active principles of *Emblica officinalis* consisting of emblicanin A (37%), emblicanin B (33%), punigluconin (12%) and pedunculagin (14%), was investigated on the basis of their effects on rat brain frontal cortical and striatal concentrations of the oxidative free radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), and lipid peroxidation in terms of thiobarbituric acid-reactive products [112].

Extract of *Tinospora cordiofolia* has been shown to inhibit the lipid peroxidation and formation of superoxide, and hydroxyl radicals *in vitro*. Administration of the extract partially reduced the elevated lipid peroxides in serum. *Tinospora* extract may be useful in reducing the chemotoxicity induced by free radical forming chemicals like

cyclophosphamides. Several glycosides with potential antioxidant activity were also isolated, as poly acetates, from the n-BuOH fraction of the *T. cordiofolia* stems [113].

Antioxidant activity of principles of *Withania somnifera*, consisting of equimolar concentrations of sitoindosides VII-X and withaferin A, was investigated for their effects on rat brain frontal cortical and striatal concentrations of superoxide dismutase, catalase and glutathione peroxidase [114]. Active glucowithanolides, administered once daily for 21 days, induced a dose-related increase in SOD, CAT and GPX activity in frontal cortex and striatum, which was statistically significant on days 14 and 21 [115].

The antioxidant properties of methanol extracts of 12 Indian medicinal plants were evaluated by two methods, namely the DPPH (1,1-diphenyl-2-picryl hydrazyl) and lipid peroxidation assay. In the later assay, seven of these extracts showed 90% or more activity compared with the standard, vitamin E and hence were studied in detail after the removal of interfering pigments. The selective pigment removal from the extracts led to an increase in free radical scavenging activity and a decrease in inhibition of lipid peroxidation [116].

Gupta *et al.* have examined the antioxidant and antitumor role of *Bahunia racemosa* against ehrlich ascites carcinoma in Swiss albino mice. They found that the methanol extract of the plant exhibited antitumor activity by modulating lipid peroxidation and augmenting antioxidant defense system in EAC bearing mice [117].

Bafana *et.al* have studied the antiulcer and antioxidant activity of a herbomineral formulation, Pepticare. They concluded that the increase in the levels of superoxide dismutase, catalase, reduced glutathione and membrane bound enzymes like Ca^{2+} ATPase, Mg^{2+} ATPase and Na^{+} K^{+} ATPase and decrease in lipid peroxidation in both the models proved the antioxidant activity of the formulation [118]. In another study, the antioxidant and antimicrobial activities of *Bahunia racemosa* was conducted on the basis of DPPH, superoxide anion radical and hydroxyl radical scavenging activity. The results obtained in the study indicate that *Bahunia racemosa* can be a potential source of natural antioxidant and antimicrobial agents [119].

2.6. Antimicrobial agents from herbal source

Antimicrobial drugs are immensely important therapeutics discovered in the last century. Their advents have changed the outlook of the physician about the power of the drugs on disease management. Many infectious diseases once considered incurable and lethal are now amenable to treatment with a few pills. The remarkable powerful and specific activity of antimicrobial drugs is due to their selectivity for highly specific targets [120].

Antimicrobial agents can be classified on basis of the following two ways:

A. Chemical structure:

- Sulfonamides and related drugs : Sulfadiazine, Dapsone.
- Diaminopyrimidine :Trimethoprim.
- β- Lactam antibiotics : Penicillin, Cephalosporin.
- Tetracycline : Doxycycline.
- Nitrobenzene derivatives : Chloramphenicol.
- Aminoglycoside : Streptomycin, Neomycin.
- Macrolides : Erythromycin, Roxythromycin.
- Polypeptides : Polymyxin-B, Bacitracin.
- Nitrofurantoin derivatives : Nitrofurantoin, Furazolidone.
- Nitroimidazole derivative : Metronidazole, Tinidazole.
- Quinolones : Nalidixic acid, Ciprofloxacin.
- Nicotinic acid derivatives : Isoniazid, Pyrazinamide.
- Polyene antibiotics : Nystatin, Amphotericin B.
- Imidazole derivatives : Miconazole, Kitoconazole.
- Miscellaneous : Rifampicin, Griseofulvin.

B. Mechanism of action

- Inhibit cell wall synthesis : Penicillin, Cephalosporin.
- Cause leakage of cell membrane : Polymixin, Colistin.
- Inhibit protein synthesis : Tetracycline, Chloramphenicol.
- Cause misreading of mRNA code : Streptomycin.
- Interfere with DNA function : Rifampicin, Metronidazole.

- Interfere DNA synthesis : Idoxuridine, Acyclovir.
- Interfere with intermediary metabolism: Para amino salycyclic acid, Ethambutol.

Agents that inhibit bacterial cell wall synthesis are essentially nontoxic to the animal and human host, since cell walls are absent in mammalian tissues. These agents inhibit synthesis of certain active enzymes resulting disruption of bacterial walls to cause loss of viability and often cell lysis; these include the penicillins and cephalosporins [121]. Drug that affect the cell membrane bring about permeability changes in the microbial cells. The other antibiotics, which affect the cell membrane, are polymyxin B, colistimethate, Nystatin and amphotericin B [122]. These antimicrobials alter the osmotic properties of the cell resulting in leakage of important cellular constituents like ammonium ions, potassium ions and nucleotides leading to their cell death [123, 124].

The major inhibitor of protein synthetic machinery are the aminoglycosides to produce two types of changes on the bacterial cell, firstly, it binds irreversibly with 30S ribosomal subunits and interacts at the recognition region causing an inhibition of amino acyl t-RNA to bind with m-RNA and 50S ribose in order to distort the codon-anticodon interaction. Secondly, it causes cyclic polysomal blockage [125]. When it interferes with the chain elongation steps, the ribosome distortion takes place, which is seen through the steps of protein synthesis, producing a bacteriostatic effect. Due to its cyclic formation of inhibition complex caused by the 'dropping off' of ribosome no protein is synthesized and the cell undergoes death [124].

The principal inhibitors of nucleic acid synthesis are rifampcin and nalidixic acid against bacteria, and griseofulvin against fungi [126]. The other antibiotics selectively inhibiting the synthesis of nucleic acid include actinomycin, kenamycin, neomycin, novomycin, olivomycin against RNA and actidin, bruneomycin, mitomycin, sereomycin against DNA[127]. Drugs that are structurally similar to cellular metabolites and can complete with the natural substrate for incorporation in functionally important antimetabolites are sulfa drugs, para aminosalicylate, trimethoprim, ethambutol, 5-fluro-cytosine, primethamine and the antibiotic antimetabolites are leucine, furanomycin [126, 128].

The use of medicinal plants and their extracts for the cure of localized and specific human and cattle infections is an age-old practice from time immemorial. As early as 1630, Europeans used natural quinine from the bark of cinchona tree to treat malaria; a dreaded disease caused by a protozoan parasite *Plasodium* species [129]. Numerous studies have been performed throughout the globe in search of newer antimicrobial agents and most of those studies are directed towards the microbes. A number of studies showed that the antimicrobial principles could also available from marine algae and higher plants, particularly among angiosperms [130]. The antimicrobial compounds isolated from higher plants are different in chemical structures. They may be flavonoids, essential oils, alkaloids, anthraquinones, triterpenoids, etc. One approach that has been used for the discovery of antimicrobial agents from higher plants is based on the evaluation of the medicinal plant extracts. The work carried out on American and Europeans folk medicines are most important in this respect [131, 132].

The World Health Organization at its meeting on Herbal Medicine for human Health at Kuwait in 1985 specified some plants, as the remedies for varieties of skin diseases. These include *Aloe vera, Ficus carica, Azadirachata indica, Fumaria officinalis, Lausonria alba, Santalum albam* [133].

The use of plants as source of remedies for the treatment of many diseases dated back to prehistory and people of all continents followed this old tradition. Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in industrialized countries derived directly or indirectly from plants [134]. However, plants used in traditional medicine are still understudied, particularly in clinical microbiology [135]. Therefore, it is a new challenge to search for the *in vitro* and *in vivo* antimicrobial activity of natural compounds from these ethnomedicinal plants on pathogenic bacteria.

Polyphenols are a group of highly hydroxylated phenolic compounds present in the extractive fraction of several plant materials. Polyphenols in plants include hydroxycoumarins, hydroxycinnamate derivatives, flavanols, flavonols, flavanones,

flavones, anthocyanins, proanthocyanidins (tannins), hydroxystilbenes, aurones, etc. Polyphenols are well documented to have microbicidal activities against a huge number of pathogenic bacteria. Oxidized polyphenols also have inhibitory activity against bacterial growth. The mechanism of polyphenols toxicity against microbes may be related to inhibition of hydrolytic enzymes (proteases and carbohydrolases) or other interactions to inactivate microbial adhesions, cell envelope transport proteins, non specific interactions with carbohydrates, etc [136]. There are numerous illustrations of plant-derived drugs used as antimicrobials. Some selected examples, are presented below.

The alcoholic extract of dry nuts of *Semecarpus anacardium* (Bhallatak) showed bactericidal activity *in vitro* against three gram-negative strains (*Escherichia coli, Salmonella typhi* and *Proteus vulgaris*) and two gram-positive strains (*Staphylococcus aureus* and *Corynbacterium diphtheriae*). Subsequent studies have shown that the alcoholic extracts of different parts of the plant (leaves, twings, green fruit) also possess antibacterial properties, especially the leaf extract. No dermatoxic effect (irritant property) was observed in the mouse skin irritant assay [137].

The acetone and alcoholic extracts of the leaves of *Cassia alata* showed significant *in vitro* antibacterial activity against *Staphylococcus aureus*, coagulase positive *Staphylococcus aureus, Baciilus sublitis, Bacillus cereus, Bacillus stearothermophilus, Escherichia coli, Salmonella typhi* and *Salmonella dysentriae*. In addition, the alcoholic extract also inhibited growth of *Klebsiellae pneumoniae* whereas the acetone extract inhibited the growth of *Vibro cholerae* [138].

Kazmi *et al.* [139] described an anthraquinone from *Cassia italica*, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*. Hypericin, an anthraquinone from St. John's wort (*Hypericum perforatum*), has received much attention in the popular press lately as an antidepressant, and Duke, P. reported the general antimicrobial properties of the plant [140].

Scalbert [141] reviewed the antimicrobial properties of tannins in 1991. He listed 33 studies, which had documented the inhibitory activities of tannins. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been found to bind cell walls of ruminal bacteria, preventing growth and protease activity [142]. Terpenenes or terpenoids are active against bacteria, fungi, viruses and protozoa [143, 144]. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Mendoza *et. al* [145] found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity. Food scientists have found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* [146].

Due to lack of ideal diffusion and evaporation from the surface it is generally difficult to assess the antibacterial properties of aromatic oils derived from plants using the agar cup methods. Hence, Agnihotri and Vaidya have developed a novel approach to study the antibacterial property of certains plants like *Eugenia caryophyllus, Thymus vulgaris, cinnamonum zeylanium* and *Cuminum ciminium.* Volatile components of the hexane extracts of these plants were tested against gram-positive and gram-negative bacteria grown on agar slants and the results were expressed as percentage inhibition of the area of the slants [147].Shirataki *et. al.* studied the medicinal significance of prenylflavanones obtained from *Sophora tomentosa* L. and *Sophora moorcroftiana* Benth. Ex Baker (Leguminosae) [148]. Further, two flavanones (YS01, YS02) and eight prenylflavanones (YS03-YS10) were investigated for both *in vitro* and *in vivo* antibacterial activity. The *in vitro* activity was shown by spot inoculation method and *in vivo* activity was carried out by determining the protection offered by Swiss albino mice against the virulent strain of *Salmonella typhimurium* NCTC 74 [149]. Owasis *et al.* evaluated the antibacterial activity of *Withania somnifera* against experimental salmonellosis in Balb/C mice and determined the bacterial load in various vital organs of the treated mice [150]. Considering the relevant literatures or depending upon the information collected from the reliable actual users of the flora in their clinical practice, the screening of herbal species for chemotherapy was felt out most essential.

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CHAPTER 3

PHYTOCHEMICAL STUDIES

3.1. Introduction

The phytochemical analysis require fresh plant tissues and the plant materials under investigation should be plunged into boiling alcohol within minutes of its collection. 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 [1]. The Flavonoids and Flavonols are also widely distributed in plants both as co-pigment to anthocyanins in petals and also in leaves of higher plants. The flavonols occur most frequently in glycosidic combination like anthocyanins. Alkaloids, steroids, triterpenoids, saponins are also present in plants [1].

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 crystallisation 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.

Flavonoids are a group of polyphenolic compounds, which are widely distributed through out the plant kingdom. They occur as aglycones, glycosides and methylated derivatives. The flavonoid aglycone consists of a benzene ring condensed with another six membered ring, in which the 2-position carries a phenyl ring as a substituent. Six membered ring condensed with the benzene ring is either a α-pyrone (flavonols and flavonones) or its dihydroderivative (flavanols and flavanones). The position of the benzoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position). Flavonols differ from flavonones by hydroxyl group at the 3-position and a C_2-C_3 double bond [2]. Flavonoids are often hydroxylated in position 3, 5, 7, 2', 3', 4', 5'. Methylethers and acetylesters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose [3].

Flavonoids are characteristic constituents of green plants with the exception of algae and hornworts. They virtually occur in all parts of plants including leaf, root, wood, bark, pollen, flowers, berries and seeds. In a few reported cases flavonoids are being found in animals. For examples in the beaver scent gland, propolis (bee secretion) and in butterfly wings, it is considered that the flavonoids originate from the plants upon which the animals feed rather than being biosynthesized *in situ*. The distribution of flavonoids in the plant kingdom is confined mainly in angiosperms. It is reported that flavonoids are being

restricted to plant groups with a degree of complexity of the Bryophyta or higher [1]. This chapter mainly deals with the preliminary identification of phytochemical groups as well as the structural elucidation by using different spectroscopic methods of flavonoid compounds isolated from the leaf and root of the plants under investigation.

3.2. Materials and Methods

3.2.1. Plant material

The leaf and root of *Colebrookea oppositifolia* (*C.oppositifolia*) Smith and *Heracleum nepalense* (*H.nepalense*) D.Don were collected from the southern district of Sikkim. It was authenticated at Botanical survey of India, Gangtok, Sikkim. The voucher specimen was preserved in our laboratory for future reference. The leaf of *C.oppositifolia* and root of *H.nepalense* were dried in shade, pulverized in mechanical grinder and passed through 40-mesh sieve to get the powder of the plant parts.

3.2.2. Extraction procedure

Coarsely powered dry leaf of *Colebrookea oppositifolia* (1kg) as well as the powered dried root of *Heracleum nepalense* (1kg) were extracted separately by cold percolation with 3 liters of 70% methanol in a percolator for 72 h at room temperature [4]. The residue was removed by filtration. The solvent was then evaporated to dryness under reduced pressure in an Eyela Rotary Evaporator (Japan) at 42-45°C. The concentrated extract of the leave and root were kept in dessicator for further use.

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, ethyl acetate and acetone. The ethyl acetate soluble fraction was subjected to thin layer chromatographic analysis. The aqueous, acetone 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 Figure 3.1.

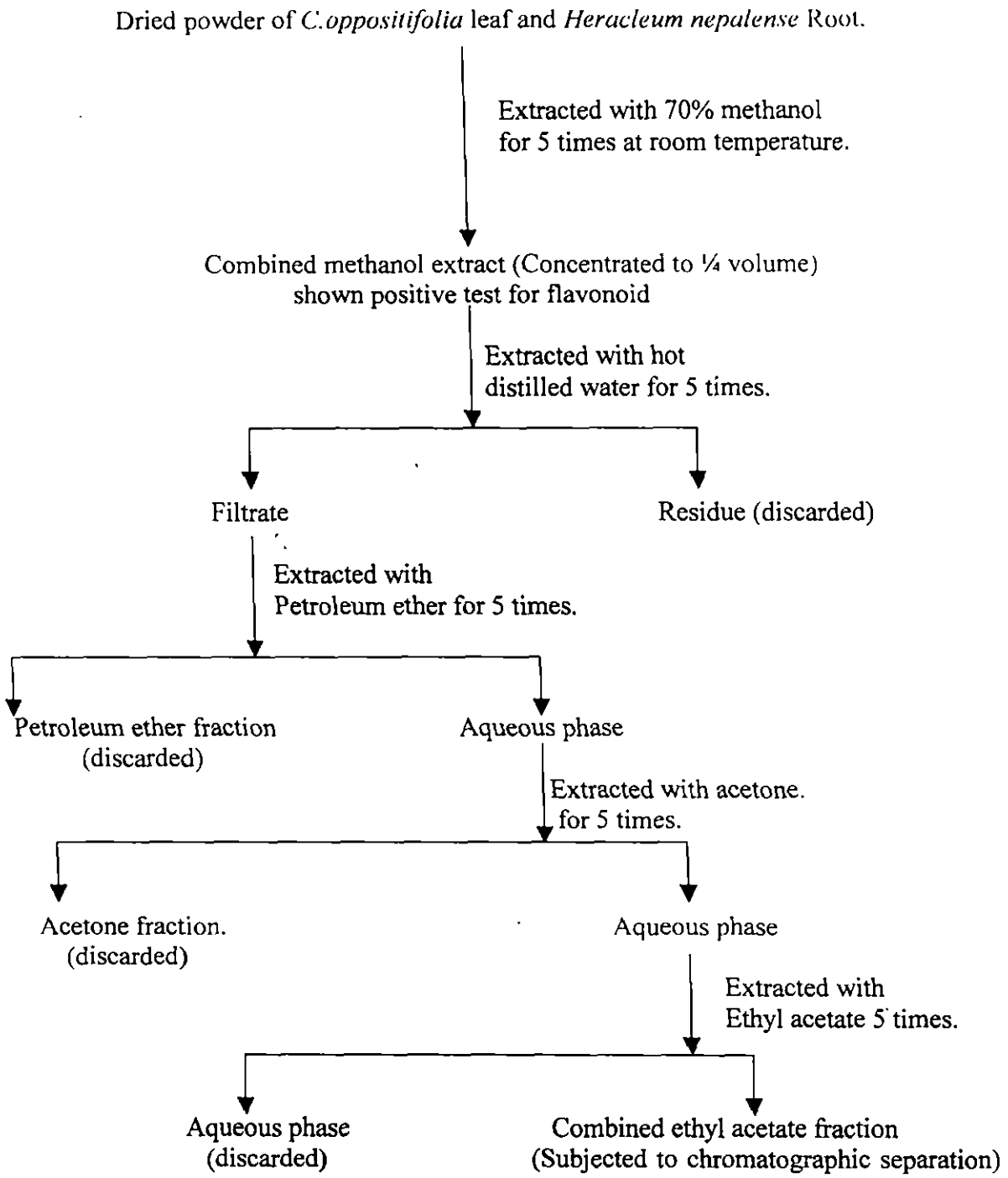


Figure 3.1. Flow chart showing the method of extraction of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root.

3.2.3. Isolation and purification of phytoconstituent from the leaf of *C. oppositifolia*

3.2.3.1. Preliminary phytochemical Test

The preliminary phytochemical group test of *C. oppositifolia* leaf extract was performed by the standard methods [5-7].

Tests for Alkaloids

- Small quantity of the leaf extract of *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* was dissolved in a few ml of distilled water and treated with Ninhydrin reagent at pH 5.0. The absence of purple coloration suggested the negative test for amino acids.
- Small quantity of leaf extract of *C.oppositifolia* 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 *C.oppositifolia* 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 coloured solution indicated the presence of proteins.

- A small quantity of leaf extract of *C.oppositifolia* 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 *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Bendict'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 *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Fehling's A&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 *C.oppositifolia* 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 *C.oppositifolia* 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 *C.oppositifolia* 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. Development of yellow colour in all the tubes demonstrated the presence of flavonoids.

- A small quantity of leaf extract of *C.oppositifolia* was dissolved in methanol. One piece of magnesium followed by concentrated hydrochloric acid was added drop wise to the test sample and heated. Appearance of magenta colour demonstrated the presence of flavonoids.

Test for Tannins

- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 10% aqueous potassium dichromate solution. Development of yellowish brown precipitate demonstrated the presence of tannins.
- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was allowed to react with 10% lead acetate solution. Formation of yellow colour precipitate indicated the positive test for tannins.
- Small quantity of leaf extract of *C.oppositifolia* 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. Formation of greenish black coloration demonstrated the presence of tannins.
- Small quantity of leaf extract of *C.oppositifolia* 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. Formation of white buff coloured precipitate demonstrated the presence of tannins.

Test for Saponins

- Small quantities of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and shaken in a graduated cylinder for 15 minutes. Formation of stable foam suggested the presence of saponins.

- A small quantity of leaf extract of *C.oppositifolia* was dissolved in methanol. 1.0 ml of extract solution was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins.

Test for Anthraquinones

- Small quantity of leaf extract of *C.oppositifolia* 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. Formation of rose pink colouration indicated the presence of anthraquinones.

Test for Gums and Mucilage

- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with equal volume of concentrated sulphuric acid. Then, it was treated 15% alcoholic solution of α-napthol (Molish's reagent). Formation of red-violet ring at the junction of the two layers indicated the positive test for gums (Molish's test).
- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 95% alcohol. Formation of precipitation indicated the presence of gums and mucilage.
- Small quantity of leaf extract of *C.oppositifolia* was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 0.008gm of ruthenium red in 10 ml of 10 % solution of lead acetate. Formation of red colour indicated the presence of gum and mucilage.

3.2.3.2. Thin Layer Chromatography of the methanol leaf extract of *C.oppositifolia*.

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. 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 plates were observed under UV light for the appearance of spots before and after spraying with spray reagents.

3.2.3.2.1. Solvent systems used

A. TLC 1. Glacial acetic acid: Water (15:85).

B. TLC 2. n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

C. TLC 3. Ethyl acetate: Formic acid: Water (10:2:3)

3.2.3.2.2. Spray reagents used

1% Aluminium chloride in ethanol.

Ammonia solution.

5% solution of 2-aminoethyl diphenyl borinate in methanol.

3.2.3.3. Coloumn Chromatography of leaf extract of *C.oppositifolia*.

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

Benzene: Ethyl acetate (with increasing amount of ethyl acetate), Benzene: Methanol (with increasing amount of methanol).

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 benzene. The solvent (benzene) 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 benzene 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 ethyl acetate fraction of *C.oppositifolia* leaf

The excess solvent on the top of the coloumn was allowed to flow down and then the dried mixture of ethyl acetate 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 benzene and 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 ethyl acetate and increasing the polarity with methanol in 10% stepwise elutions to 100% methanol. 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, yellowish 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 ethyl acetate fraction of *C.oppositifolia* 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 ethyl acetate fraction of *C.oppositifolia* 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 ethyl acetate fraction of *C.oppositifolia* 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. Glacial acetic acid: Water (15:85).

B. TLC 2. n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase).

C. TLC 3. Ethyl acetate: Formic acid: Water (10:2:3).

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 or exposing with Ammonia.

3.2.3.7. Ultraviolet-Visible absorption spectral analysis of the compound isolated from ethyl acetate fraction of *C.oppositifolia* leaf

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

and definition of the oxygenation pattern. In addition, the siting on the flavonoid nucleus of unsubstituted phenolic hydroxyl groups may be established by adding shift reagents to the sample solution and observing the resultant shifts in the absorption peaks. Thus, indirectly, the technique may be useful in determining the location of a sugar or methyl group attached to one of the phenolic hydroxyl groups.

The crystalline isolated compound was dissolved in spectroscopic grade methanol and the absorption spectra was taken in Shimadzu 1601 double beam UV-Visible spectrophotometer from 200 to 500 nm. The spectra was also recorded with the addition of Sodium methoxide (NaOMe), 5% Aluminium chloride ($AlCl_3$), sodium acetate (NaOAc) powder and (NaOAc) powder with boric acid (H_3BO_3).

3.2.3.8. Infrared spectrum of the compound isolated from ethyl acetate fraction of *C.oppositifolia* 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 ethyl acetate fraction of *C.oppositifolia* leaf

The ^{1}H and ^{13}C NMR spectra of the isolated compound from ethyl acetate fraction of *Colebrookea oppositifolia* leaves 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 spectra of the compound isolated from ethyl acetate fraction of *Colebrookea oppositifolia* leaf

Mass spectrum ($FAB^{+}MS$) of the isolated compound from the leaves of *Colebrookea oppositifolia* was recorded on a JEOL JMS 600 Spectrophotometer

3.2.3.11. Elemental analysis of the compound isolated from ethyl acetate fraction of *C.oppositifolia* leaf

The isolated compound was dried at 60°C in a high vacuum for 8 hr and the elemental analysis was performed with Perkin Elmer 2400 elemental analyzer. In the analysis, the percentage of carbon, hydrogen and nitrogen was determined.

3.2.3.12. Sugar analysis of the compound isolated from ethyl acetate fraction of *C.oppositifolia* leaf

1 mg of the isolated compound was hydrolyzed by addition of 1% hydrochloric acid, boiled under reflux for 2 hr. The hydrosylate was analyzed for sugars (co-TLC) with standard sugars by using Ethyl alcohol: Ammonium hydroxide: Water (20:1: 4) as mobile phase [14]. They were visualized by spraying with aniline phthalate and heating at 105°C.

3.2.4. Isolation and Purification of Phytoconstituent from the root of *H.nepalense*

The methods used for isolation and purification of phytoconstituents from the root of *H.nepalense* were as per the procedure followed for the isolation and purification of phytoconstituents from the leaf extract of *C.oppositifolia* described in 3.2.3.1 to 3.2.3.12 expect 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 chloroform: 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 to result an isolated flavonoidal glycoside.

3.3. Results

3.3.1. Phytochemical Study of *C.oppositifolia* leaf

The concentrated methanol extract obtained from the shade-dried leaf of *C.oppositifolia* was fractionated successively with petroleum ether, acetone and ethyl acetate. It was observed that only the ethyl acetate 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, tannins, saponins and reducing sugar (Table 3.1). The thin layer chromatographic study of the ethyl acetate fraction showed the presence of three components with selected mobile phase and spraying reagents (Table 3.2-3.4). 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 3.5). They were purified with a silica gel (60-120) coloumn, which yielded a flavonoidal glycoside (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 3.6 & Table 3.7. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data [8-12].

Compound I was obtained as yellow needle shaped crystal, soluble in water & methanol. The compound was melted at 181-185°C. The complete acid hydrolysis of compound I gave Catechin and Rhamnose. The compound showed strong absorption at 281 nm in its spectrum, which implied the presence of phenolic aromatic rings. The UV absorption spectrum of compound I is shown in Figure 3.1. The UV spectrum showed absorption bands reagents shifts of the compound to be a 7-substituted derivatives. The absence of free 7-hydroxyl group in the compound was observed in lack of shift of Band II in the presence of NaOAc (Table 3.8). The IR spectrum of the compound is presented in Figure 3.2. The IR spectrum shows the presence of absorption bands at 3480, 1029 and 1078 cm^{-1}. Other prominent peaks were 1866, 1771, 1632, 1606, 1519, 1456, 1286, 1146, 1113, 1029, 963, 731, 578 cm^{-1}. The IR spectrum confirmed the presence of aromatic ring (1632 cm^{-1}) and hydroxyl group (3480 cm^{-1}) in compound I.

The ^{1}H NMR spectrum of compound I is presented in Figure 3.3. In the ^{1}H NMR spectra in DMSO-d_6, the signals for aglycone were the same as for 7-O substituted catechin

(Table 3.9). The doublets at 6.212 ppm (J=2.0 MHz) and 6.226 ppm (J=2.0 MHz) in its ^{1}H NMR spectrum were suggestive of two aromatic protons existing at the *meta* positions[11-13]. The signals at 6.591 ppm (J=2.0 Hz, J=7.6 Hz), 6.681 ppm (J=7.6Hz) and 6.731 ppm (J=2.0 Hz) inferred the presence of another set of aromatic protons existing at 1,3,4 positions. The signals at 2.506 ppm and 2.699 ppm, and the double double doublet at 3.476 ppm suggested that the compound should be a flavan-3-ol derivative. Existence of a sugar in its structure was evidenced by the anomeric proton signal, which appeared at a 5.698 ppm (J= 3.0 Hz). The coupling constant (J= 3.0 Hz) of the anomeric proton sugar suggested that the phenoxy group be attached to the anomeric carbon atom by β-configuration. The fragmentation ion at m/z 291 in its mass spectrum, which is presented in Figure 3.5, inferred that the compound should have (+) – Catechin as an aglycone. The structure of the compound was further confirmed by ^{13}C NMR and elemental analysis of Catechin from the available literature [9, 13]. The signals of C-5, C-7 and C-9 were observed at higher value than 150 ppm, the signals at 156.75 ppm only showed correlations with these of H-6 and H-8 (at 6.212 ppm and 6.226 ppm respectively), we assigned it to C-7 (Figure 3.4). The signals at 155.68 ppm and 156.52 ppm showed correlations with those of H-6 and H-8 respectively, so we assigned them to C-5 and C-9 respectively. The correlation of the anomeric proton signals at 5.698 ppm with that of C-7 at 156.75 ppm suggested that the sugar should be attached at the C-7 position. In the elemental analysis of compound I, the percentage of carbon, hydrogen and nitrogen was found to be 54.12, 4.24 and nil respectively. The result is corresponding to the molecular formula $C_{24}H_{22}O_8$ (confirmed by elemental analysis). From these data we concluded that the structure of the isolated flavonoid compound I is (+)–Catechin-7-O-β-rhamnopyranoside.

Table 3.1. Preliminary phytochemical test of methanol extract of *C.oppositifolia* leaf and *H.nepalense* root.

	C.oppositifolia		*H.nepalense*	
Phytoconstituents	Methanol Extract	Ethyl acetate Fraction	Methanol Extract	Ethyl acetate Fraction
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 ethyl acetate fraction of the methanol extract of *C.oppositifolia* leaf.

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	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	83	59	58
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	29	52
3	–	–	Green	–	–	Yellowish green.	–	–	41

Spray reagent: 1% Aluminium chloride in ethanol.

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

Table 3.3. Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *C.oppositifolia* leaf.

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after exposure to NH_3			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	83	59	58
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	29	52
3	–	–	Green	–	–	Yellowish green.	–	–	41

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

Table 3.4. Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *C.oppositifolia* leaf.

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	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	Bright yellow	83	59	58
2	Yellow	Yellow	Yellow	Orange	Orange	Orange	64	29	52
3	–	–	Green	–	–	Yellowish green.	–	–	41

Spray reagent: 5% solution of 2-aminoethyl diphenyl borinate in methanol.

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

Table 3.5. Coloumn and Thin layer chromatography separation of ethyl acetate fraction of *C.oppositifolia* leaf.

Eluent Benzene: 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 yellowish spots. (59, 54)	Mixture of compounds
30:70	44-51	6.0	Two bright yellow spots. (58, 54)	Mixture of compounds
20:80	52-54	4.5	Two yellowish spots (59, 54)	Mixture of compounds
10:90	55-56	3.5	Three red spot (84, 57, 29)	Mixture of compounds
0:100	57-58	2.0	Nil	Colourless solid

Spray reagent: 1% Aluminium chloride in ethanol.

Table 3.6. Qualitative analysis of the compound I isolated from ethyl acetate fraction of *C. oppositifolia* leaf.

S.no	Treatment	Observation	Inference
1	1 mg of the crystalline solid was dissolved in 0.5 ml of methanol and treated with 1 ml of dilute ammonia solution.	A bright yellow colour.	Presence of flavonoid.
2	1 mg of the crystalline solid was dissolved in 1 ml of methanol and treated with 1 ml of 0.1 N sodium hydroxide solutions.	A bright yellow colour.	Presence of flavonoid.
3	1 mg of the crystalline solid was dissolved in 1 ml of methanol and treated with 1 ml of dilute sodium carbonate solution.	A bright yellow colour.	Presence of flavonoid.
4	1 mg of the crystalline solid was dissolved in 1 ml of methanol and allowed to react with 5% alcoholic ferric chloride solution.	Olive green colour.	Presence of flavonoid.
5	1 mg of the crystalline solid was treated with 1 ml of concentrated hydrochloric acid.	Intense yellow with green florescence.	Presence of flavonoid
6	1 mg of the crystalline solid was treated with magnesium hydrochloride solution (Shinoid's test)	Red to magenta colour.	Presence of flavonoid

All of the above mentioned colour reactions are identical with the reference literature for flavonoid [1, 8, 9].

Table 3.7. Thin layer chromatography of the compound I isolated from ethyl acetate fraction of *C.oppositifolia* leaf.

Solvent system	hR_f values	Colour of fluorescent produced			
		UV_{366nm}	UV_{366nm} +$AlCl_3$	UV_{366nm} +NH_3	UV_{366nm} +ADB
TLC 1	64	Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 2	59	Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 3	41	Yellow	Bright Yellow	Bright Yellow	Bright Yellow

$AlCl_3$: Aluminium chloride in ethanol; NH_3: Ammonia; ADB: 5% solution of 2-aminoethyl diphenyl borinate in methanol.

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

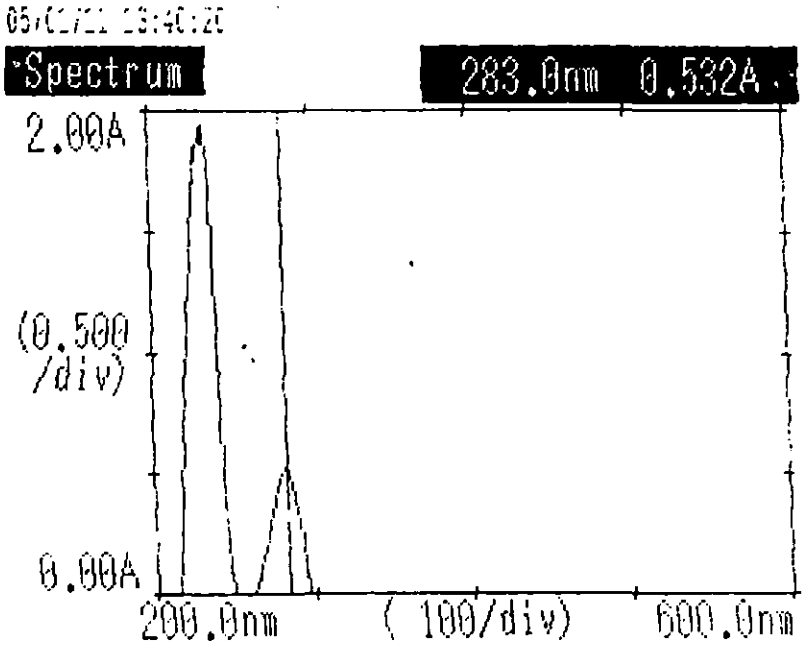
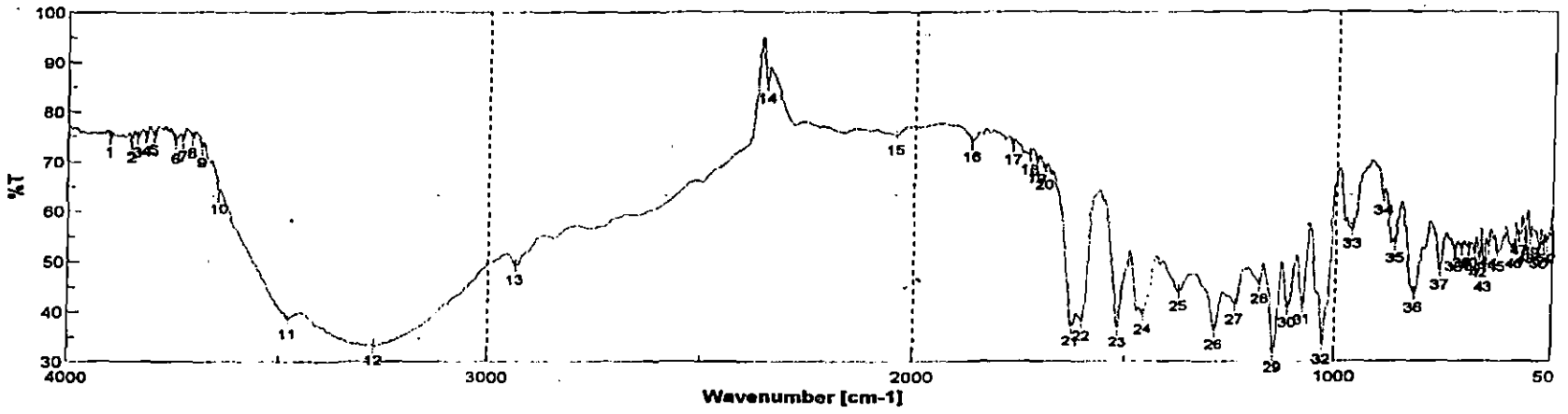


Figure 3.1. UV-Visible Spectro photometric analysis of compound I from *C.oppositifolia* leaf extract.



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	Auto (32)	Scanning Speed	Auto (2 mm/sec)
Date/Time	1/7/2005 5:49PM	Update	1/11/2005 6:16PM
Operator	msn		
File Name	sidash-c.oppositifolia.jws		
Sample Name	pymtf-1		
Comment			

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3900.32	74.6083	2	3852.11	73.7005	3	3838.61	74.6846	4	3818.36	74.9047	5	3799.08	75.1178
6	3748.94	73.5257	7	3732.55	73.8894	8	3710.37	74.5506	9	3688.19	72.6248	10	3647.7	63.4221
11	3480.88	38.5811	12	3278.47	33.1736	13	2937.06	49.2171	14	2348.87	85.4135	15	2046.1	75.0259
16	1867.72	73.5728	17	1771.3	73.0351	18	1732.73	71.0013	19	1716.34	69.4906	20	1697.05	68.1782
21	1632.45	36.9995	22	1606.41	38.0989	23	1519.83	36.812	24	1456.96	39.4063	25	1368.25	43.9113
26	1286.29	36.2951	27	1237.11	41.2983	28	1181.19	45.4316	29	1146.47	31.6571	30	1113.69	40.6449
31	1078.01	41.2623	32	1029.8	33.0952	33	963.269	56.3393	34	892.88	63.0292	35	867.81	53.4416
36	823.455	43.6056	37	764.637	48.1111	38	731.853	51.6932	39	715.461	52.5072	40	699.069	52.4487
41	685.57	51.4253	42	674.963	50.5806	43	663.393	47.8805	44	651.822	52.5055	45	631.573	51.772
46	589.147	52.3518	47	578.54	55.0131	48	562.148	53.2168	49	551.542	54.5801	50	531.293	52.317
51	521.65	52.8595	52	513.936	53.3909									

Figure 3.2. IR spectrum of compound I isolated from *C.oppositifolia* leaf extract.

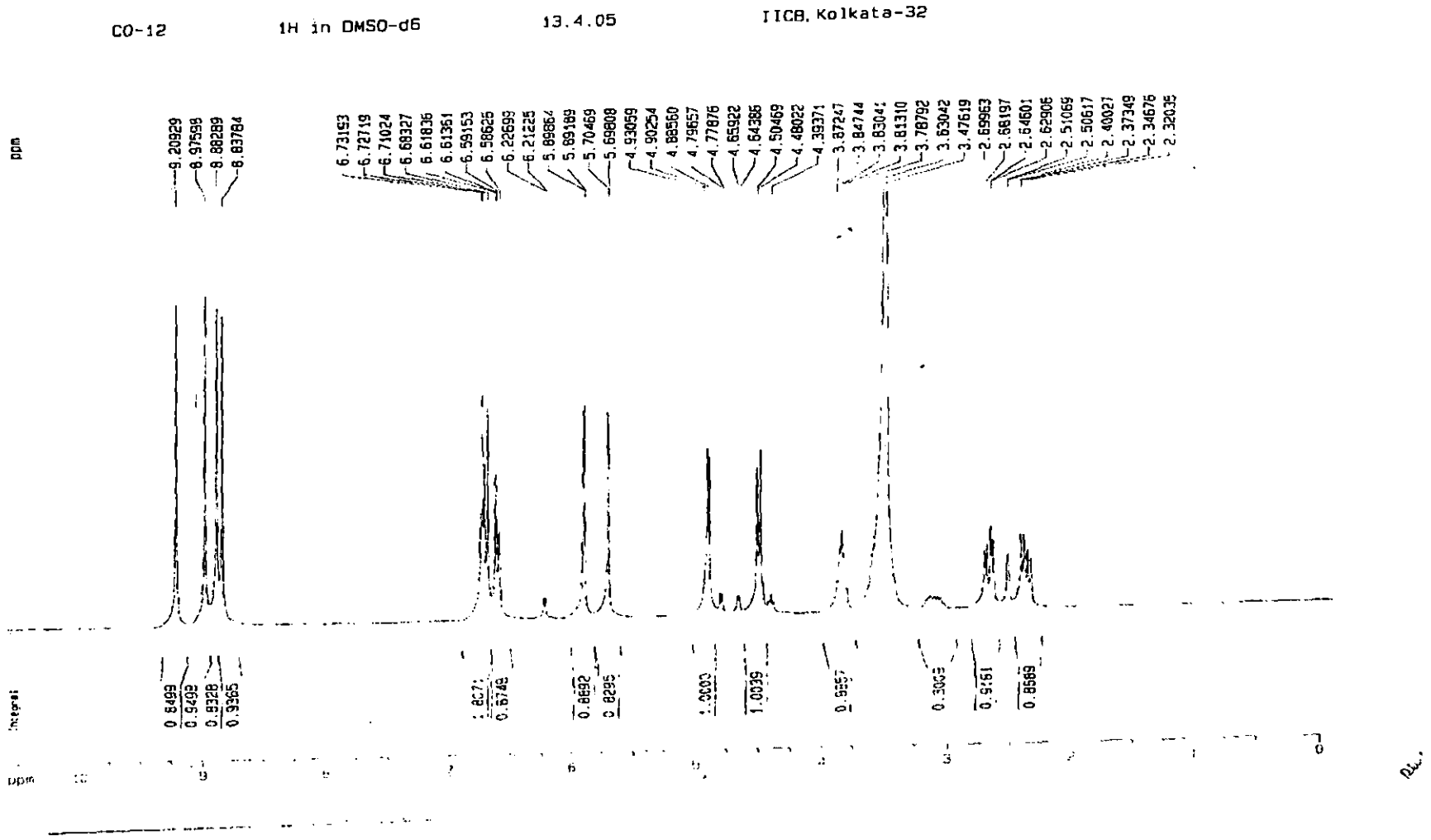


Figure 3.3. ^{1}HNMR spectrum of compound I isolated from *C.oppositifolia* leaf extract.

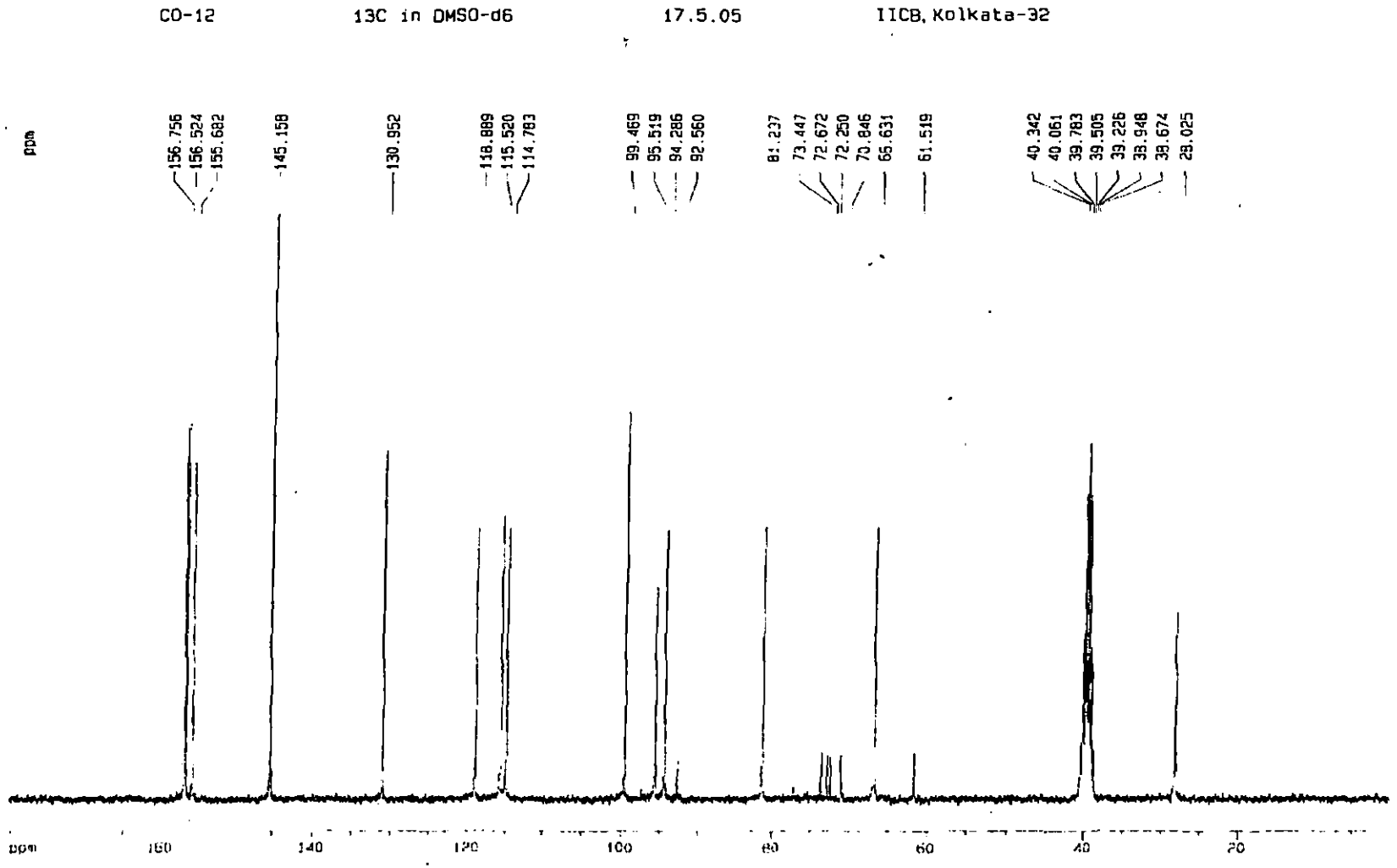


Figure 3.4. ^{13}CNMR spectrum of compound I isolated from *C.oppositifolia* leaf extract.

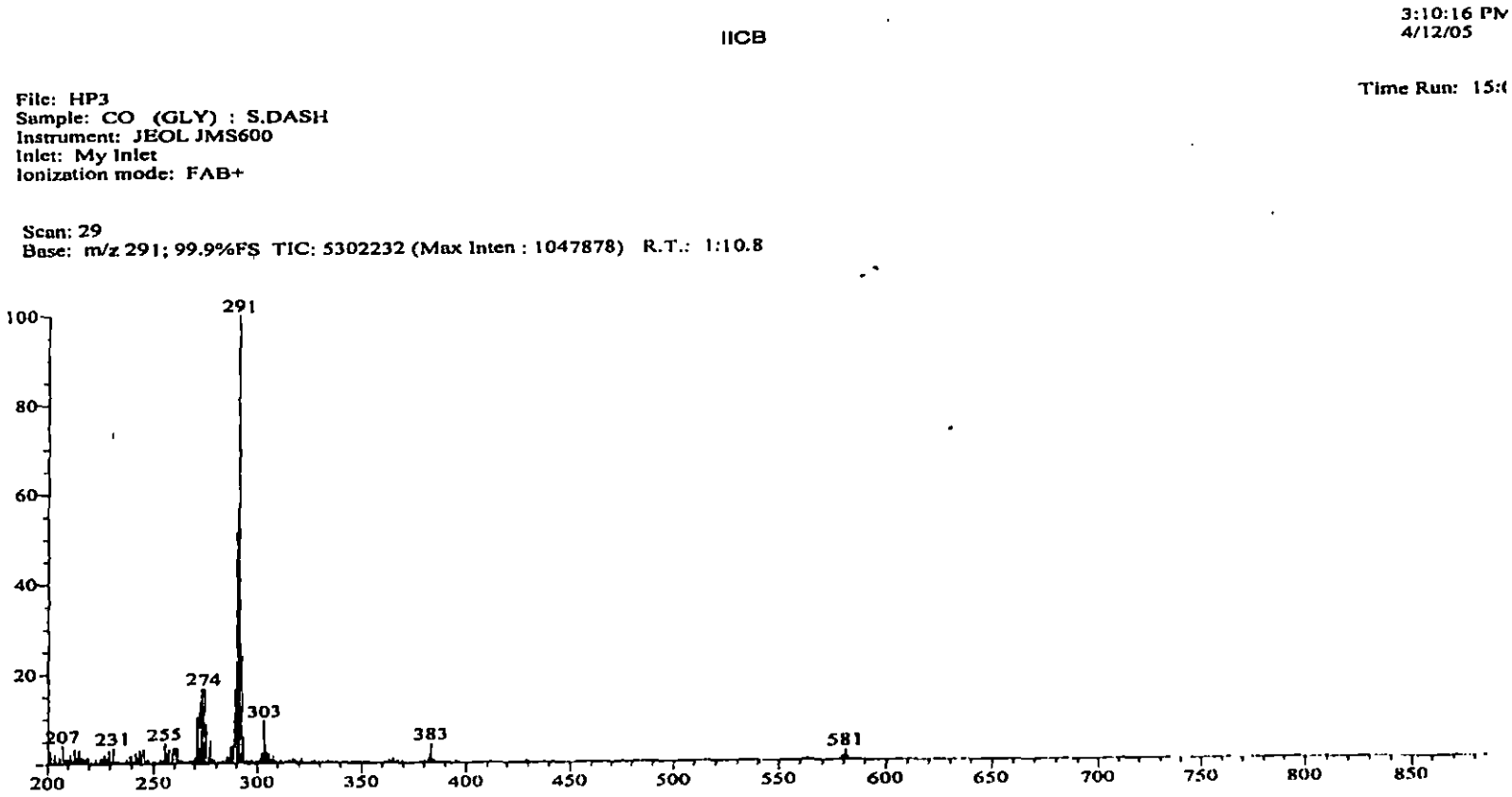


Figure 3.5. Mass spectrum of compound I isolated from *C.oppositifolia* leaf extract.

Table 3.8. UV spectral data of compound I isolated from ethyl acetate fraction of *C.oppositifolia* leaf.

Sl. No	Preparation	λ_{max} (nm) of compound I	Spectral effect	Structural diagnosis
1	Methanol solution of compound I.	241, 281		Flavan-3-ol
2	Methanol solution of compound I + 3 drops of sodium methoxide solution	250,293	69 nm Bathochromic shift in Band I	4'-OH free
3	Methanol solution of compound I + 6 drops of Aluminium chloride	248,282	25 nm Shift in Band I	5-OH Free
4	Methanol solution of compound I + 6 drops of Aluminium chloride and 3drops of Hydrochloric acid.	280,314,361	30 nm shift in Band I	Presence of di-OH in B ring
5	Methanol solution of compound I + powdered NaOAc	243, 283	Lack of shift in Band II	Absence of free 7-hydroxyl group
6	Methanol solution of compound I + powdered NaOAc and H_3BO_3	281	12 nm shift in Band I relative to methanol.	Presence of di-OH in B ring

[Note – Structural analysis was done on basis of reference literature for flavonoid.]

Table 3.9 ^{1}H and ^{13}C NMR spectral data of compound I isolated from ethyl acetate fraction of *C.oppositifolia* leaf.

Position of C/H	Department	Value of δ H	Value of δ C
2	C		145.1
3	C		130.95
4	CH	2.506, 2.699	81.23
5	C		155.68
6	CH	6.226	94.28
7	C		156.75
8	CH	6.212	92.56
9	C		156.52
10	C		99.46
1'	C		118.88
2'	CH	6.731	95.51
3'	C		115.52
4'	C		114.78
5'	CH	6.681	92.56
6'	CH	6.591	91.23
7-rha			
1"	CH	5.698	72.67
2"	CH	4.393	72.25
3"	CH		70.84
4"	CH	3.787-3.872	61.51
5"	CH	3.476	66.63
6"	CH_3	2.320	28.51

3.3.2. Phytochemical study of *H.nepalense* root.

The concentrated methanol extract prepared from the shade-dried root of *H.nepalense* was fractionated successively with petroleum ether, acetone and ethyl acetate. Different fractions of the extract were undertaken for preliminary pharmacological studies. It was observed that the ethyl acetate 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, tannins, saponins and reducing sugar (Table 3.1). The thin layer chromatographic study of ethyl acetate fraction of methanol extract showed the mixture of three components with selected mobile phase and spraying reagents (Table 3.10-3.12). This fraction was subjected to coloumn chromatography, which yielded sixty-two fractions. The fractions having identical hRf values were mixed together (Table 3.13). They were purified with a silica gel coloumn, by using chloroform: methanol as mobile phase, which yielded a flavonoidal glycoside (compound II). It was further subjected to chemical tests and TLC to confirm the chemical nature and was depicted in Table 3.14 & 3.15. 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 flavonoidal glycoside [8-20].

Compound II was obtained as yellow needle shaped crystal, partially soluble in water & soluble in methanol. The compound was melted at 210-214°C. The complete acid hydrolysis of compound II gave Quercetin and glucose. The UV analysis (Table 3.16) proved the absence of 3-hydroxyl group in the compound [47 nm bathochromic shift of Band I (in MeOH) in Aluminium chloride and hydrochloric acid] (Figure 3.6). Therefore, it was indicated that the compound was monodesmoside [15]. The IR spectrum shows the presence of absorption bands at 3648- 3611 cm^{-1} ν (OH), 2904 cm^{-1} ν (C=O) in flavone, 1615- 1507 cm^{-1} ν (aromatic rings), 1304 cm^{-1}, 1263 cm^{-1}, 1132 cm^{-1}, 942 cm^{-1} and 768 cm^{-1} (Figure 3.7). In the ^{1}HNMR spectra (Table 3.17) in DMSO- d_6, the signals for aglycone were the same as for 3-O substituted quercetin [16]. The doublet of the glucose

anomeric proton appeared (Figure 3.8) at 4.92 ppm (J= 7.5 Hz) and assigned to β-glucopyransone linked to quercetin at C-3 [17]. The fragmentation ion at 303 (98.8) in its mass spectrum inferred that the compound contains quercetin as aglycone (Figure 3.10). The structure of the compound was further confirmed by ^{13}C NMR and elemental analysis [9, 10, 15, 16, 17, 18, 20]. The observed chemical shifts for carbon atoms of aglycone and sugar at C-3 (Figure 3.9) are consistent with those for the quercetin-3-glucopyranoside at C-3 (C"-98.4 ppm) [17]. The isolated compound II showed significant peaks in its mass spectra (Figure 3.10) at m/z 495 (10.2), 415 (15.8), 371 (20.0), 327 (19.2), 303 (98.8), 301 (29.7), 287 (10.2), 277 (12.0), 239 (8.2), 207 (8.0). This data showed a molecular ion at 415 (M^+); which corresponds to be molecular formula $C_{19}H_{22}O_{12}$ as confirmed by elemental analysis. The percentage of carbon, hydrogen and nitrogen in the compound was found to be 48.70, 5.29 and nil respectively. From these data it is concluded that the structure of the isolated flavonoid was quercetin-3-O-β-D-glucopyranoside.

Table 3.10 Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *H.nepalense* root.

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	Yellow	Yellow	Yellow	Bright yellow	Bright yellow	Bright yellow	68	54	69
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	28	49
3	–	–	Green	–	–	Yellowish green.	–	–	41

Spray reagent: 1% Aluminium chloride in ethanol

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

Table 3.11 Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *H.nepalense* root

No of Spots	Colour of spots under long wave UV light			Colour of spots under long wave UV light after exposure to NH_3			hR_f values		
	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3	TLC 1	TLC 2	TLC 3
1	Yellow	Yellow	Yellow	Bright yellow	Bright yellow	Bright yellow	68	54	69
2	Yellow	Yellow	Yellow	Yellow Florescence	Yellow Florescence	Yellow Florescence	64	28	49
3	–	–	Green	–	–	Yellowish green.	–	–	41

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

Table 3.12. Thin Layer Chromatography study of ethyl acetate fraction of the methanol extract of *H.nepalense* root.

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	Yellow	Yellow	Yellow	Bright yellow	Bright yellow	Bright yellow	68	54	69
2	Yellow	Yellow	Yellow	Orange	Orange	Orange	64	28	49
3	–	–	Green	–	–	Yellowish green.	–	–	41

Spray reagent: 5% solution of 2-aminoethyl diphenyl borinate in methanol.

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

Table 3.13. Coloumn and Thin layer chromatography separation of ethyl acetate fraction of *H.nepalense* root.

Eluent Benzene: ethyl acetate	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: 1% Aluminium chloride in ethanol

Table 3.14. Qualitative analysis of the compound II from ethyl acetate fraction of *H.nepalense root*.

Sl.No	Treatment	Observation	Inference
1	1 mg of the crystalline solid was dissolved in 0.5 ml of methanol and treated with 1 ml of diluted ammonia solution.	A bright yellow colour.	Presence of flavonoid.
2	1 mg of the crystalline solid was dissolved in 1 ml of methanol and treated with 1 ml of 0.1 N sodium hydroxide solutions.	A bright yellow colour.	Presence of flavonoid.
3	1 mg of the crystalline solid was dissolved in 1 ml of methanol and treated with 1 ml of diluted sodium carbonate solution.	A bright yellow colour.	Presence of flavonoid.
4	1 mg of the crystalline solid was dissolved in 1 ml of methanol and allowed to react with 5% alcoholic ferric chloride solution.	Olive green colour.	Presence of flavonoid.
5	1 mg of the crystalline solid was treated with 1 ml of concentrated hydrochloric acid.	Intense yellow with green florescence.	Presence of flavonoid.
6	1 mg of the crystalline solid was treated with magnesium hydrochloride solution (Shinoid's test)	Red to magenta red colour.	Presence of flavonoid.

All of the above mentioned colour reactions are identical with the reference literature for flavonoid [1, 8, 9].

Table 3.15. Thin layer chromatography of the compound II isolated from ethyl acetate fraction of *H.nepalense* root.

Solvent system	hR_f values	Colour of the fluorescent produced			
		UV_{366nm}	UV_{366nm} +$AlCl_3$	UV_{366nm} +NH_3	UV_{366nm} +ADB
TLC 1	69	Pale Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 2	55	Pale Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 3	41	Pale Yellow	Bright Yellow	Bright Yellow	Bright Yellow

$AlCl_3$: Aluminium chloride in ethanol; NH_3: Ammonia; ADB: 5% solution of 2-aminoethyl diphenyl borinate in methanol.

TLC 1: Glacial acetic acid: Water (15:85).

TLC 2: n-Butanol: Glacial acetic acid: Water (4:1:5 organic phase)

TLC 3: Ethyl acetate: Formic acid: Water (10:2:3)

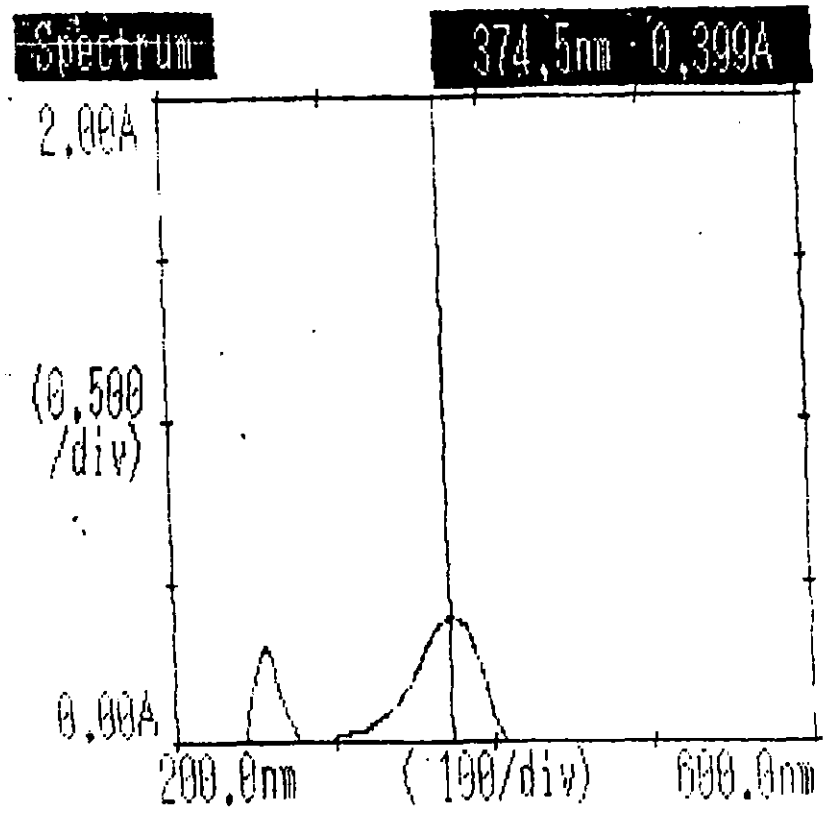
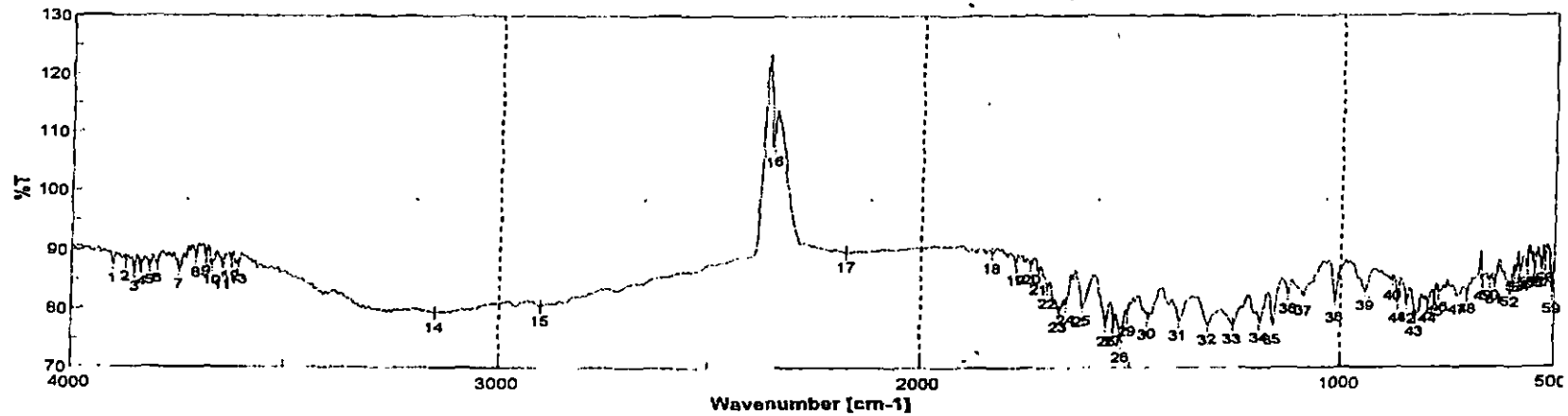


Figure 3.6. UV-Visible Spectro photometric analysis of compound II from *H.nepalense* root extract.



Accumulation	16	Resolution	4 cm-1
Zero Filling	ON	Apodization	Cosine
Gain	Auto (32)	Scanning Speed	Auto (2 mm/sec)
Date/Time	1/7/2005 5:33PM	Update	1/11/2005 5:17PM
Operator	mm		
File Name	skdsah-H. nepalense.jws		
Sample Name	pymtf-1		
Comment			

No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T	No.	cm-1	%T
1	3901.29	87.5754	2	3871.4	87.9453	3	3852.11	86.1638	4	3837.65	87.0306	5	3816.38	87.1426
6	3800.04	87.4405	7	3749.9	86.5231	8	3711.33	88.5096	9	3688.19	88.7485	10	3674.69	87.2105
11	3648.66	88.4514	12	3628.41	87.9507	13	3611.05	87.3781	14	3148.22	79.4085	15	2904.27	80.515
16	2347.91	107.41	17	2172.42	89.6264	18	1829.16	89.3982	19	1771.3	87.4239	20	1733.69	87.4787
21	1716.34	85.7855	22	1697.05	83.4544	23	1669.09	79.4463	24	1653.66	80.6909	25	1615.09	80.4333
26	1558.2	77.0754	27	1540.85	77.0906	28	1522.52	74.2389	29	1507.1	78.6935	30	1456.96	78.2279
31	1384.64	77.9328	32	1319.07	77.3197	33	1263.15	77.3191	34	1199.51	77.6034	35	1168.65	77.2709
36	1132.01	82.4384	37	1093.44	82.1123	38	1015.34	80.9954	39	942.056	82.9564	40	877.452	84.6844
41	864.917	80.8617	42	843.704	80.8026	43	823.455	78.5791	44	794.528	80.7525	45	779.101	81.9739
46	768.494	82.6587	47	725.104	82.2054	48	701.962	82.2845	49	685.321	84.4869	50	648.929	84.5789
51	637.358	83.9686	52	602.646	83.4754	53	589.147	86.1189	54	579.504	86.0268	55	562.148	86.8273
56	545.758	86.5099	57	528.4	86.699	58	520.686	87.426	59	504.294	82.2061			

Figure 3.7. IR spectrum of compound II isolated from *H.nepalense* root extract.

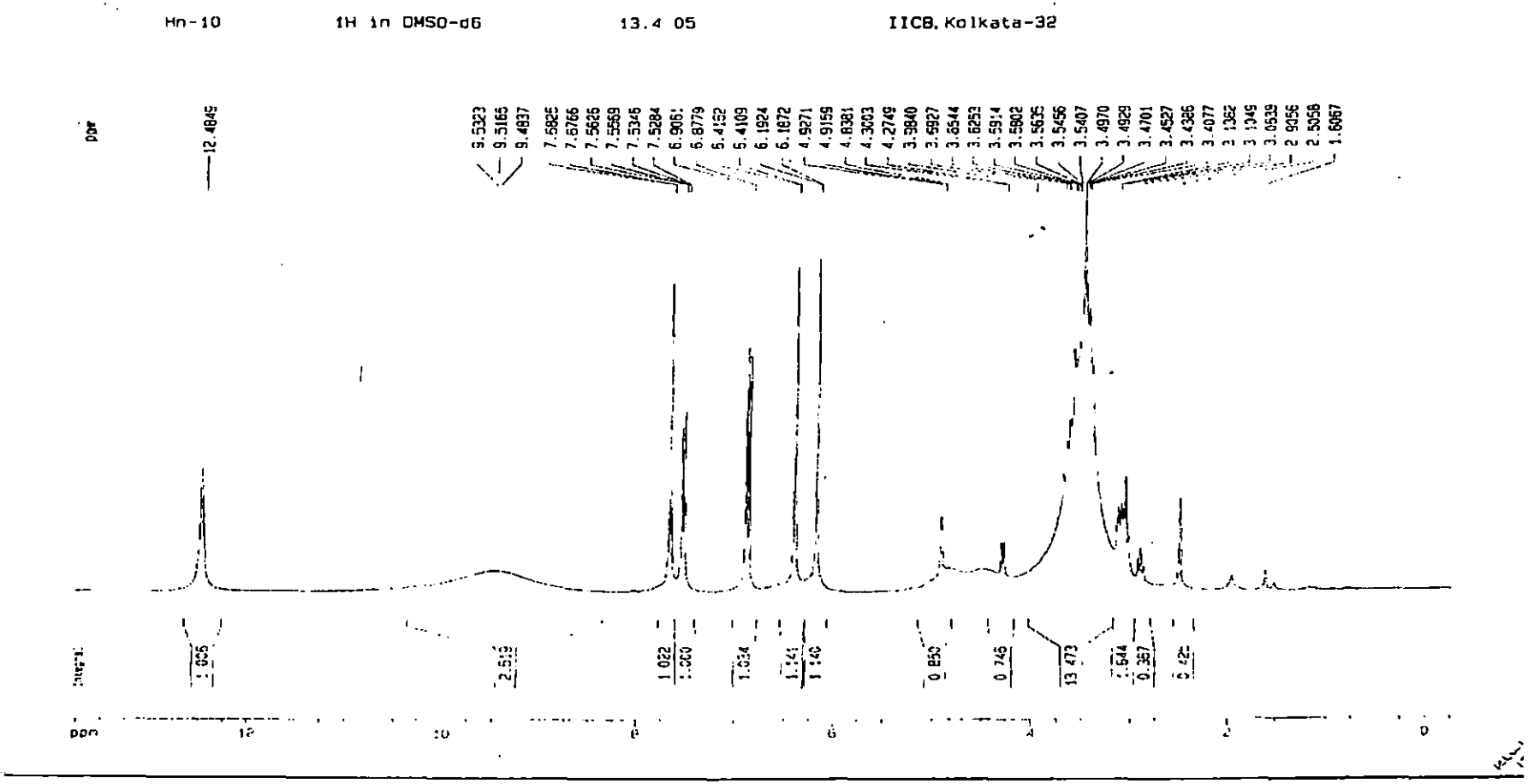


Figure 3.8. ^{1}H NMR spectrum of compound II isolated from *H.nepalense* root extract.

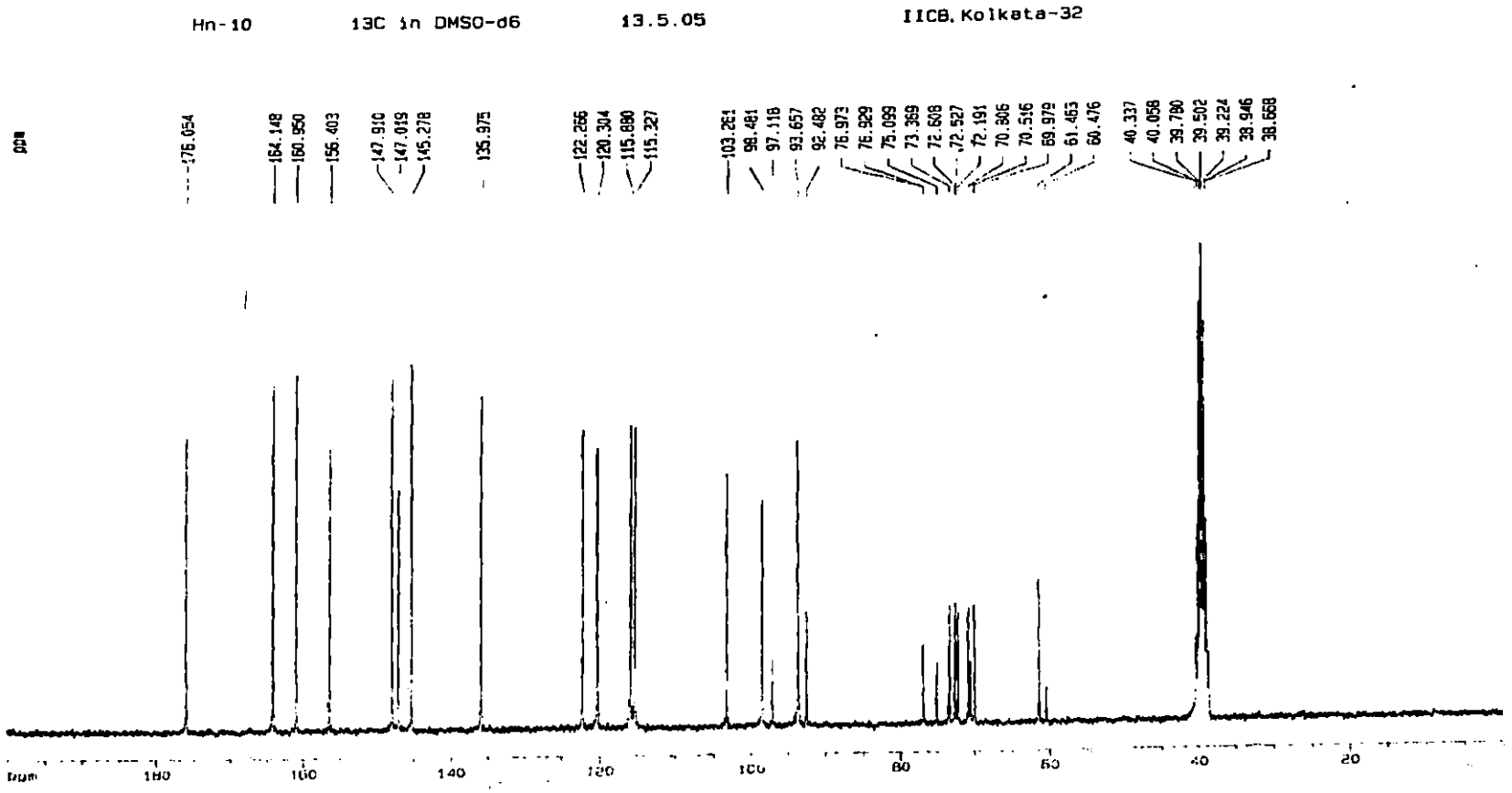


Figure 3.9. ^{13}C NMR spectrum of compound II isolated from *H.nepalense* root extract.

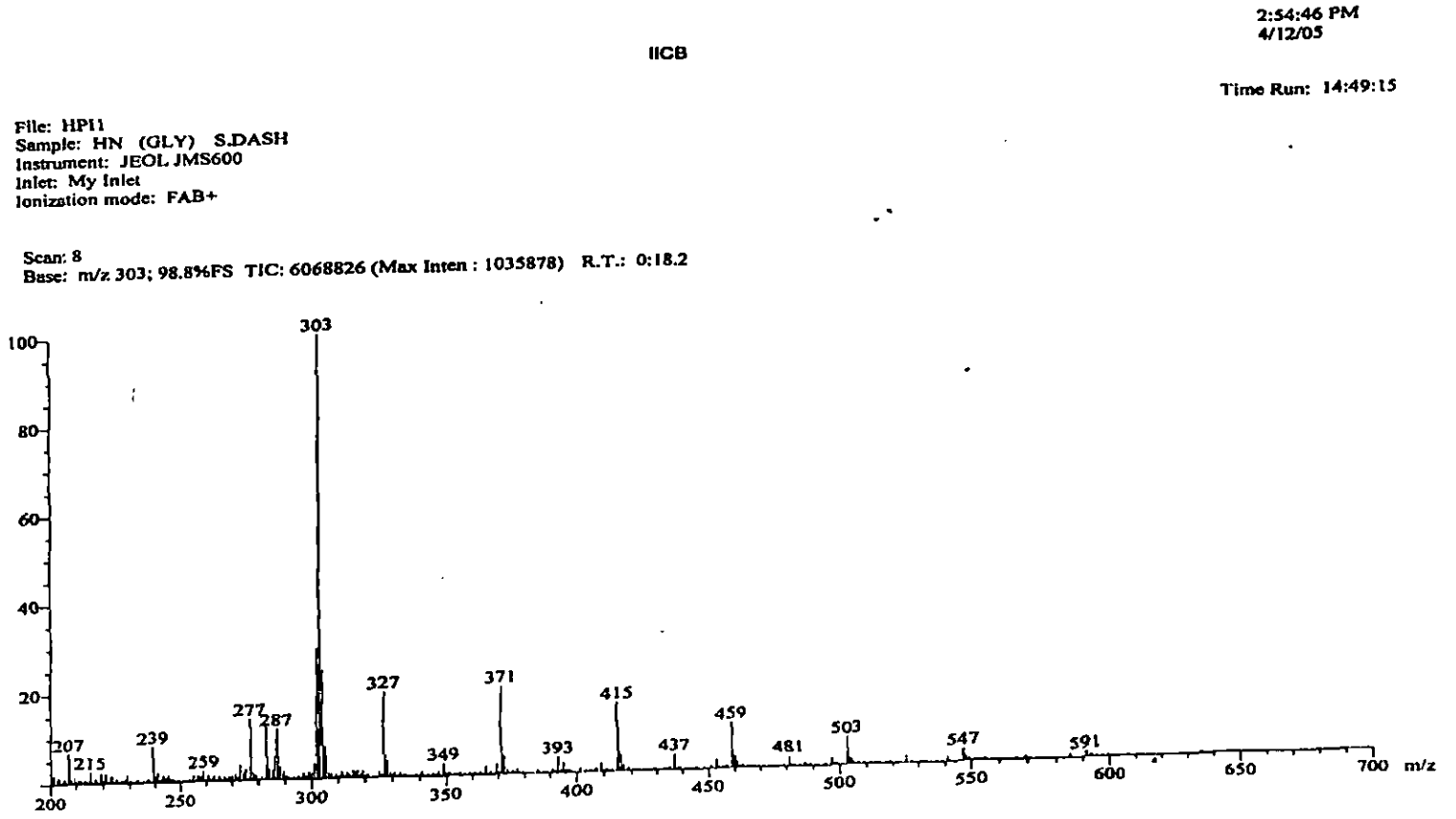


Figure 3.10. Mass spectrum of compound II isolated from *H.nepalense* root extract.

Table 3.16. UV spectral data of isolated compound II isolated from ethyl acetate fraction of *H.nepalense* root.

Sl.No	Preparation	λ_{max} (nm) of compound II	Spectral effect	Structural diagnosis
1	Methanol solution of compound II.	251, 374	–	Flavonol
2	Methanol solution of compound II + 3 drops of sodium methoxide solution	246, 328, 425	Rapid degradation of spectrum	3', 4'-OH free
3	Methanol solution of compound II + 6 drops of Aluminium chloride	272, 310	35 nm Shift in Band I	5-OH Free
4	Methanol solution of compound II + 6 drops of Aluminium chloride and 3drops of Hydrochloric acid.	268, 304, 361	47 nm shift in Band I	Lack of free 3-OH
5	Methanol solution of compound II + powdered NaOAc	269, 314	35 nm shift in Band II	7-OH
6	Methanol solution of compound II + powdered NaOAc and H_3BO_3	268, 300	12 nm shift in Band I relative to methanol.	Presence of di-OH in B ring

[Note – Structural analysis was done on basis of reference literature for flavonoid.]

Table 3.17. ^{1}H and ^{13}C NMR spectral data of compound II isolated from ethyl acetate fraction of *H.nepalense* root.

Position of C/H	Department	Value of δ H	Value of δ C
2	C		156.403
3	C		135.975
4	C		176.054
5	C	12.48 (OH)	160.950
6	CH	6.18	93.657
7	C		164.148
8	CH	6.41	92.482
9	C		147.910
10	C		103.261
1'	C		122.266
2'	CH	7.56	115.327
3'	C		145.278
4'	C		147.910
5'	CH	6.87	115.327
6'	CH	7.56	120.304
3-Glu			
1"	CH	4.92	98.481
2"	CH		75.099
3"	CH		76.929
4"	CH		69.979
5"	CH		76.973
6"	CH_2	1.60	60.472

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CHAPTER 4

ACUTE TOXICITY STUDIES

4.1. Introduction

Toxicology is the science that deals with the adverse effect of chemicals on living organisms. Toxicity tests are focused at discerning the compilations arising from the therapeutic use of the drug. Special attention should be paid to the solvent and dispersing agents as the toxic effect may arise from these sources. Animal care during the period of the toxicity tests is also of paramount importance.

Traditional use of herbal drugs may broadly be divided into three categories as follows

- Those are well known and have been widely used for many years.
- Those are not well known in the country but for which international experience is available.
- That represent a new compound hitherto not evaluated as to its safety and efficacy.

The first category consists chiefly of foodstuff product (s), which have been in use for a long time as traditional herbal remedies, and the requirements are limited. In general, it seems unnecessary to require the proof of safety of these products. For the second category, views concerning the type of documents required to be presented may differ from country to country. So it is necessary that varieties of requirements will be elaborated for these products covering anything from reference in scientific literature confirming that the product is safe. To satisfy the demands for limited or shortened toxicological testing of these products, an investigation must be carried out on toxicity profile. The third group, where the authority is faced with a product not previously screened for its toxicological properties, toxicity studies of those product must have to be undertaken.

The index of the acute toxicity is LD_{50} (median lethal dose at which 50 percent of the population dies), which should not be regarded as a biological constant, since different results are observed on different sets of tests or when the investigations are carried out in different laboratories. This has been indicated very clearly in multicentric study carried out in the European community with five substances [1, 2]. Historically, the LD_{50} was determined with high degree of precision and was used to compare toxicities of

compounds relative to their therapeutic doses. It is now realized that high degree of precision may not be necessary to compare toxicities [3]. Therefore, the median lethal dose is now estimated from the smallest number of animals possible [4].

The median lethal dose or LD_{50}

This is the dose (mg/kg body weight), which would be expected to kill one-half of an unlimited population of the same species and strain. The median effective dose or ED_{50} is the dose (mg/kg body weight), which produces a desired therapeutic action in 50 percent of the test population [5]. In this present study lethal dose (LD_{50}) of the methanol extracts of *Colebrookea oppositifolia* leaves and *Heracleum nepalense* roots has been determined by oral as well as intraperitoneal route of administration on mice.

4.2. Materials and Methods

4.2.1. Plant materials

Methanol extracts of *Colebrookea oppositifolia* leaves and *Heracleum nepalense* roots (described in **Chapter 3**) were used as test drug in these experiments and propylene glycol was used as control vehicle.

4.2.2. Animals

Swiss albino mice of either sex weighing between 18-20 g were used for the study. The mice were housed in standard stainless steel cages having solid bottom in well-ventilated animal room. Sawdust was spread on the bottom of the cage to absorb urine and moisture from feces. Noisy atmosphere was avoided as much as possible for healthy living condition of mice, as mice are very sensitive to noise. The mice were fed with standard pellet diet of the following composition:

Wheat flour - 63%
Casein - 15%
Sucrose - 10%
Groundnut oil - 05%
Salt mixture - 04%

Shark liver oil - 02%

Vitamin mixture - 01%

Water was given *ad libitum*

4.2.3. Methods of evaluation

Ten groups of animals were used taking ten animals in each group. Different doses of methanol extract of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root suspended in propylene glycol were administered orally to nine groups of animals. One group of animal was treated as control and was fed with propylene glycol. The method of Lorke [6] was followed to determine the acute toxicity study of the extracts. The method was repeated with ten groups of animals separately following intraperitoneal route of administration. The animals were kept under observation in open field condition for 72 hrs after the administration of extracts of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root in both of the routes as mentioned earlier, and the number of deaths and signs of clinical toxicity were recorded. The median lethal dose (MLD) and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon [7].

4.3. Results

The results of the acute toxicity studies of leaf have been presented in Table 4.1 and 4.2. The MLD of methanol extract of *Colebrookea oppositifolia* leaf was found to be 3.0 g/kg body weight and 4.5 g/kg body weight in intraperitoneal and oral route respectively. On the other hand the MLD of the methanol extract of *Heracleum nepalense* root was to be 4.5 g/kg body weight and more than 5.5 g/kg body weight in intraperitoneal and oral route respectively. These results have been presented in Table 4.3 & 4.4.

Table 4.1. Determination of LD_{50} of methanol extract of *C.oppositifolia* leaf after intraperitoneal administration in mice.

Group	Dose (mg/kg)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)	X^2	Xy	Y
1	250	2.39	10	0	0	2	2.95	5.71	7.05	2.88
2	500	2.69	10	1	10	10	3.72	7.23	10.00	3.56
3	1000	3.00	10	2	20	20	4.16	9.00	12.48	4.27
4	2000	3.30	10	4	40	30	4.75	10.89	15.67	4.95
5	3000	3.47	10	5	50	50	5.00	12.04	17.35	5.34
6	4000	3.60	10	6	60	60	5.25	12.96	18.90	5.63
7	4500	3.65	10	7	70	70	5.52	13.32	20.14	5.75
8	5000	3.69	10	9	90	90	5.84	13.61	21.54	5.84
9	5500	3.74	10	10	100	98	7.05	13.98	26.36	5.95

$\Sigma X = 29.52$ $\Sigma y = 44.24$ $\Sigma X^2 = 98.74$ $\Sigma Xy = 149.49$

$x = 3.28$ $\bar{y} = 4.91$

$$b = \frac{\Sigma Xy - (\Sigma X. \Sigma y) / n}{\Sigma X^2 - \Sigma X^2/n} = \frac{149.49 - (29.52 \times 44.24) / 9}{98.74 - (29.52)^2 / 9} = \frac{149.49 - 145.10}{98.74 - 96.82} = 2.28.$$

Linear regression equation

$Y = \bar{y} + b(X - x)$, where x, $\bar{y}$ are the mean values of X and y, b is known as the regression coefficient

$Y = 4.91 + 2.28 (X - 3.28)$

Corrected formula: for 0% dead = $100 \times 0.25 / n$; for 100% dead = $100 (n - 0.25) / n$, where n is the number of animals in each group.

Table 4.2. Determination of LD_{50} of methanol extract of *C.oppositifolia* leaf after oral administration in mice.

Group	Dose (mg/kg)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)	X^2	Xy	Y
1	500	2.69	10	0	0	2	2.95	7.23	7.93	2.67
2	1000	3.00	10	1	10	10	3.72	9.00	11.16	3.57
3	2000	3.30	10	2	20	20	4.16	10.89	13.72	4.45
4	3000	3.47	10	3	30	30	4.48	12.04	15.54	4.94
5	4000	3.60	10	4	40	40	4.75	12.96	17.10	5.32
6	4500	3.65	10	5	50	50	5.00	13.32	18.25	5.47
7	5000	3.69	10	7	70	70	5.52	13.61	20.36	5.58
8	5500	3.74	10	8	80	80	5.84	13.98	21.84	5.73
9	6000	3.77	10	10	100	98	7.05	14.21	26.57	5.81

$\Sigma X = 30.91$ $\Sigma y = 43.47$ $\Sigma X^2 = 107.24$ $\Sigma Xy = 152.47$

$x = 3.43$ $\tilde{y} = 4.83$

$$b = \frac{\Sigma Xy - (\Sigma X.\ \Sigma y)/n}{\Sigma X^2 - \Sigma X^2/n} = \frac{152.47 - (30.91 \times 43.47)/9}{107.24 - (30.91)^2/9} = \frac{152.47 - 149.29}{107.24 - 106.15} = 2.91$$

Linear regression equation

$Y = \tilde{y} + b\,(X - x)$. where x, $\tilde{y}$ are the mean values of X and y. b is known as the regression coefficient

$Y = 4.83 + 2.91\,(X - 3.43)$

Corrected formula: for 0% dead = 100 × 0.25 / n: for 100% dead = 100 (n – 0.25) / n. where n is the number of animals in each group.

Table 4.3 Determination of LD_{50} of methanol extract of *H.nepalense* root after intraperitoneal administration in mice.

Group	Dose (mg/kg)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)	X^2	Xy	Y
1	250	2.39	10	0	0	2	2.95	5.71	7.05	2.39
2	500	2.69	10	0	0	2	2.95	7.23	7.93	3.11
3	1000	3.00	10	1	10	10	3.72	9.00	11.16	3.86
4	2000	3.30	10	2	20	20	4.48	10.89	13.72	4.58
5	3000	3.47	10	3	30	30	4.75	12.04	15.54	4.99
6	4000	3.60	10	4	40	40	5.00	12.96	17.10	5.31
7	4500	3.65	10	5	50	50	5.52	13.32	18.25	5.43
8	5000	3.69	10	8	80	80	5.84	13.61	21.54	5.52
9	5500	3.74	10	10	100	98	7.05	13.98	26.36	5.64

$\Sigma X = 29.53$ $\Sigma y = 40.90$ $\Sigma X^2 = 98.74$ $\Sigma Xy = 138.65$

$x = 3.28$ $\bar{y} = 4.54$

$$b = \frac{\Sigma Xy - (\Sigma X . \Sigma y)/n}{\Sigma X^2 - \Sigma X^2/n} = \frac{138.65 - (29.53 \times 40.90)/9}{98.74 - (29.53)^2/9} = \frac{138.65 - 134.19}{98.74 - 96.89} = 2.41$$

Linear regression equation

$Y = \bar{y} + b(X - x)$, where x, $\bar{y}$ are the mean values of X and y, b is known as the regression coefficient

$Y = 4.54 + 2.41(X - 3.28)$

Corrected formula: for 0% dead = $100 \times 0.25 / n$; for 100% dead = $100 (n - 0.25) / n$, where n is the number of animals in each group.

Table 4.4 Determination of LD_{50} of methanol extract of *H.nepalense* root after oral administration in mice.

Group	Dose (mg/kg)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)	X^2	Xy	Y
1	500	2.69	10	0	0	2	2.95	7.23	7.93	3.02
2	1000	3.00	10	0	0	2	2.95	9.00	8.85	3.64
3	2000	3.30	10	1	10	10	3.72	10.89	12.27	4.24
4	3000	3.47	10	2	20	20	4.16	12.04	14.43	4.58
5	4000	3.60	10	3	30	30	4.48	12.96	14.97	4.84
6	5000	3.69	10	4	40	40	4.75	13.61	17.52	5.02
7	5500	3.74	10	5	50	50	5.00	13.98	18.70	5.12
8	6000	3.77	10	8	80	80	5.84	14.21	22.00	5.18
9	6500	3.81	10	10	100	98	7.05	14.51	26.86	5.26

$\Sigma X = 31.07$ $\Sigma y = 40.90$ $\Sigma X^2 = 108.43$ $\Sigma Xy = 143.53$

$x = 3.45$ $\tilde{y} = 4.54$

$$b = \frac{\Sigma Xy - (\Sigma X.\ \Sigma y)/n}{\Sigma X^2 - \Sigma X^2/n} = \frac{143.53 - (31.07 \times 40.90)/9}{108.43 - (30.07)^2/9} = \frac{143.53 - 141.19}{108.43 - 107.26} = 2.00$$

Linear regression equation

$Y = \tilde{y} + b(X - x)$, where x, $\tilde{y}$ are the mean values of X and y, b is known as the regression coefficient

$Y = 4.54 + 2.00(X - 3.45)$

Corrected formula: for 0% dead = $100 \times 0.25 / n$; for 100% dead = $100 (n - 0.25) / n$, where n is the number of animals in each group.

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CHAPTER 5

ENHANCEMENT OF BIOAVAILABILITY OF SOME ANTIBIOTICS

5.1. Introduction

There is a great importance in the therapeutic regimen for the improvement of bioavailability of a large number of drugs which are (a) poorly biologically available (b) given for long periods, and (c) toxic and expensive. Maximizing oral bioavailability is therapeutically important as the extent of bioavailability directly influences plasma concentration and consequently therapeutic efficacy and dose related toxic effects resulting from oral administration. Any significant improvement in bioavailability will result in lowering the dose and the frequency of doses of that particular drug. Besides, inter-subject variability is inversely correlated with the extent of bioavailability.

Several approaches have been adopted in the past to maximize oral bioavailability. However, based on the clues from Ayurvedic literature, a new approach of increasing the bioavailability of drugs has been conceptualized [1]. There is a growing awareness that herbal medicines and other phytochemicals could severely affect the disposition of certain drugs and pharmaceuticals. Well-known examples are concurrent administration of St. John's wort rendering antiviral treatment less efficient and grapefruit juice increases the bioavailability of number of drugs [2]. Recent studies have shown that the presence of flavonoids, furanocoumarins and other phytoconstituents present in the grapefruit are found to be involved in the enhancement of therapeutic efficacy of number of drugs [3, 4]. It was determined that in combination with piperine the dose of rifampicin can be reduced by about 50% while retaining the therapeutic efficacy at par with the standard dose [5]. Based on these findings other reputed plants were evaluated for enhancing bioavailability of various drugs.

Amoxycillin and cefixime are β - lactam antibiotics exhibiting a marked bactericidal effect against different Gram-positive and Gram-negative bacteria. They are useful in the treatment of respiratory tract, urinary tract and soft tissue infection [6]. Rifampicin, the semisynthetic hydrazine derivatives of rifamycin B, is one of the most potent and powerful mycobactericidal drugs and used mainly for the treatment of tuberculosis and leprosy [7]. It is also indicated for the prophylactic treatment of *H.influenza* (type B)

causing meningitis. It is potent inducers of hepatic microsomal enzyme and its administration results in a decrease of its own half-life and number of other drugs [6].

All the three antibiotics are absorbed rapidly after oral administration, but, when taken with food, the rate and sometimes extent of absorption are decreased. Besides the systemic metabolism and poor patient compliance the insufficient rate and extent of absorption affected the success of their antibacterial therapies. After considering all these factors it was felt necessary for the structural and effective change in the regimen of these drug therapies. The present study reports the effect of methanol extract of *Colebrookea oppositifolia* leaf, *Heracleum nepalense* root and their isolated compounds on the bioavailability and pharmacokinetic of amoxycillin, cefixime and rifampicin in rabbits.

5.2. Materials and Methods

5.2.1. Plant materials

Methanol extracts of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

5.2.2. Test compound formulations

Oral suspensions of the extract and isolated compounds were prepared by suspending them separately in 1% solution of sodium carboxy methylcellulose to prepare suitable dosage forms.

5.2.3. Drugs and chemicals

Amoxycillin was purchased from Libra Drugs (India) Ltd, Pune. Cefixime and rifampicin were kindly gifted by Blue Cross (India) Ltd, Mumbai and Lupin Laboratories Ltd, Pimpiri, Pune respectively. HPLC grade acetonitrile, methanol, water were purchased from S.D Fine Chemicals Ltd, Mumbai. Potassium dihydrogen phosphate, Potassium hydroxide and Zinc sulphate were obtained from Qualigens Fine Chemicals, Mumbai.

5.2.4. Animals

Albino rabbits weighing between 1.5 – 2 kg of either sex were used. They were housed under standard conditions of temperature (23 ±10°C) and relative humidity (55±10%); 12hr/12hr light/dark cycle and fed with standard pellet diet and water *ad libitum*. Rabbits were fasted for at least 24 hr prior to the experiments.

5.2.5. HPLC method of analysis of antibiotics

5.2.5.1. Chromatographic condition

An isocratic HPLC system (Shimadzu) consisting of LC – 20 at liquid pump, SPD – 20 AL UV – Visible detector and spinchrome software was used.

- **Column:** An ODS C-18 RP column (4.6 mml. D × 250 mm) with Hamilton 702 NR (25 µl) injecting syringe.
- **Mobile Phase:** Acetonitrile: Phosphate buffer (0.01M), The pH was adjusted to 5.0 with potassium hydroxide solution (45% w/v).
- **Column Temperature:** Ambient
- **Flow rate:** 1.0 ml/min
- **Injection volume:** 20 µl
- **Detector:** UV
- **Wave length:** 230 nm

5.2.5.2. Validation of assay method

The system suitability was evaluated by the intraday and interday precision and accuracy of triplicates. The accuracy of this method was further assessed with recovery study by spiking the antibiotics separately into blank plasma and phosphate buffer (pH 6) to afford

5.0, 10.0 and 20 μg/ml in triplicates, respectively, and the concentration obtained in blank plasma to the corresponding ones were compared. The LOQ (Limit of quantification) represents the lowest concentration of analysis in a sample that can be determined with acceptable precision and accuracy.

To verify the suitability of method the following analytical variables were analysed:

1. **Precision:** the precision of the method was assessed by repeated analysis of plasma containing known concentrations of all antibiotics separately.

2. **Recovery:** The absolute recovery from plasma was measured in the following way: The drug was added to drug free plasma to achieve the midpoint concentrations and were analysed carefully. Measured aliquot of the acetonitrile layer injected and the peak areas were measured. Absolute recovery was calculated by comparing these peak areas with the peak area obtained by the direct injection of the pure drug standard.

3. **Linearity and Sensitivity:** Concentration and peak area of standard antibiotics in plasma correlated linearly with each other.

5.2.5.3. Quantitative analysis

External standard calibration method was used for quantitative analysis of antibiotics. The external standard was the same substance as that being analysed in the plasma sample. In this method, by injecting standard solution of antibiotic in different concentrations, peak response was plotted versus concentration. Unknown samples were analysed in similar manner and their concentrations were determined from calibration curve. The calibration curve has well covered the range of unknown sample.

$$R_f = \frac{\text{Standard peak area}}{\text{Concentration of the sample}}$$

$$\text{Unknown concentration} = \frac{\text{Peak response of the sample}}{R_f}$$

5.2.6. Preparation of standard curve of amoxycillin, cefixime and rifampicin.

Thawed and drug free plasma from rabbit was pipetted into a disposable test tube and spiked with 50 µl of standard solution of amoxycillin to make the concentration of the drug up to 10, 20, 40, 60, 80, 100, 120 µg/ml of solution with distilled water. The solutions were vortex mixed for 30 sec. After the addition of 300 µl HPLC grade methanol and 200 µl zinc sulphate (0.7M), the tubes were vortex mixed for 30 sec and then centrifuged at 3000 rpm for 5 mins. A 20 µl aliquot of the supernatant was injected into HPLC system and eluted with mobile phase at the rate of 1.0 ml/min at ambient temperature. The column output is monitored at 230 nm using UV detector. The standard curve with peak area on Y-axis and concentration on X-axis was prepared. Similar procedure was followed for preparation of standard curve of cefixime and rifampicin.

5.2.7. Effect of *C.oppositifolia* leaf extract and compound I on bioavailability and pharmacokinetics of antibiotics.

5.2.7.1. Administration schedule of test drugs.

Rabbits were divided into seven groups, each containing six animals. Group I served as control and received only amoxycillin at a dose of 100 mg/kg, p.o. Group II was coadministered 450 mg/kg, p.o. dose of methanol extract of *C.oppositifolia*, and group III & IV were coadministered 25 mg/kg and 50 mg/kg, p.o. dose of compound I respectively with amoxycillin at a dose of 100 mg/kg. Group V – VII were given 450 mg/kg, p.o. dose of methanol extract of *C.oppositifolia*, 25 mg/kg and 50 mg/kg, p.o. dose of compound I respectively and 30 mins later amoxycillin trihydrate was administered at a dose of 100 mg/kg, p.o. Similar type of treatment schedule was followed for cefixime and rifampicin.

The dose of amoxycillin, cefixime and rifampicin was chosen as 100 mg/kg to keep plasma concentrations above the lower limit of detection. As the minimum lethal dose of methanol extract of *C.oppositifolia* was reported to be 4.5 g/kg body weight (described in **Chapter 4**), one tenth of the MLD was selected for evaluation of the activity [8]. Compound I was tested at different doses (25 and 50 mg/kg) for evaluation of the activity.

5.2.7.2. Preparation of plasma samples and determination of plasma levels of antibiotics

Each rabbit was anaesthetized with ether. The right ear marginal vein was cannulated with polyethylene tubing for blood sampling. Blood samples (2 ml) were withdrawn at 0, 0.5, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0 hrs after administration of the drugs and centrifuged at 5000 rpm for 5 min to obtain the plasma samples (1 ml). The samples were labeled properly and stored at – 20°C up to the HPLC analysis.

Thawed plasma samples (900 µl) were mixed with 300 µl HPLC grade methanol and 200 µl zinc sulphate solution (0.7 M). The mixtures were centrifuged at 3000 rpm for 5 min and the supernatants were (20 µl) assayed in the same manner as described in the preparation of standard curve of antibiotics in plasma.

5.2.7.3. Pharmacokinetic analysis

The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (t_{max}) were determined by a visual inspection of the experimental data. The area under the plasma concentration-time curve (AUC) was calculated by trapezoidal rule method. The elimination rate constant (k_{el}) was calculated by regression analysis from the slope of the line, and the half -life ($t_{1/2}$) of the antibiotics was obtained by applying the equation $0.693/k_{el}$. The absorption rate constant (k_a) was calculated by the residual method. The relative bioavailability of the antibiotics (RB%) after oral administration was calculated as follows:

$$\text{Relative bioavailability (RB\%)} = \frac{AUC_{\text{coadministered group}}}{AUC_{\text{control group.}}} \times 100$$

5.2.8. Effect of *H.nepalense* root extract and compound II on bioavailability and pharmacokinetics of antibiotics.

The effect of methanol extract of *H.nepalense* root and its isolated compound II on bioavailability and pharmacokinetics of the tested antibiotics was determined as per the methods described above in **5.2.7** for *C.oppositifolia* leaf.

5.3. Results

5.3.1. Effects of *C.oppositifolia* leaf extract and compound I on bioavailability and pharmacokinetics of antibiotics.

The HPLC method of analysis for amoxycillin, cefixime and rifampicin in rabbit plasma was developed and validated. The limit of quantification was 0.2 μg/ml with recovery >90% for all the three antibiotics. The coefficient of variation for within a day precision ranged from 0.6 to 9.6%. The percent relative standard deviation (% RSD) was calculated and found to be 0.05% (Table 5.1 to 5.3). The coefficient of correlation of the assay was found to be 0.999 in amoxycillin, cefixime and rifampicin. Chromatographic elution was undertaken for 30 mins and the average retention time for amoxycillin, cefixime and rifampicin were about 2.6, 3.9 and 6.9 min respectively (Figure 5.1 to Figure 5.3). The plasma concentrations of all antibiotics after administration of test compounds were calculated from the standard calibration curve as presented in Table 5.4 to Table 5.6 and Figure 5.4 to Figure 5.5.

The plasma concentration-time profile after oral administration of amoxycillin with or without *C.oppositifolia* and compound I is presented in Table 5.7. The data was fitted to a one compartment open model, which followed first order kinetics. Comparative pharmacokinetic parameters calculated from these data are summarized in Table 5.8. In all types of the administration absorption process was completed within median t_{max} 1.5 hr. When amoxycillin (100 mg/kg) were co-administered or given 30 min after the administration of methanol leaf extract peak plasma concentration C_{max} was found to be 6.14 ± 0.39 μg/ml and 6.72 ± 1.04 μg/ml respectively as compared to 5.28 ± 0.32 μg/ml for the control group. But the preadministration of methanol leaf extract shifted the t_{max} 0.5 hr earlier than the control group (1.0 ± 0.41 hr versus 1.5 ± 0.62 hr). The relative bioavailability of amoxycillin preadministered with methanol leaf extract (211%) was higher than the co-administration of the same dose of methanol leaf extract (163%). Comparison of other pharmacokinetic parameters of both the groups with control showed significant difference in plasma half-life ($t_{1/2}$) and elimination rate constant (k_{el}). The absorption rate constants (k_a) of both co-administered and preadministered with methanol

extract groups were increased compared with the control but not statistically significant. When compound I was co-administered at 25 and 50 mg/kg dose with amoxycillin at 100 mg/kg, C_{max} was determined to be 6.52 ± 0.52 μg/ml and 6.71 ± 0.12 μg/ml respectively. In addition, the t_{max} was attained 1.0 hr sooner than the t_{max} for the control group. The C_{max} values of amoxycillin was found to be increased in a dose dependent manner when co-administered or preadministered with compound I at 25 and 50 mg/kg body weight. The absorption rate constants (k_a) of amoxycillin were increased but not statistically significant. Comparison of AUC showed that higher plasma levels of amoxycillin were achieved in the group administered with compound I and amoxycillin 30 min after. The relative bioavailability (RB %) of amoxycillin in preadministered group with compound I at a dose of 50 mg/kg is higher than the co-administered group of the same compound at the same dose. Comparison of other pharmacokinetic parameters with co-administered or preadministered group showed significant increase in elimination half-life ($t_{1/2}$) and elimination rate constant (k_{el}) than the control group.

The result outlined in Table 5.9 showing the mean plasma concentration versus time profile of cefixime administration alone and in combination with methanol extract of *C.oppositifolia* and compound I. The bioavailability and the pharmacokinetic parameters of cefixime after co-administration and preadministered with methanol leaf extract and compound I are shown in Table 5.10. Following co-administration and preadministration of methanol extract of *C.oppositifolia* resulted the peak plasma concentration C_{max} of 4.03 ± 0.42 μg/ml and 4.12 ± 0.22 μg/ml respectively as compared to the control group where it is found to be 4.01 ± 0.36. The time to reach maximum concentration (t_{max}) failed to show any significant differences as compared with the control. Comparison of other pharmacokinetic parameters also failed to show any significant differences in the relative bioavailability (RB%), absorption rate constant (k_a) and mean elimination half- life ($t_{1/2}$). There is no significant difference between the respective AUC values of the groups administered with the test compared with the control group. However the preadministration of compound I 30 mins before the administration of cefixime (100 mg/kg) increased the C_{max} (4.93 ± 0.64 μg/ml at 25 mg/kg and 5.21 ± 0.24 μg/ml at 50 mg/kg); $t_{1/2}$ (3.64 ± 0.61 hr at 25 mg/kg and 4.0 ± 0.32 hr at 50 mg/kg) and AUC (18.05 ±

1.62 μg.hr/ml at 25 mg/kg and 21.06 ± 2.56 μg.hr/ml at 50 mg/kg) as compared to the control group (C_{max} 4.01± 0.36 μg/ml, $t_{1/2}$ 3.01 ± 0.21 hr, AUC 15.17 ± 3.58 μg.hr/ml). The relative bioavailability (RB%) of the cefixime with the preadministered compound I at 50 mg/kg was higher (139%) than the co-administered group at the same dose of compound I (106%).

The mean plasma concentration versus time profile of rifampicin administration with or without methanol extract of *C.oppositifolia* (350 mg/kg), compound I (25mg/kg) is shown in Table 5.11. The data fitted to a one compartment open model, which followed the first order kinetic, and other pharmacokinetic patterns derived from these data are summarized in Table 5.12. In all type of administration the absorption process was completed with t_{max} of 2.5 ± 0.78 hr, 2.0 ± 0.34 hr and 1.5 ± 0.36 hr for rifampicin (100 mg/kg), rifampicin (100 mg/kg) coadministered with methanol extract of *C.oppositifolia* (450 mg/kg) and rifampicin (100 mg/kg) after 30 min of administration of methanol extract of *C.oppositifolia* (450 mg/kg) respectively. The methanol extract of *C.oppositifolia* preadministration induced a significant shift in C_{max} of rifampicin, which was statistically significant ($P<0.05$). The distribution phase was fairly short in all the groups and a fall of concentration of the antibiotic being evident within 2.5 hr of drug administration. Comparison of other pharmacokinetic parameters of the coadministered & preadministered groups with the control showed significant increases in the C_{max} and the mean elimination half-life ($t_{1/2}$). Comparison of AUC showed that higher plasma level of rifampicin was achieved in the group administered with methanol extract of *C.oppositifolia* and postadministration of rifampicin (30 min). The relative bioavailability (RB %) of rifampicin was increased in the groups co-administered and preadministration with methanol extract compared with the control. The absorption rate constants (k_a) of rifampicin were also increased but not statistically significant in both the groups. Following co-administration and preadministration of compound I at 25 and 50 mg/kg the C_{max} was further increased significantly to 8.14 ± 0.84 μg/ml and 8.67 ± 0.52 μg/ml with t_{max} of 2.0 ± 0.32 hr and 1.5 ± 0.31 hr respectively. The half-life ($t_{1/2}$) was prolonged significantly in the groups where compound I was administered 30 min before the administration of rifampicin. All other pharmacokinetic parameters including AUC,

which was increased, and the k_{el}, which was decreased significantly when compound I was preadministered and co-administered with rifampicin compared to the control group. The absorption rate constants (k_a) of both co-administered and preadministered group with compound I were increased compared to the control but not statistically significant.

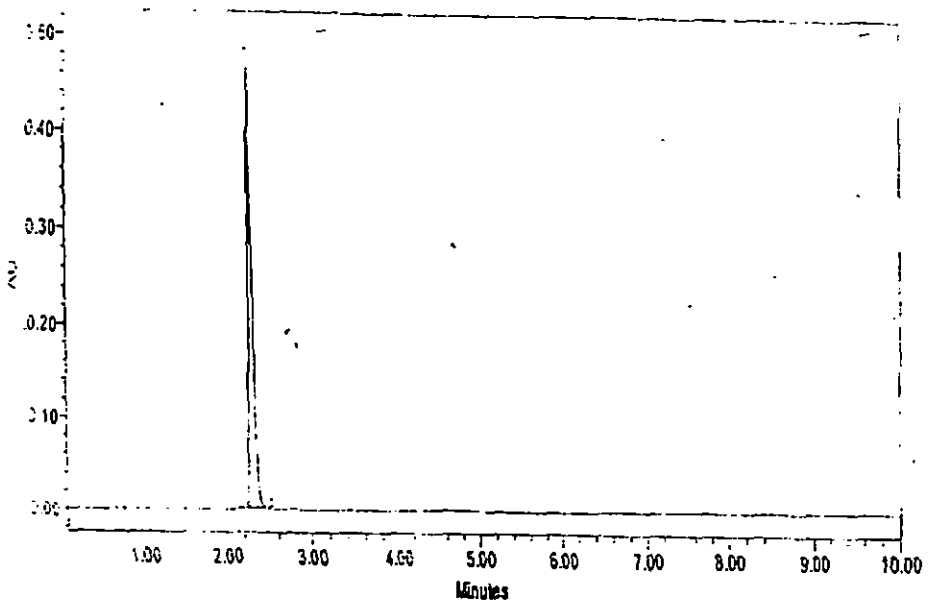

Figure 5.1. Typical HPLC Chromatogram of amoxycillin.

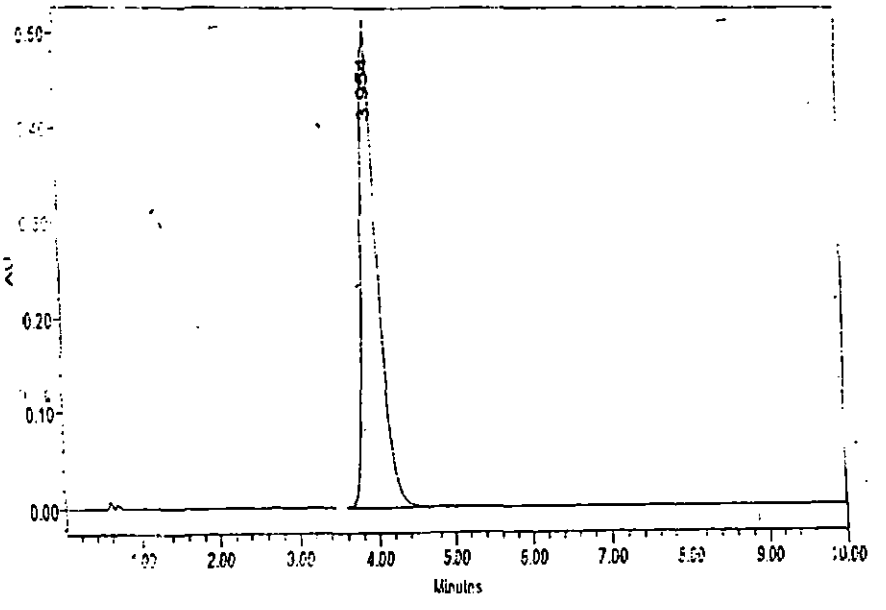

Figure 5.2. Typical HPLC Chromatogram of cefixime.

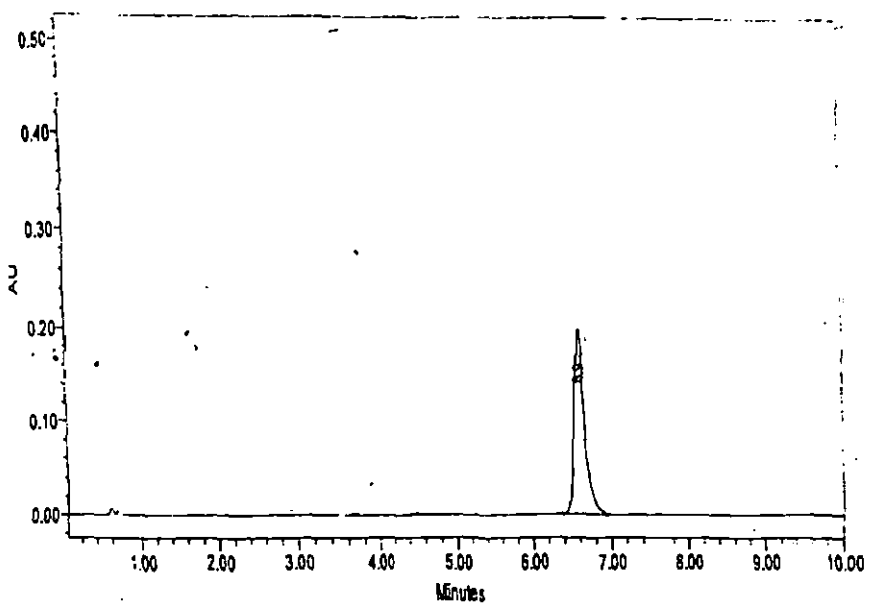

Figure 5.3. Typical HPLC Chromatogram of rifampicin.

Table 5.1 Percent relative standard deviation of amoxycillin.

Injection number	Retention time (min)	Peak area observed
1	2.60	3680192
2	2.61	3679562
3	2.60	3680242
4	2.59	3682469
5	2.60	3678292

Concentration used to calculate % RSD is 10 µg/ml

$$\% \text{ RSD} = \frac{\text{Average peak area} - \text{Minimum peak area}}{\text{Average peak area}}$$

Average peak area = 3680151, Minimum peak area = 36782922

% RSD = 0.05

Table 5.2 Percent relative standard deviation of cefixime.

Injection number	Retention time (min)	Peak area observed
1	3.41	3597423
2	3.40	3596727
3	3.41	3597872
4	3.42	3595512
5	3.40	3599621

Concentration used to calculate % RSD is 10 µg/ml

$$\% \text{ RSD} = \frac{\text{Average peak area} - \text{Minimum peak area}}{\text{Average peak area}}$$

Average peak area = 3597431, Minimum peak area = 3595512

% RSD = 0.05

Table 5.3 Percent relative standard deviation of rifampicin.

Injection number	Retention time (min)	Peak area observed
1	5.92	3694178
2	5.92	3696232
3	5.90	3694583
4	5.91	3694247
5	5.92	3692352

Concentration used to calculate % RSD is 10 µg/ml

$$\% \text{ RSD} = \frac{\text{Average peak area} - \text{Minimum peak area}}{\text{Average peak area}}$$

Average peak area = 3694318, Minimum peak area = 3692352

% RSD = 0.05

Table 5.4 Data for standard graph of amoxycillin in plasma.

Sl. No.	Concentration (µg/ml)	Mean peak area
1	1	365154.37
2	2	742367.24
3	3	1181562.72
4	4	1486482.62
5	5	1902923.54
6	10	3680195.30
7	15	6169852.13
8	20	7323146.77

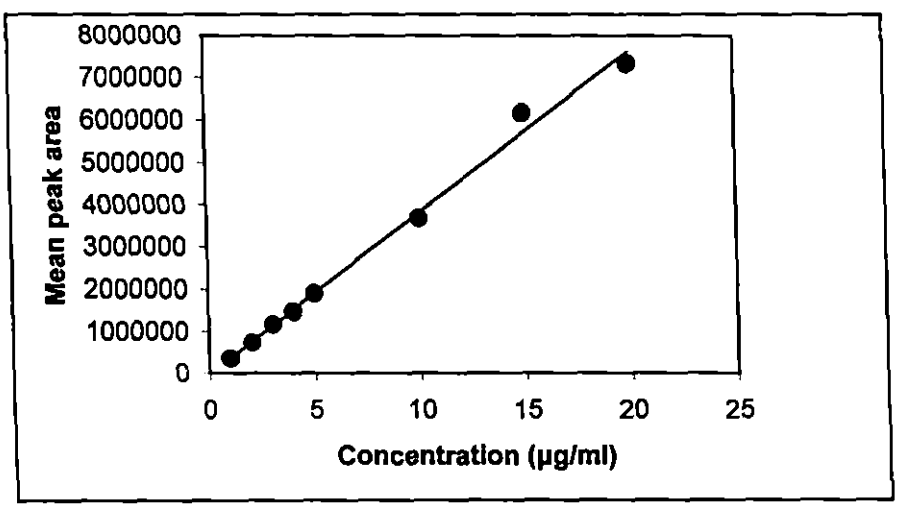

Figure 5.4. Standard curve of amoxycillin in plasma.

Table 5.5 Data for standard graph of cefixime in plasma.

Sl. No.	Concentration (µg/ml)	Mean peak area
1	1	352642.14
2	2	714624.55
3	3	1162436.11
4	4	1483727.57
5	5	1832420.66
6	10	3598969.18
7	15	6437894.30
8	20	7290055.15

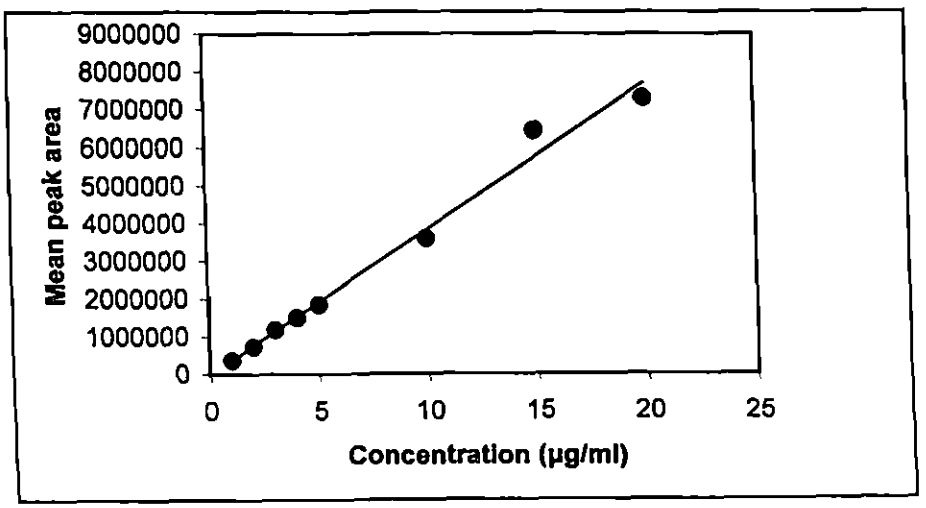

Figure 5.5. Standard curve of cefixime in plasma.

Table 5.6 Data for standard graph of rifampicin in plasma.

Sl. No.	Concentration (µg/ml)	Mean peak area
1	1	388819.52
2	2	777254.24
3	3	1106453.62
4	4	1481278.12
5	5	1826435.62
6	10	3693142.32
7	15	6242572.39
8	20	7369821.66

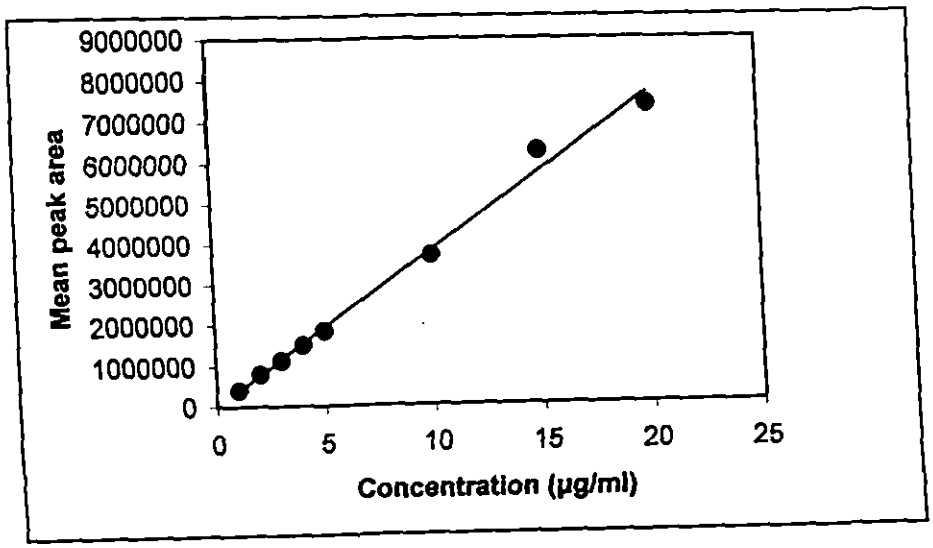

Figure 5.6. Standard curve of rifampicin in plasma.

Table 5.7 Mean plasma concentration of amoxycillin after oral administration of amoxycillin (100 mg/kg), co-administration with methanol extract of *C.oppositifolia* (450 mg/kg) and compound I (25, 50 mg/kg) and preadministered with *C.oppositifolia* (450 mg/kg) and compound I (25, 50 mg/kg) in rabbit.

Sl. No	Time of collection (hr)	(Group I) Amoxycillin (100 mg/kg)	(Group II) methanol extract (450 mg/kg) + Amoxycillin (100 mg/kg)	(Group III) Compound I (25 mg/kg) + Amoxycillin (100 mg/kg)	(Group IV) Compound I (50 mg/kg) + Amoxycillin (100 mg/kg)	(Group V) methanol extract (450 mg/kg) + Amoxycillin (100 mg/kg) 30 min later	(Group VI) Compound I (25 mg/kg) + Amoxycillin (100 mg/kg) 30 min later	(Group VI) Compound I (50 mg/kg) + Amoxycillin (100 mg/kg) 30 min later
1	0.5	1.67 ± 0.191	2.12 ± 0.231	2.86 ± 0.112	3.06 ± 0.352	3.84 ± 0.392	3.09 ± 0.154	3.85 ± 0.212
2	1.0	3.12 ± 0.393	3.86 ± 0.352	4.62 ±0. 321	5.12 ± 0.252	6.72 ± 1.041	7.07 ± 0.742	7.51 ± 0.332
3	1.5	5.26 ± 0.323	6.14 ± 0.391	6.52 ± 0.522	6.71 ± 0.121	6.08 ± 0.726	6.45 ± 0.332	6.87 ± 0.543
4	2.0	4.23 ± 0.546	5.84 ± 0.655	5.79 ± 0.423	6.03 ± 0.211	5.83 ± 0.521	5.94 ± 0.141	6.06 ± 0.412
5	2.5	3.56 ± 0.522	4.62 ± 0.312	4.82 ± 0.152	5.01 ± 0.432	4.92 ± 0.132	5.04 ± 0.112	5.28 ± 0.645
6	3.0	2.34 ± 0.761	3.17 ± 0.231	3.82 ± 0.111	4.67 ± 0.311	3.86 ± 0.432	4.69 ± 0.462	4.16 ± 0.322
7	4.0	1.33 ± 0.234	2.22 ± 0.145	2.74 ± 0.243	3.68 ± 0.512	3.12 ± 0.321	3.71 ± 0.562	3.67 ± 0.515
8	5.0	1.12 ± 0.645	1.84 ± 0.532	2.12 ± 0.521	2.74 ± 0.234	2.46 ± 0.645	2.75 ± 0.732	2.92 ± 0.843
9	6.0	0.92 ± 0.074	1.12 ± 0.191	1.34 ± 0.512	1.94 ± 0.512	1.92 ± 0.685	1.93 ± 0.113	2.03 ± 0.752
10	8.0	0.46 ± 0.012	0.96 ± 0.321	0.99 ± 0.015	1.02 ± 0.112	1.24 ± 0.213	1.03 ± 0.115	1.16 ± 0.119
11	10.0	0.29 ± 0.065	0.82 ± 0.031	0.64 ± 0.034	0.78 ± 0.055	0.98 ± 0.012	0.72 ± 0.032	0.89 ± 0.016
12	12.0	0.11 ± 0.087	0.49 ± 0.012	0.52 ± 0.021	0.54 ± 0.012	0.64 ± 0.032	0.56 ± 0.011	0.74 ± 0.042
13	24.0	0.03 ± 0.009	0.22 ± 0.011	0.21 ± 0.012	0.22 ± 0.021	0.44 ± 0.011	0.22 ± 0.017	0.24 ± 0.043

Values are expressed in µg/ml, and mean ± SEM; n=6 in each group. $P<0.05$ in comparison with control

Table 5.8 Comparison of pharmacokinetic parameters of amoxycillin alone and in combination with methanol extract of *C.oppositifolia* and compound I

Sl.No	Parameters	(Group I) Amoxycillin (100 mg/kg)	(Group II) methanol extract (450 mg/kg) + Amoxycillin (100 mg/kg)	(Group III) Compound I (25 mg/kg) + Amoxycillin (100 mg/kg)	(Group IV) Compound I (50 mg/kg) + Amoxycillin (100 mg/kg)	(Group V) methanol extract (450 mg/kg) + Amoxycillin (100 mg/kg) 30 min later	(Group VI) Compound I (25 mg/kg) + Amoxycillin (100 mg/kg) 30 min later	(Group VI) Compound I (50 mg/kg) + Amoxycillin (100 mg/kg) 30 min later
1	C_{max} (µg/ml)	5.28 ± 0.32	6.14 ± 0.39	6.52 ± 0.52	6.71 ± 0.12	6.72 ± 1.04	7.07 ± 0.74	7.51 ± 0.33
2	t_{max} (hr)	1.5 ± 0.61	1.5 ± 0.31	1.5 ± 0.42	1.5 ± 0.54	1.0 ± 0.41	1.0 ± 0.40	1.0 ± 0.36
3	AUC (µg.hr/ml)	17.32 ± 2.34	28.05 ± 1.81	30.1 ± 2.59	34.43 ± 3.36	36.59 ± 3.21	35.38 ± 2.46	38.20 ± 1.92
4	$t_{1/2}$ (hr)	1.54 ± 0.47	1.69 ± 0.43	1.72 ± 0.63	1.84 ± 0.67	1.83 ± 0.32	2.13 ± 0.39	2.34 ± 0.67
5	k_{el} (hr^{-1})	0.45 ± 0.023	0.41 ± 0.024	0.40 ± 0.036	0.37 ± 0.041	0.37 ± 0.032	0.32 ± 0.021	0.29 ± 0.051
6	RB (%)	100	163	173	199	211	204	221
7	k_a (hr^{-1})	2.71 ± 0.32	2.83 ± 0.41^{NS}	2.95 ± 1.11^{NS}	2.97 ± 0.83^{NS}	2.96 ± 1.12^{NS}	3.09 ± 1.10^{NS}	3.23 ± 0.43^{NS}

Values are in mean ± SEM; n=6 in each group; P<0.05 in comparison with control; C_{max}: peak concentration; t_{max}: time to reach peak concentration; AUC: area under plasma concentration time curve from 0 hrs to 24 hr; $t_{1/2}$: plasma half life; k_{el}: elimination rate constant; RB (%): relative bioavailability; ka: absorption rate constant; NS: not significant.

Table 5.9 Mean plasma concentration of cefixime after oral administration of cefixime (100 mg/kg), co-administration with methanol extract of *C.oppositifolia* (450 mg/kg) and compound I (25, 50 mg/kg) and preadministered with *C.oppositifolia* (450 mg/kg) and compound I (25, 50 mg/kg) in rabbit.

Sl. No	Time of collection (hr)	(Group I) Cefixime (100 mg/kg)	(Group II) methanol extract (450 mg/kg) + cefixime (100 mg/kg)	(Group III) Compound I (25 mg/kg) + cefixime (100 mg/kg)	(Group IV) Compound I (50 mg/kg) + cefixime (100 mg/kg)	(Group V) methanol extract (450 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VI) Compound I (25 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VII) Compound I (50 mg/kg) + cefixime (100 mg/kg) 30 min later
1	0.5	1.76 ± 0.142	1.78 ± 0.232	1.56 ± 0.242	1.82 ± 0.374	1.84 ± 0.462	1.94 ± 0.413	3.32 ± 0.469
2	1.0	2.98 ± 0.132	2.97 ± 0.231	2.86 ±0. 232	2.99 ± 0.371	2.98 ± 0.241	3.23 ± 0.422	5.12 ± 0.243
3	1.5	4.01 ± 0.362	4.03 ± 0.426	3.97 ± 0.412	4.11 ± 0.251	4.12 ± 0.221	4.93 ± 0.645	4.92 ± 0.453
4	2.0	3.56 ± 0.546	3.69 ± 0.131	3.54 ± 0.321	3.63 ± 0.653	3.61 ± 0.582	3.98 ± 0.411	3.93 ± 0.142
5	2.5	2.84 ± 0.612	2.31 ± 0.321	3.04 ± 0.523	3.09 ± 0.184	3.01 ± 0.312	3.17 ± 0.237	3.19 ± 0.512
6	3.0	2.01 ± 0.341	1.94 ± 0.124	2.28 ± 0.234	2.77 ± 0.322	2.34 ± 0.322	2.46 ± 0.612	2.48 ± 0.242
7	4.0	1.92 ± 0.541	1.86 ± 0.245	1.94 ± 0.463	1.98 ± 0.157	1.96 ± 0.301	2.04 ± 0.643	2.02 ± 0.145
8	5.0	1.12 ± 0.523	1.02 ± 0.342	1.08 ± 0.341	1.06 ± 0.321	1.05 ± 0.146	1.29 ± 0.342	1.74 ± 0.413
9	6.0	0.84 ± 0.024	0.72 ± 0.431	0.84 ± 0.031	0.84 ± 0.101	0.81 ± 0.021	0.98 ± 0.043	1.03 ± 0.212
10	8.0	0.31 ± 0.032	0.31 ± 0.262	0.42 ± 0.046	0.56 ± 0.039	0.49 ± 0.014	0.67 ± 0.054	0.86 ± 0.019
11	10.0	0.19 ± 0.015	0.18 ± 0.042	0.23 ± 0.061	0.24 ± 0.051	0.21 ± 0.014	0.34 ± 0.036	0.39 ± 0.014
12	12.0	0.08 ± 0.037	0.09 ± 0.002	0.07 ± 0.007	0.09 ± 0.011	0.07 ± 0.005	0.11 ± 0.026	0.14 ± 0.010
13	24.0	0.01 ± 0.005	0.01 ± 0.005	0.03 ± 0.004	0.01 ± 0.002	0.02 ± 0.011	0.02 ± 0.005	0.03 ± 0.002

Values are expressed in μg/ml, and mean ± SEM; n=6 in each group. $P<0.05$ in comparison with control

Table 5.10 Comparison of pharmacokinetic parameters of cefixime alone and in combination with methanol extract of *C.oppositifolia* and compound I

Sl.No	Parameters	(Group I) Cefixime (100 mg/kg)	(Group II) methanol extract (450 mg/kg) + cefixime (100 mg/kg)	(Group III) Compound I (25 mg/kg) + cefixime (100 mg/kg)	(Group IV) Compound I (50 mg/kg) + cefixime (100 mg/kg)	(Group V) methanol extract (450 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VI) Compound I (25 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VII) Compound I (50 mg/kg) + cefixime (100 mg/kg) 30 min later
1	C_{max} (µg/ml)	4.01 ± 0.36	4.03 ± 0.42	3.97 ± 0.41	4.11 ± 0.25	4.12 ± 0.22	4.93 ± 0.64	5.12 ± 0.24
2	t_{max} (hr)	1.5 ± 0.42	1.5 ± 0.32	1.5 ± 0.24	1.5 ± 0.44	1.5 ± 0.31	1.5 ± 0.41	1.0 ± 0.21
3	AUC (µg.hr/ml)	15.17 ± 3.58	14.49 ± 2.65	15.41 ± 3.61	16.17 ± 3.34	15.44 ± 2.65	18.05 ± 1.62	21.06 ± 2.56
4	$t_{1/2}$ (hr)	3.01 ± 0.21	2.97 ± 0.35	3.01 ± 0.17	3.15 ± 0.52	3.01 ± 0.23^{NS}	3.64 ± 0.61	4.0 ± 0.32
5	k_{el} (hr^{-1})	0.23 ± 0.062	0.22 ± 0.024	0.23 ± 0.042	0.21 ± 0.032	0.23 ± 0.034	0.19 ± 0.016	0.17 ± 0.015
6	RB (%)	100	96	101	106	101	119	139
7	k_a (hr^{-1})	2.31 ± 0.16	2.29 ± 0.87^{NS}	2.31 ± 0.27	2.34 ± 0.83^{NS}	2.32 ± 0.42^{NS}	2.43 ± 1.10	2.86 ± 0.52

Values are in mean ± SEM; n=6 in each group; P<0.05 in comparison with control; C_{max}: peak concentration; t_{max}: time to reach peak concentration; AUC: area under plasma concentration time curve from 0 hrs to 24 hr; $t_{1/2}$: plasma half life; k_{el}: elimination rate constant; RB (%): relative bioavailability; ka: absorption rate constant; NS: not significant

Table 5.11 Mean plasma concentration of rifampicin after oral administration of rifampicin (100 mg/kg), co-administration with methanol extract of *C.oppositifolia* (450 mg/kg) and compound I (25, 50 mg/kg) and preadministered with *C.oppositifolia* (450 mg/kg) and compound I (25, 50 mg/kg) in rabbit.

Sl. No	Time of collection (hr)	(Group I) Rifampicin (100 mg/kg)	(Group II) methanol extract (450 mg/kg) + rifampicin (100 mg/kg)	(Group III) Compound I (25 mg/kg) + rifampicin (100 mg/kg)	(Group IV) Compound I (50 mg/kg) + rifampicin (100 mg/kg)	(Group V) methanol extract (450 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VI) Compound I (25 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VII) Compound I (50 mg/kg) + rifampicin (100 mg/kg) 30 min later
1	0.5	2.62 ± 0.143	3.14 ± 0.356	3.42 ± 0.126	4.17 ± 0.318	3.32 ± 0.311	3.89 ± 0.486	4.67 ± 0.238
2	1.0	3.52 ± 0.241	4.72 ± 0.278	4.91 ±0. 158	5.23 ± 0.247	4.82 ± 0.416	5.17 ± 0.422	7.18 ± 0.247
3	1.5	4.14 ± 0.221	7.06 ± 0.369	7.12 ± 0.242	7.37 ± 0.251	7.97 ± 0.672	7.99 ± 0.236	8.67 ± 0.524
4	2.0	5.82 ± 0.152	7.87 ± 0.681	7.89 ± 0.412	8.14 ± 0.846	7.36 ± 0.415	7.42 ± 0.162	7.98 ± 0.126
5	2.5	6.97 ± 1.321	6.98 ± 0.247	6.99 ± 0.177	7.88 ± 0.415	7.03 ± 0.423	7.07 ± 0.134	7.16 ± 0.443
6	3.0	6.12 ± 0.243	6.15 ± 0.244	6.18 ± 0.158	7.02 ± 0.151	6.29 ± 0.314	6.37 ± 0.434	6.67 ± 0.242
7	4.0	5.66 ± 0.748	5.72 ± 0.243	5.71 ± 0.434	6.16 ± 0.126	5.82 ± 0.231	5.94 ± 0.552	5.96 ± 0.145
8	5.0	4.72 ± 0.417	4.87 ± 0.381	4.88 ± 0.566	5.31 ± 0.458	4.99 ± 0.498	5.11 ± 0.342	5.16 ± 0.241
9	6.0	3.54 ± 0.726	3.96 ± 0.248	3.89 ± 0.124	4.23 ± 0.268	4.03 ± 0.523	4.14 ± 0.177	4.24 ± 0.284
10	8.0	2.32 ± 0.476	2.46 ± 0.252	2.52 ± 0.146	3.13 ± 0.142	3.02 ± 0.213	3.16 ± 0.162	3.52 ± 0.163
11	10.0	1.01 ± 0.242	1.06 ± 0.310	1.07 ± 0.328	1.37 ± 0.246	1.42 ± 0.172	1.51 ± 0.034	2.32 ± 0.136
12	12.0	0.62 ± 0.043	0.67 ± 0.014	0.68 ± 0.021	0.74 ± 0.012	0.94 ± 0.071	1.01 ± 0.013	1.21 ± 0.128
13	24.0	0.11 ± 0.027	0.14 ± 0.016	0.17 ± 0.013	0.23 ± 0.013	0.17 ± 0.026	0.19 ± 0.012	0.24 ± 0.021

Values are expressed in µg/ml, and mean ± SEM; n=6 in each group. $P<0.05$ in comparison with control

Table 5.12 Comparison of pharmacokinetic parameters of rifampicin alone and in combination with methanol extract of *C.oppositifolia* and compound I

Sl.No	Parameters	(Group I) Rifampicin (100 mg/kg)	(Group II) methanol extract (450 mg/kg) + rifampicin (100 mg/kg)	(Group III) Compound I (25 mg/kg) + rifampicin (100 mg/kg)	(Group IV) Compound I (50 mg/kg) + rifampicin (100 mg/kg)	(Group V) methanol extract (450 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VI) Compound I (25 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VII) Compound I (50 mg/kg) + rifampicin (10C mg/kg) 30 min later
1	C_{max} (µg/ml)	6.97 ± 1.32	7.87 ± 0.68	7.89 ± 0.41	8.14 ± 0.84	7.97 ± 0.67	7.99 ± 0.23	8.67 ± 0.52
2	t_{max} (hr)	2.5 ± 0.78	2.0 ± 0.34	2.0 ± 0.22	2.0 ± 0.32	1.5 ± 0.36	1.5 ± 0.24	1.5 ± 0.31
3	AUC (µg.hr/ml)	43.37 ± 2.43	48.56 ± 3.56	49.06 ± 2.59	54.94 ± 2.36	53.20 ± 1.42	55.33 ± 2.17	61.22 ± 3.82
4	$t_{1/2}$ (hr)	3.01 ± 0.45	3.31 ± 0.43	3.32 ± 0.63^{NS}	3.62 ± 0.64^{NS}	3.46 ± 0.47	3.74 ± 0.72	3.93 ± 0.74
5	k_{el} (hr^{-1})	0.23 ± 0.016	0.21 ± 0.021	0.21 ± 0.016	0.19 ± 0.041	0.20 ± 0.012	0.18 ± 0.014	0.17 ± 0.016
6	RB (%)	100	112	113	126	123	128	141
7	k_a (hr^{-1})	2.66 ± 0.28	2.69 ± 0.32^{NS}	2.70 ± 1.27^{NS}	2.76 ± 0.34^{NS}	2.74 ± 0.18^{NS}	2.77 ± 0.42	2.81 ± 1.10^{NS}

Values are in mean ± SEM; n=6 in each group; P<0.05 in comparison with control; C_{max}: peak concentration; t_{max}: time to reach peak concentration; AUC: area under plasma concentration time curve from 0 hrs to 24 hr; $t_{1/2}$: plasma half life; k_{el}: elimination rate constant; RB (%): relative bioavailability; ka: absorption rate constant; NS: not significant

5.3.2. Effects of *H.nepalense* root extract and compound II on bioavailability and pharmacokinetics of antibiotics.

The validated HPLC assay methods were applied to determine the plasma concentration of antibiotics (discussed in **5.3.1**) in rabbit. The plasma concentration of amoxycillin after oral administration of amoxycillin co-administered and administered 30 min after the administration of methanol root extract and compound II are shown in Table 5.13. The data fitted to a one compartment open model, which followed first order kinetics. The bioavailability and the pharmacokinetic parameters of amoxycillin after co-administration and preadministered with methanol root extract and compound II are shown in Table 5.14. In all types of the administration absorption process was complete within median t_{max} of 1.5 hr.

When amoxycillin (100 mg/kg) were co-administered or administered 30 min after the administration of the root extract, C_{max} of amoxycillin were failed to show any significant differences when compared with the control. The $t_{1/2}$ of amoxycillin after both types of the administration of extract were remained unchanged as 1.5 hr. Comparison of the other pharmacokinetic parameters failed to show any differences in elimination rate constant (k_{el}), percentage of relative bioavailability (RB %) and area under plasma concentration-time curve (AUC). The absorption rate constants (k_a) were within 2.32 (hr^{-1}) compared to 2.31 (hr^{-1}) of the control group. After the co-administration of amoxycillin with compound II at 25 and 50 mg/kg body weight, C_{max} of amoxycillin were increased significantly ($P<0.05$) to 6.14 ± 0.42 μg/ml and 6.97 ± 0.29 μg/ml respectively compared with 5.27 ± 0.22 μg/ml for the control. However, the t_{max} were remained unchanged in the entire groups. When compound II was preadministered 30 min before the administration of amoxycillin at the same dose of 25 and 50 mg/kg body weight the C_{max} of amoxycillin were further increased significantly to 6.13 ± 0.37 μg/ml and 6.96 ± 0.22 μg/ml respectively with unchanged t_{max}. In addition, the co-administration and preadministration of compound II at 50 mg/kg body weight with amoxycillin has prolonged the plasma half-life ($t_{1/2}$) and increased the area under curve (AUC) compared to the control. The relative bioavailability of amoxycillin administered 30 min after the administration of compound II at 50 mg/kg (181 %) was higher than co-administration of

the same dose of compound II (164 %). The absorption rate constant (k_a) was increased but was not statistically significant.

The result outlined in Table 5.15 shows mean plasma concentration versus time profile of cefixime administered alone and in combination with methanol root extract and isolated compound II. The bioavailability and the pharmacokinetic parameters of cefixime after co-administration or preadministration with compound II are shown in Table 5.16. In all types of the administration the absorption process was complete with unchanged median t_{max} of 1.5 hr. The t_{max} was not statistically significant ($P>0.05$). The distribution phase was short in all the groups, a fall of concentration of the antibiotic being evident within 2 hr of its administration. Comparison of other pharmacokinetic parameters of the coadministered and preadministered groups with the control group failed to show any differences either in peak drug levels (C_{max}) or mean elimination half-life ($t_{1/2}$) attained. There is no significant difference between the respective AUC values. But the group in which compound II was administered 30 min before administration of amoxycillin significantly increased the AUC value to 17.03 ± 2.15 μg/ml compared with 14.73 ± 3.58 μg/ml of the control group. The absorption rate constant (k_a) and relative bioavailability (RB %) was increased in the preadministered groups with compound II in a dose dependent manner. The absorption rate constant (k_a) was increased but was not statistically significant.

The plasma concentrations of rifampicin after oral administration of the rifampicin administered with or without methanol root extract and compound II is outlined in Table 5.17. The data fitted to a one compartment open model which followed first order kinetics and other pharmacokinetic pattern derived from these data are summarized in Table 5.18. In all types of the administration absorption process was complete within median t_{max} of 2.5 hr. When rifampicin (100 mg/kg) co-administered and preadministered with root extract, C_{max} of rifampicin was increased up to 7.11 ± 0.25 μg/ml and 7.14 ± 0.43 μg/ml compared to control where it was observed to be 6.95 ± 0.34 μg/ml. On the other hand, the t_{max} of both the group was remained unchanged as 2.5 hr as in the control group. Comparison of other pharmacokinetic parameters of the coadministered and

preadministered groups with the control group also failed to show any differences in the elimination half-life ($t_{1/2}$), elimination rate constant (k_{el}), area under curve (AUC) and relative bioavailability (RB %). There was also no significant difference in the absorption rates constant (k_a) of the groups with the control. After co-administration of rifampicin with compound II at 25 and 50 mg/kg body weights, C_{max} of rifampicin was increased significantly ($P<0.05$) to 7.93 ± 0.18 μg/ml and 8.96 ± 0.62 μg/ml respectively. In addition, the t_{max} was attained 0.5 hr sooner than the t_{max} of the control group (2.0 versus 2.5 hr). The AUC of rifampicin was significantly increased in both the co-administered and preadministered groups up to 54 43 ± 4.61 μg.hr/ml and 65.33 ± 2.36 μg.hr/ml respectively in a dose dependent manner compared to 43.43 ± 3.49 μg.hr/ml for the control group. The half-life ($t_{1/2}$) of rifampicin in which it was co-administered with compound II was prolonged significantly (4.07 ± 0.7 hr) compared to the control group (3.01 ± 0.42 hr). The absorption rate constant (k_a) was increased but not statistically significant. In the same way, the groups in which compound II at 25 and 50 mg/kg body weight was preadministered 30 mins before the administration of rifampicin, C_{max} of rifampicin was further increased significantly to 8.14 ± 0.34 μg/ml and 9.30 ± 0.20 μg/ml respectively compared to the control group (6.95 ± 0.34) μg/ml with reduction of t_{max} to 1.5 ± 0.46 hr & 1.5 ± 0.18 hr respectively compared to control group (2.5 ± 0.27 hr). The AUC value of rifampicin was increased significantly in the entire groups compared to the control group. Comparison of other pharmacokinetic parameters showed significant difference in half-life ($t_{1/2}$) and elimination rate constant (k_{el}). The relative bioavailability of rifampicin administered 30 min before the administration of compound II at 25 and 50 mg/kg body weight was found to be 129 and 165% compared to 124 and 149% for co-administration of the same doses of compound II with rifampicin. The absorption rate constants (k_a) was increased in all the groups compared to that of control group but not statistically significant.

Table 5.13 Mean plasma concentration of amoxycillin after oral administration of amoxycillin (100 mg/kg), co-administration with methanol extract of *H.nepalense* (550 mg/kg) and compound II (25, 50 mg/kg) and preadministered with *H.nepalense* (550 mg/kg) and compound II (25, 50 mg/kg) in rabbit.

Sl. No	Time of collection (hr)	(Group I) Amoxycillin (100 mg/kg)	(Group II) methanol extract (550 mg/kg) + amoxycillin (100 mg/kg)	(Group III) Compound II (25 mg/kg) + amoxycillin (100 mg/kg)	(Group IV) Compound II (50 mg/kg) + amoxycillin (100 mg/kg)	(Group V) methanol extract (550 mg/kg) + amoxycillin (100 mg/kg) 30 min later	(Group VI) Compound II (25 mg/kg) + amoxycillin (100 mg/kg) 30 min later	(Group VII) Compound I (50 mg/kg) + amoxycillin (100 mg/kg) 30 min later
1	0.5	1.78 ± 0.161	1.74 ± 0.322	2.23 ± 0.256	3.17 ± 0.347	1.78 ± 0.362	2.26 ± 0.414	3.22 ± 0.351
2	1.0	3.16 ± 0.245	3.17 ± 0.361	4.37 ± 0.228	5.12 ± 0.235	3.19 ± 0.452	4.41 ± 0.234	5.14 ± 0.228
3	1.5	5.27 ± 0.227	5.30 ± 0.234	6.14 ± 0.426	6.97 ± 0.291	5.34 ± 0.712	6.13 ± 0.378	6.96 ± 0.227
4	2.0	4.24 ± 0.172	4.28 ± 0.612	5.32 ± 0.128	5.88 ± 0.243	4.27 ± 0.152	5.31 ± 0.196	5.89 ± 0.128
5	2.5	3.58 ± 0.261	3.52 ± 0.277	4.19 ± 0.147	4.93 ± 0.329	3.54 ± 0.423	4.18 ± 0.434	4.97 ± 0.426
6	3.0	2.34 ± 0.231	2.36 ± 0.264	3.37 ± 0.135	3.86 ± 0.521	2.41 ± 0.312	3.38 ± 0.619	3.84 ± 0.239
7	4.0	1.36 ± 0.418	1.39 ± 0.411	2.62 ± 0.342	3.02 ± 0.246	1.36 ± 0. 311	2.64 ± 0.512	3.07 ± 0.432
8	5.0	1.11 ± 0.451	1.12 ± 0.381	1.47 ± 0.521	2.18 ± 0.438	1.18 ± 0.464	1.44 ± 0.247	2.14 ± 0.272
9	6.0	0.95 ± 0.026	0.96 ± 0.024	1.02 ± 0.115	1.62 ± 0.252	0.99 ± 0.023	1.04 ± 0.187	1.61 ± 0.256
10	8.0	0.51 ± 0.047	0.53 ± 0.052	0.94 ± 0.042	1.03 ± 0.032	0.61 ± 0.013	0.96 ± 0.162	1.04 ± 0.126
11	10.0	0.32 ± 0.024	0.33 ± 0.012	0.43 ± 0.052	0.64 ± 0.036	0.32 ± 0.026	0.44 ± 0.052	0.65 ± 0.042
12	12.0	0.14 ± 0.043	0.15 ± 0.012	0.29 ± 0.021	0.31 ± 0.022	0.16 ± 0.021	0.31 ± 0.018	0.34 ± 0.013
13	24.0	0.08 ± 0.009	0.09 ± 0.006	0.12 ± 0.015	0.16 ± 0.034	0.10 ± 0.014	0.14 ± 0.012	0.15 ± 0.021

Values are expressed in μg/ml, and mean ± SEM: n=6 in each group. $P<0.05$ in comparison with control

Table 5.14 Comparison of pharmacokinetic parameters of amoxycillin alone and in combination with methanol extract of *H.nepalense* and compound II

Sl.No	Parameters	(Group I) Amoxycillin (100 mg/kg)	(Group II) methanol extract (550 mg/kg) + amoxycillin (100 mg/kg)	(Group III) Compound II (25 mg/kg) + amoxycillin (100 mg/kg)	(Group IV) Compound II (50 mg/kg) + amoxycillin (100 mg/kg)	(Group V) methanol extract (550 mg/kg) + amoxycillin (100 mg/kg) 30 min later	(Group VI) Compound II (25 mg/kg) + amoxycillin (100 mg/kg) 30 min later	(Group VII) Compound I (50 mg/kg) + amoxycillin (100 mg/kg) 30 min later
1	C_{max} (µg/ml)	5.27 ± 0.22	5.30 ± 0.23^{NS}	6.14 ± 0.42^{NS}	6.97 ± 0.29	5.34 ± 0.71	6.13 ± 0.37	6.96 ± 0.22
2	t_{max} (hr)	1.5 ± 0.32	1.5 ± 0.36^{NS}	1.5 ± 0.29^{NS}	1.5 ± 0.42^{NS}	1.5 ± 0.54^{NS}	1.5 ± 0.22^{NS}	1.5 ± 0.28^{NS}
3	AUC (µg.hr/ml)	17.75 ± 2.29	18.0 ± 3.62^{NS}	24.67 ± 2.54	29.17 ± 4.32	18.44 ± 2.46	25.11 ± 1.23	30.22 ± 3.54^{NS}
4	$t_{1/2}$ (hr)	1.5 ± 0.21	1.5 ± 0.36	1.9 ± 0.18	2.3 ± 0.46	1.5 ± 0.25	2.0 ± 0.32	2.4 ± 0.29
5	k_{el} (hr^{-1})	0.46 ± 0.028	0.46 ± 0.032^{NS}	0.36 ± 0.027	0.30 ± 0.032	0.46 ± 0.034	0.34 ± 0.018	0.28 ± 0.016
6	RB (%)	100	101	139	164	103	141	181
7	k_a (hr^{-1})	2.31 ± 0.12	2.29 ± 0.87^{NS}	2.47 ± 0.23^{NS}	2.78 ± 0.74^{NS}	2.32 ± 0.42^{NS}	2.48 ± 1.10^{NS}	2.94 ± 0.47^{NS}

Values are in mean ± SEM; n=6 in each group; P<0.05 in comparison with control; C_{max}: peak concentration; t_{max}: time to reach peak concentration; AUC: area under plasma concentration time curve from 0 hrs to 24 hr; $t_{1/2}$: plasma half life; k_{el}: elimination rate constant; RB (%): relative bioavailability; ka: absorption rate constant; NS: not significant.

Table 5.15 Mean plasma concentration of cefixime after oral administration of cefixime (100 mg/kg), co-administration with methanol extract of *H.nepalense* (550 mg/kg) and compound II (25, 50 mg/kg) and preadministered with *H.nepalense* (550 mg/kg) and compound II (25, 50 mg/kg) in rabbit.

Sl. No	Time of collection (hr)	(Group I) Cefixime (100 mg/kg)	(Group II) methanol extract (550 mg/kg) + cefixime (100 mg/kg)	(Group III) Compound II (25 mg/kg) + cefixime (100 mg/kg)	(Group IV) Compound II (50 mg/kg) + cefixime (100 mg/kg)	(Group V) methanol extract (550 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VI) Compound II (25 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VII) Compound II (50 mg/kg) + cefixime (100 mg/kg) 30 min later
1	0.5	1.76 ± 0.218	1.81 ± 0.241	1.74 ± 0.365	1.76 ± 0.374	1.79 ± 0.435	1.79 ± 0.423	1.92 ± 0.423
2	1.0	2.97 ± 0.165	2.94 ± 0.235	2.90 ± 0.263	2.95 ± 0.371	2.91 ± 0.262	3.02 ± 0.233	2.99 ± 0.327
3	1.5	4.03 ± 0.428	4.04 ± 0.247	4.02 ± 0.427	4.11 ± 0.271	4.08 ± 0.264	4.24 ± 0.325	4.14 ± 0.356
4	2.0	3.54 ± 0.479	3.56 ± 0.238	3.58 ± 0.395	3.58 ± 0.503	3.57 ± 0.824	3.63 ± 0.128	3.67 ± 0.473
5	2.5	2.81 ± 0.597	2.79 ± 0.361	2.78 ± 0.223	2.79 ± 0.360	2.76 ± 0.172	2.84 ± 0.253	2.83 ± 0.763
6	3.0	2.09 ± 0.332	2.07 ± 0.321	2.22 ± 0.218	2.16 ± 0.147	2.11 ± 0.322	2.28 ± 0.423	2.24 ± 0.536
7	4.0	1.82 ± 0.451	1.87 ± 0.483	1.84 ± 0.436	1.79 ± 0.261	1.81 ± 0.328	1.81 ± 0.522	1.87 ± 0.345
8	5.0	1.10 ± 0.263	1.11 ± 0.321	1.11 ± 0.451	1.13 ± 0.156	1.12 ± 0.491	1.22 ± 0.024	1.23 ± 0.175
9	6.0	0.86 ± 0.033	0.91 ± 0.332	0.79 ± 0.034	0.84 ± 0.036	0.82 ± 0.064	0.86 ± 0.063	0.94 ± 0.026
10	8.0	0.42 ± 0.014	0.44 ± 0.625	0.41 ± 0.063	0.42 ± 0.025	0.43 ± 0.035	0.44 ± 0.032	0.49 ± 0.018
11	10.0	0.17 ± 0.021	0.16 ± 0.032	0.19 ± 0.062	0.21 ± 0.013	0.18 ± 0.054	0.23 ± 0.058	0.23 ± 0.015
12	12.0	0.08 ± 0.065	0.09 ± 0.004	0.08 ± 0.003	0.11± 0.012	0.09 ± 0.005	0.14 ± 0.046	0.16 ± 0.012
13	24.0	0.02 ± 0.004	0.01 ± 0.005	0.01 ± 0.005	0.01 ± 0.006	0.02 ± 0.003	0.05 ± 0.008	0.09 ± 0.005

Values are expressed in μg/ml and mean ± SEM; n=6 in each group. $P<0.05$ in comparison with control

Table 5.16 Comparison of pharmacokinetic parameters of cefixime alone and in combination with methanol extract of *H.nepalense* and compound II

Sl. No	Parameters	(Group I) Cefixime (100 mg/kg)	(Group II) methanol extract (550 mg/kg) + cefixime (100 mg/kg)	(Group III) Compound II (25 mg/kg) + cefixime (100 mg/kg)	(Group IV) Compound II (50 mg/kg) + cefixime (100 mg/kg)	(Group V) methanol extract (550 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VI) Compound II (25 mg/kg) + cefixime (100 mg/kg) 30 min later	(Group VII) Compound II (50 mg/kg) + cefixime (100 mg/kg) 30 min later
1	C_{max} (µg/ml)	4.03 ± 0.42	4.04 ± 0.24	4.02 ± 0.42	4.11 ± 0.27	4.08 ± 0.26	4.24 ± 0.32	4.14 ± 0.35
2	t_{max} (hr)	1.5 ± 0.22	1.5 ± 0.31 ^{NS}	1.5 ± 0.27 ^{NS}	1.5 ± 0.38 ^{NS}	1.5 ± 0.17 ^{NS}	1.5 ± 0. 57 ^{NS}	1.5 ± 0.46 ^{NS}
3	AUC (µg.hr/ml)	14.73 ± 3.58	15.33 ± 1.92^{NS}	15.12 ± 4.68^{NS}	15.61 ± 3.34 ^{NS}	15.23 ± 2.37^{NS}	16.37 ± 4.12	17.03 ± 2.15
4	$t_{1/2}$ (hr)	3.01 ± 0.21	3.01 ± 0.35	3.00 ± 0.14	3.02 ± 0.56	3.01 ± 0.22	3.11 ± 0.61	3.2 ± 0.15
5	k_{el} (hr^{-1})	0.23 ± 0.046	0.23 ± 0.024	0.23 ± 0.018	0.22 ± 0.032	0.23 ± 0.015	0.22 ± 0.016	0.21 ± 0.018
6	RB (%)	100	104	102	105	103	111	115
7	k_a (hr^{-1})	2.31 ± 0.12	2.31 ± 0.67^{NS}	2.31 ± 0.19	2.34 ± 0.42^{NS}	2.33 ± 0.38^{NS}	2.38 ± 1.15^{NS}	2.40 ± 0.28^{NS}

Values are in mean ± SEM; n=6 in each group; P<0.05 in comparison with control; C_{max}: peak concentration; t_{max}: time to reach peak concentration; AUC: area under plasma concentration time curve from 0 hrs to 24 hr; $t_{1/2}$: plasma half life; k_{el}: elimination rate constant; RB (%): relative bioavailability; ka: absorption rate constant; NS: not significant.

Table 5.17 Mean plasma concentration of rifampicin after oral administration of rifampicin (100 mg/kg), co-administration with methanol extract of *H.nepalense* (550 mg/kg) and compound II (25. 50 mg/kg) and preadministered with *H.nepalense* (550 mg/kg) and compound II (25, 50 mg/kg) in rabbit.

Sl. No	Time of collection (hr)	(Group I) Rifampicin (100 mg/kg)	(Group II) methanol extract (550 mg/kg) + rifampicin (100 mg/kg)	(Group III) Compound II (25 mg/kg) + rifampicin (100 mg/kg)	(Group IV) Compound II (50 mg/kg) + rifampicin (100 mg/kg)	(Group V) methanol extract (550 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VI) Compound II (25 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VII) Compound II (50 mg/kg) + rifampicin (100 mg/kg) 30 min later
1	0.5	2.68 ± 0.236	2.61 ± 0.335	3.12 ± 0.289	3.64 ± 0.747	2.64 ± 0.321	3.26 ± 0.411	3.82 ± 0.346
2	1.0	3.49 ± 0.424	3.58 ± 0.282	3.97 ± 0.158	4.12 ± 0.224	3.57 ± 0.136	4.04 ± 0.269	5.87 ± 0.462
3	1.5	4.08 ± 0.351	4.54 ± 0.721	5.26 ± 0.462	5.89 ± 0.251	4.56 ± 0.342	8.14 ± 0.376	9.23 ± 0.204
4	2.0	5.81 ± 0.365	6.12 ± 0.147	7.93 ± 0.182	8.96 ± 0.623	6.16 ± 0.223	7.79 ± 0.661	8.86 ± 0.262
5	2.5	6.95 ± 0.342	7.11 ± 0.258	7.17 ± 0.621	8.02 ± 0.536	7.14 ± 0.431	7.21 ± 0.492	8.08 ± 0.213
6	3.0	6.16 ± 0.427	6.38 ± 0.225	6.69 ± 0.548	7.78 ± 0.542	6.32 ± 0.174	6.69 ± 0.324	7.79 ± 0.425
7	4.0	5.64 ± 0.439	5.79 ± 0.237	5.83 ± 0.364	6.97 ± 0.254	5.69 ± 0.245	5.92 ± 0.352	6.96 ± 0.420
8	5.0	4.68 ± 0.157	4.86 ± 0.169	4.78 ± 0.525	5.83 ± 0.424	4.93 ± 0.364	4.76 ± 0.313	5.87 ± 0.413
9	6.0	3.52 ± 0.236	3.67 ± 0.493	3.92 ± 0.264	4.92 ± 0.668	3.65 ± 0.274	3.89 ± 0.621	4.94 ± 0.815
10	8.0	2.36 ± 0.428	2.42 ± 0.221	2.86 ± 0.671	3.76 ± 0.446	2.48 ± 0.245	2.91 ± 0.121	3.78 ± 0.309
11	10.0	1.05 ± 0.412	1.23 ± 0.109	1.98 ± 0.282	2.52 ± 0.462	1.25 ± 0.163	1.97 ± 0.018	2.34 ± 0.386
12	12.0	0.66 ± 0.017	0.76 ± 0.021	1.14 ± 0.018	1.32 ± 0.024	0.79 ± 0.011	1.22 ± 0.035	1.86 ± 0.252
13	24.0	0.12 ± 0.032	0.19 ± 0.011	0.26 ± 0.025	0.37 ± 0.026	0.18 ± 0.023	0.25 ± 0.016	0.52 ± 0.018

Values are expressed in μg/ml and mean ± SEM; n=6 in each group. $P<0.05$ in comparison with control

Table 5.18 Comparison of pharmacokinetic parameters of rifampicin alone and in combination with methanol extract of *H.nepalense* and compound II

Sl. No	Parameters	(Group I) Rifampicin (100 mg/kg)	(Group II) methanol extract (550 mg/kg) + rifampicin (100 mg/kg)	(Group III) Compound II (25 mg/kg) + rifampicin (100 mg/kg)	(Group IV) Compound II (50 mg/kg) + rifampicin (100 mg/kg)	(Group V) methanol extract *H.nepalense* (550 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VI) Compound II (25 mg/kg) + rifampicin (100 mg/kg) 30 min later	(Group VII) Compound II (50 mg/kg) + rifampicin (100 mg/kg) 30 min later
1	C_{max} (µg/ml)	6.95 ± 0.34	7.11 ± 0.25	7.93 ± 0.18	8.96 ± 0.62	7.14 ± 0.43	8.14 ± 0.37	9.23 ± 0.20
2	t_{max} (hr)	2.5 ± 0.27	2.5 ± 0.32 ^{NS}	2.0 ± 0.24	2.0 ± 0.23	2.5 ± 0.31 ^{NS}	1.5 ± 0.46	1.5 ± 0.18
3	AUC (µg.hr/ml)	43.93 ± 3.49	46.66 ± 3.25^{NS}	54.43 ± 4.61	65.33 ± 2.36	46.9 ± 2.36	56.52 ± 3.18	72.35 ± 3.62^{NS}
4	$t_{1/2}$ (hr)	3.01 ± 0.42	3.01 ± 0.32	3.62 ± 0.42	3.85 ± 0.42	3.01 ± 0.16	3.64 ± 0.31	4.07 ± 0.78
5	k_{el} (hr $^{-1}$)	0.23 ± 0.024	0.23 ± 0.018	0.19 ± 0.016	0.18 ± 0.041	0.23 ± 0.015	0.19 ± 0.021	0.17 ± 0.013
6	RB (%)	100	106	124	149	107	129	165
7	k_a (hr $^{-1}$)	2.66 ± 0.22	2.68 ± 0.46^{NS}	2.73 ± 1.27 ^{NS}	2.86 ± 0.34^{NS}	2.68 ± 0.18^{NS}	2.74 ± 0.76^{NS}	2.89 ± 0.37^{NS}

Values are mean ± SEM; n=6 in each group; P<0.05 in comparison with control; C_{max}: peak concentration; t_{max}: time to reach peak concentration; AUC: area under plasma concentration time curve from 0 hrs to 24 hr; $t_{1/2}$: plasma half life; k_{el}: elimination rate constant; RB (%): relative bioavailability; ka: absorption rate constant; NS: not significant.

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CHAPTER 6

IMMUNOSTIMULATION ACTIVITY

6.1. Introduction

Ayurveda, the Indian traditional system of medicine lays emphasis on promotion of health as a concept of prevention of diseases and strengthening of both physical and mental health [1]. It was recognized in the Ayurveda that the immune system was involved in the etiology and pathophysiologic mechanisms of various inflammatory diseases of the skin, gut, respiratory tract, joints and central organs as well as in infectious diseases. It held the doctrine that modulation of the immune response would alleviate the diseases and the concept of Rasayana in Ayurveda was based on related principles [2].

Immunostimulants or immunopotentiators are drugs leading predominantly to a non-specific stimulation of immunological defense mechanisms. Most of them are not real antigens but antigenomimetics or so-called mitogens. Non-specific and non-antigen dependent stimulants do not affect immunological memory cells and, since their pharmacological efficacy fades comparatively quickly, they have to be administered either in intervals or continuously. Some immunostimulants may also stimulate T-suppressor cells and thereby reduce immune resistance, hence the term immunomodulation or immunoregulation, denoting any effect on, or change of, immune responsiveness [3, 4].

The human body is continuously exposed to a series of stress factors, which more or less weaken the function of the immune system and hereby generate immunosupression. Immunosupression can be generated by severe bacterial and viral infections, cancer, environmental agents such as pesticides or allergens, excessive long-term chemo- or radiotherapy, malnutrition, psychic stress or endogenic autoimmune reactions [5]. Day by day dramatic increase in microorganisms resistant to antibiotics and chemotherapeutic agents are being recorded. Also the emerge of new plagues such as AIDS and return of the old diseases like tuberculosis has been observed in the modern world. We have no effective vaccines against some severe infections or parasitic diseases, and many chronic diseases are the consequences of an unbalanced or impaired immune response. Among them, the recurrent opportunistic infections, skin and intestine inflammations are the most

important ones, and we should realize that the incidence of some severe infections such as AIDS might be positively influenced by a restoration of the chronically suppressed immune system. In view of the above, the present investigation was undertaken to evaluate the immunostimulatory potentiality of *H. nepalense* and *C.oppositifolia* both by *in vitro* and *in vivo* model system based on the evidence of ethnomedicinal use of these plants in such diseases of immunosupression state in Sikkim.

6.2. Materials and Methods

6.2.1. Plant materials

Methanol extracts of *C.oppositifolia* leaf and *H.nepalense* root as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

6.2.2. Test compound formulations

Oral suspensions of the leaf & root extract and isolated compounds were prepared by suspending them separately in 1% solution of sodium carboxy methylcellulose to obtain suitable dosage forms.

6.2.3. Animal used

Swiss Albino mice of either sex, weighing 17–25 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 diet and water *ad libitum*.

6.2.4. Drugs and Chemicals

EDTA, RPMI- 1640, Hank's balanced salt solution (HBSS), Dextran, Phosphate buffered saline, Fetal calf serum, Streptomycin, Penicillin, Amphotericin, Trypan blue were purchased from Himedia Laboratories Pvt. Ltd, Mumbai. Phythaemagglutinin, Ficoll Hypaque, L-glutamine were purchased from Sigma diagnostic, USA. Interferon α-2b and levamisole were obtained as gift samples from Fulford (I) Ltd and Khandelwal Laboratories Ltd, Mumbai respectively.

6.2.5. Antigen

Fresh blood of a healthy sheep was collected from the local slaughterhouse. Sheep red blood cells (SRBCs) were washed three times with normal saline and adjusted to a concentration of 1×10^8 cells in 0.1 ml for immunization and challenge.

6.2.6. Immunostimulant activity of *C.oppositifolia* leaf

6.2.6.1. *In vivo* carbon clearance test

Mice were divided into eight groups, each containing ten animals. Group I (Control) was given 1% Sodium carboxy methyl cellulose in water (0.3 ml/mouse) for seven days, Group II- V were administered different concentration of methanol extract (250- 1000 mg/kg, p.o.), Group VI was administered standard drug (Levamisole 50 mg/kg, p.o.) and Group VII & VIII were given compound I (25 and 50 mg/kg, p.o.) for seven days. At the end of seven days, mice of all the groups were injected via the tail vein the carbon ink suspension (10 µl/gm body weight). Blood samples were drawn (in EDTA solution 5µl) from the retro-orbital vein at intervals of 0 and 15 min, a 25 µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and their absorbance were measured at 660 nm. The carbon clearance was calculated using the equation: $(\text{Log}_e\ OD_1 - \text{Log}_e\ OD_2)/\ 15$, where OD_1 and OD_2 are the optical densities at 0 and 15 min, respectively [6].

6.2.6.2. *In vivo* humoral antibody titer and delayed type hypersensitivity response

6.2.6.2.1. Humoral antibody titer test (HA)

Mice were divided into eight groups, each group containing six mice. Group I (Control) was given 1% Sodium carboxy methylcellulose in water (0.3 ml/mouse) for seven days, Group II- V were given different concentration of methanol extract (250- 1000 mg/kg, p.o.). Group VI was administered standard drug (Levamisole 50 mg/kg, p.o.) and Group VII & VIII were given compound I (25 and 50 mg/kg, p.o.) for seven days.

The animals of all the groups were immunized by injecting 0.1 ml of SRBCs suspension containing 1×10^8 cells intraperitoneally on day 0. Blood samples were collected in microcentrifuge tubes from individual animal of all the groups by retro-orbital vein

puncture on day 8. The blood samples were centrifuged and serum was separated. Antibody levels were determined by the haemagglutination technique [6]. Briefly, equal volumes of (50 µl) individual serum samples of each group were polled. To serial two fold dilutions of pooled serum samples made in 50 µl volumes of RPMI-1640 in microtitration plates, 50 µl of 1% suspension of SRBC in RPMI-1640 was added. After mixing, the plates were incubated at 37°C for 1 h and examined for haemagglutination under microscope (button formation). The reciprocal of highest dilution, just before the button formation, was observed as the titre values of the test samples.

6.2.6.2.2. Delayed type hypersensitivity test (DTH)

The experiment of DTH was commenced after the Humoral Antibody titre model in the same animals. On day 8, the thickness of the right hind footpad was measured using vernier calliper. The mice were then challenged by injection of 1×10^8 SRBCs in right hind footpad. The footpad thickness was measured again after 24 h of the challenge. The difference between the pre and post challenge footpad thickness expressed in mm was taken as a measure of DTH response [7].

6.2.6.3. *In vitro* phagocytic index determination

6.2.6.3.1. Preparation of microorganism

Escherichia coli 832 *(E. coli)* was grown and kept on a slope of solid agar medium. Before use, microorganism is cultured in 100 ml of 2.5% nutrient broth (Oxoid) for about 18 h at 37°C. The culture was then washed twice with phosphate buffer saline and re-suspended in gelatin- Hank's balanced salt solution (HBSS) to a concentration of (1×10^7 cells/ml). To determine the exact number of microbes used during each time the number of viable microorganism was determined microbiologically by counting colony forming units (cfu) using nutrient agar plates followed by incubation at 37°C for 18h [8].

6.2.6.3.2. Preparation of human polymorphonuclear leukocyte (PMNCs)

Human blood was collected from local blood bank and the red blood cells were removed by sedimentation in 5% (w/v) solution of dextran in buffered saline (m.w 200,000; 3 ml

of solution to 10 ml of blood) for 30 min at 37°C. The PMNCs rich supernatant layer was washed twice with heparin-saline, concentrated by centrifugation (10 min at 110 g), counted with a haemocytometer, and suspended in gelatin in HBSS to a concentration of (1×10^7 cells/ml).

6.2.6.3.3. Microbiological assay for the phagocytosis

For assessing phagocytosis, different concentrations of methanol extract (250-1000 µg/ml), compound I (25 and 50 µg/ml) and standard drug Interferon α-2b (0.5 million IU) in the final volume of 0.1ml were incubated respectively with 2ml of the PMNCs suspension (1×10^7 cells/ml), 2ml of the suspended microorganisms (1×10^7 cells/ml) and 0.4 ml of fetal calf serum at 37°C for 1 h in 5% CO_2 atmosphere in slanting position. At 30 min intervals up to 120 min, 0.5 ml aliquot of the suspension was removed and added to 1.5 ml of the ice-cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 110 g for 4 min. Under this condition the non-ingested microorganisms were remained in the supernatant fluid. The viable count of the microorganisms was done by using the colony counter [8]. Phagocytosis was expressed as the percentage decrease in the initial number of viable extra cellular bacteria according to the following formula: $P(t) = (1 - N_t/N_0) \times 100$, where P (t) is the phagocytic index at time t=t, N_0 and N_t are the number of viable extra cellular bacteria at time t = 0 and t = t, respectively [9].

6.2.6.4. *In vitro* Cell proliferation assay

This test was performed with total peripheral mononuclear blood cells, following their separation from the blood by using ficoll-hypaque gradient centrifugation, according to manufacture's instructions (Sigma diagnostic, USA). The rate of proliferation of mononuclear cells under the influence of mitogens was measured by the method of Sriwanthana [10] with minor modification. Briefly, under sterile conditions, the cells were diluted to 1×10^7 cells/ml with RPMI-1640 (supplemented with 20% fetal calf serum). The suspension of cells (2 ml) was transferred into sterile culture tube and to each sample different concentration of the plant extract (250-1000 µg/ml, filtered through 0.22 µ pore

size filter), compound I (25 and 50 µg/ml) and standard drug Interferon α-2b (0.5 million IU) in the final volume of 0.1 ml were added respectively. Proliferation of cells was induced by 50 µl phythaemagglutinin (PHA, 0.1 mg/ml). The prepared samples were incubated for 72 h at 37°C in a CO_2 atmosphere, supplemented with 2 mM L- Glutamine solution, streptomycin 100 µg/ml of sample, 100 units/ml samples of penicillin and 0.25 µg/ml sample of amphotericin. The control was kept for incubation with cells without the plant extract. The viability of the cells was assessed after incubation with test compounds with the help of trypan blue dye exclusion method [11]. Briefly, 20 µl of the incubation mixture was mixed with 20 µl of 10% w/v solution of Trypan blue dye. The total number of mononuclear cells and mononuclear dead cells (stained blue) were counted under inverted microscope (Olympus, Japan), using haemocytometer. The percentage of cell viability was taken as a measure of cell proliferation and calculated as per the following formula [12].

$$\% \text{ of viability} = \frac{\text{Total number of cells} - \text{Total number of dead cells} \times 100}{\text{Total number of cells.}}$$

Similarly, the percentage of cell stimulation was calculated as per the following formula[5].

$$\% \text{ of cell stimulation} = \frac{\% \text{ Viability of test compound} - \% \text{ Viability of control} \times 100}{\% \text{ Viability of control}}$$

6.2.7. Immunostimulant activity of *H.nepalense* root.

The immunostimulation activity of methanol extract of *H.nepalense* root and its isolated compound II was determined as per the methods described above in **6.2.6** for *C.oppositifolia* leaf.

6.3. Results

6.3.1. Immunostimulant activity of *C.oppositifolia* leaf

The results presented in Table 6.1 showed the immunostimulatory activity of methanol extract of *C.oppositifolia* leaf and compound I in mice. The results revealed that animals

treated with lower doses of methanol extract i.e. 250 – 750 mg/kg did not show much increase rate of in carbon clearance from blood. However at higher dose (1000 mg/kg) increase in the rate of carbon clearance was evident though the values were not statistically significant. Administration of isolated compound I at 50mg/kg resulted in significant increase in the rate of carbon clearance compared with control group. On the other hand the rate of carbon clearance value of compound I was found to be slightly lower than that of the standard compound levimasole at the same dose.

Next the ability of methanol extract and compound I to induce the HA titer and DTH response has been determined. The results of HA titer is presented in Figure 6.1. The HA titer and DTH response are believed to be related with humoral mediated and cell mediated immunity. Treatment of mice with methanol extract at 250 – 1000 mg/kg dose did not show much increase in HA titer value as evident from haemagglutination after incubation of serum with SRBCs. While the compound I and levimasole at 50 mg/kg showed 228.6 ± 3.23 and 432.02 ± 6.5 HA titer value respectively. In DTH response test the methanol extract at higher dose (1000 mg/kg) showed statistically significant increase in mean paw edema (0.38 ± 0.34) in mice, as compared with (0.42 ± 0.21) the standard drug levamisole at a concentration of 50 mg/kg. The isolated compound I at 50 mg/kg dose showed significant DTH response (0.40 ± 0.32) which is considered to be almost equal to the standard drug.

The effect of methanol extract (250 – 1000 μg/ml) and compound I (25 – 50 μg/ml) on phagocytic index is presented in Table 6.2. The phagocytosis of microorganism was assessed at 30 min interval up to 120 min incubation with the test substances. The results showed that the methanol extract at 1000 μg/ml possesses moderate phagocytic activity (94.34 ± 1.24) compared with the control (89.78 ± 1.84) after 120 min incubation. Maximum phagocytic index was observed at 50 μg/ml concentration of isolated compound (96.24 ± 2.14), which is slightly lower than that of the standard drug Interferon α-2b (99.54 ± 1.62) after 120 min of incubation.

Results concerning the proliferative response of the test compound on the viability of mononuclear cells in presence of the PHA mitogen were presented in Table 6.3. Percentage of viability of PHA activated mononuclear cells has found to be not increased at all the concentrations of the methanol extract tested and hence there was stimulation of cells viability. However, the compound I at 50 μg/ml concentrations showed 56.2 ± 6.54 % cell viability compared to the control (51.5 ± 4.62 %). Further, the standard drug Interferon α-2b at 0.5 millions IU and compound I at 50 μg/ml concentration showed 22.26 % and 9.70 % cell stimulation respectively.

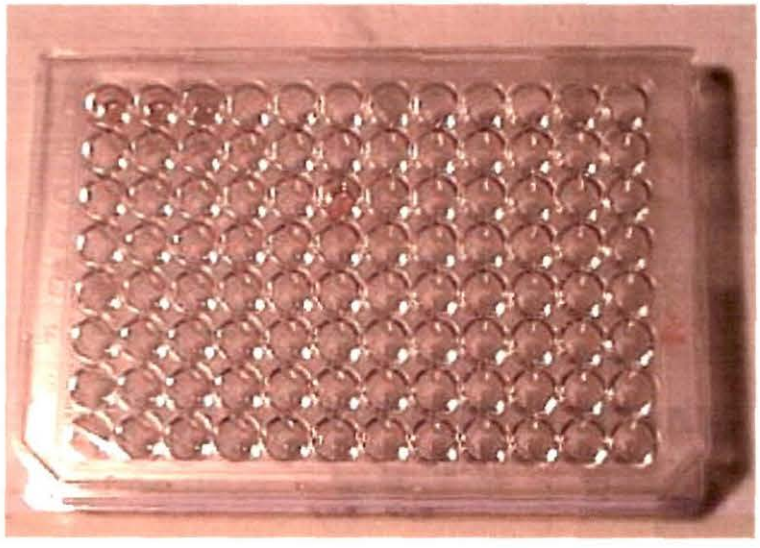

Figure 6.1. Photograph showing effect of methanol extract of *C.oppositifolia* (1000 mg/kg) on haemagglutination antibody titer in mice.

Table 6.1. Effect of *C.oppositifolia* leaf extract and compound I on immunostimulant activity in mice.

Group number	Treatment (mg/kg)	Carbon clearance	HA titer	DTH response (mm)
I	Control	0.069 ± 0.014	82.23 ± 2.24	0.28 ± 0.12
II	Extract (250)	0.073 ± 0.015[a]	86.4 ± 2.12[a]	0.29 ± 0.14
III	Extract (500)	0.076 ± 0.013[a]	96.8 ± 2.34[a]	0.29 ± 0.15
IV	Extract (750)	0.077 ± 0.016[a]	112.4 ± 6.34[a]	0.31 ± 0.12[a]
V	Extract (1000)	0.094 ± 0.014	132.2 ± 2.32[a]	0.38 ± 0.24[a]
VI	Levamisole (50)	0.158 ± 0.014[a]	432.02 ± 6.5[a]	0.42 ± 0.21[a]
VII	IC 25	0.116 ± 0.014[a]	218.4 ± 2.3	0.39 ± 2.23
VIII	50	0.124 ± 0.015[a]	228.6 ± 3.23[a]	0.40 ± 0.32[a]

Values are mean ± SEM; n=6 in each group. [a]: $P < 0.05$ in comparison with control.
IC: Isolated Compound I.

Table 6.2. Effect of *C.oppositifolia* leaf extract and compound I on phagocytic index of polymorphonuclear leukocyte.

Concentration (μg/ml)	Phagocytic index (%)			
	30 min	60 min	90 min	120 min
Control	61.38 ± 2.21	64.18 ± 1.32	78.18 ± 1.49	89.78 ± 1.84
250	62.24 ± 1.62[a]	66.54 ± 1.28[a]	79.54 ± 1.41[a]	90.14 ± 1.56[a]
500	63.62 ± 1.48[a]	69.15 ± 1.21[a]	84.67 ± 1.21[a]	92.28 ± 1.33[a]
750	64.54 ± 1.34[a]	72.23 ± 1.22[a]	86.23 ± 1.42[a]	93.14 ± 1.26[a]
1000	66.52 ± 1.22[a]	78.52 ± 1.17[a]	89.24 ± 1.14[a]	94.34 ± 1.24[a]
Interferon α-2b (0.5 millions IU)	71.54 ± 1.24[a]	82.92 ± 1.46[a]	93.38 ± 1.72[a]	98.54 ± 1.62[a]
IC 25	67.23 ± 1.42[a]	80.21 ± 1.44[a]	89.14 ± 1.62[a]	93.46 ± 2.12[a]
50	68.24 ± 1.14[a]	81.24 ± 1.52[a]	90.85 ± 1.52[a]	96.24 ± 2.14[a]

Values are mean ± SEM; n=6 in each group. [a]: $P<0.05$ in comparison with control. IC: Isolated Compound I.

Table 6.3. Effect of *C.oppositifolia* leaf extract and compound I on proliferation of mononuclear cell

Concentration (μg/ml)		Viability of cells (%)	Stimulation of cells (%)
Control		51.2 ± 4.62	0
250		42.2 ± 5.32[a]	(– 17.57)
500		46.4 ± 4.52[a]	(– 9.37)
750		49.5 ± 4.26[a]	(– 3.32)
1000		51.6 ± 3.64[a]	(+0.78)
Interferon α-2b (0.5 millions IU)		62.6 ± 4.58[a]	(+22.26)
IC	25	54.6 ±3.75[a]	(+6.64)
	50	56.2 ± 6.54[a]	(+9.76)

Values are mean ± SEM; n=6 in each group. [a]: $P<0.05$ in comparison with control. IC: compound I. "+": Indicates increase and "–": Indicates decrease of cell stimulation.

6.3.2. Immunostimulant activity of *H.nepalense* root

The results presented in Table 6.4 showed that the methanol extract at 250 – 1000 mg/kg, p.o. and its isolated compound at 25 and 50 mg/kg, p.o. doses exhibited significant increase in carbon clearance from the blood in a dose dependent manner. The methanol extract at 1000 mg/kg exhibited ($P<0.05$) maximum carbon clearance (0.158 ± 0.018) and its isolated compound at 50 mg/kg dose showed (0.160 ± 0.018) carbon clearance ($P<0.05$); while levamisole showed (0.164 ± 0.016) clearance value. The results presented in Table 6.4 indicated that animals treated with doses of 250, 750 and 1000 mg/kg produced significant increase in HA titre (Humoral mediated immunity) as evident from haemagglutination after incubation of serum with SRBCs (Figure 6.2). While the isolated compound at 50 mg/kg and levamisole at the same dose showed 328.6 ± 10.4 and 430.06 ± 8.3 HA titer respectively, which is evident from the Figure 6.2. In DTH response (Cell mediated immunity) test the methanol extract at higher doses (750 and 1000 mg/kg) showed statistically significant increase in mean paw edema in mice. The isolated compound at a dose of 50 mg/kg on the other hand exhibited maximum DTH response of 0.50 ± 0.22 which is almost comparable to the result obtained for the standard drug levamisole (0.57 ± 0.21) at the same dose.

The effect of methanol extract (250 –1000 µg/ml) and its isolated compound (25 – 50 µg/ml) on phagocytic index model is presented in Table 6.5. The results showed that phagocytic index was increased ($P< 0.05$) on dose dependent manner after 30, 60, 90 and 120 min intervals in the presence of methanol extract and isolated compound. Maximum phagocytic index was observed at 1000 µg/ml of methanol extract (97.86 ± 1.67) and 50 µg/ml of isolated compound (97.24 ± 1.23) after 120 min of incubation; whereas the standard compound Interferon α–2b at 0.5 million IU concentration exhibited maximum phagocytic index (99.23 ± 1.11) after 120 min. Results obtained for the proliferative response of the test samples on basis of the viability of mononuclear cells to the PHA mitogen were presented in Table 6.6. Percentage of viability of PHA activated mononuclear cells was significantly increased by 56.3 ± 8.94 % at 1000 µg/ml concentration of root extract, compared with the control, in which it was observed to be 50.7 ± 10.42 %. The maximum viability (60.4 ± 4.58 %) was noticed with the standard

drug Interferon α-2b. The isolated compound at 50 μg/ml concentration has showed 58.2 ± 4.53 % viability, which is also a comparable value to the standard compound Interferon, α–2b. Further, the methanol extract at 1000 μg/ml and isolated compound at 50 μg/ml concentration showed 11.04 % and 14.79% cell stimulations respectively as compared with Interferon α-2b, for which it was observed to be 19.13%.

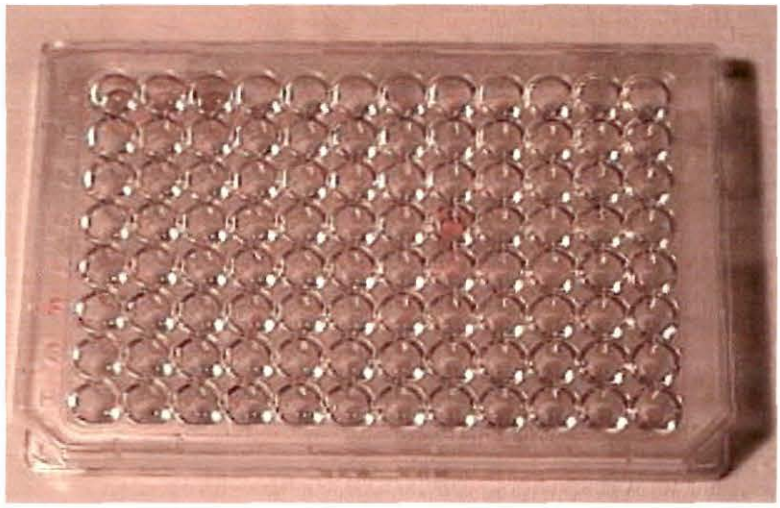

Figure 6.2. Photograph showing effect of methanol extract of *H.nepalense* (1000 mg/kg) on haemagglutination antibody titer in mice.

Table 6.4. Effect of methanol root extract of *H.nepalense* and compound II on immunostimulant activity in mice.

Group number	Treatment (mg/kg)	Carbon clearance	HA titer	DTH response (mm)
I	Control	0.068 ± 0.012	80.43 ± 2.21	0.28 ± 0.10
II	Extract (250)	0.122 ± 0.016[a]	185.3 ± 3.31[a]	0.39 ± 0.13
III	Extract (500)	0.132 ± 0.014[a]	172.7 ± 0.1[a]	0.28 ± 0.13
IV	Extract (750)	0.146 ± 0.016[a]	282.6 ± 8.1[a]	0.43 ± 0.10[a]
V	Extract (1000)	0.158 ± 0.018[a]	320.8 ± 10.6[a]	0.48 ± 0.21[a]
VI	Levamisole (50)	0.164 ± 0.016[a]	430.06 ± 8.3[a]	0.57 ± 0.21[a]
VII	IC 25	0.156 ± 0.016[a]	324.2 ± 8.3	0.48 ± 0.22
VIII	50	0.160 ± 0.018[a]	328.6 ± 10.4[a]	0.50 ± 0.22[a]

Values are mean ± SEM; n=6 in each group. [a]: $P < 0.05$ in comparison with control.
IC: Isolated compound II.

Table 6.5. Effect of methanol root extract of *H.nepalense* and compound II on phagocytic index of polymorphonuclear leukocyte.

Concentration (μg/ml)	Phagocytic index (%)			
	30 min	60 min	90 min	120 min
Control	61.14 ± 1.26	67.26 ± 1.52	84.32 ± 1.69	92.25 ± 1.83
250	63.53 ± 1.52[a]	78.54 ± 1.18[a]	85.74 ± 1.43[a]	94.27 ± 1.86[a]
500	64.12 ± 1.18[a]	79.23 ± 1.21[a]	86.23 ± 1.24[a]	95.67 ± 2.33[a]
750	67.38 ± 1.24[a]	81.52 ± 1.42[a]	88.15 ± 1.86[a]	96.55 ± 1.83[a]
1000	69.54 ± 1.12[a]	83.26 ± 1.37[a]	91.18 ± 2.14[a]	97.86 ± 1.67[a]
Interferon α-2b (0.5 millions IU)	70.27 ± 1.64[a]	84.14 ± 1.32[a]	94.52 ± 1.65[a]	99.23 ± 1.11[a]
IC 25	69.54 ± 1.12[a]	82.86 ± 1.45[a]	90.96 ± 1.14[a]	96.42 ± 1.42[a]
50	70.27 ± 1.64[a]	83.09 ± 1.51[a]	91.14 ± 1.62[a]	97.24 ± 1.23[a]

Values are mean ± SEM; n=6 in each group. [a]: $P<0.05$ in comparison with control.
IC: Isolated compound.

Table 6.6. Effect of methanol root extract of *H.nepalense* and compound II on proliferation of mononuclear cell

Concentration (µg/ml)		Viability of cells (%)	Stimulation of cells (%)
Control		50.7 ± 10.42	0
250		47.6 ± 4.42[a]	(– 6.11)
500		49.8 ± 5.42[a]	(– 1.77)
750		49.0 ± 4.45[a]	(– 3.35)
1000		56.3 ± 8.94[a]	(+11.04)
Interferon α-2b (0.5 millions IU)		60.4 ± 4.58[a]	(+19.13)
IC	25	56.9 ±6.62[a]	(+12.22)
	50	58.2 ± 4.53[a]	(+14.79)

Values are mean ± SEM; n=6 in each group. [a]: $P<0.05$ in comparison with control. IC: Isolated compound. "+": Indicates increase and "–": Indicates decrease in cell stimulation.

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CHAPTER 7

ANTIOXIDANT ACTIVITY

7.1. Introduction

Free radicals are chemical species possessing an unpaired electron that can be considered as fragment of molecules and which are generally extremely reactive and short lived. They are produced continuously in cells either as accidental byproducts of metabolism or deliberately during different pathological disorders and phagocytosis. Free radicals can be formed in mechanisms.

- By the homolytic cleavage of a covalent bond of a molecule, with each fragment retaining one unpaired electron.
- By the loss of a single electron from a normal molecule.
- By the addition of a single electron to a normal molecule.

The electron transfer is a far more common process in biological systems, than that of first mechanism. The most important free radicals in biological systems are the radicals derived from oxygen. With increasing acceptance of free radicals as common and important biochemical intermediates, they have been implicated in a large number of human diseases.

Free radical reaction is an important pathway in wide range of unrelated biological systems. It has been implicated in causing diabetes, liver cirrhosis, nephrotoxicity etc [1]. Reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radical ($OH^\bullet$) and nitric oxide (NO) inactivate enzymes and damage important cellular components causing tissue injury through covalent bonding and lipid peroxidation [2], and thus have been shown to augment collagen synthesis and fibrosis. The increased production of toxic oxygen derivatives is considered to be a universal feature in stress conditions. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human body against infection and degenerative diseases. Presently, there is an increasing realization that herbs can influence the oxidative stress related diseases by maintaining equilibrium between rates of generation of ROS and their neutralization by endogenous antioxidant enzymes [3]. Use of herbs as a source of antioxidant has opened a novel lead for the development of new modalities of treatments.

The aim of the present study is to determine the antioxidant and free radical scavenging activities of the methanol extract of *C.oppositifolia* leaf and *H.nepalense* root and their isolated compounds. For this purpose, plant extracts and their isolated compounds were tested for different free radical scavenging activities and their capacity to reduce lipid peroxidation.

7.2. Materials and Methods

7.2.1. Plant materials

Methanol extracts of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

7.2.2. Chemicals

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. Deoxy ribose was obtained from Merck India. Dimethyl sulphoxide, ethylene diamine tetra acetic acid (EDTA), ferrous sulphate, trichloroacetic acid, hydrogen peroxide, ascorbic acid, mannitol, potassium dihydrogen phosphate, potassium hydroxide, deoxy ribose, phenazine methosulphate were of analytical grade and were obtained from Ranbaxy fine chemicals.

7.2.3. Antioxidant activity of *C.oppositifolia* leaf

7.2.3.1. Lipid peroxidation model

The extent of lipid peroxidation in goat liver homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method [4] with minor modifications [5]. Goat liver was purchased from local slutter house. Its lobes were dried between blotting papers (to remove excess blood) and were cut into small pieces with a heavy-duty blade. They were then homogenized in glass-teflon homogenizer tube in cold phosphate buffer saline (pH 7.4). 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 [6]. To study the comparative response, the experiment was performed in nine glass petri dishes (35 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 & 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 [7]. 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 1 (molar extinction coefficient of TBARS) and expressed as nanomoles (nM)/mg of tissue [8, 9]. The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the Equation 2.

$$\text{nM of TBARS/mg of tissue} = \frac{\text{OD} \times \text{volume of homogenate} \times 100 \times 10^3}{(1.56 \times 10^5) \times \text{volume of extract taken}} \quad \text{---------- Eqn 1}$$

$$\% \text{ Inhibition} = \frac{[(\text{OD of control} - \text{OD of test}) \times 100]}{\text{OD of control}} \quad \text{---------- Eqn 2.}$$

7.2.3.2. DPPH radical scavenging activity

DPPH scavenging activity of *C.oppositifolia* was measured by spectrophotometric method [7]. To each of the nine glass tubes methanolic solution of DPPH (100 µM, 2.95ml) was taken. Tube No. one 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 *C.oppositifolia*

(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 2 mentioned in 7.2.3.1.

7.2.3.3. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of *C.oppositifolia* was measured by studying the competition between deoxy ribose and test compounds for hydroxyl radical generated by the Fe^{3+}– ascorbate – EDTA – H_2O_2 system according to the method of Kunchandy and Rao [10]. The reaction mixture was containing of 100 µl of 2-deoxy-ribose, 500 µl of the various concentrations of the methanol extract (200 to 1000 µg/ml) as well as compound I (25, 50 µg/ml) and standard compound (Mannitol 50 mM) in KH_2PO_4- KOH buffer (20mM, pH 7.4), 200 µl of 1.04 mM H_2O_2 and 100 µl of 1.0mM ascorbic acid. The final volume of the solution was made up to 1.0 ml by adding phosphate buffer. The tubes were incubated at 37°C for 1 hr. One ml of 1% trichloroacetic acid was added to each test tube and incubated at 100°C for 20 min. After cooling to room temperature, absorbance of the content of the tubes was measured at 532nm against a control preparation containing 2-deoxyribose and buffer. Percent inhibition was determined by comparing the results of the test and control samples applying the Equation 2 as mentioned in 7.2.3.1.

7.2.3.4. Superoxide scavenging activity

The superoxide scavenging activity of *C.oppositifolia* was determined by the method described by Nishimik et al [11], 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 2 mentioned in 7.2.3.1.

7.2.4. Antioxidant activity of *H.nepalense* root.

The antioxidant activity of methanol extract of *H.nepalense* root and its isolated compound II was determined as per the methods described above in **7.2.3** for *C.oppositifolia* leaf.

7.3. Results

7.3.1. Antioxidant activity of *C.oppositifolia* leaf

The results presented in Table 7.1 showed the effect of methanol extract of *C.oppositifolia* leaf and compound I on ferrous sulphate induced lipid peroxidation in goat liver homogenate. The results revealed that at 1000 μg/ml concentration of methanol extract had the maximum inhibition percentage (58.67 ± 2.21) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 72.94 ± 2.12. The same results have also been presented in Figure 7.1, which revealed that compound I at 50 μg/ml showed 77.46 ± 2.8 % inhibitions, greater than the inhibition produced by vitamin E. The IC_{50} value, (concentration of the test substances at which 50 % of inhibition produced) was found to be 845.68 ± 3.98 μg/ml. From the results of quantitative estimation of TBARS levels it appears that both the methanol extract at 1000 μg/ml and compound I at 25 μg/ml concentration decreases the levels of TBARS in liver homogenate to a similar level (0.71 ± 0.046), which is highly comparable to the results obtained from the well known antioxidant vitamin E ($p< 0.05$).

The effect of methanol extract and compound I on scavenging of DPPH radical is presented in Table 7.2. The results showed that the DPPH scavenging capacity of the extract was found to be 71.52 ± 2.32 % at 1000 μg/ml. The compound I at 50 μg/ml

concentration, on the other hand, exhibited 80.54 ± 1.34 % compared to 79.69 ± 1.36 % for the standard drug vitamin E at 5mM. The IC_{50} value of the extract was found to be 6.8 mg/ml. The percentage of activity was also dependent on time as depicted from Figure 7.2.

The effect of leaf extract and compound I on scavenging of hydroxyl radical is presented in Table 7.3. The results revealed that the extract significantly inhibited (79.12 ± 3.8 %) degradation of deoxy-ribose mediated by hydroxyl radical at the concentration of 1000 µg/ml, compared to that of known scavenger mannitol (88.14 ± 1.42 %). Here the compound I at 50 µg/ml concentration exhibited inhibition of 81.42 ± 1.36 % compared to 88.14 ± 1.42 % for the standard drug at a concentration of 50 mM. The concentration of the methanol extract needed for 50 % inhibition was 593.64 µg/ml.

The effect of leaf extract and compound I on superoxide scavenging model is presented in Table 7.4. It was found that the methanol extract at concentration of 1000 µg/ml caused significant increase of the inhibition up to 58.46 ± 1.28 %. The compound I at 50 µg/ml concentration exhibited 68.23 ± 1.8 % inhibition of superoxide radicals. IC_{50} was found to be 8.75 mg/ml. Inhibition was found directly proportional to the amount of the extract added.

Table 7.1 Effect of methanol extract of *C.oppositifolia* and compound I on ferrous sulphate induced lipid peroxidation in goat 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)	72.94 ± 2.12		0.70 ±0.02[a]
3	200	14.02 ± 1.84		1.06 ± 0.012[a]
4	400	25.34 ± 1.67		1.04 ± 0.026[a]
5	600	34.18 ± 1.52		1.00 ± 0.016[a]
6	800	48.54 ± 1.21	845.68 ± 3.98	0.92 ± 0.038[a]
7	1000	58.67 ± 2.21		0.71 ± 0.12[a]
	Compound I			
8	25	72.12 ± 4.3		0.71 ± 0.046[a]
9	50	77.46 ± 2.8		0.71 ± 0.036[a]

Values are mean ± SEM of 3 replicates [a]$p < 0.05$.

Table 7.2 Free radical scavenging activity of *C.oppositifolia* leaf extract and compound I by DPPH reduction.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	–	
2	Vitamin E (5 mM)	79.69 ± 1.36	
3	200	03.42 ± 1.45	
4	400	24.16 ± 2.12	
5	600	45.51 ± 3.31	685.08 ± 3.67
6	800	64.14 ± 1.82	
7	1000	71.52 ± 2.32	
	Compound I		
8	25	78.52 ± 2.32	
9	50	80.54 ± 1.34	

Values are mean ± SEM of 3 replicates

Table 7.3 Hydroxyl radical scavenging activity of *C.oppositifolia* leaf extract and compound I.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	–	
2	Mannitol (50 mM)	88.14 ± 1.42	
3	200	24.28 ± 1.42	
4	400	39.46 ± 1.82	593.64 ± 1.16
5	600	49.54 ± 2.14	
6	800	59.68 ± 1.32	
7	1000	79.12 ± 3.8	
	Compound I		
8	25	79.34 ± 3.14	
9	50	81.42 ± 1.36	

Values are mean ± SEM of 3 replicates

Table 7.4 Superoxide radical scavenging activity of *C.oppositifolia* leaf extract and compound I.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	–	
2	Vitamin E (5mM)	69.23 ± 1.45	
3	200	12.28 ± 1.42	
4	400	26.42 ± 2.13	
5	600	32.14 ± 1.32	875.95 ± 3.74
6	800	44.14 ± 2.82	
7	1000	58.46 ± 1.28	
	Compound I		
8	25	66.34 ± 3.2	
9	50	68.23 ± 1.8	

Values are mean ± SEM of 3 replicates

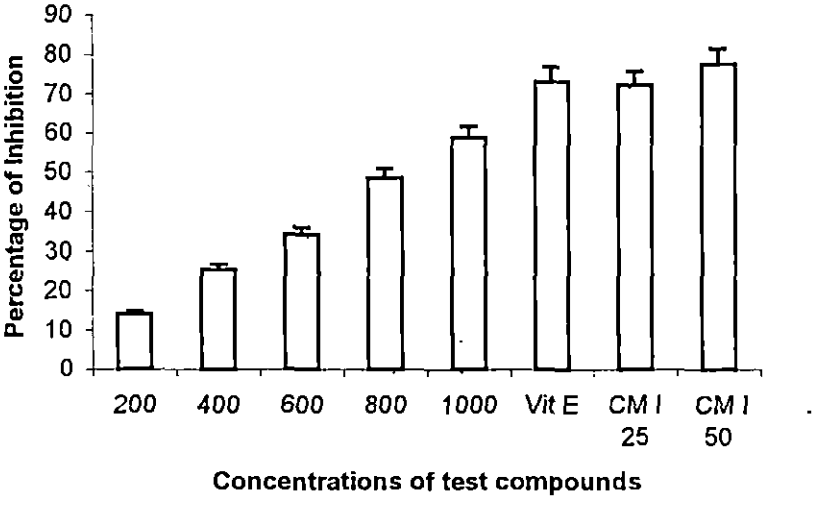

Fig 7.1 Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *C. oppositifolia* leaf, Compound I (CM I) and Vitamin E (Vit E).

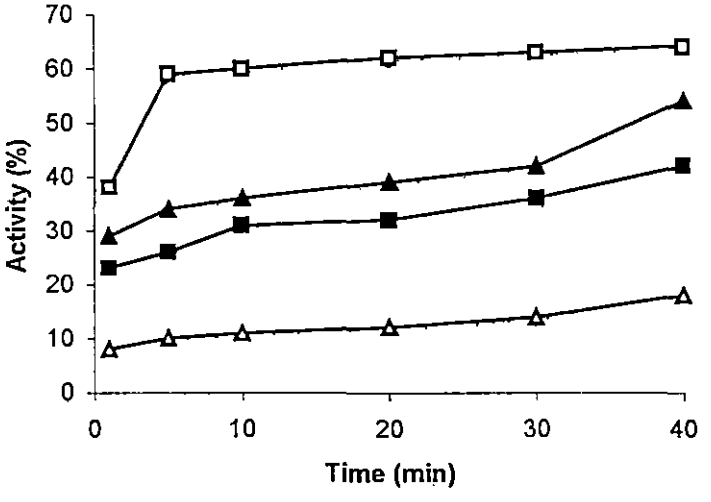

Figure 7.2 DPPH scavenging activity of methanol extract of *C.oppositifolia* leaf, 10mg/ml (□), 7.5mg/ml (▲), 5mg/ml (■) and 2.5 mg/ml (Δ).

7.3.2. Antioxidant activity of *H.nepalense* root

The results presented in Table 7.5 showed the effect of methanol extract of *H.nepalense* root and compound II on ferrous sulphate induced lipid peroxidation in goat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition (69.25 ± 1.21 %) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 73.42 ± 2.3. The same result have also been presented in Figure 7.3 which revealed that compound II at 50 µg/ml showed 72.38 ± 1.9 % inhibitions, which is almost equal to that of the inhibition produced by vitamin E. The IC_{50} value was found to be 745.5 ± 3.16 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 0.99 ± 0.09 to 0.63 ± 0.11 nmoles/mg of tissue and its compound II at 25 µg/ml concentration showed nearly similar level of TBARS (0.63 ± 0.034 nmoles/mg of tissue), which is much higher rate of decrease than that of the results obtained from the well known antioxidant vitamin E ($p< 0.05$).

The effect of methanol extract and compound II on scavenging of DPPH radical is presented in Table 7.6. The result showed that the DPPH scavenging capacity of the extract was found to be 72.38 ± 3.92 % at 1000 µg/ml concentration. The compound II at 50 µg/ml concentration, on the other hand, exhibited 76.38 ± 5.12 % compared to 80.46 ± 4.62 % for the standard drug vitamin E at 5mM. The IC_{50} value of the extract was found to be 6.0 mg/ml. The percentage of activity was also dependent on time as depicted from Figure 7.4.

The effect of root extract and compound II on scavenging of hydroxyl radical is presented in Table 7.7. The results revealed that the extract significantly inhibited (80.38 ± 2.28 %) degradation of deoxy-ribose mediated by hydroxyl radical at the concentration of 1000 µg/ml, compared to that of known scavenger mannitol (89.64 ± 4.62 %). Here the compound II at 50 µg/ml concentration exhibited inhibition of 79.68 ± 2.62 % compared with 89.64 ± 4.62 % for the standard drug at a concentration of 50 mM. The concentration of the methanol extract needed for 50 % inhibition was 615.57 µg/ml.

The effect of root extract and compound II on superoxide scavenging model is presented in Table 7.8. It was found that the methanol extract at 1000 µg/ml concentration caused significant increase of the inhibition up to 60.57 ± 2.34 %. The compound II at 50 µg/ml exhibited 68.24 ± 1.86 % inhibition of superoxide radicals. IC_{50} was found to be 8.91 mg/ml. Inhibition was found to be directly proportional to the amount of the extract added.

Table 7.5 Effect of methanol extract of *H.nepalense* and compound II on ferrous sulphate induced lipid peroxidation in goat 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 (5mM)	73.42 ± 2.3		0.69 ± 0.02[a]
3	200	16.08 ± 4.3		0.98 ± 0.014[a]
4	400	26.57 ± 3.8		0.97 ± 0.017[a]
5	600	36.36 ± 1.84		0.95 ± 0.015[a]
6	800	51.74 ± 1.92	747.5 ± 3.16	0.89 ± 0.018[a]
7	1000	69.25 ± 1.21		0.63 ± 0.11[a]
	Compound II			
8	25	70.15 ± 1.64		0.63 ± 0.034[a]
9	50	72.38 ± 1.9		0.61 ± 0.016[a]

Values are mean ± SEM of 3 replicates [a]$p < 0.05$.

Table 7.6 Free radical scavenging activity of *H.nepalense* root extract and compound II by DPPH reduction.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	–	
2	Vitamin E (5mM)	80.46 ± 4.62	
3	200	8.47 ± 1.83	
4	400	36.46 ± 2.32	
5	600	45.52 ± 3.73	600.52 ± 3.46
6	800	69.42 ± 3.86	
7	1000	72.38 ± 3.92	
	Compound II		
8	25	71.32 ± 1.85	
9	50	76.38 ± 5.12	

Values are mean ± SEM of 3 replicates

Table 7.7 Hydroxyl radical scavenging activity of *H.nepalense* root extract and compound II.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	–	
2	Mannitol (50mM)	89.64 ± 4.62	
3	200	32.14 ± 2.24	
4	400	46.28 ± 1.89	
5	600	65.37 ± 2.26	615.57 ± 2.16
6	800	69.42 ± 3.86	
7	1000	80.38 ± 2.28	
	Compound II		
8	25	72.35 ± 2.93	
9	50	79.68 ± 2.62	

Values are mean ± SEM of 3 replicates

Table 7.8 Superoxide radical scavenging activity of *H.nepalense* root extract and compound II.

Tube number	Concentration of methanol extract (μg/ml)	Inhibition (%)	IC_{50} and confidence interval (μg/ml)
1	Control	–	
2	Vitamin E (5mM)	68.36 ± 2.73	
3	200	12.23 ± 1.42	
4	400	24.12 ± 1.38	
5	600	40.26 ± 2.16	891.9 ± 14.42
6	800	52.27 ± 3.18	
7	1000	60.57 ± 2.34	
	Compound II		
8	25	68.36 ± 2.73	
9	50	68.24 ± 1.86	

Values are mean ± SEM of 3 replicates

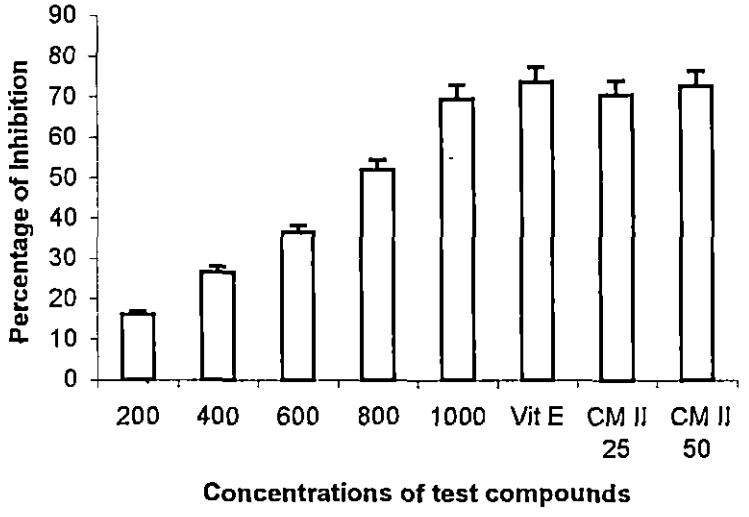

Fig 7.3 Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *H.nepalense* root, Compound II (CM II) and Vitamin E (Vit E).

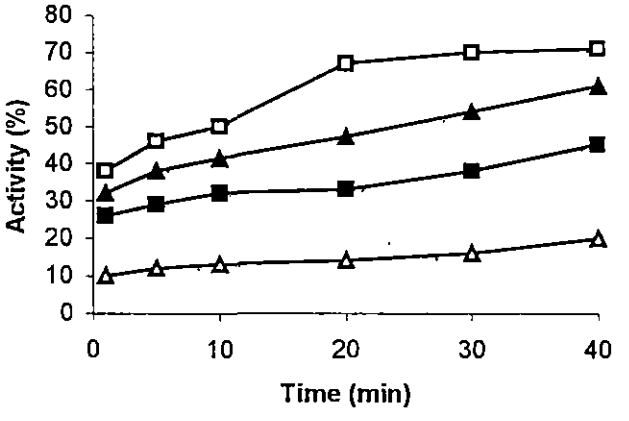

Figure 7.4. DPPH scavenging activity of *H.nepalense*. 10mg/ml (□), 7.5mg/ml (▲), 5mg/ml (■) and 2.5 mg/ml (Δ).

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CHAPTER 8

ANTIMICROBIAL ACTIVITY

8.1. Introduction

The use of plants as source of medicine for the treatment of many diseases dated back to prehistory and people of all continents follow this old tradition. Despite the remarkable progress of synthetic organic chemistry in the twentieth century, over 25% of prescribed medicines in industrialized countries derived directly or indirectly from plants [1]. However, plants used in traditional medicine are still understudied, particularly in clinical microbiology [2].

Infectious diseases are responsible for one-quarter of all the deaths occur in the world, second to the cardiovascular diseases [3]. In the modern world filled with infectious disease due to poor hygiene and insufficient sewage distribution systems the advent of antibiotics was a boon to modern medicine. Myriad infectious diseases continue to kill millions of people every year, especially in developing nations. Many of these diseases are effectively treated with antibiotics or antimicrobial agents. However, the over use of antibiotics, as well as the prevalence of antibiotics introduced to human through the food chain, is contributing to a problem that may result in future plagues and uncontrollable epidemics.

Bacteria and microbes, like human being, are very adept at adapting. When continually exposed to the same antibiotic agent, microbes eventually develop a resistance to its killing effects. Emergence of multiple drug resistant strains has appeared as a real problem in the field of medical science. The primary cause of the development of resistance is occurrence of random mutations. Mutations may occur in genes responsible for conferring sensitivity against a drug. With a relative dearth of new antibiotics with novel mode of action we may find ourselves on the verge of a medical disaster. It is high time to revive the hidden wonders of plant molecules with the modern tools of target based screening to develop newer advanced generation drugs and antibiotics with novel modes of actions [4, 5]. Combinations of active molecules from natural sources like plants need to be systematically explored failing which the consequences are destined to be devastating and out of control for the human race in the new millennium. Among the most promising advances in the field of drug development is discovering new molecules

or novel uses of the already available compounds with known efficacy and without any side effects. Such active biomolecules combined with other antibiotics can kill the drug resistant bacteria and simultaneously check further development of resistance in the infectious microbes. Based on this information the present study was carried out to evaluate the antimicrobial activity of the methanol extract of leave of *C.oppositifolia* and root of *H.nepalense* as well as their isolated compound on different pathogenic bacterial strains by using *in vitro* and *in vivo* model system.

8.2. Materials and Methods

8.2.1. Plant materials

Methanol extracts of *Colebrookea oppositifolia* leaves and *Heracleum nepalense* roots as well as their isolated compounds (described in **Chapter 3**) were used as test drug in these experiments.

8.2.2. 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 and Institute of Microbial Technology (IMTECH), Chandigarh, India. All the strains are of human origin and were isolated in our Institute, as well from different parts of India and abroad. Three multiresistant *Staphylococcus* strains (MRSC) (*Staphylococcus aureus* ML 275, *Staphylococcus aureus* NCTC 8530 and *Staphylococcus epidermidis* 865) were kindly provided by Prof. (Mrs.) Sujata Ghosh Dastidar, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

8.2.3. Media

8.2.3.1. Liquid media

8.2.3.1.1. 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.

8.2.3.1.2. Alkaline peptone water

This alkaline medium used for the cultivation of *Vibrio cholerae*, was prepared as per the following composition:

Bacteriological peptone (Oxoid) - 1.0%

Sodium chloride (Analar) - 0.5%

pH adjusted to - 8.5 to 9.0

8.2.3.1.3. 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

8.2.3.2. Solid media.

8.2.3.2.1. 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

8.2.3.2.2. 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.

8.2.4. 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 4°C and also in freeze dried state. All these strains were checked for purity and identified where necessary. Routine subculturing of the Gram-positive bacteria was carried out on nutrient agar and Gram-negative strains on bromoothymol blue lactose agar [6].

8.2.5. Standard antibiotics

The standard antibiotics used in the studies were amoxycillin (Lyka Labs, India) and gentamicin (Hindustan Antibiotics, Pimpri, Pune) obtained from the respective manufacturer.

8.2.6. Animals

Swiss adult albino mice weighing 20-25 g were used and provided with food (Chakan oil mill) and tap water. The mice exhibited genetic homogeneity and were susceptible to the infecting bacterium. The tests involved only male mice, as the female mice are reported to be more resistant to different infective organisms [8]. Batches each containing 20 mice were kept in standard stainless steel cages having solid bottom in well ventilated animal house with standard conditions of temperature (27°C±3°C), 12h/12h light/dark cycles.

The techniques used for collection of blood samples, injection as well as sacrifice of the animals, were approved by the Animal Ethical Committee of the institute.

8.2.7. 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 160°C for an hour in batches of 100 in screw capped Bijou bottles. The dried extract (semisolid) of *C.oppositifolia* leaf and *H.nepalense* 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 4°C 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 discs adsorbed 0.01 ml of the solution, so the entire 1.0 ml volume was adsorbed by the 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.

8.2.8. Antimicrobial activity of *C.oppositifolia* leaf

8.2.8.1. *In vitro* model

Microbial sensitivity tests were performed by disc diffusion method [7]. 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 without 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 h. 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 [8]. 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 upto 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 agar plate containing only sterile distilled water and Amoxycillin was served as negative and positive control respectively.

8.2.8.2. *In vivo* model

Salmonella typhimurium NCTC 74, naturally virulent to mice as well as sensitive to the extract and compound I, was selected as the challenge strain for this study. Virulence of this strain was enhanced by repeated mouse passages by administering intraperitoneally and recovering from the heart blood. The median lethal dose (MLD or LD_{50}) of the mouse-passaged strain was determined by using graded challenges on 5 groups of mice each having 6 mice and recording mortality up to 100 h [8]. The virulent strain, thus prepared, was preserved by freeze-drying. The basic assumptions behind this method is that the animals dying at a stated dose of the challenge would also have been killed by a greater dose of the challenges and conversely the animals surviving at a stated dose of the challenge would have also been survived at a smaller dose of the challenge. An accumulated value for the affected mice was obtained by adding the number of mice dying at a certain dilution to the number killed by lesser doses; a similar reverse addition was made for the survivors.

Experiments on the rate of mortality in Swiss albino mice with or without the methanol extract of *C.oppositifolia* leaf and compound I were carried out by challenging mice with 50 LD_{50} dose of the virulent strain of *Salmonella typhimurium* NCTC 74; which corresponded to 1.95×10^9 cfu in 0.5ml peptone water followed by mouse passaged. Reproducibility of challenge dose was ensured by choosing a fixed value of its optical density at 640nm in a calorimeter corresponding to a predetermined number of cfu on nutrient agar. For the test 05 groups of mice 20 in each group, each weighing 18 – 20 gm, kept in separate cages. Each mouse of the first group received 50 μg/g body weight dose of the extract in 0.1 ml of the stock solution by intraperitoneal (*i.p.*) injection. The second group received 128 μg/g body weight dose of the extract *i.p.* In the same way each mouse in the third and fourth group received 256, 512 μg/g body weight doses of the extract respectively. The control group (fifth group) was also challenged with the same organism and received 0.1 ml sterile saline in place of the extract. Similar procedure was carried out for the compound I with 05 groups of mice at doses of 25, 50, 100 & 200 μg/g body weight and 0.1 ml saline respectively. The protective capacity of the extract and the compound I was assessed on the basis of the following: (i) when both the infective challenge, as well as, the antibacterial test compounds doses are administered, (ii) when the test compounds alone were administered, (iii) and when the bacterial challenge plus 0.1ml normal saline (instead of test compounds) was administered. In a similar experiment 15mice were divided into 3 groups 5 in each, all of which received the bacterial challenged dose but group I and II received methanol extract and compound I respectively while group III received only normal saline 3h before the challenge. All animals of group I, II and III were autopsied 18 h after the bacterial challenge. Their spleen and liver was removed, homogenized in a glass homogenizer and preserved at -20°C for subsequent determination of cfu/ml counts. At the same time 0.2 to 0.4ml of heart blood samples were also collected aseptically from those animals, allowed to clot and analyzed for the size of bacteriaemia by clot culture method [9, 11].

8.2.8.3. Determination of mode of action of the extract of *C.oppositifolia* & compound I

A multiresistant and highly sensitive strain *S.aureus* 8530 was cultured in nutrient broth overnight, 2ml of the culture was added to 4 ml of nutrient broth and incubated for 2 hours so that the culture could reach logarithmic growth phase. At this stage, the cfu count of the culture was determined and the extract as well as the compound I was added separately at a concentration higher than the MIC. The cfu counts were determined again at the time interval of 2, 4, 8 and 18 h [10].

8.2.9. Antimicrobial activity of *H.nepalense* root.

The antibacterial activity of methanol extract of *H.nepalense* root and its isolated compound II was determined as per the methods described above in **8.2.8** for *C.oppositifolia* leaf.

8.3. Results

8.3.1. Antimicrobial activity of *C.oppositifolia* leaf

8.3.1.1. *In vitro* model

The methanol extract of *C.oppositifolia* leaf exhibited a significant *in vitro* antimicrobial activity against 257 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 leave extract presented in Table 8.1 showed that out of 257 bacteria, the growth of 171 isolates were inhibited by the extract at a concentration of 128 – 512 μg/ml. 79 isolates were resistant at <1000μg/ml, while remaining 07 isolates where resistant up to <2000 μg/ml, the highest concentration of the extract tested. The MICs tests revealed that 63 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, 94 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 8.2, which revealed that all the isolates were sensitive at 128-256 μg/ml concentration of the compound I. It was interesting to note that all the MRSC strains were susceptible to compound I at concentration of 128 μg/ml, while they are resistant to both the standard antibiotics used.

8.3.1.2. *In vivo* model

The result of the median lethal dose (MLD or LD_{50}) of the mouse passaged strain *S. typhimurium* NCTC 74 depicted in Table 8.3 show that in group I where the highest challenge dose was administered (cfu 5.0×10^9) corresponding to 130 O.D. reading, all the 06 animals died. In group II where the challenge dose contained 4.1×10^8 viable cells per ml with 112 O.D reading, 04 mice died. In the following two groups (III and IV), which contained 3.9×10^7 (O.D-95) and 2.5×10^6 (O.D-87) viable cells per ml respectively, only 03 and 02 mice died, whereas at the lowest challenge dose of 3.9×10^5 cfu ml^{-1} (O.D-76) in the group V no animal died. It was noted from the accumulated values that 100% of the animals died in group I, while 50% of the animals died in group III. Therefore MLD of *S.typhimurium* NCTC 74 was found to be 3.9×10^7 cfu/ml and 50 LD_{50} dose was calculated to be 1.95×10^9 cfu/ml for the same organism.

The protective capacity of *C.oppositifolia* and compound I against *S.typhimurium* NCTC 74 is presented in Table 8.4. The result showed that only 04 out of 20 mice were died in the bacterial challenge and actual test dose of the extract at 256 μg/g body weight, while no mice was died in a control group receiving only the extract. When the challenge dose of *S.typhimurium* NCTC 74 and the extract at a dose of 50 μg/g body weight level was administered, 10 mice were died, followed by 08 mice at 128 μg/g, 18 mice at 512 μg/g body weight in the test groups. In the control group where only bacterial challenge dose was administered 16 out of 20 mice were died. Further study with compound I revealed that only 02 out of 20 mice died in the bacterial challenged group along with a test dose of 100 μg of compound I per gram body weight of mice, while no mice died in the control group received only compound I. When compound I was administered at a dose of 50 μg/g body weight level with bacterial challenge, 06 animals died followed by 10 animals at dose of 25 μg/g and 12 died at 200 μg/g in the test groups. In the control group

which received only the bacterial challenge 15 out of 20 mice were died. The protection test turned out to be highly significant at ($p<0.01$ in Chi square test) 256,128 µg/g doses of the extract and 100, 50 µg doses of compound I compared with the control without the test compounds but with the bacterial challenge.

The results of *in vivo* activity of the extract and the compound I against *S.typhimurium* NCTC 74 in mice have been presented in Table 8.5. The viable counts of the test organism, *S. typhimurium* NCTC 74, in mice, which received the methanol extract and the challenge, yielded 1.4×10^3 to 3.4×10^5, 2.4×10^3 to 1.2×10^5 and 4.8×10^2 to 4.8×10^5 cfu ml^{-1} for spleen, liver homogenate and heart blood respectively when autopsied and tested after 18 hrs of administration. Similarly the endogenous cfu counts in the homogenate of mouse spleen and liver and heart blood on 18^{th} h of post bacterial challenge with compound I found to be 1.8×10^3 to 1.5×10^4, 1.1×10^3 to 1.2×10^4 and 1.8×10^3 to 2.1×10^4 respectively. The groups which received the usual challenge dose without the methanol extract or compound I (control), yielded 1.0×10^7 to 2.0×10^8, 3.1×10^7 to 9.0×10^8 and 2.5×10^6 to 5.0×10^8 ranges of cfu counts of the organism in spleen, liver homogenate and heart blood respectively.

8.3.1.3. Determination of mode of action

The determination of mode of antibacterial action of the extract and compound I on multiresistant strain of *S. aureus* 8530 is presented in Figure 8.1. At the logarithmic growth phase of culture, at 6.4×10^8 cfu counts of the strain, 128 µg ml^{-1} (MIC value) of the extract and compound I was added separately. The cfu counts ml^{-1} in the culture with extract were determined and found out to be 2.5×10^8, 6.4×10^6, 7.0×10^5 and 3.0×10^2 at 2,4,8 and 18 h respectively. Similarly the cfu count in the culture with compound I was found to be 2.8×10^7, 4.2×10^5, 6.8×10^3, 5.9×10^1 at the same time intervals.

Table 8.1 *In vitro* antimicrobial spectrum of methanol extract of *C. oppositifolia*.

Bacterial Species	Number Of strain	MIC of leaf extracts (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	>2000	0.25	0.5	8	64	128	256	>1
E.Coli	70	03	25	21	20	01	-	07	03	03	05	15	3
Klebsiella spp.	12	-	-	03	09	-	-	-	-	-	-	02	1
Salmonella Spp.	18	-	01	06	11	-	-	-	-	-	01	04	
Shigella spp.	34	-	02	10	20	02	-	-	-	-	02	10	2
Vibro cholerae	15	-	06	04	05	-	-	-	-	-	-	03	1
Citrobacter spp.	15	-	02	03	08	02	-	-	-	-	-	01	
Pseudomonas aeruginosa	15	-	06	04	03	02	-	-	-	-	-	02	
Bacillus subtilis	06	1	03	02	-	-	-	-	04	01	-	01	
Staphylococcus aureus	62	19	30	10	03	-	01	27	12	13	-	09	
Streptococcus faecalis	10	4	06	-	-	-	01	05	04	-	-	-	
Total	257	27	81	63	79	07	02	39	23	17	08	47	1

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 8.2 The MIC of 15 sensitive bacteria against methanol extract of *C.oppositifolia* and Compound I.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	ME	CM I	Amoxycillin	Gentamycine	
E.Coli 832	512	512	0.50	0.25	+
E.Coli TG1	512	512	0.50	>256	+
E.Coli 871	256	512	0.50	0.50	+
E.Coli HD10	128	128	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	512	512	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	1000	1000	0.125	0.50	+
Bacillus subtilis 8241	128	128	0.50	256	++
S.typhimurium NCTC 74	256	512	8.0	>256	+
V.Cholerae 14033	128	128	8.0	>256	+
Klebsiella pneumoniae.	512	512	256	0.50	+
Pseudomonas aeruginosa	1000	1000	2.0	0.50	+

ME: Methanol extract of *C.oppositifolia*; 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 means ± S.E.M (n=3).

Table 8.3 Median lethal dose (LD_{50}) of *Salmonella typhimurium* NCTC 74.

Batch	cfu/ml	Optical density (O.D)*	No of animals		Mortality	Accumulated values from mortality data				LD_{50}	50 LD_{50}
			Dead	Survived		Died (D)	Survived (S)	Mortality ratio ** [D/(D+S)]	Percent [D×10/ D+S]		
I	5.0×10^{9}	130	06	0	6 / 6	15	0	15 / 15	10%		
II	4.1×10^{8}	112	04	02	4 / 6	09	02	09 / 11	81.81%		
III	3.9×10^{7}	95	03	03	3 / 6	05	05	05 / 10	50%	3.9×10^{7} cfu/ml	1.95×10^{9}
IV	2.5×10^{6}	87	02	04	2 / 6	02	09	02 / 11	18.18%		
V	3.9×10^{5}	76	0	06	0 / 6	0	15	0 / 15	0%		

* : Readings were taken in Klett Summerson Colorimeter at 640 nm.

**: Mortality ratio was calculated by the formula of Reed and meunch.

Table 8.4 Determination of *in vivo* protective capacity of methanol extract of *C.oppositifolia* and compound I

Group	Test Groups[a]		Control Groups[b]		Test groups[c]		Control groups[d]	
	Extract (µg/g)	Mice died	Extract (µg/g)	Mice died	Compound I (µg/g)	Mice died	Compound I (µg/g)	Mice died
I	50	10	50	0	25	10	25	0
II	128	08[e]	128	0	50	06	50	0
III	256	04[f]	256	0	100	02[f]	100	0
IV	512	18	512	3	200	12[e]	200	2
V	Bacterial challenge dose (1.95 × 10^9)	16	-	-	Bacterial challenge dose (1.95 × 10^9)	15	-	-

a. Received challenge dose of 1.95 × 10^9 cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the extract.

b. Received only the extract and saline but no challenge dose.

c. Received challenge dose of 1.95 × 10^9 cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the compound I.

d. Received only the compound I and saline but no challenge dose.

e. $P < 0.05$

f. $P < 0.01$ according to the chi-square test, after elimination of the effect due to the extract and compound I.

Table 8.5 *In vivo* antimicrobial spectrum of the methanol extract of *C.oppositifolia* and compound I against *S.typhimurium* NCTC 74 in mice [a].

Group	Time of Sampling (hr)	Mouse Number	Treated with	cfu/ml Count in		
				Liver	Spleen	Heart blood
Group I	18	1	Methanol Crude extract of *Colebrookea oppositifolia* (256µg/g)	2.4×10^3	1.8×10^5	4.8×10^5
		2		6.8×10^4	3.4×10^5	1.5×10^5
		3		1.2×10^5	3.4×10^3	4.8×10^2
		4		5.0×10^4	1.4×10^3	4.8×10^3
		5		1.2×10^4	1.45×10^5	4.8×10^2
Group II		1	Compound I (100µg/g)	1.2×10^4	1.5×10^4	2.1×10^4
		2		3.0×10^3	1.8×10^3	1.8×10^3
		3		3.5×10^3	6.4×10^3	3.8×10^4
		4		1.1×10^3	2.5×10^3	3.6×10^4
		5		1.4×10^3	2.3×10^3	4.3×10^3
Group III		1	Sterile Saline	1.0×10^7	3.1×10^7	5.0×10^8
		2		1.5×10^7	4.5×10^7	2.5×10^6
		3		1.9×10^8	9.0×10^8	3.0×10^7
		4		2.0×10^8	8.3×10^8	5.0×10^8
		5		2.5×10^7	3.3×10^7	6.3×10^7

a. Variable counts between 3 groups significant, $P < 0.001$ (Student 't' test).

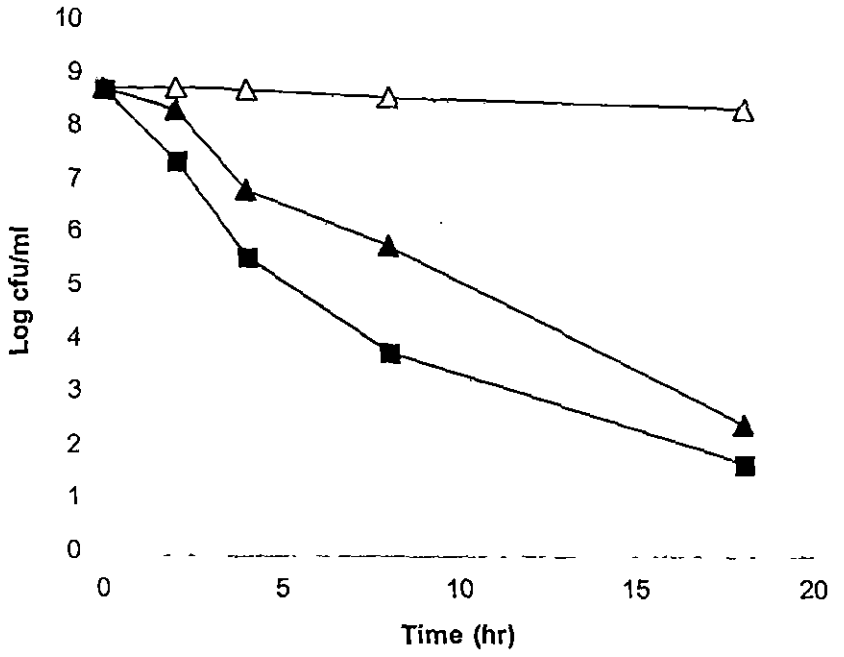

Figure 8.1 The mode of action of methanol extract and compound I of *C.oppositifolia* leaf on resistant strain of *S.aureus* 8530. (-Δ-): bacterial suspension in methanol (control); (-▲-): Methanol extract of the leave at 128 µg/ml; (-■-): Compound I at 128 µg/ml concentrations.

8.3.2. Antimicrobial activity of *H.nepalense* root.

8.3.2.1. *In vitro* model

The methanol extract of *H.nepalense* 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 8.6 showed that out of 257 bacteria, the growth of 197 isolates were inhibited by the extract at a concentration of 128 – 512 µg/ml, 57 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 63 out of 75 Gram-positive bacteria were sensitive between 128 and 256 µg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 120 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 methanol extracts was directed both against Gram-positive and Gram-negative bacteria. The isolated compound II was also tested for antimicrobial activity. The result is presented in Table 8.7, which revealed that all the isolates were sensitive at 128-256 µg/ml concentration of the compound II. It was interesting to note that all the MRSC strains were susceptible to compound II at a concentration of 128 µg/ml, while they were resistant to the two standard antibiotics used.

8.3.2.2. *In vivo* model

The result of the median lethal dose (MLD or LD_{50}) of the mouse passaged strain *S. typhimurium* NCTC 74 was same as per the result depicted in *C.oppositifolia* leave (Discussed in **8.3.1.2**). The protective capacity of methanol extract of *H.nepalense* and compound II against *S.typhimurium* NCTC 74 is presented in Table 8.8. The result showed that only 3 out of 20 mice were died in the bacterial challenge and actual test dose of the extract at 128 µg/g body weight, while no mice was died in a control group receiving only the extract. When the bacterial challenge and extract at a dose of 50 µg/g body weight level was administered, 08 mice were died, followed by 05 at a dose of 256

μg/g, 12 mice at 512 μg dose per gram body weight in the test groups. In the control group 15 out of 20 mice were died which received only the bacterial challenge. Further study with compound II revealed that only 02 out of 20 mice died in the bacterial challenge and actual test dose of 100 μg/g body weight of mice, while no mice died in a control group which received only compound II. When bacterial challenge dose and compound II was administered at a dose of 50 μg/g body weight level, 04 animals were died followed by 06 at dose of 25 μg/g, 10 animals at 200 μg/g body weight level in the test groups. The protection test turned out to be highly significant at ($p<0.01$ in Chi square test) 128, 256 μg/g doses of the extract and 100, 50 μg/g doses of compound II compared with the control without the test compounds but with the bacterial challenge.

The results of *in vivo* activity of the extract and the compound II against *S.typhimurium* NCTC 74 in mice have been presented in Table 8.9. The viable counts of the test organism, *S. typhimurium* NCTC 74, in mice, which received the methanol extract and the challenge, yielded 2.1×10^2 to 2.8×10^4, 2.6×10^3 to 7.0×10^4 and 2.5×10^3 to 2.8×10^4 cfu/ml for spleen, liver homogenate and heart blood respectively when autopsied and tested after 18 h of administration. Similarly the endogenous cfu counts in mouse spleen and liver homogenate and heart blood on 18th hours of post bacterial challenge with compound II found to be 2.6×10^3 to 6.4×10^5, 5.2×10^3 to 6.4×10^4 and 1.2×10^3 to 3.8×10^4 respectively. The groups which received the usual challenge dose without the methanol extract or compound II (control), yielded 1.6×10^8 to 6.8×10^9, 3.9×10^8 to 4.8×10^9 and 5.2×10^8 to 7.2×10^8 ranges of cfu count of the organism in spleen, liver and heart blood respectively.

8.3.2.3. Determination of mode of action

The mode of antibacterial action was determined by selecting a multiresistant strain of *S. aureus* 8530 is presented in Figure 8.2. At the logarithmic growth phase of culture, at 6.4×10^8, cfu count of the strain 128 μg/ml (MIC level) of the extract and compound II was added separately. The cfu counts/ml in the culture with extract were determined and found to be 1.2×10^8, 1.4×10^6, 6.8×10^4 and 1.2×10^2 at 2,4,8 and 18 hours respectively.

Similarly the cfu count in the culture with compound II was found to be 3.4×10^{6}, 1.8×10^{4}, 1.4×10^{2}, 0.4×10^{2} at the same time intervals.

Table 8.6 In *vitro* antimicrobial spectrum of *H.nepalense* root extract.

Bacterial Species	Number Of strain	MIC of leaf extracts (µg/ml)					MIC of amoxycillin (µg/ml)						
		128	256	512	1000	>2000	0.25	0.5	8	64	128	256	>1
E.Coli	70	08	12	21	28	01	-	07	03	03	05	15	3
Klebsiella spp.	12	01	02	04	05	-	-	-	-	-	-	02	1
Salmonella Spp.	18	02	04	06	06	-	-	-	-	-	01	04	1
Shigella spp.	34	07	09	12	06	-	-	-	-	-	02	10	2
Vibro cholerae	15	02	02	08	02	01	-	-	-	-	-	03	1
Citrobacter spp.	15	02	02	06	04	01	-	-	-	-	-	01	1
Pseudomonas aeruginosa	15	01	06	05	03	-	-	-	-	-	-	02	1
Bacillus subtilis	06	03	02	01	-	-	-	-	04	01	-	01	1
Staphylococcus aureus	62	29	20	10	03	-	01	27	12	13	-	09	
Streptococcus faecalis	10	04	06	-	-	-	01	05	04	-	-	-	
Total	257	59	65	73	57	03	02	39	23	17	08	47	1

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 8.7 The MIC of 15 sensitive bacteria against methanol extract of *H.nepalense* and Compound II.

Name of the Organism	MIC (μg/ml)				Diameter of zone of inhibition (mm) in methanol extract
	ME	EA	Amoxycillin	Gentamicin	
E.Coli 832	256	256	0.50	0.25	+
E.Coli TG1	512	512	0.50	>256	+
E.Coli 871	256	512	0.50	0.50	+
E.Coli HD10	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	1000	2.0	0.50	+

ME: Methanol extract of *H.nepalense*; EA: ethyl acetate fraction; +: ≤ 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 8.8 Determination of *in vivo* Protective capacity of *H.nepalense* root extract and compound II.

Group	Test Group[a]		Control Group[b]		Test group[c]		Control group[d]	
	Extract (μg/g)	Mice died	Extract (μg/g)	Mice died	Compound II (μg/g)	Mice died	Compound II (μg/g)	Mice died
I	50	08	50	0	25	06	25	0
II	128	03[e]	128	0	50	04[f]	50	0
III	256	05[f]	256	1	100	02[e]	100	0
IV	512	12	512	2	200	10	200	2
V	Bacterial challenge dose (1.95×10^9)	15	-	-	Bacterial challenge dose (1.95×10^9)	15	-	-

a. Received challenge dose of 1.95×10^9 cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the extract.

b. Received only the extract and saline but no challenge dose.

c. Received challenge dose of 1.95×10^9 cfu/ml of *S.typhimurium* NCTC 74, 3h after administration of the compound II.

d. Received only compound II and saline but no challenge dose.

e. $P < 0.05$

f. $P < 0.01$ according to the chi-square test, after elimination of the effect due to the extract and compound II.

Table 8.9 *In vivo* antibacterial spectrum of methanol extract of *H.nepalense* and compound II against *S.typhimurium* NCTC 74 in mice [a].

Group	Time of Sampling (hr)	Mouse Number	Treated with	cfu/ml Count in		
				Liver	Spleen	Heart blood
Group I	18	1	Methanol Crude extract of *H.nepalense root* (128µg/g)	3.1×10^4	5.4×10^3	2.8×10^4
		2		2.6×10^3	2.8×10^4	5.8×10^3
		3		3.0×10^3	4.2×10^3	4.0×10^3
		4		7.2×10^3	1.4×10^3	3.2×10^3
		5		7.0×10^4	2.1×10^2	2.5×10^3
Group II		1	Compound II (100µg/g)	2.1×10^4	3.0×10^4	1.2×10^3
		2		6.4×10^4	4.2×10^4	2.4×10^3
		3		3.2×10^4	6.4×10^5	2.6×10^3
		4		5.2×10^3	3.2×10^3	3.8×10^4
		5		2.4×10^4	2.6×10^3	7.1×10^3
Group III		1	Sterile Saline	7.4×10^3	1.6×10^8	6.3×10^8
		2		4.8×10^9	4.4×10^8	5.2×10^8
		3		3.9×10^8	6.8×10^9	7.2×10^9
		4		5.4×10^8	6.4×10^9	4.4×10^9
		5		3.2×10^9	4.2×10^8	5.4×10^9

[a]: Variable counts between 3 groups significant, $P < 0.001$ (Student 't' test).

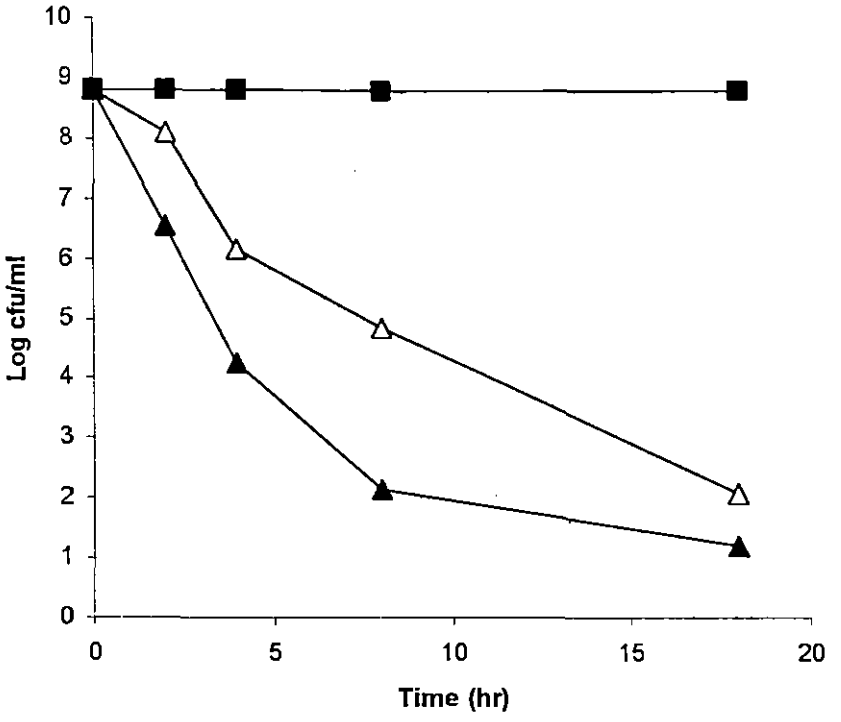

Figure 8.2 The mode of action of methanol extract and compound I of *H.nepalense* root on resistant strain of *S.aureus* 8530. (-■-): bacterial suspension containing methanol Control; (-Δ-): methanol extract of leave at 128 µg/ml; (-▲-): Compound I at 128 µg/ml.

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CHAPTER 9

DISCUSSION

Herbal drugs are probably the most common source of samples for evaluation in high-throughput screens of natural products. They yielded many useful compounds and ingredients derived from plant, which are important components of modern phytopharmaceuticals. Certain types of these substances not only help the body in providing sources of energy but also assist in the plastic repairs as well as interfere the complicated chemical reactions of life processes. In many developing countries therapeutic lists commonly contain a number of plant-based drugs, because modern synthetic drugs are beyond the reach of the population in rural areas and heavy financial burden of expensive synthetic drugs.

Phytotherapy provides a complimentary form of medicinal agents and an alternative to modern orthodox medicine. When medicinal plant products are subjected to the rigorous application of science, the experiments reveals the usefulness and clarifies the pharmacodynamic basis for the use of such plants in medical practice. These re-evaluations would provide a rationale for the use of those plants [1].

The herbal drugs can be continually used in chemotherapeutics as (i) the plants drugs provide a desirable therapeutic effect with reduced risk of iatrogerrous diseases associated with allopathic medicine; (ii) the infusion dosages of herbal preparation have advantages of complete bioavailability over conventional dosage forms; (iii) the enhanced solubility and dispersion of active molecules at gastro-intestinal absorption sites reduced the problems encountered in the pharmacodynamic phase of drug therapy; (iv) herbal drugs are most suitable for the diseases require long-term medication such as immunosupression, arthritis, hepato-biliary deficiency, spleen diseases, etc, because most plant extracts are used in repeated low doses; (v) compared to the synthetic compounds herbal drugs have a reduced chance of acute toxicity and secondary drug effects; and (vi) the multiple component feature of phytotherapeutic agents is considered to be a positive attribute as biodynamic agents since the same extract often include compounds that have synergistic activity [2].

It is therefore important to study the phytotherapeutic agents as they play vital roles in clinical medicine. The through study of medicinal plant is very much important because some of the adverse effect occasionally observed with herbal drugs. Hence it is of immense clinical importance to determine the acute and chronic toxicity of therapeutically useful plant products and to know whether they exhibit the expected pharmacological action. This approach to the discovery and development of plant products as medicines holds a lot of promise for people of developing countries. The increase in the acceptance of the 'holistic' philosophy of therapy by medical profession, which the traditional systems exemplify, is a further indication that there will be continually growing demand for drugs based on traditional medicine derived from natural products in future too [3].

We have studied the phytochemicals, their toxicities and pharmacological activities of two well-known plant used in traditional medicines *Colebrookea oppositifolia* Smith of the family Labiatae and *Heracleum nepalense* D.Don. DC of the family Umbelliferae from Sikkim Himalayan region. Both of these plants were extensively used by the Sikkimese for their primary health care such as skin infections, digestive, stimulant, aphrodisiac as described in the literature review. The present study on the plants was undertaken, for the first time, with the following aims (i) to evaluate the bioactivity of *Colebrookea oppositifolia* and *Heracleum nepalense*, both *in vitro* and *in vivo*, for justification of their use as ethnomedicine (ii) to isolate and identify the bioactive principle(s) in pure form. Standard methods were followed for the collection and processing of the plants and their useful parts. Authentication of the plants was made with the help of qualified scientists from the BSI, Gangtok branch, Sikkim, India. The extractions of the plant parts and prescreening of the extracts were done by standard protocols and the universally accepts methodologies, as described in Materials and Methods.

The phytochemical studies of the plants were covered in chapter 3. The extractions of the plant parts were made at room temperature using water and methanol as solvent and the collected extracts were concentrated under reduced pressure. The extracts were then

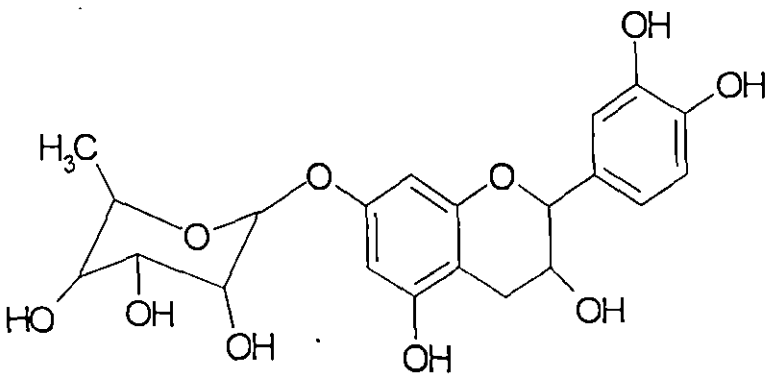
concentrated, suspended in hot distilled water, cooled and the blast precipitate was filtered off. The water-soluble component was fractionated by extracting it successively with petroleum ether, ethyl acetate and acetone. The ethyl acetate soluble fraction was subjected to chromatographic analysis [4,5].

Using multistep coloumn chromatography technique with various developing phases compound I and compound II were isolated from ethyl acetate fractions of *C.oppositifolia* leaf and *H.nepalense* root respectively. Their structures were determined by study of acid hydrolysis products with the help of spectral analysis of their UV, ^{1}HNMR, ^{13}C NMR and MS spectra.

The crystalline material isolated from the leaf of *C.oppositifolia* yielded a flavonoid glycoside (compound I). The UV absorption spectrum confirmed that compound I was a 7-substituted derivative with the presence of phenolic aromatic rings. The IR spectrum confirmed the presence of hydroxyl group and aromatic ring in the compound. Acid hydrolysis of the compound gave rhamnose and is identified by sugar tests [7]. Existence and identification of the sugar moiety is further confirmed by the ^{1}H NMR study. ^{1}H and ^{13}C NMR spectrum confirmed that compound I was identical with + (–) Catechin-7-O-β-rhamnopyranoside [7-11]. In the mass spectrum the presence of fragmentation ion at m/z 291 inferred that the compound should have + (–) Catechin as an aglycone. The structure of the compound was further confirmed by elemental analysis of the available literature of catechin [9, 11]. All the recorded spectral and elemental analysis and evidence discussed in **Chapter 3** (3.3.1) conclusively prove the identity of the isolated compound as + (–) Catechin-7-O-β-rhamnopyranoside. This confirmed that catechin is one of the major bioconstituent of the plant.

Catechin (5, 7, 3′, 4′ - tetrahydroxy – flavan – 3ols) is a polyphenol compound, generally found in green tea, catechu and rarely in other bark and leaf of plant kingdom. The traditional uses of plants containing catechin in folk medicine are multiple. Contemporary research also revealed that catechin isolated from different herbal sources and green teas possess diverse *in vivo* and *in vitro* pharmacological actions like anticancerous [9],

antioxidant, anti-inflammatory and antimicrobial properties [12, 13, 14]. The catechin polyphenols have also been shown to markedly inhibit digestive lipase *in vitro*, resulting in decreased lipolysis of triglycerides, which may translate to reduce fat digestion in humans [15].

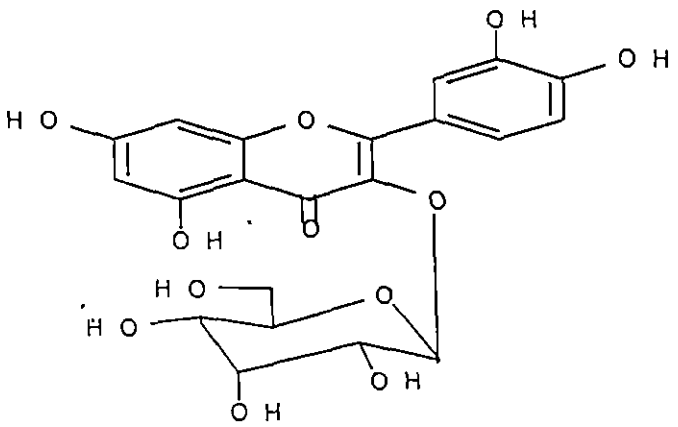


(+) – Catechin-7-O-β-rhamnopyranoside (Compound I).

The crystalline material isolated as compound II from the root of *H.nepalense* demonstrated the positive tests for flavonoid. The chemical nature was characterized by comparison of its physical parameters and UV, IR, ^{1}H NMR, ^{13}C NMR and MS spectral data with that of flavonoid glycoside [16-22]. The complete acid hydrolysis of compound II gave glucose as sugar moiety and is confirmed by sugar tests. The UV analysis proved the substitution of hydroxyl group in the compound by the bathochromic shift of band I in presence of aluminium chloride and hydrochloric acid. Therefore it was indicated that the compound was monodesmoside [23]. All the experimental evidences discussed in **Chapter 3** (3.3.2) suggest that the compound II isolated as quercetin-3-O-β-D-glucopyranoside. The isolation is a mark significance since no flavonoid has previously been isolated from the plant under study.

Quercetin (3, 3', 4', 5, 7-pentahydroxy flavone) is widely distributed in the plant kingdom and is the most abundant in the flavonoid molecules. It is found in many consumed foods, including apple, onion, tea, berries and brassica vegetables as well as many seeds, nuts,

flowers, roots, barks and leaves. It is reported for having many beneficial effects on human including cardiovascular protection, anticancer activity, antiulcer effects, anti-allergy activity, catharact prevention, antimicrobial activity, antioxidant activity and anti-inflammatory effects [24, 25]. It is reported to inhibit metabolic enzyme systems and P-glycoprotein [26]. Recent reports indicate that quercetin and its glycoside stimulate human peripheral blood leukocyte proliferation and significantly increase the helper T cells [27].



Quercetin-3-O-β-D-glucopyranoside (Compound II).

Determination of acute toxicity is the primary step in toxicological investigation of unknown substances which has been included in chapter 4 of the present studies. Fifty percent lethal dose of LD_{50} (acute oral/ i.p toxicity) is performed in Swiss albino mice following standard protocol [6]. The median lethal dose (MLD) of the methanol extract of *Colebrookea oppositifolia* leaf was found to be 3.0 g/kg body weight and 4.5 g/kg body weight in intraperitoneal and oral route respectively. While the MLD of the methanol extract of *Heracleum nepalense* roots was to be 4.5 g/kg body weight and more than 5.5 g/kg body weight in intraperitoneal and oral route respectively. Therefore, the doses of 600 mg/kg and 100 mg/kg p.o. were fixed as the highest and least doses to carry out all pharmacological experiments. These selected doses are well tolerated in the system tested and no untoward effect was observed with the methanol extract of either *C.oppositifolia* leaf or *H.nepalense* root.

We have studied several pharmacological activities of the methanol extracts and isolated compounds of *Colebrookea oppositifolia* leaf and *Heracleum nepalense* root on the basis of their ethnomedicinal uses. The bioactivities studied included enhancement of bioavailability of some antibiotics, immunostimulant, antioxidant and antimicrobial activities, which have been covered in chapter 5 to chapter 8.

Improvement of bioavailability of a large number of poorly biologically available, long-term treatments, toxic and expensive drugs may be proved as having greater clinical value and thus the problems with their therapy can be overcome. In the first part of the present research work (chapter 5) attempt has been made to study the effect of *C.oppositifolia* leaf and *H.nepalense* root with their isolated compounds on bioavailability and pharmacokinetics of some widely used antibiotics in rabbits. The present experimental findings clearly demonstrated that methanol extract of *C.oppositifolia* leaf and compound I enhanced the bioavailability of amoxycillin and rifampicin. On the other hand, methanol extract of *H.nepalense* root failed to show any significant increase in the bioavailability of these antibiotics but compound II isolated from root of the plant has moderately increased the bioavailability of these drugs.

The results of methanol extract of *C.oppositifolia* and compound I on bioavailability of amoxycillin showed that co-administration or pre-administration of methanol extract and compound I increased all the pharmacokinetic parameters of the amoxycillin compared to control group. Based on the literature data the mechanism by which the extract and compound I increases the bioavailability of amoxycillin is due to increased gastro intestinal blood supply, increased gastrointestinal motility or influence on epithelial cell wall modification [28, 29]. However, it cannot be ruled out for their direct inhibitory effect on microsomal enzymes or enzyme systems. The pre-administration of methanol extract of *C.oppositifolia* and compound I shifted the time to reach maximum concentration (t_{max}) 30 min and 1.0 hr respectively earlier than the control group. Similarly the relative bioavailability (RB %) of the pre-administration group of methanol extract and compound I was found to be higher than the co-administration group. This may be due to the effects of the extract and compound I on the permeability of intestinal epithelium to

promote absorption of antibiotic [30]. Intestinal epithelial cells represent the tight junctions and achieve the high transepithelial electrical resistance (> 700 ohm cm^2) in confluent monolayer. The methanol extract and compound I may have direct effect on the fragmentation of the tight junction between the cells and thus increased the permeability of the antibiotic [30].

From the experiments performed, it was observed that the elimination rate constant (k_{el}) is reduced by the extract and compound I and there is corresponding increase in the plasma half-life of the antibiotic. The results were consistent with the reported literature i.e. Choi et al. [31] Scambia et.al. [32], Woo et al. [33], and Zhang et al. [34] in that flavonoids and P-glycoprotein (Pgp) inhibitor KR 30031 increased the bioavailability of different drugs by inhibiting either the Pgp efflux pump or cytochrome P-450. Methanol extract of *C.oppositifolia* and compound I affected the bioavailability of amoxycillin in similar manner to those of quercetin, flavone, which are Cyp3A and Pgp efflux pump inhibitors.

The effect of methanol extract of *C.oppositifolia* and compound I on the bioavailability of cefixime indicated that the methanol leaf extract failed to change the bioavailability of cefixime after preadministration or coadministration of the extract. However the preadministration of compound I 30 min before the administration of cefixime increases all the pharmacokinetic parameters of cefixime including Area under curve (AUC) and relative bioavailability (RB %). This could be attributed to the fact that compound I may alter the permeation coefficient of intestinal epithelium and increases the absorption of cefixime[30].

The results of effect of methanol extract of *C.oppositifolia* and compound I on bioavailability of rifampicin indicated that coadministration or preadministration of methanol extract and compound I enhanced the bioavailability of rifampicin significantly. The analysis of various pharmacokinetic parameters t_{max}, C_{max}, $t_{1/2}$, AUC and RB % revealed that methanol extract and compound I might influence the different physiological process like alternation in gastric milieu or transportation and absorption process [28]. Rifampicin is a hepatic microsomal enzyme inducer and helps its own

metabolism in liver. So the direct inhibitory effect of *C.oppositifolia* and compound I on microsomal metabolizing enzyme systems cannot be ruled out [35]. The presence of high flavonoidal content of *C.oppositifolia* is responsible for the enhancement of bioavailability of rifampicin.

The effect of *H.nepalense* root extract and compound II on bioavailability and pharmacokinetics of amoxycillin, cefixime and rifampicin revealed that the methanol extract of *H.nepalense* failed to produce any effect on the bioavailability of all the antibiotics. All the pharmacokinetic parameters of antibiotics are unchanged with preadministration or coadministration of methanol root extract of the plant. However the coadministration of compound II enhanced the bioavailability of amoxycillin and rifampicin. The analysis of various pharmacokinetic parameters t_{max}, C_{max}, $t_{1/2}$, k_{el}, AUC and RB % indicated that compound II might influence the different physiological process like alternation in gastric milue or transport and absorption process [28]. The results were consistent with the result reported by Choi et al. [31] Scambia et.al. [32], Woo et al. [33], and Zhang et al. [34], in that the flavonoid (quercetin, naringin and flavone) and p-glycoprotein inhibitor KR 30031 increase the bioavailability of different drugs by inhibiting either the Pgp efflux pump or cytochrome P-450. This result was also consistent with the results reported by Bailey *et.al* [36], Rashid *et.al.* [37], Lasker *et. al* [38] that quercetin and their glycoside increased the bioavailability of rifampicin, tetracycline and sulphadiazine. Rifampicin and amoxycillin has been reported to be metabolized by cytochrome P-450 both in the liver and epithelial cells of the small intestine [39]. In addition P-glycoprotein efflux pump inhibited the absorption of these antibiotics in the intestinal mucosa [40]. Compound II might affect the bioavailability of amoxycillin and rifampicin in similar manner to those of other CYP3A4 and Pgp inhibitors.

Immunostimulatory therapy, which relates to stimulation of immune response of the host, is now being recognized as an alternative to conventional chemotherapy for variety of diseased conditions, especially when host's defense mechanisms have to be activated under condition of impaired immune response of the host [41]. The human body is continuously exposed to a series of stress factors, which more or less weaken the function

of the immune system and thereby generate immunosupression. Immunostimulators have been known to support T-cell function, activate macrophages, granulocytes, complement, natural killer cells and also help the production of various effector molecules generated by activated cells (Para immunity) [42].

The results of immunostimulatory activity of the methanol extract of *C.oppositifolia* leaf and compound I, as explained in chapter 6, indicated that the methanol extract at higher dose (1000 mg/kg body weight) moderately increased in the rate of carbon clearance from blood. However, administration of compound I at 50 mg/kg body weight dose resulted significant increased in the rate of carbon clearance compared to the rate of clearance produced by the standard immunostimulating drug levamisole at the same dose level. Hence methanol extract of *C.oppositifolia* and compound I at higher dose found to stimulate the phagocytic activity of macrophages as evidenced by an increase in the rate of carbon clearance.

The results of HA titer and DTH response model showed that the methanol extract increased the antibody titer and mean paw edema in mice at a dose of 1000 mg/kg body weight. Compound I also pronounced significant activity at dose of 50 mg/kg body weight. The HA titer and DTH response are believed to be related to humoral mediated and cell mediated immune response. The presence of flavonoid could be responsible for the stimulation of the immune response [41].

The *in vitro* immunostimulatory activity of methanol extract and its isolated compound I was tested on human polymorphonuclear and mononuclear cells. The phagocytosis and intracellular killing of microorganisms by polymorphonuclear phagocytes was determined by direct measurement of the microbicidal activity [42]. Phagocytosis was expressed as the phagocytic index, in which the percentage of decrease in number of viable extracellular bacteria was determined microbiologically after incubation with polymorphonuclear leukocytes. The phagocytic index of *C.oppositifolia* leaf extract was found to have moderate phagocytic activity as compared with the control group.

However, compound I showed maximum phagocytic index, which is slightly lower than that of the standard drug Interferon-α-2b.

Further, the immunostimulatory effect of extract and its isolated compound I was tested in mitogen activated cultured mononuclear cells. PHA was used for activating mononuclear cells in the culture. The mitogenic PHA is polyclonal activators, in that they activate mononuclear cells including memory type cells, irrespective of their antigenic specificity[43]. The effect of methanol extract of *C.oppositifolia* failed to produce any stimulation of PHA activated mononuclear cells and hence there was no increase of cells viability compared with the control. However, the compound I at 50 µg/ml concentration showed moderate immunostimulatory activity as compared with standard drug.

Prophylactic treatment of *H.nepalense* and its isolated compound enhanced the clearance of carbon from the blood in the rate of (more than 2 fold) when compared with the control group. The result is due to a mechanism related to phagocytosis by macrophages. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b leading to more rapid clearance of foreign particulate matter from blood [44] *H.nepalense* was found to stimulate the phagocytic activity of the macrophages as evidenced by increase in the rate of carbon clearance.

The results of the HA titer experiment indicated that administration of methanol extract at a dose of 1000 mg/kg body weight increase in the HA titer value almost four times as compared to the untreated control animals. The isolated compound also pronounced significant activity at a dose of 50 mg/kg body weight. This is owing to the augmentation of the humoral response by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis [45]. The DTH response, which is a direct correlation of cell-mediated immunity (CMI), was found to be the highest at the maximum dose of root extract tested (1000 mg/kg body weight). The mechanism behind this elevated DTH during the CMI responses is due to sensitized T-lymphocytes, when challenged by the antigen, which are converted to lymphoblasts and secret lymphokines, attracting more

scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive (inflammatory) reaction [46]. Increase in DTH response indicates that root extract of *H.nepalense* and isolated compound II has a stimulatory effect on lymphocytes and a type of accessory cell required for the initiation of the reaction. [47].

The phagocytic index of *H. nepalense* root extract has found to be increased in a time and dose dependent manner. The isolated compound II at 50 μg/ml concentration showed significant activity nearly equal to the phagocytic index of root extract at 1000 μg/ml concentration after 120 min incubation as compared to control. The result of cell proliferation model indicated that methanol extract of *H.nepalense* at low concentration failed to show any immunostimulating effect. However, at higher concentrations increased cell stimulation was observed. Similarly, the standard drug and the isolated compound II demonstrated a significant stimulation of the mononuclear cells at 0.5 million IU and 50 μg/ml of tested concentrations respectively. This is attributed to the fact that the methanol extract and isolated compound may stimulate the PHA activated mononuclear cells and induce the release of cell proliferating factors like Interleukin and TNFα [48].

Earlier reports on the phytochemistry of *H. nepalense* indicate the presence of compounds like steroids and coumarins. We have isolated a known flavonoid quercetin glycoside from the plant. However, there are no report on the chemical nature and pharmacological activity of the plant. Contemporary research revealed that quercetin glycoside isolated from different herbal sources, has several pharmacological actions like antioxidant, anticancer, antiulcer, anti-inflammatory and antiviral [49]. Recent reports indicate that several types of flavonol stimulate human peripheral blood leukocyte proliferation, and significantly increase the activity of helper T cells, cytokines, interleukin 2, γ-interferon and macrophages and thereby useful in the therapy of several diseases caused by immune dysfunction [50]. It is thus apparent that the immunostimulatory effect produced by methanol extract of *H.nepalense* containing quercetin glycoside may be due to the cell mediated and humoral antibody mediated immune response.

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing[51]. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent diseases [52].

The results of ferrous sulphate induced lipid peroxidation, from the experiments performed in chapter 7, showed that methanol extract of *C.oppositifolia* leaf at 1000 µg/ml concentration produced maximum percentage inhibition (58.67 %) of lipid peroxidation as compared with the standard antioxidant Vitamin E while compound I at 50 µg/ml showed an inhibition of 77.46 % greater than the inhibition produced by Vitamin E i.e.72.94 %. The inhibition could be attributed to the prevention of ferryl-perferryl complex or by changing the ratio of Fe^{3+} / Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself or combination thereof [53].

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 [54]. 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 *C.oppositifolia* and compound I in DPPH scavenging model revealed that the methanol extract of *C.oppositifolia* at 1000 µg/ml concentration showed an inhibition to DPPH reduction by 71.52 % as compared to the inhibition produced by Vitamin E (79.69 %). Compound I at 50 µg/ml showed higher inhibition of DPPH radical, 80.54 % compared to the standard drug Vitamin E i.e. 79.69 % at 5 mM concentration. The percentage of activity was also dependent on time.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [55]. Ferric-EDTA solution was incubated with H_2O_2 and

ascorbic acid at pH 7.4. Free hydroxyl radicals were formed in the solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH [56]. When the test compounds were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented their degradation. The extract of *C.oppositifolia* significantly inhibited degradation of deoxy-ribose mediated by hydroxyl radical by 79.12 % at the concentration of 1000 µg/ml, compared to that of known scavenger mannitol (88.14 %). Here the compound I at 50 µg/ml concentration exhibited an inhibition of 81.42 % compared to 88.14 % for the standard drug mannitol at a concentration of 50 mM.

Superoxide radical O_2^- is highly toxic specie, 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 [57]. Reduced phenazine methosulfate assay was used to measure the superoxide dismutase activity of methanol extract of *C.oppositifolia* and compound I. The methanol extract showed significant scavenging capacity of superoxide free radical at a concentration of 1000 µg/ml by 58.46 %. The compound I at 50 µg/ml concentration showed maximum inhibition of superoxide radicals (68.23 %) as compared with standard drug Vitamin E (69.23 %) at 5mM concentration.

The results of antioxidant activity of methanol root extract of *H.nepalense* on ferrous sulphate induced lipid peroxidation showed that the extract of *H.nepalense* inhibited the lipid peroxidation in a dose dependent manner. Compound II at 50 µg/ml concentration exhibited 72.38 % inhibition, which is comparable to the inhibition produced by vitamin E (73.42 %) at 5 mM concentration. The DPPH scavenging capacity of the extract was found to be 72.38% at the maximum tested concentration (1000 µg/ml). The compound II at 50 µg/ml, on the other hand, exhibited 76.38 % inhibition compared with 80.46 % for the standard drug vitamin E at 5mM. The extract of *H.nepalense* significantly inhibited 80.38 % of degradation of deoxy-ribose mediated by hydroxyl radicals at the concentration of 1000 µg/ml, compared to that of a known scavenger mannitol at 50mM

(89.64 %). The concentration of the methanol extract needed for 50% inhibition (IC_{50}) was 615.57 μg/ml. The compound II at 50 μg/ml on the other hand, exhibited 79.68 % inhibition compared to 89.64 % inhibition for the standard mannitol at 50mM concentration. Reduced phenazine methosulfate assay was followed to measure the superoxide dismutase activity of *H.nepalense* root extract and compound II. The results indicated that the scavenging capacity of the extract was 60.57 % at 1000 μg/ml concentration as compared with standard drug Vitamin E (68.36 %) at 5 mM concentration. The compound II at 25 μg/ml exhibited equal inhibition of superoxide radicals as compared with the standard drug (68.36 %). IC_{50} was found to be 8.9 mg/ml. Inhibition was proportional to the amount of the extract added.

Several epidemiological studies support that consumption of fruits and vegetables, rich in antioxidant compounds, flavonoids are associated with a lower incidence of diseases induced by oxidative stress [58]. It has been reported that flavonoids prevents injury and cell death caused by oxidative via several mechanisms, such as scavenging oxygen radicals, protecting against lipid peroxidation and chelating metal ions [59]. Here methanol extracts of *C.oppositifolia* leaf and *H.nepalense* root as well as compound I and compound II showed significant antioxidant effects in concentration dependent manner in all the models tested. Thus, the flavonoids present in the extracts are responsible for observed antioxidant activity.

Antimicrobial drugs have received immense importance in the therapeutic list of last century. Many infectious diseases sometimes considered incurable and lethal are now amenable to cure with a few doses of some drugs. The remarkable powerful and specific activity of anti-microbial drugs is due to their selectivity for specific targets that are either unique to microorganisms or much more important in terms of human use [60]. The antimicrobial compounds mainly isolated from microbes are structurally different from the compounds isolated from plant sources. The antimicrobial of plant source 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 [61, 62].

The methanol leaf extract of *C.oppositifolia* demonstrated significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multi resistance *Staphylococcus* strains (MRSC). The antimicrobial activity spectrum of *C.oppositifolia* leaf extract, as explained in chapter 8, revealed that out of 257 bacterial strains 171 (68 %) isolates were inhibited by the extract at a concentration up to 512 μg/ml. The experiment determining the minimum inhibitory concentration (MIC) of methanol extract revealed that 63 (81 %) out of 78 Gram-positive bacteria were sensitive within 512 μg/ml concentration while 94 (53 %) out of 179 Gram-negative isolates were sensitive within the same concentration. The disc diffusion test also demonstrated significant degree of antibacterial activity as compared with standard drug amoxycillin and gentamicin. The isolated compound I was also screened against several bacterial strains of *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp* including three MRSC, most of them being inhibited at 128 μg/ml concentration of compound I.

The *in vitro* studies were followed by extensive *in vivo* tests. When methanol extract and compound I were injected to Swiss albino mice infected with a virulent dose of *Salmonella typhimurium* NCTC 74, offered significant protection to the animals at dose of 256 and 100 μg/ml respectively for the test organism. Thus methanol extract and compound I elucidated remarkable *in vivo* antimicrobial activity as evident from the reduction in initial bacterial cfu count in different vital organs determined on 18h post infection.

The methanol leaf extract and compound I were found to be bactericidal against *S.aureus* 8530. When methanol leaf extract & compound I were added to the logarithmic growth phase of the broth cultures of *S.aureus* 8530 at a concentration of their MIC values, the viable counts were sharply reduced from the culture as observed at 2,4,8 and 18 h.

The methanol root extract of *H.nepalense* also exhibited a significant *in vitro* activity against 257 Gram-positive and Gram-negative bacteria including MRSC strains. The antimicrobial activity spectrum of *H.nepalense* root extract revealed that out of 257 bacterial strains 197 (77 %) isolates were inhibited by the extract at a concentration up to 512 µg/ml. The results revealed that the methanol root extract was most effective against Gram-positive bacteria (84 %) than Gram-negative bacteria (67 %). The isolated compound II was also effective within the concentration of 256 µg/ml against several species of *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp*. All the three MRSC strains were susceptible to compound II at a concentration of 128 µg/ml, while they were resistant to the two standard antibiotics Amoxycillin and Gentamicin.

The *in vitro* studies were followed by extensive *in vivo* tests. When methanol root extract and compound II were injected to Swiss albino mice infected with a virulent dose of *Salmonella typhimurium* NCTC 74, offered significant protection to the animals at dose of 128 and 100 µg/ml respectively for the test organism. Hence, the result demonstrated that the treatment with methanol root extract of *H.nepalense* and compound II successfully obliterate the severity of infection, as evident from the reduction in initial bacterial cfu count in different vital organs determined on 18h post infection.

The methanol root extract and compound II were found to be bactericidal against *S.aureus* 8530. When methanol root extract & compound II were added to the logarithmic growth phase of the broth cultures of *S.aureus* 8530 at a concentration of their MIC values, thee viable counts were sharply reduced from the culture as observed at 2,4,8 and 18h.

The present investigation therefore reveals that the methanol extract of *C.oppositifolia* leaf and *H.nepalense* root have a significant degree of antimicrobial activity, which may be due to the presence of compound I and compound II as evident by the *In vitro* and *in vivo* tests.

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CHAPTER 10

SUMMARY AND CONCLUSION

The major aim of the investigation was to explore two popular ethnomedicines used by traditional healer of Sikkim in their health care system since time immemorial. The medicinal plants used by the ethnic community of Sikkim are neither systemically documented nor properly tested by scientific approaches. The systemic scientific studies of these ethnomedicines can lead to promising phytochemicals (drugs) for many health problems. In view of the above, it was thought to be much worthy to take up the present research project i.e. "chemical and pharmacological evaluation of *Colebrookea oppositifolia* Smith and *Heracleum nepalense* D.Don DC" so as to explore the therapeutic values and development of some novel drugs from those indigenous plants.

The Chapter 1 of this thesis deals with the importance and development of herbal medicine in the present scenario. Herbal medicines include herbs, herbal preparations and finished herbal products that contain active ingredients, parts of plant, or other materials, or their combination. Traditional use of herbal medicines refers to the long historical use of these medicines. Their use is well established and widely acknowledged to be safe and effective, and accepted by national authorities. They contain pharmacologically active compounds. Furthermore, sometimes these are recommended as dietary supplements or valuable nutraceuticals. The actions of herbal preparation are often different from those of pharmaceuticals containing isolated single compound of the original plant preparation. The ultimate objective in drug discovery and drug development should be the production of safe and effective remedies. In the herbal preparations the molecules accompanying the active compound may help in stabilization, reduced toxicity or enhance the therapeutic value of active component. Further, different approaches used for the development of drug from natural sources, methods of drug evaluation, scope and aim of phytomedicine in the near future has thoroughly been discussed in this chapter.

In Chapter 2, various reports on phytochemical and pharmacological analysis of *C.oppositifolia* and *H.nepalense* have been presented. The current updated status of natural origin used as bioavailability enhancer, immunostimulators, antioxidant and antimicrobials have been elaborated in the form of comprehensive review of literature. Though some reports on phytochemical and pharmacological analysis of *C.oppositifolia*

and *H.nepalense* 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 *C.oppositifolia* and *H.nepalense* along with phytochemical group tests have been presented. The isolated bioactive compounds were analysed by using physical spectroscopic methods like UV, IR, ^{1}HNMR, ^{13}CNMR, Mass and melting point determination etc. The evidence presented conclusively proved that the isolated bioactive compounds isolated were (+) – catechin-7-O-β-rhamnopyranoside from *C.oppositifolia* leaf and quercetin-3-O-β-D-glucopyranoside from *H.nepalense* root. The isolations bear much significance in the present context because both the isolated phytoconstituents have not been reported from the plants under the study till date.

In Chapter 4, toxicological investigation of methanol extract of *C.oppositifolia* leaf and *H.nepalense* root are described. The MLD of methanol extract of *C.oppositifolia* leaf was found to be 3.0 g/kg body weight and 4.5 g/kg body weight in intraperitoneal and oral route respectively. On the other hand the MLD of the methanol extract of *H.nepalense* root was to be 4.5 g/kg body weight and more than 5.5 g/kg body weight in intraperitoneal and oral route respectively. The MLD values of both the extracts were found to be much higher indicating the wide safety range of action.

Chapter 5 deals with the effects of methanol extract of *C.oppositifolia* and *H.nepalense* root and their isolated compound on bioavailability of antibiotics like amoxycillin, cefixime and rifampicin. A sensitive, rapid and precise High Performance Liquid Chromatographic (HPLC) method was used to measure the concentrations of antibiotics in plasma samples collected for 24 h following different administration of drugs in rabbits. Pharmacokinetic parameters, including peak plasma concentration (C_{max}), time to reach peak concentration (t_{max}), area under curve (AUC), plasma half-life ($t_{1/2}$) elimination rate constant (k_{el}), relative bioavailability (RB %) were calculated following the one compartment open model. When *C.oppositifolia* was either coadministered or preadministered with amoxycillin, and rifampicin, C_{max}, AUC, $t_{1/2}$, RB % of amoxycillin

and rifampicin were increased significantly than that of the control used. But the preadministration or coadministration of the extract with cefixime failed to show any change in all the pharmacokinetic parameters. Further study with compound I at dose of 50 mg/kg body weight had significantly increased the bioavailability of amoxycillin and rifampicin. However, preadministration of compound I 30 min before the administration of cefixime increases all the pharmacokinetic parameters of the antibiotic.

The effects of the methanol root extract of *H.nepalense* and compound II were also studied for their effect on bioavailability of amoxycillin, cefixime and rifampicin in rabbits. The results revealed that the methanol extract failed to produce any effect on the bioavailability of all the three antibiotics. However the coadministration of compound II has moderately increased the bioavailability of these drugs.

In conclusion, the presence of flavonoid could be attributed to the enhancement of bioavailability of these antibiotics. However, the role of microsomal enzyme systems and efflux pump inhibitor P-glycoprotein (Pgp) cannot be ruled out. Based on the results acquired from the rabbit model it is proposed that further *in vitro* and *in vivo* studies are required with Caco-2 cell, isolated microsomal enzymes and by using human volunteers. If these studies show positive results of enhancement of bioavailability of antibiotics by *C.oppositifolia* and *H.nepalense* along with their compounds will be a great breakthrough in the field of bioenhancers and its application in pharmaceutical industry and therapeutics.

In Chapter 6, immunostimulatory activity of methanol extract of *C.oppositifolia* leaf as well as compound I has been presented. The immunostimulatory potential was investigated by *in vitro,* phagocytic index and lymphocyte viability tests, using interferon-α-2b, a known immunostimulant drug, as standard. Other tests such as carbon clearance, antibody titer and delayed type hypersensitivity were studied in mice, using levamisole as the standard. The results revealed that the leaf extract at higher dose (1000 mg/kg) moderately increased the rate of carbon clearance, humoral antibody titer and delayed type hypersensitivity in mice. The extract also showed moderate increase in

phagocytic index at higher concentration (1000 µg/ml), but failed to produce any stimulation of PHA activated mononuclear cells.

The immunostimulatory activity of the methanol root extract of *H.nepalense* and compound II also has been studied by different *in vitro* and *in vivo* test models. To investigate immunostimulatory potential, phagocytic index and lymphocyte viability tests were performed *in vitro* using interferon-α-2b, a known immunostimulant drug as standard while *in vivo* carbon clearance, antibody titer and delayed type hypersensitivity parameters were studied in mice using levamisole as standard drug. The results demonstrated that: (1) at a concentration of 1000 µg/ml dried root extract and isolated quercetin glycoside at 50 µg/ml increased significantly i*n vitro* phagocytic index and lymphocyte viability in all assays. (2) The root extract at oral doses of 1000 mg/kg and the isolated quercetin glycoside at 50 mg/kg showed a significant increase of *in vivo* antibody titer, carbon clearance and delayed type hypersensitivity response in mice. The immunostimulatory effect was pronounced in dose dependent manner.

In conclusion, the immunostimulant effect of *C.oppositifolia* leaf and *H.nepalense* root could be attributed to the flavonoid content or due to the combination with other component (s). The present finding provides scientific evidence to the ethnomedicinal use of this plant by the tribal group of Sikkimese as antimicrobial and aphrodisiac. The plant *H.nepalense* may thus find new therapeutic application in the future as immunostimulant.

In Chapter 7, antioxidant activities of methanol extract of *C.oppositifolia* and *H.nepalense* root as well as compound I and II 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, hydroxyl radical, superoxide radical scavenging assay using vitamin E (5mM) and mannitol (50 mM) as standard drugs. The percentages of inhibitions were calculated as compared with standard drugs. It was observed that the methanol extracts of both the plants exhibited a considerable inhibition of lipid peroxidation and possessed DPPH radical, hydroxyl 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 concentration showed significant antioxidant effects in concentration dependent manner in all the models tested. In conclusion, the presence of flavonoid isolated from the plants could responsible for observed antioxidant activity.

In Chapter 8, antimicrobial activity of methanol extract of *C.oppositifolia* leaf and compound I has been studied both *in vitro* and *in vivo* model system. The *in vitro* test was carried out by agar dilution and disc diffusion method. The *in vivo* study was performed by determining the protection offered to Swiss albino mice against the virulent strain of *Salmonella typhimurium* NCTC 74 and the mode of actions were determined in Oxoid brand nutrient broth at 2, 4, 8 and 18 h. The crude methanol extract of *C.oppositifolia* leaf was found to be active against 257 bacterial isolates comprising of 12 genera of both Gram-positive and Gram-negative organisms including multiresistant *Staphylococcus* (MRSC) strains. The minimum inhibitory concentration (MIC) ranges were found to be from 128-1000 µg/ml for most of the bacteria. The endogenous cfu counts in mouse spleen, liver homogenate and heart blood on 18th post-bacterial challenge hour with crude methanol extract demonstrated significant antibacterial effect. Inhibitory activity was based on bactericidal action and viable cell number reduced significantly after 18h incubation with the extract. The bactericidal activity was observed at the MIC value. Compound I showed similar activity against *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp* including three MRSC, most of them being inhibited at 128 µg/ml concentration of the agent.

The antimicrobial activity of *H.nepalense* root and compound II were also studied by *in vitro* and *in vivo* model system. These are found to be active against several pathogenic strains of *Escherichia coli*, *Klebsiella spp*, *Shigella spp*, *Citrobacter spp*, *Pseudo aeruginosa*, *Bacillus spp*, *Streptococcus spp*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp* including three MRSC strains. The minimum inhibitory concentration (MIC) ranges from 128-512 µg/ml for most of the bacteria. The endogenous cfu counts in mouse spleen, liver homogenate and heart blood on 18th post-bacterial challenge hour with crude methanol extract demonstrated significant antibacterial effect. Inhibitory activity was

based on bactericidal action and viable cell number reduced significantly after 18h of incubation with the extract. The bactericidal activity was observed at the MIC value. Compound II showed similar activity against *Escherichia coli*, *Staphylococcus spp*, *Salmonella spp* and *Vibrio spp* including three MRSC, most of them being inhibited at 128 μg/ml concentration of compound II. The study confirms the possible antimicrobial potentiality of *C.oppositifolia* and *H.nepalense*. The present finding provides scientific evidence to the ethnomedicinal use of this plant by the tribal group of Sikkimese in skin infection and diarrhoea. However, further studies are required with different isolates of fungus to confirm the spectrum of antimicrobial activity.

All the experiments were performed both by using *in vitro* and *in vivo* model to established that the plants of the Himalayan region of Sikkim investigated provide valuable proof 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 of 21st century. Many drug discoveries resulted 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 could contribute a little in the development of some newer molecules, having therapeutic value. Most of the work presented in this thesis has been authenticated with concomitant publications in national and international journals.

LEGENDS OF PUBLICATIONS

1. Dash, S., Bhise, S.B. and Dhachinamoorthi, D. (2004). Effect of aqueous extract of *Colebrookea oppositifolia* on bioavailability of amoxycillin in rabbits. *Ind Drugs* 41 (4), 231-235.
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EFFECT OF AQUEOUS EXTRACT OF *COLEBROOKEA OPPOSITIFOLIA* ON BIOAVAILABILITY OF AMOXYCILLINE IN RABBITS

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ABSTRACT

This study was aimed to evaluate the effect of *Colebrookea oppositifolia* on bioavailability of amoxycilline. A sensitive, rapid and precise High Performance Liquid Chromatography (HPLC) method was used to measure the concentration of amoxycilline in plasma samples collected for 24 hours following different administration of drugs in rabbits. Pharmacokinetic parameters, including E_{max}, C_{max}, K_{el}, AUC, $t_{1/2}$ were calculated as per one compartment model. Analysis of data revealed that the variations in all pharmacokinetic parameters in different administration were statistically significant ($P<0.05$). This concludes that co-administrations of aqueous extract of *C. oppositifolia* enhance the bioavailability of amoxycilline. The enhanced bioavailability could be attributed to the effect of the extract on microsomal enzymes or enzymes systems.

Keywords: *Colebrookea oppositifolia*, Bioavailability, Cyp_s, PgP, Flavonoids.

INTRODUCTION

Colebrookea oppositifolia (Family: *Lamiaceae*) Commonly known as 'Bansa' or 'Dosul' is a monotypic genus of evergreen shrubs or small tree, 1.2 to 3.6m tall growing widely in Northern and Southern slopes of the Himalayan range of Sikkim at elevation ranging between 3000-5000 ft. It has been reported to be useful in the treatment of various diseases like, asthma[1], epilepsy[2] tumor[3], and CNS disorders[4]. Various substances are present in the plant e.g. flavonoids, which have recently attracted interest due to their various beneficial pharmacological effects and additional abilities to modulate the bioavailability of different xenobiotics. In the present study we have made an attempt to investigate the effect of high flavonoidal content of aqueous extract of *C. oppositifolia* on bioavailability of amoxycilline in rabbit.

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MATERIALS AND METHODS

Preparation of Extract

The fresh dried leaves (3.0 kg) of *C. oppositifolia* were collected from the southern district of the Sikkim. It was authenticated by Botanical survey of India, Gangtok, Sikkim. After collection, the leaves were washed thoroughly in tap water and dried in shade for about 3-4 weeks. The dried leaves were powdered and stored in a well-closed container. A suspension of 10g of dried leaf powder in 100ml of distilled water was stirred magnetically for 4 hr at room temperature. The residue was removed by filtration and the filtrate was used as such for oral feeding or dried by freeze-drying. The yield of the water extract was about 9g from 10g dried leaves. Chemical tests were performed to confirm the flavonoid in the extract.[5]

Animals

Albino rabbits, (Himalayan Pharmacy Institute) weighing between 1.5 - 2 kg of either sex were used in the bioavalability studies and albino mice, (Himalayan Pharmacy Institute) between 20 - 25g for toxicity studies. All the animals were housed under standard conditions of temperature (27°C ± 3°C), 12hr/12hr light/dark cycles and fed with standard pellets diet (Chakan oil mill) and tap water. The

experiments were performed after getting the experimental protocols approved by the institutional Animal Ethics Committees of the Jadavpur University, Kolkatta.

Drugs and Chemicals

Amoxycilline from Libra Drugs (India), Pune. Sodium hydrogen phosphate from S.D. Fine Chemicals Ltd., Boisar, HPLC grade methanol and water from Qualigens Fine Chemicals, Mumbai, orthophosphoric acid and acetonitrile from E.merck, Mumbai were used during present investigation.

Acute Toxicity Studies

The extract was administered orally to different groups (5mice/group) of mice in doses ranging from 300mg/kg. No lethality was observed in any of the groups. Mice, which received extract of doses above 6000 mg/kg exhibited ptosis (dropping of upper eyelids) and were found lethargic. One tenth of the maximum dose of the extracts tested for toxicity study (800 mg/kg p.o) was selected for evaluation of activity.[3]

Acute toxicity studies in rabbits were also carried out by administering *C. oppositifolia* 30 min prior to amoxycilline treatment to find out whether *C.oppositifolia* potentiate the toxicity of the antibiotic.

Preparation of Test Drugs and Treatment Schedule

Amoxycilline was prepared as suspension in carboxy methyl cellulose (0.5%w/v) using pestle and mortar. Rabbits were randomly divided into 3 groups (6 rabbits/group). The first group served as control and received only amoxycilline (100mg/kg) orally. The second group received Extract of *C. oppositifolia* (800 mg/kg) orally with amoxycilline. The third group received *C. oppositifolia* (800mg/kg) extract orally and 30min later amoxycilline (100mg/kg).

Blood Collection and Determination of Blood Levels of Amoxycilline

Blood samples of rabbits treated with antibiotic alone and with *C. oppositifolia* were collected from the marginal ear vein by 1ml syringe with 26½ gauge needle (Disposable) at different time intervals (0, 0.1, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 hrs) following the antibiotic treatment. Blood samples were centrifuged at 2000 rpm for 20min. Thereafter the samples were separated and transferred to clean, dry vials and kept at 20°C until analyzed by developed HPLC method. All the samples were coded and labeled properly.

Pharmacokinetic Analysis

C_{max} and t_{max} for antibiotic were determined directly from the raw data. The area under curve (AUC) was calculated using the trapezoidal rule method. The apparent elimination rate constant (Kel) was determined by the regression analysis of the serum concentration verses time data of the terminal phase. The apparent half-life for the drug in plasma was calculated from relationship $t_{½}$=0.693/Kel equation. The significance between the respective treatment groups was calculated by paired student's 't' test.

RESULTS

The serum concentration versus time profile of amoxycilline administered with or without aqueous extract of *C. oppositifolia* is outlined in Table I. The study was taken in single dose, cross over design. The data was fitted to a one compartment open model with first order kinetics. Comparative pharmacokinetic parameters of three sets of the experiments have been shown in the Table II. In this interactive study, in all three treatment categories the absorption process was completed with median t_{max} of 2.58 (S.D. ± 0.08), 1.98 (S.D ± 0.15) and 1.75 (S.D. ± 0.10) hrs for amoxycilline, aqueous extract of *C. oppositifolia* + amoxycilline and *C. oppositifolia* + amoxycilline (30minutes later) respectively.

Aqueous extract of *C. oppositifolia* pretreatment induced a significant ($P<0.05$) shift in t_{max} of amoxycilline. This indicates that the prior treatment of the aqueous extract of *C. oppositifolia* has altered the t_{max} significantly as compared to *C. oppositifolia* + amoxycilline together. Thus the aqueous extract

Table I: Effect of Co-administration of Aqueous Extract of *Colebrookea oppositifolia* on Serum Concentration of Amoxycilline [Values expressed as μg/ml, are mean ± SD]

Sl. No.	Time of collection (hrs)	Amoxycilline (100mg/kg)	*C.oppositifolia* (800mg/kg)+ Amoxycilline (100mg/kg)	*C.oppositifolia* (800mg/kg) + Amoxycilline (100mg/kg) 30 mins later
1.	0	0	0	0
2.	0.5	8.85±0.195	18.15±0.194	28.70±0.701
3.	1.0	18.26±0.477	28.81±0.391	48.69±0.963
4.	1.5	26.28±0.410	45.50±0.267	62.34±0.591
5.	2.0	32.39±0.607	58.21±0.237	58.41±0.656
6.	2.5	37.08±0.394	54.47±0.227	55.35±0.408
7.	3.0	34.22±0. 390	50.57±0.293	51.61±0.516
8.	4.0	28.48±0.226	43.69±o.360	44.48±0.489
9.	5.0	22.81±0.153	36.68±0.575	36.67±0.409
10.	6.0	18.52±0.301	29.35±0.277	30.77±0.486
11.	8.0	12.81±0.162	21.62±0.265	23.18±0.294
12.	10.0	8.39±0.159	14.56±0.121	15.63±0.282
13.	12.0	5.05±0.115	10.80±0.270	11.68±0.443
14.	24.0	0.48±0.058	1.30±0.112	1.67±0.136

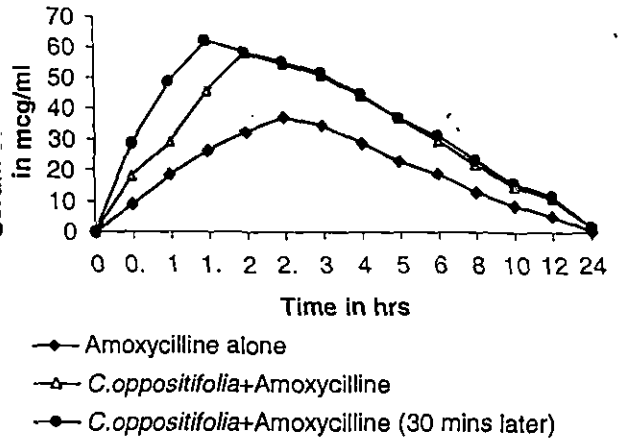
The data based on three separate experiments with six animals in each group. P values. <0.05.

Table II: Comparison of Pharmacokinetic Parameters (Values expressed as μg/ml, are mean ± SD)

Sl. No	Treatment	t_{max} (hr)	c_{max} (μg/ml)	$t_{1/2}$ (hr)	AUC (μg.hr/ml)	k_{el}
1.	Amoxycilline (100mg/kg)	2.58±0.08	37.08±0.34	3.39±0.08	248.69±1.723	0.2044± 0.008
2.	*C.oppositifolia* (800mg/kg) + Amoxycilline (100mg/kg)	1.98±0.15	58.38±0.316	4.015±0.139	429.29±1.968	0.1727± 0.006
3.	*C.oppositifolia* (800mg/kg) + Amoxycilline (100mg/kg) 30minutes later	1.58±0.04	62.34±0.591	4.29±0.140	475.15±3.28	0.1616± 0.005

The data based on three separate experiments with six animals in each group. P values. <0.05.

C. oppositifolia shows the time dependent effect the gastrointestinal absorption of the drug. The ribution phase of the amoxycilline in all three s of the experiments was fairly short i.e. centration decay being evident within 2 hr. of the g administration. Comparison of other rmacokinetic parameters (Table II) revealed t there were significant differences in peak ibiotic levels attained (c_{max}) or mean elimination f life ($t_{½}$). Comparison of AUC showed that higher um levels of amoxycilline were achieved in the up treated with *C. oppositifolia* + amoxycilline. mparison of the serum concentration time curve g.1) of each administration shows significant ferences of amoxycilline present in the blood.



g. 1 : Comparative Mean Plasma Amoxycilline (100mg/g) Concentration versus Time Profile for Rabbits treated ith Amoxycilline (100mg/kg)and Aqueous Extracts of *olebrookea oppositifolia* at various Time Intervals

ISCUSSION

The present experimental findings in animal xperiments were clearly indicated that concurrent dministration of aqueous extract of *C. oppositifolia* ignificantly enhances the bioavailability of moxycilline. The analysis of various harmacokinetic parameters tmax, Cmax, K_{el}, $t_{½}$ nd AUC point out that aqueous extract of *C. oppositifolia* might influence the different hysiological process like alteration in gastric ransport or absorption process. Again it was reported hat aqueous extract of *C. oppositifolia* has flavonoid as the major chemical constituent[6-8]. In recent years, flavonoids have attracted increasing interest due to their various pharmacological effects and additional abilities to modulate cyp_s and P-glycoprotien (PgP)[9-12]. Therefore direct inhibitory effect of high flavonoidal content of *C. oppositifolia* on Cyp_s and PgP can not be ruled out.

The studies conclude that aqueous extract of *C. oppositifolia* co-administration with amoxycilline improves the bioavailability. This is reflected from various pharmacokinetic parameters studied. Since the increased bioavailability of amoxycilline has great implication in clinical medicine, co-administration of isolated products like flavonoids from *C.oppositifolia* reduce the amount of the dose of amoxycilline and its dosing frequency. It is directly related with patient compliance and adverse effect of the drug. However careful bioavailability studies using human volunteers and specific isolated flavonoid from the plant need to be under taken to confirm the results obtained.

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Research Article

Antioxidant and antimicrobial activities of *Heracleum nepalense* D Don root

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Abstract

Purpose: The aim of the present study was to investigate antioxidant and antimicrobial effects of the methanol extract of *Heracleum nepalense* D.Don roots.
Method: The antimicrobial effect was determined by agar dilution and disc diffusion method. The free radical scavenging potential was studied by using different antioxidants models of screening using vitamin E (5mM) as standard.
Results: The crude methanol extract of *H.nepalense* root was found to be active against both Gram-positive and Gram-negative organisms. The ethyl acetate soluble fraction of the extract showed similar activity against these organisms. Similarly, the methanol extract at 1000 μg. ml^{-1} and the ethyl acetate fraction at 50 μg. ml^{-1} exhibited significant antioxidant activity in ferrous sulphate induced lipid peroxidation, 1,1- diphenyl- 2-picryl hydrazyl (DPPH), Hydroxyl radical and Superoxide scavenging models.
Conclusions: The study confirms the possible antioxidant and antimicrobial potentiality of the plant extract. Presence of flavonoid alone or in combination with its other components could be responsible for the activity.

Keywords: *Heracleum nepalense*, Lipid peroxidation, Superoxide scavenging, DPPH assay, Antimicrobial effect, Flavonoid.

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Introduction

Reactive oxygen species (ROS) such as superoxides, peroxides and hydroxyl radicals are known to play an important role and have been identified as major contributors to all cell and tissue damage in many disease conditions[1]. In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, alcohol, pesticides, certain pollutants and microbial infections [2-3].

In recent years, there has been increased incidence of antibiotic resistance in pathogenic organisms and the persistence of pathogens in immune compromised individuals is of great concern[4]. Therefore, actions must be taken to reduce this problem such as controlling the use of antibiotics, carrying out research to better understand the genetic mechanisms of resistance and continuing investigations aimed at the development of drugs from natural sources.

The recent years have witnessed resurgence of interest in herbal drugs globally as more people are turning to the use of herbal medicinal products in health care. About 80% of individuals from developing countries use traditional medicine, which involves compounds derived from medicinal plants. It is high time the hidden wonders of plant molecules were revived with the modern tools of target-based screening to develop newer advanced generation antioxidants and antimicrobials with novel modes of action. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to human body against infection and degenerative diseases.

Heracleum nepalense D.Don (Apiaceae) is a small shrub occurring in Nepal and Sikkim [5]. The plant is used in veterinary medicine. It exhibits stimulant property and increases blood pressure in goats [6]. The roots of the plant are used in folk medicine as digestive, carminative and antidiarrhoeal (Authors personal experience). The roots of the plant are reported to have coumarins [7] and steroids [8]. The present study was aimed at evaluating antioxidant and antimicrobial properties of *Heracleum nepalense*.

Experimental

Plant materials and phytochemical screening

The fresh dried roots of *Heracleum nepalense* (HNSE) were collected from the southern district of Sikkim. The plant was authenticated by Botanical Survey of India, Gangtok, Sikkim. The voucher specimen was preserved in our laboratory for future reference. The dried roots were powdered and stored in a well-closed container. 1 kg of powder (40 mesh size) was extracted by cold percolation with 3 liters of 70% v/v methanol in a percolator for 72 h at room temperature [9] .The residue was removed by filtration. The extract was then evaporated to dryness under reduced pressure in a rotary evaporator at 42-45°C. The concentrated extract of leaves was kept in a dessicator for further use.

The preliminary phytochemical tests of the root extract were done by Pollock and Stevense method [10]. Concentrated methanol extract was suspended in hot distilled water, cooled and the blast precipitate was filtered off. The filtrate (aqueous solution) was fractioned by extracting it successively with petroleum ether, ethyl acetate and acetone. The ethyl acetate fraction of the crude extract on purification yielded one major fraction A (flavonoid) with some fatty substances.

Chemicals

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. Deoxy ribose was obtained from Merck India. Dimethyl sulphoxide, ethylene diamine tetra acetic acid, ferrous sulphate, trichloroacetic acid, hydrogen peroxide, ascorbic acid, mannitol, potassium dihydrogen phosphate, potassium

hydroxide, deoxy ribose, phenazine methosulphate were of analytical grade and were obtained from Ranbaxy fine chemicals.

Determination of antioxidant activity

Assay of lipid peroxidation

The extent of lipid peroxidation in goat liver homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method [11] with minor modifications [12]. Goat liver was purchased from local slutter house. Its lobes were dried between blotting papers (to remove excess blood) and were cut into small pieces with a heavy-duty blade. They were then homogenized in glass-Teflon homogenizing tube in cold phosphate buffer saline (pH 7.4). It 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 [13]. To study the comparative response, the experiment was divided into nine groups. Liver homogenate (5%, 3ml) was aliquoted to nine different 35mm glass petri dishes. The first two groups were treated as control and standard where buffer and vitamin E were added. In the third to seventh group, different concentrations of methanol extract (200-1000 $\mu g.ml^{-1}$) and fraction A (25, 50 $\mu g.ml^{-1}$) were added. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate [14]. After 30 minutes, 100 µl of this reaction mixture was taken in a tube 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 percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the following formula:

Inhibition (%) = [(control – test) ×100] / control---- --- Eqn 1.

DPPH radical scavenging activity

DPPH scavenging activity was measured by spectrophotometric method[14]. To a methanolic solution of DPPH (100 µM, 2.95ml), 0.05 ml of methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and Standard compound (vitamin E) were added at different concentrations. Equal amount (0.05 ml) of methanol was added to a control. Absorbance was recorded at 517nm at regular intervals of 1 to 5 min. The percentage of scavenging was calculated by comparing the control and test samples with the Eqn 1.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxy ribose and test compounds for hydroxyl radical generated by the Fe^{3+}– ascorbate – EDTA – H_2O_2 system (Fenton reaction) according to the method of Kunchandy & Rao [15]. The reaction mixture containing, a final volume of 1.0 ml, 100 µl 2-deoxy-ribose, 500 µl of the various concentrations of the methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and standard compound (Mannitol 50 mM) in KH_2PO_4- KOH buffer (20mM, pH 7.4), 200 µl 1.04 mM H_2O_2 and 100 µl 1.0mM ascorbic acid was incubated at 37°C for 1 hour. One milliliter 1% trichloroacetic acid was added to each test tube and incubated at 100°C for 20 min. After cooling to room temperature, absorbance was measured at 532nm against a control preparation containing deoxyribose and buffer. Percent inhibition was determined by comparing the results of the test and control samples with the above mentioned Eqn 1.

Superoxide scavenging activity

The superoxide scavenging activity of methanol extract was determined by the method described by Nishimik et al [16], with slight modification. About 1.0 ml NBT solution containing 156 µM NBT dissolved in 1.0 ml 100 mM phosphate buffer, pH 7.4, 1.0 ml NADH solution containing 468 µM NADH dissolved in 1.0 ml 100mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of the methanol extract as well as fraction A (25, 50 $\mu g.ml^{-1}$) and standard compound (vitamin E) were 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 at 560 nm was measured against control sample. Percent inhibition was determined by comparing the results of the test and control samples with the above mentioned Eqn 1.

Antimicrobial activity

Bacteria

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 and Institute of Microbial Technology (IMTECH), Chandigarh, India. . Three multiresistant *Staphylococcus* strains (*Staphylococcus aureus* ML 275, *Staphylococcus aureus*', NCTC 8530 and *Staphylococcus epidermidis* 865) were kindly provided by Prof. (Mrs.) Sujata Ghosh Dastidar, Department of Pharmacy, Jadavpur University, and Kolkata, India. They were aseptically isolated and identified by the Barrow and Feltham's method and preserved in the freeze-dried state [17]. Gram-positive strains were grown in nutrient broth (NB, Oxoid brand) and Gram-negative bacteria were grown in peptone water (PW, Oxoid brand, bacteriological peptone plus Nacl 0.5%) for 18 h before use.

Determination of antimicrobial activity

Sensitivity tests were performed by disc diffusion method[18]. The nutrient agar plates (Oxoid brand), containing an inoculum size of 10^5-10^6 cfu.ml^{-1} of bacteria were used. Previously prepared crude methanol extract (Concentration 128-2000 μg.ml^{-1}) and fraction A (Concentration 128-2000 μg.ml^{-1}) discs were placed aseptically on sensitivity plates. The discs containing methanol and known antibiotics (Amoxycillin and Gentamicin) served as negative and positive controls. All the plates were then incubated at 37°C±2°C for 18 h. 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 [19]. All the test compounds were dissolved in methanol. These were then individually added at each final concentrations of 0-2000 μg.ml^{-1}, to molten agar (Oxoid brand), 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 10^4 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 agar plate containing only methanol and Amoxycillin was served as negative and positive control.

Statistical analysis

Data are reported as the mean ± SD of three measurements. Statistical analysis was performed by the student *t*-test and by ANOVA. IC_{50} values for all the above experiments were determined by linear regression method. A p-value less than 0.05 was considered as indicative of significance.

Results and Discussion

Antioxidant activity

Assay of lipid peroxidation

The results presented in Table-1 showed that the methanol extract of the HNSE inhibited $FeSO_4$ induced lipid peroxidation in a dose dependent manner. The extract at 1000 μg.ml^{-1} exhibited maximum inhibition (69.25 ± 1.21%) of lipid peroxidation, on the other hand fraction A at 50 μg.ml^{-1} concentrations showed (72.38 ±1.9%) inhibition, nearly equal to the inhibition produced by vitamin E (Fig 1). The IC_{50} value was found to be 747.5 ±3.16 μg.ml^{-1}. The inhibition could be caused by the absence of ferryl-perferryl complex or by changing the ratio of Fe^{3+} / Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself or combination thereof [20].

DPPH scavenging activity

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

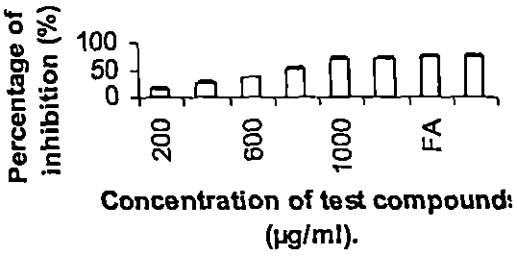

Fig 1: Inhibition (%) of lipid peroxidation by different concentrations of methanol extract of *H. nepalense*, Ethyl acetate fraction (FA) and Vitamin E (Vit E).

absorption at 517 nm. DPPH radical reacts with suitable reducing agents and then electrons become paired off and the solution loses colour stoichometrically with the number of electrons taken up [21]. Such reactivity has been widely used to test the ability of compound/plant extracts to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. The DPPH scavenging capacity of the extract was found to be 72.38 ± 3.92 % at 1000 $\mu g.ml^{-1}$. The fraction A at 50 $\mu g.ml^{-1}$ doses, on the other hand, exhibited 76.38 ± 5.12 % inhibition compared with 80.46 ± 4.62 % for the standard drug vitamin E at 5mM (Table 1). The IC_{50} value was found to be 6.5 $mg.ml^{-1}$. The activity was also dependent on time (Fig 2).

Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [22]. Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH [23]. When the test compounds were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented their degradation. The extract of HNSE significantly inhibited (80.38 ± 2.28 %) degradation of deoxy-ribose mediated by hydroxyl radicals at the dose of 1000 $\mu g.ml^{-1}$ (Table 1), compared to that of a known scavenger mannitol (50mM). The concentration of the methanol extract needed for 50% inhibition was 615.57 $\mu g.ml^{-1}$. The fraction A at 50 $\mu g.ml^{-1}$ on the other hand, exhibited (78.68 ± 2.62 %) inhibition compared with (89.64 ± 2.84 %) for the standard mannitol.

Superoxide scavenging activity

Superoxide radical O_2 is a highly toxic specie, which is generated by numerous biological and photochemical reactions. Both aerobic and anaerobic organisms possess superoxide dismutase enzymes, which catalyse the

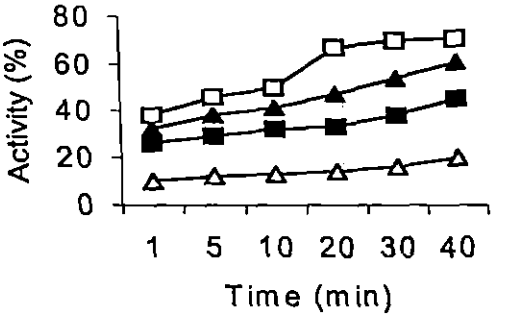

Figure 2: DPPH scavenging activity of *H.nepalense*. 10mg/ml (□), 7.5mg/ml (▲), 5mg/ml (■) and 2.5 mg/ml (Δ).

breakdown of superoxide radical [24]. Reduced phenazine methosulfate assay was used to measure the superoxide dismutase activity of HNSE. The results presented in Table 1 showed that the scavenging capacity of the extract was 60.57 ± 2.34% at 1000 $\mu g.ml^{-1}$. The fraction at 25 $\mu g.ml^{-1}$ exhibited 68.36 ± 2.73 % inhibition of superoxide radicals. IC_{50} was found to be 8.9 $mg.ml^{-1}$. Inhibition was proportional to the amount of the extract added.

Antimicrobial activity

The methanol extract of HNSE roots exhibited a significant *in vitro* antimicrobial activity against 257 Gram-positive and Gram-negative bacteria including multiresistant *Staphylococcus* (MRSC)

strains. All the three reference strains of bacteria were found to be sensitive within 1000 μg.ml^{-1}. The preliminary biological screening of the fractions showed that the ethyl acetate part was more active than other fractions (Data not shown). The results of the antimicrobial spectrum of the roots extract presented in Table 2 showed that out of 257 bacteria, the growth of 197 isolates were inhibited at a concentration of 128 – 512 μg.ml^{-1}. 57 isolates were resistant up to 1000μg.ml^{-1}, while the remaining 03 isolates where resistant up to >2000 μg.ml^{-1}, the highest concentration tested. The MICs tests revealed that 63 out of 75 Gram-positive bacteria were sensitive between 128 and 256 μg/ml (zone diameter 10–16 mm); while out of 179 Gram-negative isolates, 120 were sensitive between 256-512 μg.ml^{-1} (zone diameter 10-14 mm). Hence, it appears that the antimicrobial activity of the methanol extracts was directed both against Gram-positive and Gram-negative bacteria. The ethyl acetate fraction (fraction A) was also tested for antimicrobial activity. The result revealed that all the isolates were sensitive at 128-256 μg.ml^{-1} (Table 3). It was interesting to note that all the MRSC were susceptible to fraction A at a concentration of 128 μg.ml^{-1}, while they were resistant to the two test antibiotics. Thus, ethyl acetate fraction may become clinically relevant, particularly for antibiotic-resistant strains. However, the activity has to be studied using isolated pure compounds from the fraction to which the more resistant strains were susceptible in order to confirm these findings.

Table: 1 Effect of *H.nepalense* on different antioxidant models.

Concentration (μg/ml)	Percentage of Inhibition (%)			
	Lipid Peroxidation	DPPH	Hydroxyl radical	Superoxide radical
1000	69.25 ± 1.21	72.38 ± 3.92	80.38 ± 2.28	60.57 ± 2.34
800	51.74 ± 1.92	69.42 ± 3.86	79.54 ± 1.24	52.27 ± 3.18
600	36.36 ± 1.84	45.52 ± 3.73	65.37 ± 2.26	40.26 ± 2.16
400	26.57 ± 3.8	36.46 ± 2.32	46.28 ± 1.89	24.12 ± 1.38
200	16.08 ± 4.3	8.47 ± 1.83	32.14 ± 2.24	12.23 ± 1.42
Vitamin E (5mM)	73.42 ± 2.3	80.46 ± 4.62	NT	68.36 ± 2.73
Mannitol (50mM)	NT	NT	89.64 ± 4.62	NT
Fraction A				
	70.15 ± 1.64	71.32 ± 1.85	72.35 ± 2.93	68.36 ± 2.73
25	72.38 ± 1.9	76.38 ± 5.12	79.68 ± 2.62	68.24 ± 1.86
50				
IC_{50} (μg/ml)	747.5 ± 3.16	600.52 ± 3.46	615.57 ± 2.16	891.9 ± 14.42

Values are mean ± S.E.M of 3 replicates. NT: Not tested.

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NOTE

A Flavonoid from the Roots of *Heracleum nepalense* D. Don

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A known flavonol glycoside, namely, quercetin-3-O-β-D-glucopyranoside, was isolated from the roots of *Heracleum nepalense* D. Don. The structure was determined on the basis of UV, IR, FAB$^+$, MS, ^{1}H & ^{13}C NMR spectral data. The isolation is significant since a flavonoid has not been previously reported from the plant.

Key Words: Isolation, Quercetin, *Heracleum nepalense*.

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Heracleum nepalense D. Don[1-3] (Apiaceae) is a small shrub occurring in Nepal and Sikkim. The plant is used in veterinary medicine. It exhibits stimulant property and increases the rate of respiration and blood pressure in goats. The roots of the plant are used in folk medicine as digestive, carminative and antidiarrhoeal. In our earlier work we have reported the plant having antimicrobial property. The roots of the plant reported for having coumarins[4, 5] and steroids[6]. Flavonoids are more common throughout the family Apiaceae than other constituents[7]. Very little research has been conducted on the roots of *H. nepalense* and nothing has been found concerning the flavonoids, it was of interest to examine the flavonoid patterns of the species. In this report, we describe the isolation and structure elucidation of a flavonoidal glycoside from the roots of *H. nepalense*. It was identified by spectroscopic techniques[8].

The air-dried roots (1 kg) were extracted with methanol (70%) and the extract was concentrated, treated with hot distilled H_2O and filtered. The water-soluble component was fractionated by extracting it successively with petroleum ether, ethyl acetate and acetone. The ethyl acetate soluble fraction was concentrated and then on TLC over silica gel showed three spots. This fraction was submitted to column chromatography on sephadex LH-20 column using benzene : ethyl acetate as eluent with increasing polarity. Fractions 32–46 were combined, evaporated under reduced pressure, dissolved in MeOH and purified on a silica

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gel (60–120) column. From the dry residue of the flavonoid fraction compound 1 (75 mg) was obtained by recrystallization from MeOH.

Compound 1 (Quercetin 3-O-β-D-glucopyranoside)

It was obtained as a pale yellow amorphous powder, m.p 210–214°C; UV $_{max}\lambda^{MeOH}$ nm: 251, 374; +NaOMe 246, 328, 425; +NaOAc-H_3BO_3 229, 268, 320 sh, 383; IR (KBr, cm^{-1}) ν_{max}: 3648–3611 ν(OH), 2904 ν(C-H), 1653 ν(C=O in flavone), 1615–1507 ν(aromatic rings), 1456, 1304, 1263; ^1H NMR (DMSO-d_6), δ (ppm): 12.48 (1H, S, OH-5), 7.56 (2H, dd, J = 2.2 Hz, H-2' and H-6'), 6.87 (1H, d, J = 8.5 Hz, H-2, H-5'), 6.41 (1H, d, J = 1.9 Hz, H-2, H-8), 6.18 (1H, d, J = 1.9Hz, H-6), 4.92 (1H, d, J = 7.3Hz, H-1''), 3.98–3.06 (m, the remaining protons of glucose); ^{13}C NMR (DMSO-d_6), δ (ppm): 176.05 (C-4), 164.14 (C-7), 160.95 (C-5), 156.4 (C-2), 147.9 (C-4'), 147.01 (C-3'), 135.9 (C-3), 125.8 (C-1'), 122.2 (C-6'), 115.3 (C-2), 103.2 (C-10), 98.48 (C-1''), 76.97 (C-5''), 76.92 (C-3''), 75.0 (C-2''), 69.97 (C-4''), 60.47 (C-6''); MS data m/z (rel. int.): 459 (10.2), 415 (15.8), 371 (20.0), 327 (19.2), 303 (98.8), 301 (29.7), 287 (10.2), 277 (12.0), 239 (8.2), 207 (8.0).

The structure of quercetin was established by comparison of measured UV, NMR and mass spectral data with spectroscopic data available from literature[9, 10]. The quercetin 3-O-β-D-glucopyranoside showed ^1H and ^{13}C NMR data in full agreement with those given by Olszewska *et al.*[11] and Irena *et al.*[12]

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Isolation and Characterization of a Catechin Glycoside from the Leaves of *Colebrookea oppositifolia* Smith

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Abstract

Phytochemical investigation on methanol extract of leaves of *Colebrookea oppositifolia* led to the isolation of a known flavan-3-ol. Ethyl acetate fraction of the extract was subjected to coloumn chromatography and monitored by TLC, resulted a single compound. The compound was established on the basis of UV, IR, FAB$^+$MS, ^{1}H, ^{13}C NMR spectral and elemental analysis. The isolated compound was identified as (+)– Catechin-7-O-β-rhamnopyranoside. This confirmed that Catechin is one of the major bioconstituent of the plant.

Key Words: Isolation, Catechin, Flavan-3-ol, *Colebrookea oppositifolia*.

Introduction

Colebrookea oppositifolia Smith. (Lamiaceae) locally known as "Bansa" or "Dosul" is a monotypic genus of ever green shrubs or small trees, 1.2-3.6m tall growing widely in northern and southern slopes of the Himalayan range of Sikkim at elevation ranging between 3000 to 5000 ft. Ethnobotanical studies indicate that the decoctions of *C.oppositifolia* leaves and stem bark is widely used among the tribal populations of Sikkim to treat skin infections, indigestion (Gurung G, 1999), diarrohea (Jain SP, 1984) wounds and cuts (Manadhar NP, 1995). Alcoholic extracts of leaves is reported to be useful in asthma (Singh V, 1995), epilepsy (Paul SR, 1997) and helminthes (Ansari S, 1982). Earlier reports on the phytochemistry of *C. oppositifolia* indicate the presence of compounds like β-Sitosterol, Stearic acid (Ansari S, 1982), Quercetin (Mukherjee PK, 2001), and Baicalein (Ahmad SA, 1974). The present phytochemical studies for the first time showed the presence of (+) – Catechin in the ethyl acetate fraction of the methanol extract of *C. oppositifolia* leaves.

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Materials and Methods

UV-Visible spectral analysis was carried out on a Shimadzu model 1601 Spectrophotometer. TLC was carried out on polyamide (E.merck & Co) coloumn using n-Butanol: Acetic acid: Water (4:1:5), Phenol:Water (3:1) and Concentrated Hcl: Acetic acid: Water (3:30:10) . For coloumn chromatography Sephadex LH-20 and Silica gel 60-120 (SRL, India) were used. Sugar analysis was carried out according to standard procedure on Whatman paper No.1 along with authentic standard sugar samples (Markham KR, 1982). FAB^+MS was recorded on a JEOL JMS 600 Spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 400 MHz on Brucker WM 400 Spectrophotometer. The Infrared absorption spectra of the isolated compound were taken with Perkin Elmer FTIR spectrophotometer. Elemental analysis was made with a Carlo Erba EA 1110 apparatus. The structure of catechin was established by comparison of measured UV, NMR and Mass spectral data with spectroscopic data available from the literature (Markham KR, 1982, Ta-Chen Lin, 1999).

Extraction and fractionation

The fresh dried leaves of *C. oppositifolia* were collected from the southern district of Sikkim. It was authenticated by Botanical survey of India, Gangtok, Sikkim. The voucher specimen was preserved in our laboratory for future reference. The dried leaves was powdered and stored in a well-closed container.1 kg of powdered plant (40 mesh size) was extracted by cold percolation with 3 liters of 70% methanol in a percolator for 72 h at room temperature. The residue was removed by filtration. The solvents were then evaporated to dryness under reduced pressure in a rotary evaporator at 42-45°C. The concentrated extract of leaves was kept in dessicator for further use. 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, ethyl acetate and acetone. The ethyl acetate soluble fraction was subjected to coloumn chromatography on Sephadex LH-20 coloumn using benzene: ethyl acetate as eluent with increasing polarity. Fifty-eight fractions were collected. Identical fractions were combined, evaporated under reduced pressure, dissolved in methanol and purified on a Silica gel 60-120 coloumn. From the dry residue of the fractions compound-I was obtained by recrystallisation from methanol.

Compound I was obtained as yellow amorphous solid; m.p. 181-185°C; UV (MeOH) λ_{max} 241, 281; +NaOMe 250, 293; +$AlCl_3$ 248, 282; +$AlCl_3$+HCl 280, 314sh, 361sh; +NaOAc 243, 283; +NaOAc 281; IR (KBr) γ_{max} cm^{-1}. 3480, 1771, 1632, 1606, 1519, 1456, 1029; ^1H NMR (DMSO-d_6 400 MHz) δppm 2.506 (1H, d, J= 2.0 Hz, 4H), 2.699 (1H,dd, J=16 Hz, 4H), 3.476 (2H, m, 5"), 3.787- 3.872 (1H, m, 4"H), 4.393 (1H, d, J=3.0 Hz, 2"H), 5.698 (1H, d, J=3.0 Hz, 1"H), 6.212 (1H, d, J=2.0 Hz, 8H), 6.226 (1H, d, J=2.0 Hz, 6H), 6.591 (1H, dd, J=2.0 Hz, J=7.6 Hz, 6'H), 6.681 (d, J=7.6Hz, 5'H), 6.731 (d, J=2.0 Hz, 2'H); ^{13}C NMR (DMSO, d_6, 400 MHz): 145.1 (C-2), 130.95 (C-3), 81.23 (C-4), 155.68 (C-5), 94.28 (C-6), 156.75 (C-7), 92.56 (C-8), 156.52 (C-9), 99.46 (C-10), 118.88 (C-1'), 95.51 (C-2'), 115.52 (C-3'), 114.78 (C-4'), 92.56 (C-5'), 91.23 (C-6'), 72.67 (C-1"), 72.25 (C-2"), 70.84 (C-3"), 61.51 (C-4"), 66.63 (C-5"), 28.51 (C-6"); FAB^+MS data (m/z) 581 (3.2), 383 (4.5), 303 (10.0), 291 (100), 274 (18.0), 255 (4.2), 231 (3.3), 207 (5.0). Elemental analysis (Found: C-54.12; H-4.24. $C_{24}H_{22}O_8$. Calc. for C-54.13; H-4.23 %)

Results and Discussion

Compound I was isolated by repeated coloumn chromatography on a Sephadex LH-20 and silica gel 60-120 from ethyl acetate fraction of *C.oppositifolia* leaves. It was

obtained as yellow amorphous solid. The complete acid hydrolysis gave Catechin and glucose (co- TLC). The compound showed strong absorption at 281 nm in its spectrum, which implied the presence of phenolic aromatic rings. The UV spectrum showed absorption bands reagents shifts of the compound to be a 7-substituted derivatives. The absence of free 7-hydroxyl group in the compound was observed in lack of shift of band II in the presence of NaOAc. The IR spectrum confirmed the presence of aromatic ring (1632 cm^{-1}) and hydroxyl group (3480 cm^{-1}). The doublets at 6.212 ppm (J=2.0 MHz) and 6.226 ppm (J=2.0 MHz) in its ^{1}H NMR spectrum were suggestive of two aromatic protons existing at the *meta* positions. The signals at 6.591 ppm (J=2.0 Hz, J=7.6 Hz), 6.681 ppm (J=7.6Hz) and 6.731 ppm (J=2.0 Hz) inferred the presence of another set of aromatic protons existing at 1,3,4 positions. The signals at 2.506 ppm and 2.699 ppm and the double double doublet at 3.476 ppm suggested that the compound should be a flavan-3-ol derivative. Existence of a sugar in its structure was evidenced by the anomeric proton signal, which appeared at a 5.698 ppm (J= 3.0 Hz). The coupling constant (J= 3.0 Hz) of the anomeric proton sugar suggested that the phenoxy group be attached to the anomeric carbon atom by β-configuration. The fragmentation ion at m/z 291 in its mass spectrum inferred that the compound should have (+) – Catechin as an aglycone. The structure of the compound was further confirmed by $^{13}CNMR$ and elemental analysis of the available literature. The signals of C-5, C-7 and C-9 were observed at higher value than 150 ppm, the signals at 156.75 ppm only showed correlations with these of H-6 and H-8 (at 6.212 ppm and 6.226 ppm respectively), we assigned it to C-7. The signals at 155.68 ppm and 156.52 ppm showed correlations with those of H-6 and H-8 respectively, so we assigned them to C-5 and C-9 respectively. The correlation of the anomeric proton signals at 5.698 ppm with that of C-7 at 156.75 ppm suggested that the sugar should be attached at the C-7 position. The sugar analysis was carried out along with authentic standard sugar sample. The result is corresponding to the molecular formula $C_{24}H_{22}O_8$ (confirmed by elemental analysis). From these data we concluded that the structure of the isolated flavonoid was (+)-catechin-7-O-β-rhamnopyranoside.

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Research Paper

Stimulation of immune function activity by the alcoholic root extract of *Heracleum nepalense* D. Don

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ABSTRACT

Objective: This study was designed to assess the immunostimulatory activity of *H. nepalense*, using different *in vitro* and *in vivo* experimental models.

Materials and Methods: The immunostimulatory potential of the test compound was investigated by *in vitro*, phagocytic index and lymphocyte viability tests, using interferon-α-2b, a known immunostimulant drug, as the standard. Other tests such as carbon clearance, antibody titer and delayed type hypersensitivity were studied in mice, using levimasole as the standard.

Results: The dried root extract (1000 µg/ml) and isolated quercetin glycoside (50 µg/ml) significantly increased the *in vitro* phagocytic index and lymphocyte viability in all assays. They also showed a significant increase in antibody titer, carbon clearance and delayed type hypersensitivity in mice.

Conclusion: *H. nepalense* exhibited a dose-dependent immunostimulant effect, which could be attributed to the flavonoid content or due to the combination with other component(s).

KEY WORDS: Cell proliferation, immunostimulation, HIV.

Introduction

Heracleum nepalense D.Don (Apiaceae) is a small shrub which grows in Nepal and Sikkim. It is used in veterinary medicine. It exhibits stimulant property and increases the rate of respiration and blood pressure in goats.[1] The root of the plant is used as a digestive, an aphrodisiac, a carminative and an antidiarrheal in folk medicine.[2] We have earlier reported the antioxidant and antimicrobial properties of the plant.[3] *H. nepalense* was studied for its potential immunomodulatory activity, driven by the presence of its antimicrobial property, and its usage in folk medicine.

Immunostimulatory therapy is now being recognised as an alternative to conventional chemotherapy for a variety of disease conditions, involving the impaired immune response of the host.[4] Immunostimulators have been known to support T-cell function, activate macrophages and granulocytes, and complement natural killer cells apart from affecting the production of various effector molecules generated by activated cells (Paraimmunity).[5] It is expected that these non-specific effects offer protection against different pathogens, including bacteria, fungi, viruses and so on, and constitute an alternative to conventional chemotherapy.[6] In view of the above, the present investigation was undertaken to evaluate the immunostimulatory potential of *H. nepalense* roots, using *in vitro* and *in vivo* models.

Materials and Methods

Plant material and preparation of extract

The fresh roots (9 kg) of *H. nepalense* were collected from the southern district of Sikkim between September-November, 2003. The roots were authenticated by the Botanical Survey of India, Gangtok, Sikkim. A voucher specimen was preserved in our laboratory for future reference. The shade-dried root was ground and yielded 2.5 kg of powder. It was stored in an air-tight, hard polyethylene container with silica pouch up to 10-12 days. One kg of powdered plant (40 mesh size) was extracted by cold percolation with 3 liters of 70% methanol in a percolator for 72 h, at room temperature. The residue was removed by filtration. The solvent was then evaporated to dryness, under reduced pressure, in a rotary evaporator at 42-45°C and yielded 400 mg of extract. The concentrated methanol extract was kept in a dessicator for further use.

Phytochemical studies

The chemical constituents of the methanol extract were identified by qualitative chemical tests for the presence of flavones, tannins, sterols, triterpenoids and saponins (Data

presented).[7] The methanol extract was concentrated, spended in hot distilled water, cooled and the blast cipitate was filtered. The water-soluble component was ctionated by extracting it successively with petroleum ether, yl acetate and acetone. The ethyl acetate soluble fraction s subjected to column chromatography on Sephadex LH- column using benzene:ethyl acetate as eluent with creasing polarity. Fractions 32-46 were combined, aporated under reduced pressure, dissolved in MeOH and rified, on a silica gel (60-120) column. A known flavonol coside, namely quercetin-3-O-β-D-glucopyranoside, was olated along with some minor compounds.[8]

st compound formulations

Oral suspensions of the extract and isolated compound ercetin-3-O-β-D-glucopyranoside were prepared by spending them separately in 1% solution of sodium carboxy ethyl cellulose to prepare suitable dosage forms.

imals used

Swiss Albino mice of either sex, weighing 17–25 g each, ere used. They were housed under standard conditions of mperature (23±10°C) and relative humidity (55±10%), 2/12 h light/dark cycle, and fed with standard pellets and ater *ad libitum*. The Institutional Animal Ethics Committee eviewed the animal protocols prior to the experiments.

rugs and chemicals

EDTA, RPMI-1640, Hank's balanced salt solution (HBSS), extran, phosphate buffered saline, fetal calf serum, treptomycin, penicillin, amphotericin, and Trypan blue were urchased from Himedia. Phytohaemagglutinin, ficoll ypaque and L-glutamine were purchased from Sigma iagnostic, USA. Interferon α-2b and levimasole were btained as gift samples from Fulford (I) Ltd. and Khandelwal aboratories Ltd., Mumbai, respectively.

ntigen

Fresh blood was collected from a healthy sheep from the ocal slaughter house. Sheep red blood cells (SRBCs) were vashed thrice with normal saline and adjusted to a oncentration of 0.1 ml containing $1X10^8$ cells for mmunisation and challenge.

n vivo carbon clearance test

The mice were divided into 8 groups, each consisting of 0 animals. Group I (Control) was given 1% sodium carboxy methyl cellulose in water (0.3 ml/mouse, i.p.) for 7 days, Group II-VIII were given different concentrations of methanol extract (250-1000 mg/kg, p.o.), isolated compound (25, 50 mg/kg, p.o.) and standard drug (Levimasole 50 mg/kg, p.o.) for 7 days. At the end of 7 days, the mice were injected, via the tail vein, with carbon ink suspension (10 μl/g, body weight). Blood samples were drawn (in EDTA solution 5 μl), from the retroorbital vein, at intervals of 0 and 15 min, a 25 μl sample was mixed with 0.1% sodium carbonate solution (2 ml) and the absorbance measured at 660 nm. The carbon clearance was calculated using the following equation: $(Log_e OD_1 - Log_e OD_2)/15$, where OD_1 and OD_2 are the optical densities at 0 and 15 min, respectively.[9]

In vivo humoral antibody (HA) titer and delayed type hypersensitivity (DTH) response

Humoral antibody (HA)

The mice were divided into 8 groups, each consisting of 6 mice. Group I (Control) was given 1% sodium carboxy methyl cellulose in water (0.3 ml/mouse) for 7 days. Group II-VIII were given drug treatment which was exactly the same as with the carbon clearance test.

The animals were immunised by injecting 0.1 ml of SRBCs suspension, containing $1X\ 10^8$ cells, intraperitoneally, on day 0. Blood samples were collected in microcentrifuge tubes from individual animals of all the groups by retroorbital vein puncture on day 8. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the haemagglutination technique.[9] Briefly, equal volumes of 50 μl individual serum samples of each group were pooled. Serial two-fold dilutions of pooled serum samples were made in 50 μl volumes of RPMI-1640 in microtitration plates. To this 50 μl of 1% suspension of SRBC in RPMI-1640 was added. After mixing, the plates were incubated at 37°C for 1 h and examined for haemagglutination under the microscope (button formation). The reciprocal of highest dilution, just before the button formation, was observed and titre values were calculated.

Delayed type hypersensitivity test (DTH)

On Day 8, the thickness of the right hind footpad was measured using a Vernier calliper. The mice were then challenged by injection of $1\ X\ 10^8$ SRBCs in the right hind footpad. The footpad thickness was measured again after 24 h of challenge. The difference between the pre- and post challenge footpad thickness, expressed in mm, was taken as a measure of the DTH response.[10]

In vitro phagocytic index

Preparation of microorganism

Escherichia coli 832 (*E. coli*) was grown and kept on a slope of solid agar medium. Before use, the microorganism was cultured in 100 ml of 2.5% nutrient broth (oxoid) for about 18 h at 37°C. The culture was then washed twice with phosphate buffer saline and re-suspended in gelatin-HBSS to a concentration of $1\ X\ 10^7$ cells/ml. During each experiment, the number of viable microorganisms was determined microbiologically by counting colony forming units (cfu), using nutrient agar plates after incubation, at 37°C for 18 h.[11]

Preparation of human polymorphonuclear leukocytes (PMNCs)

Human blood was collected from a local blood bank and the RBCs removed by sedimentation in 5% (w/v) solution of dextran in buffered saline (m.w. 200,000; 3 ml of solution to 10 ml of blood) for 30 min at 37°C. The PMNC-rich supernatant layer was washed twice with heparin-saline, concentrated by centrifugation (10 min at 110 g), counted with a hemocytometer, and suspended in gelatin-HBSS to make up a concentration of $1\ X\ 10^7$ cells/ml.

Microbiological assay for the phagocytosis

To assess phagocytosis, different concentrations of methanol extract (250-1000 μg/ml), its isolated compound (25, 50 μg/ml) and the standard drug, Interferon α-2b (0.5 million IU), in the final volume of 0.1ml, were incubated respectively with 2 ml of the PMNCs suspension ($1\ X\ 10^7$ cells/ml), 2 ml of the suspended microorganisms ($1\ X\ 10^7$ cells/ml) and 0.4 ml of fetal calf serum at 37°C for 1 h in 5% CO_2 atmosphere in a slanting position. At 30 min intervals up to 120 min, 0.5 ml aliquot of the suspension was removed

and added to 1.5 ml of the ice-cooled gelatin-HBSS to stop phagocytosis. The control was run using gelatin-HBSS in place of the test compounds. These samples were centrifuged at 110 g for 4 min. Under this condition, the non-ingested microorganisms remained in the supernatant fluid. The viable count of the microorganisms was undertaken using the colony counter[11]. Phagocytosis was expressed as the percentage decrease in the initial number of viable extracellular bacteria according to the formula: $P(t) = (1 - N_t/N_0) \times 100$, where P (t) is the phagocytic index at time t = t, N_0 and N_t are the number of viable extra cellular bacteria at time t = 0 and t = 30, 60, 90 and 120 min, respectively.[12]

In vitro cell proliferation assay

This test was performed with peripheral mononuclear blood cells, following their separation from the blood by using ficoll-hypaque gradient centrifugation, according to manufacture's instructions (Sigma Diagnostic, USA). The rate of proliferation of mononuclear cells, under the influence of mitogens, was measured by the method of Sriwanthana,[13] with minor modification. Briefly, under sterile conditions, the cells were diluted to 1 X 10^7 cells/ml with RPMI-1640 (supplemented with 20% fetal calf serum). The cell suspension (2 ml) was transferred into a sterile culture tube and to each sample. Different concentrations of the plant extract (250-1000 μg/ml, filtered through 0.22 μ pore size filter), isolated compound (25, 50 μg/ml) and standard drug Interferon α-2b (0.5 million IU), in the final volume of 0.1 ml, were added, respectively. The proliferation of cells was induced by 50 μl phythaemagglutinin (PHA, 0.1 mg/ml). The prepared samples were incubated for 72 h at 37°C in a CO_2 atmosphere, supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 μg/ml amphotericin. The control incubated with cells minus the plant extract. The viability of the cells was assessed after incubation with test compounds, using the Trypan blue dye exclusion method.[14] Briefly, 20 μl of the incubation mixture was mixed with 20 μl of Trypan blue dye. The total number of mononuclear cells and mononuclear stained blue (dead cells) were counted under an inverted microscope (Olympus, Japan), using the hemocytometer. The percentage of cell viability was taken as a measure of cell proliferation and calculated as per the following formula:[15]

$$\% \text{ of cell viability} = \frac{\text{Total number of cells} - \text{Total number of dead cells}}{\text{Total number of cells}} \times 100$$

Similarly, the percentage of cell stimulation was calculated as per the following formula:[15]

$$\% \text{ of cell stimulation} = \frac{\text{Viability of test} - \text{Viability of control}}{\text{Viability of control}} \times 100$$

Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Dunnett's test. The significance in difference was accepted at $P < .05$.

Results

The methanol extract, 250-1000 mg/kg, p.o. and its isolated compound, 25 and 50 mg/kg, p.o. exhibited a significant increase in carbon clearance from the blood in a dose-dependent manner. [Table 1] The doses of test drugs, for which maximum carbon clearance were seen, are methanol extract (1000 mg/kg) and its isolated compound (50 mg/kg). The results (Table 1) also indicate that animals treated with 250, 750 and 1000 mg/kg of methanol root extract produced a significant increase in HA titer (humoral immunity) as evident from hemagglutination after incubation of serum with SRBCs, while the isolated compound at 50 mg/kg and levimasole (50 mg/kg) showed 328.6±10.4 and 430.06±8.3 HA titer, respectively. In the DTH response (cell mediated immunity) test, the methanol extract at higher doses (750 and 1000 mg/kg) showed a statistically significant increase in mean paw edema in mice. The isolated compound at 50 mg/kg, on the other hand, exhibited the maximum DTH response of 0.50±0.22 compared with 0.57±0.21 for the standard drug, levimasole.

The effects of methanol extract (250–1000 μg/ml) and its isolated compound (25–50 μg/ml) on the phagocytic index model are presented in Table 2. The phagocytic index was significantly increased in a dose-dependent manner after 30, 60, 90 and 120 min intervals with methanol extract as well as its isolated compound. Maximum phagocytic index was observed at 1000 μg/ml (97.86±1.67) of methanol extract and 50 μg/ml (97.24±1.23) of its isolated compound after 120 min of incubation; whereas the standard compound Interferon α-2b exhibited maximum phagocytic index (99.23±1.11) after 120 min. The results of the proliferative response, on the basis of the cell viability of mononuclear cells to the PHA mitogen, are presented in Table 3. The percentage of viability of PH- activated mononuclear cells was significantly increased at 1000 μg/ml of the root extract, compared with the control. The maximum viability (60.4±4.58 %) was noticed at standard drug Interferon α-2b; whereas

Table1

Effect of methanol root extract of *Heracleum nepalense* and isolated compound on immunostimulatory activity in mice

Treatment (mg/kg)	*Carbon clearance*	*HA titer*	*DTH response*
Control	0.068±0.012	80.43 ±2.21	0.28 ±0.10
Extract 250	0.122±0.016*	185.3 ±3.31*	0.39 ±0.13
500	0.132±0.014*	172.7 ±0.1*	0.28 ±0.13
750	0.146±0.016*	282.6 ±8.1*	0.43 ±0.10*
1000	0.158±0.018*	320.8 ±10.6*	0.48 ±0.21*
Levimasole (50)	0.164±0.016*	430.06 ±8.3*	0.57 ±0.21*
IC (25)	0.156±0.016*	324.2 ±8.3	0.48 ±0.22
IC (50)	0.160±0.018*	328.6 ±10.4*	0.50 ±0.22*
One-way F	230	5231	6.432
ANOVA P	0.05	0.05	0.05

Values are mean±SEM; n=6 in each group; df=7,40. *P<0.05 in comparison with control. IC: Isolated compound.

Table 2

Effect of methanol root extract of *Heracleum nepalense* and isolated compound on phagocytic index of polymorphonuclear leukocyte

Concentration (μg/ml)	Phagocytic index (%) 30 min	60 min	90 min	120 min
Control	61.14±1.26	97.26±1.52	84.32±1.69	92.25±1.83
Extract 250	63.53±1.52*	78.54±1.18*	85.74±1.43*	94.27±1.86*
500	64.12±1.18*	79.23±1.21*	86.23±1.24*	95.67±2.33*
750	67.38±1.24*	81.52±1.42*	88.15±1.86*	96.55±1.83*
1000	69.54±1.12*	83.26±1.37*	91.18±2.14*	97.86±1.67*
Interferon α-2b	70.27±1.64*	84.14±1.32*	94.52±1.65*	99.23±1.11*
IC 25	69.54±1.12*	82.86±1.45*	90.96±1.14*	96.42±1.42*
50	70.27±1.64*	83.09±1.51*	91.14±1.62*	97.24±1.23*
One-way ANOVA F	1.123	1.154	1.272	1.526
One-way ANOVA P	0.05	0.05	0.05	0.05

Values are mean±SEM; n=6 in each group; df=7,40. *P<0.05 in comparison with control. IC:Isolated compound.

Table 3

Effect of methanol root extract of *Heracleum nepalense* and isolated compound on mononuclear cell proliferation

Treatment (μg/ml)	Percentage viability of cells (%)	Stimulation (%)
Control	50.7±10.42	0
Extract 250	47.6±4.42*	(– 6.11)
500	49.8±5.42*	(– 1.77)
750	49.0±4.45*	(– 3.35)
1000	56.3±8.94*	(+11.04)
Interferon α-2b	60.4±4.58*	(+19.13)
IC 25	56.9±6.62*	(+12.22)
50	58.2±4.53*	(+14.79)
One-way ANOVA F	5.630	
One-way ANOVA P	0.05	

Values are mean±SEM; n=6 in each group; df=7,40. *P<0.05 in comparison with control. IC:Isolated compound. "+" indicates increase and "–" indicates decrease in cell stimulation.

the isolated compound at 50 μg/ml concentration showed 58.2±4.53 % viability. Further, the methanol extract at 1000 μg/ml and its isolated compound at 50 μg/ml concentration showed 11.04 % and 14.79% cell stimulations, respectively, as compared with Interferon α-2b, for which it was observed as 19.13%.

Discussion

The present study established the immunostimulatory activity of the methanol extract and its isolated compound. Prophylactic treatment of *H.nepalense* and its isolated compound enhanced the rate of carbon clearance from the blood (more than a two-fold increase) when compared with the control group. The result is owing to a mechanism related to phagocytosis by macrophages. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b, leading to a more rapid clearance of foreign particulate matter from the blood[12]. *H.nepalense* was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance.

The methanol extract, at a dose of 1000 mg/kg, body weight showed almost a four-fold increase in HA titer, compared to untreated controls. The isolated compound also pronounced significant activity at a dose of 50 mg/kg body weight. This could be due to the presence of flavonoids which augment the humoral response, by stimulating the macrophages and B-lymphocytes subsets involved in antibody synthesis.[10] The DTH response, which directly correlates with cell-mediated immunity (CMI), was found to be the highest at the maximum dose tested in the root extract (1000 mg/kg). The mechanism behind this elevated DTH during the CMI responses could be due to sensitised T-lymphocytes. When challenged by the antigen, they are converted to lymphoblasts and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction.[16] The infiltrating cells are probably immobilised to promote defensive (inflammatory) reaction.[17] An increase in DTH response indicates that the root extract of *H.nepalense* and its isolated compound have a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction.[18]

The *in vitro* immunostimulatory activity of the methanol extract and its isolated compound was tested on human polymorphonuclear and mononuclear cells. The phagocytosis and intracellular killing of microorganisms by polymorphonuclear phagocytes was determined by the direct measurement of the microbicidal activity.[12] Phagocytosis was expressed as the phagocytic index, in which the percentage decrease in the initial number of viable extracellular bacteria was determined microbiologically after incubation with polymorphonuclear leukocytes. In our study, the phagocytic index of *H. nepalense* root extract was found to be increased in a time- and dose-dependent manner. The isolated compound (50 μg/ml) and root extract (1000 μg/ml) showed significant phagocytic index as compared to control.

Further, the immunostimulatory effect of the extract and its isolated compound was tested in mitogen-activated cultured mononuclear cells. PHA was used to activate the mononuclear cells in the culture. The mitogenic PHA are polyclonal activators, in that they activate mononuclear cells including memory type cells, irrespective of their antigenic specificity.[19] The root extract (higher concentrations) and its isolated compound caused a significant stimulation of the mononuclear cells. This is attributed to the fact that the methanol extract and its isolated compound may stimulate the PHA-activated mononuclear cells and induce the release of cell proliferating factors such as interleukin and TNFα.[19]

Earlier reports on the phytochemistry of *H. nepalense*

indicate the presence of compounds such as steroids and coumarins. We have isolated a known flavonoid, quercetin glycoside, from the plant.[8] However, there is no report on the pharmacological activity of the plant. Contemporary research revealed that quercetin glycoside, isolated from different herbal sources, has several pharmacological actions such as antioxidant, anticancer, antiulcer, antiinflammatory and antiviral.[20] Recent reports indicate that several types of flavonols stimulate human peripheral blood leukocyte proliferation. They significantly increase the activity of helper T cells, cytokines, interleukin 2, γ-interferon and macrophages and are thereby useful in the treatment of several diseases caused by immune dysfunction.[21] It is thus apparent that the immunostimulatory effect produced by the methanol extract of *H.nepalense*, containing quercetin glycoside, may be due to cell mediated and humoral antibody mediated immune responses.

The present finding provides scientific evidence to the ethnomedicinal use of this plant by tribals in Sikkim. The plant *H.nepalense* has the potential for new therapeutic applications in the future.

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