STUDIES ON PHARMACOLOGICAL SCREENING OF THE BIOACTIVE MOLECULES FROM SOME SELECTED PLANTS

THESIS SUBMITTED FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (PHARMACY)
OF

UNIVERSITY OF NORTH BENGAL

By

Mrs. Jyochhana Priya Mohanty
M. PHARM

Himalayan Pharmacy Institute
East Sikkim

University of North Bengal
Raja Rammohunpur, Dist- Darjeeling
West Bengal
2009

Dr. L. K. Nath, Professor
Deptt. of Pharmaceutical Sciences
Dibrugarh University
Dibrugarh - 786 004
Tel - 0373 - 2370254
Fax- 0373 - 2370323

DIBRUGARH UNIVERSITY

Residence :
Tekela Chiring Gaon
(Near Dibrugarh University)
Tel : 0373 - 2370355
e-mail :lknath@yahoo.com

Ref. No. .....

Date.....

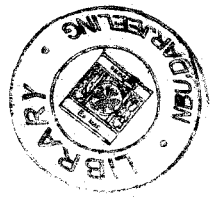
CERTIFICATE

This is to certify that the thesis entitled *"Studies on Pharmacological Screening of the Bioactive Molecules from some Selected Plants"* Submitted by **Mrs. Jyochhana Priya Mohanty**, Sr. Lecturer, Himalayan Pharmacy Institute, Majhitar, Rangpo, E.Sikkim – 737 136 for the award of the Degree of Doctor of Philosophy (Pharmacy) of University of North Bengal, is absolutely based upon her own research work under the supervision of **Dr. Lila Kanta Nath**, Professor in Pharmacy, Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh – 786 004, Assam, and that neither her thesis nor any part of her thesis has been submitted for any degree or any other academic award anywhere before.

(Dr. L. K. Nath)
Supervisor,

Date- 20/08/2009
Seal-

Professor
Deptt. of Pharmaceutical Sciences
Dibrugarh University
DIBRUGARH - 786 004



DECLARATION

The research work entitled **"Studies on Pharmacological Screening of the Bioactive Molecules from some Selected Plants"** has been carried out by me in the Department of Pharmacognosy, Himalayan Pharmacy Institute, Majhitar, E. Sikkim under the supervision of **Prof. (Dr.) Lila Kanta Nath.** I hereby declare that the work is original and has not been submitted so far, in part or full for any other degree or diploma of any university.

Date: - 20.08.2009

Place: - HPI, Majhitar

Jyochhana Priya Mohanty
(JYOCHHANA PRIYA MOHANTY)

DEDICATED TO MY FAMILY

ACKNOWLEDGEMENT

It gives me great pleasure to acknowledge my deep sense gratitude and indebtedness to my guide ***Dr. L.K. NATH****, Professor Dibrugarh University for his excellent guidance, aesthetic suggestions, constant encouragement and innovative ideas, that give me the impetus to complete this work.*

I express my regards and heartfelt thanks to ***Dr. H.P. Chhetri****, Director, Himalayan Pharmacy Institute, Majhitar, Sikkim for providing me the necessary infrastructural and laboratory facilities, valuable suggestions and encouragement during the course of study.*

I express my heart felt gratitude to ***Dr. K. Gauthaman,*** *Ex-Principal, Himalayan Pharmacy Institute for his judicious help.*

I am extremely indebted to ***Dr. B.P. Saha,*** *HOD, Pharmaceutical chemistry of Himalayan Pharmacy Institute for his constructive and philosophical suggestions and kind co- operation.*

I take this opportunity to express my gratitude towards ***Dr. Santosh Mahapatra*** *and* ***Mr. Ranabir chanda*** *for their immense help, helpful suggestion and for helping me at the various stages of work.*

I express my profound gratitude towards ***Mr. G. Mariappan, Mr. S. Dewanjee*** *and* ***Ms. S. Ganguli*** *who helped in every possible way.*

I take this opportunity with immense pride and pleasure to offer my deep sense of gratitude, heartiest love, ample thanks and appreciation to the wonderful person ***Nihar*** *my husband who stood by unfailingly my side all throughout as a source of inspiration and moral booster for my life.*

I offer my cordial thanks to ***Mr. Sandip Ghosh*** *and* ***Mr. Ratan Biswas*** *the faculty of Computer Application who have assisted me to make this work successful. I take this page to record my heartfelt and ample thanks to* ***Uday Nayak*** *for his constant co-operation and help.*

I would like to express my deep gratitude to persons of Himalayan Pharmacy institute connected directly or indirectly in this regard for providing facilities.

Last but not least I would like to pay highest regards to my parents and in laws, for their sincere encouragement, blessings and inspiration during my doctoral thesis. The silent encouragement and great deal patience of my remaining family members are precious in this regard.

Jyochhana Priya Mohanty.

JYOCHHANA PRIYA MOHANTY

PREFACE

During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world. Documenting the indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources. Today according to the World Health Organization (WHO), as many as 80% of the world's people depend on traditional medicine for their primary healthcare needs.

Plants have been used in traditional medicine for several thousand years. The knowledge of medicinal plants has been accumulated in the course of many centuries based on different medicinal systems such as Ayurveda, Unani and Siddha etc. Sikkim Himalayan region is characterized by a rich diversity of ethno medicinal plants as well as a rich heritage of traditional medicine practices. Earlier studies on traditional medicinal plants also revealed that the economically backward local and tribal people of Sikkim prefer folk medicine due to low cost and sometimes it is a part of their social life and culture. Now, the time has come to compile and document available knowledge on our valuable plant resources and to prove their utility scientifically through detailed phytochemical, biological and pharmacological investigations.

The work embodied in this thesis is a humble and sincere effort to explore and screen two ethnomedicine *Kaempferia rotunda* Linn. and *Eupatorium cannabinum* Linn. used by traditional healer of Sikkim in their health care since time immemorial. In this research work the constituents of these plants were isolated, purified and analyzed the chemical structures of the pure compounds as well as their activities were evaluated. The thesis has been segregated into 10 well-defined chapters and finally a summary and the conclusion has been drawn along with the summary of the whole work. At the end of each chapter the necessary references have listed which are superscripted in the text. Thus the investigated works deals with a scientific approach to explore the pharmacological activities of the extracts and some activities from isolated compound from *K. rotunda* and *E. cannabinum* two popular ethnomedicine from Himalayan Sikkim region, for the benefit of the entire human beings.

CONTENTS

CHAPTER - 1

INTRODUCTION

1.1. Herbal medicine: An introduction

By definition, the word *natural* is an adjective referring to something that is present in or produced by nature and not artificial or man-made. The term *natural products* today is quite commonly understood to refer to herbs, herbal concoctions, dietary supplements, traditional Chinese medicine, or alternative medicine[1].

Medicinal herbs are moving from fringe to mainstream use with a greater number of people seeking remedies and health approaches free from side effects caused by synthetic chemicals. Recently, considerable attention has been paid to utilize eco-friendly and biofriendly plant-based products for the prevention and cure of different human diseases. Considering the adverse effects of synthetic drugs[2,3] the western population is looking for natural remedies which are safe and effective.

India in a supreme position with respect to richness of medicinal flora and is sitting on a gold mine of well-recorded and traditionally well-practiced knowledge of herbal medicine. This country is perhaps the largest producer of medicinal herbs and is rightly called the botanical garden of the world. It is generally estimated that over 6000 plants in India are in use in traditional, folk and herbal medicine, representing about 75% of the medicinal needs of the third world countries[4]. Three of the ten most widely selling herbal medicines in the developed countries, namely preparations of *Allium sativum*, *Aloe barbedensis* and *Panax* spp. are available in India. Medicinal herbs have been in use in one form or another, under indigenous systems of medicine like Ayurveda, Sidha and Unani. India, with its traditional background, needs to increase its share in the world market. But unlike China, India has not been able to capitalize on this herbal wealth by promoting its use in the developed world, despite their renewed interest in herbal medicines. This can be achieved by judicious product identification based on diseases prevalent in the developed world for which no medicine or palliative therapy is available. India is one of the twelve-mega biodiversity countries in the world. The total number of plant species of all groups recorded from India is 45,000 (the total number may be even close to 60,000 as several parts of India are yet to be botanically explored). Of these, number of seed bearing plants account for nearly 15,000-18,000. India enjoys the benefits of varied climate, from alpine in the Himalaya to tropical wet in the south and arid in

Rajasthan. Such climatic conditions have given rise to rich and varied flora in the Indian subcontinent. In order to promote Indian herbal drugs, there is an urgent need to evaluate the therapeutic potentials of the drugs as per WHO guidelines[5].

The World Health Organization (WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. The traditional preparations comprise medicinal plants, minerals, organic matter, etc. Herbal drugs constitute only those traditional medicines, which primarily use medicinal plant preparations for therapy. The classical Indian texts include *Rigveda, Atherveda, Charak Samhita,* and *Sushruta Samhita.* The herbal medicines/traditional medicaments have, therefore, been derived from rich traditions of ancient civilizations and scientific heritage.

WHO estimates that 4 billion people, 80 percent of the world population, presently use herbal medicine for some aspect of primary health care. Herbal medicine is a major component in all indigenous peoples' traditional medicine and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental and Native American Indian medicine. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74 percent are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value[6].

1.2. History of herbal medicine[7-11]

Herbal medicine is considered to be the most ancient form of healing. It is the oldest and purest form of medicine known to mankind. Herbs have been used in most traditional cultures and have had an extraordinary influence on many systems of medicine. The first generally accepted use of plants as healing agents was depicted in the cave paintings discovered in the Lascaux caves in France, which have been radiocarbon-dated between13,000-25,000 BC.

No one knows, for sure, when humans began using herbs for medicinal purposes. The first written record of herbal medicine use showed up in 2800 B.C. in China by the

Chinese emperor Shen Nong. He wrote an authoritative treatise on herbs that is still in use today. Shen Nong recommended the use of Ma Huang (known as ephedra in the Western world) against respiratory distress. Since then the use of herbs has gained, and fallen out of, favor many times in the medical field. The timeline that follows shows some of the key dates and major points in the history of herbal medicine.

Prehistoric Era

The oldest herb in history may be ginkgo biloba. Fossils from the Paleozoic era tell us that the ginkgo biloba tree has been on earth for millions of years. Prehistoric tribes of hunter-gatherers would include in their diet any berries, leaves, roots, mushrooms, cacti, seeds, herbs or any parts of any plants that were found to be edible. Over many millennia, various herbs and plants were chewed, made into teas, pounded into pastes, made into oils, gargles and snuffs, and added to various foods and drinks. Over the eons of time, effects of herbs were learned by trial and error. Some plants, such as the hemlock tree, were found to be poison. Herbs such as valerian roots were found to make a person drowsy, while other herbs such as tea, contained caffeine and would help people to feel more awake. Somewhere along the way in history, white willow bark was boiled in water and made into a tea that somehow helped relieve aches and pains and lower fevers. In recent times it was discovered that this tree bark contained salicin, which was later synthesized into acetylsalicylic acid, better known as aspirin. Today aspirin has become the number one selling over the counter remedy around the world.

Ancient Babylon

An ancient Babylonian burial site that was discovered by archeologists contained various preserved medicinal herbs, including marshmallow root. These are thought to be some 60,000 years old and are the oldest intact examples of herbal remedies. In ancient cultures, people were often buried with treasures and items that would help them in the afterlife.

Egypt

Some of the oldest and most complete written records about the use of herbs as supplements were recorded thousands of years ago in Egyptian hieroglyphics. The most famous of these writings is known as the Papyrus Ebers, which note the medicinal uses of

over 700 herbs and plants. Many of the herbs recorded in this document back in 1500 BC are still in use today, aloe vera being one well-known example.

China

Chinese medicine is famous for its extensive use of herbs and plants. For over five thousand years, Chinese herbalists have used ginkgo biloba tree leaves, ginseng roots, Cordyceps mushrooms, teas and many other herbs and health tonics to support good health. China has the longest history of continuous use and learning about medicinal herbs. The first recorded Chinese herbal study, called Ben Cao, is believed to have been written around 2000 B.C. by Emperor Shen Nong. The Emperor studied and wrote about over 300 plants and herbal remedies.

India

In early times, herbs and spices were so valuable that their trade was one of the first forms of organized commerce. India played an important part in the herb and spice trade. In India, the study of medicinal herbs has been ongoing since around 2000 B.C. and is known as Ayurveda which means the "science of life". The herb Gotu kola has been used to help memory throughout the history of India. *Gymnema sylvestre* leaves have been used to help diabetics and dieters overcome sugar cravings.

Biblical Times

In the Bible we read about how Kings traveled from afar to bring frankincense and myrrh as gifts for Jesus. In Ezekiel, Jesus says "the fruit of the tree is for man's meat, and the leaves for man's medicine." In the book of Genesis, God declares "I have given you every herb bearing seed, which is upon the face of the earth, and every tree, in which is the fruit of a tree yielding seed; to you it shall be for meat".

Greece

Herbal medicine and the history of herbs in Greece would not be complete without discussing Hippocrates (460 BC - 377 BC), a Greek physician. He made a system of much that was known in his era and extended that knowledge. Hippocrates used many herbs in his treatment of illness because he believed that disease had natural causes contrary to many contemporaries who held that it was inflicted by Gods. In his day

Parsley was used to treat rheumatism and relieve kidney pain while Tarragon was used to treat toothaches. In the first century AD, the Greek physician Dioscorides made a thorough record of the medicinal uses of over 500 herbs and plants. This record, named De Materia Medica, informed and influenced herbalists for centuries afterward.

Rome

Herbal remedies were widely used in the Roman Empire, including crushed mint leaves, basil, oregano and mandrake herb. Other early uses of plants in Rome were for the poisoning of political opponents, and for antidotes to poisons. The Roman emperor Nero created a kind of cure-all potion which remained in use for over 2000 years and may have been the first "patent medicine." Much of the Roman knowledge was lost when libraries and schools were destroyed by warfare. Many years later, Italy would be home to the first standardized dosage of ingredients. A pharmacopoeia called the Nuovo Receptario was published around 1500 B.C and became a standard for pharmacists of the time.

The Middle Ages

After the fall of the Roman Empire, during the "dark ages," much of the learning and culture of European civilizations was lost. While barbarians ravaged the continent, monks and scribes in unconquered Ireland hand-copied books and written works. This history is told in the book titled "How The Irish Saved Civilization." Most monasteries also had gardens where Monks grew medicinal herbs. The liquor named Benedictine contains 27 herbs, plants & spices and was thought to be a health elixir. It is named after the religious Benedictine monks that invented it.

The Americas

In the mid 1800's, American Shakers grew and gathered over 200 kinds of medicinal herbs with religious zeal. They pressed the herbs into bricks and sold them to doctors and pharmacists. People all over the world trusted the Shaker label for honesty and quality. Native North Americans used black cohosh for women's symptoms of menstruation, and now modern scientists have found it to offer an estrogen-like effect upon hormones. In Peru, the bark of the cinchona tree proved useful against malaria and later became the source of quinine sulfate.

Important events in the history of herbal medicine

2800 BC : The first written record of herbal medicine use showed up. (Titled: The Pen Ts'ao by Shen Nung).

2600 BC : Babylonians recorded the uses for honey, poppy juice, essential oil of cypress and cedar, myrrh, licorice and other remedies which today are used every day by people all around the world.

1800 BC : The records of King Hammurabi of Babylon include instructions for using medicinal plants. Hammurabi prescribed the use of mint for digestive disorders. Modern research has confirmed that peppermint does indeed relieve nausea and vomiting by mildly anesthetizing the lining of the stomach

400 BC : The Greeks joined the herbal medicine game. Hippocrates stressed the ideas that diet, exercise and overall happiness formed the foundation of wellness.

50 AD : The Roman Empire spread herbal medicine around the Empire, and with it the commerce of cultivating herbs.

200 AD : The first classification system that paired common illnesses with their herbal remedy appeared. This was prepared by the herbal practitioner Galen.

800 AD : Monks took over the herbal field with herbal gardens at most monasteries and infirmaries for the sick and injured.

1100 AD : The Arab world became a center of medicinal influence. Physician Avicenna wrote the Canon of Medicine, which gave mention to herbal medicines.

1200 AD : Black Death spread across Europe and herbal medicines were used along side "modern" methods such as bleeding, purging, arsenic and mercury with equal, or better, results

1500 AD : Herbal medicine and herbalists were promoted and supported by Henry VII and the Parliament, due to the large number of untrained apothecaries giving substandard care.

1600 AD : Herbs were used in treating the poor, while extracts of plant, minerals, and animals (the "drugs"), were used for the rich. The English Physician, an herbal explaining the practice of herbal medicine, was written during this time

1700 AD : Herbal medicine got another high profile endorsement from Preacher Charles Wesley. He advocated for sensible eating, good hygiene and herbal treatments for healthy living

1800 AD : Pharmaceuticals began to hit the scene and herbal treatments took a back seat. As side effects from the drugs began to be documented, herbal remedies came into favor again

1900 AD : Lack of availability of drugs during World War I increased the use of herbal medicines again. After the war pharmaceutical production increased and penicillin was discovered.

2000 AD : EU legislation advocates all herbal medicines should be subject to compulsory clinical testing comparable to that undertaken for conventional drugs. Thus all herbal medicines would be licensed.

Herbal medicines have been documented for almost 4000 years. These medicines have survived real world testing and thousands of years of human testing. Some medicines have been discontinued due to their toxicity, while others have been modified or combined with additional herbs to offset side effects. Many herbs have undergone changes in their uses. Studies conducted on the herbs and their effects keep changing their potential uses. The healing power of plants has been acknowledged by many cultures for thousands of years, and aromatherapy can be said to stem from the various systems of traditional medicine developed by ancient civilizations. Primitive peoples used plants in both their healing traditions as well as in their religious rituals. Indian medicine is traditionally plant based. The most ancient of Indian religious writings contain prescriptions and formulae, as well as invocations and prayers, which address the healing plants themselves. The medicinal plants of India became famous throughout Asia, and many have now found their way into Western medical treatments and aromatherapy. India's age-old Ayurvedic medical system is increasingly popular in the West as more people become disillusioned with chemical preparations and turn instead to

traditional and holistic forms of healing. Herbs and plants have been used for centuries to improve health. Plants that have demonstrated beneficial effects include herbs, roots, cacti, mushrooms, trees, succulents, seeds, flowers and mosses. Herbal ingredients are used in nutritional supplements, Chinese medicine, cosmetics, perfumes, herbal remedies and in many health foods and beverages.

1.3. Types of herbal medicines[12-14]

Herbal Medicine can be broadly classified into various basic systems:

- *Traditional Chinese Herbal medicine*, which is part of Traditional Oriental Medicine.
- *Ayurvedic Herbal medicine*, which is derived from Ayurveda,
- *Western Herbal medicine*, which originally came from Greece and Rome to Europe and then spread to North and South America.

There are more sophisticated traditional herbal healing methods in Chinese medicine, Eclectic medicine, Cherokee Medicine, Unani Medicine and Ayurveda.

In Chinese medicine herbs (which may include animal and mineral parts) are divided into Superior (food grade), Moderate (to be taken for disease for a short time) and Inferior (toxic, short term) grades.

Cherokee medicine tends to divide herbs into foods, medicines and toxins and to use seven plants in the treatment of disease, which is defined with both spiritual and physiological aspects. Auyrvedic medicine has quite complex formulas with 30 or more ingredients, including a sizable number of ingredients that have undergone alchemical processing, chosen to balance Vata, Pitta or Kapha.

Western Herbal system is today primarily a system of folk medicine.

1.4. Use of herbal medicines[15]

Herbs releases volatile oils, antibiotics, aromatics, and other healing chemicals contained within them. Herbs can be prepared in a variety of forms depending on their purpose. Such techniques include:

- Juice squeezed from herbs.
- Mashing herbs into a paste.
- Decoction or extracting the active ingredients by boiling down the herb in water.
- Hot infusion (like hot tea)- Herb is steeped in hot water.
- Herbal wine made by adding the herb to water and sugar and letting it ferment.
- Tincture, made by combining ground herbs with alcohol, glycerin or vinegar and used internally.
- Liniment - Made like a tincture except it is used externally.
- Syrups - Made by adding herb to a medium such as honey, sugar or glycerin.
- Poultice - Herb is applied directly to a wound or body part and held in place with a cloth.
- Herbal Oil - Usually made with common base oil, such as olive, almond, grape seed, or sesame oils.

1.5. Styles of herbal medicine in practiced[16-19]

1.5.1. Western herbal medicine

Herbal Medicine is a general term for the use of plant material, minerals and possibly even animal substances administered individually or in combinations to improve ones health and heal disease. The practice of western herbal medicine is distinguished primarily by the use of herbs commonly found in the west. St. John's Wort, Black Cohosh, Chamomile and Feverfew are some examples of herbs commonly used in western herbal medicine. In general, western herbal practitioners treat your condition as it is defined by western medicine, using St. John's Wort to treat depression, for example. This is in contrast to many other systems of herbal medicine, which diagnose and treat a particular condition according to the theories unique to that medicine. The herbs administered within Western herbal medicine may be raw and cooked into a tea, taken in pill form, extracted into a tincture, or applied externally.

1.5.2. Traditional Chinese herbal medicine

Herbal medicine has been used in China for centuries and is backed by a long and rich history of development, use and research. Chinese herbal medicine is unique in that the diagnosis and treatments are based on the theories of traditional Chinese medicine. Besides discussing, the health issues with the patient, a practitioner of herbal medicine use other signs and symptoms such as those found in tongue and pulse to form a TCM (Traditional Chinese Medicine) diagnosis. The common cold, for example, may be diagnosed as "wind-cold invasion" and herbs, which dispel wind and warm cold, may be prescribed. Herbs administered within Chinese herbal medicine are usually raw and cooked into a tea, in a powder form and taken with hot water or in a pill form.

1.5.3. Kampo herbal medicine

Kampo herbal medicine is a system of using herbs based on the theories behind Traditional Chinese Medicine as they have been developed in Japan. As with Japanese acupuncture, the Japanese system of herbal medicine is a refined version of Chinese Herbal Medicine in that they have taken the time to look at a variety of theories and applications of Chinese medicine and determined what works best clinically. Kampo practitioners use well-defined formulas of herbs for conditions based on a person's Chinese medicine-based diagnosis. This is in contrast to a practitioner of Chinese herbal medicine, which may choose from a much wider range of herbs when developing a prescription. Kampo herbs are generally administered in a pill form.

1.5.4. Homeopathy

Homeopathy is a unique form of western medicine that is in use around the world. The theories behind homeopathy are based on the "law of similar" and a homeopathic practitioner uses theory unique to homeopathy to form a diagnosis and decide a course of treatment. Homeopathy attempts to stimulate the body to heal itself by using small amounts of medicines (from plants, minerals, animals or chemicals), which in large amounts might cause the condition for which you are being treated. The idea is loosely related to that behind vaccines. Homeopathic remedies are generally administered in a tiny pill form, which come in a variety of strengths.

1.5.5. Ayurvedic medicine

Ayurvedic medicine is a complete system of medicine from India, which has been used for thousands of years. As with Traditional Chinese Medicine, an Ayurvedic practitioner uses their own unique theories of diagnosis and treatment to heal disease and promote health and wellbeing. Many of the herbs used within Ayurvedic medicine, such as Neem and Arjuna, are commonly found in India and are relatively unique to this system, whereas more common herbs such as Ginger may be used which are also used in other herbal systems. The herbs may be incorporated into meals, prepared as teas or taken in a pill form.

1.6. Biological background of herbal medicine[20,21]

All plants produce chemical compounds as part of their normal metabolic activities. These can be split into primary metabolites, such as sugars and fats, found in all plants, and secondary metabolites found in a smaller range of plants, some only in a particular genus or species.

The autologous functions of secondary metabolites are varied. For example: as toxins to deter predation, or to attract insects for pollination. It is these secondary metabolites which can have therapeutic actions in humans and which can be refined to produce drugs. The word drug itself comes from the Swedish word "drug", which means 'dried plant'. Some examples are inulin from the roots of dahlias, quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove. The active ingredient in Willow bark, once prescribed by Hippocrates, is salacin, or salicylic acid alpha-hydroxybenzoic acid, led to the development of aspirin, acetyl-salicylic acid, originally a trade name, patented by Bayer. In 2004 the National Center for Complementary and Alternative Medicine began funding clinical trials into the effectiveness of herbal medicine. Surveys of a scientific approach to herbal medicine can be found in the books Evidence-based herbal medicine and Herbal and traditional medicine: molecular aspects of health.

1.7. Role of herbal medicine in human society[22]

People on all continents have used hundreds to thousands of indigenous plants for treatment of ailments since prehistoric times. There is evidence from the Shanidar Cave in Iraq that suggests Neanderthals living 60,000 years ago used medicinal plants. A body that was unearthed there had been buried with eight species of plants, which are still widely used in ethnomedicine around the world.

Anthropologists theorize that animals evolved a tendency to seek out bitter plant parts in response to illness. This behavior arose because bitterness is an indicator of secondary metabolites. The risk benefit ratio favored animals and protohumans that were inclined to experiment in times of sickness. Over time, and with insight, instinct, and trial-and-error, a base of knowledge would have been acquired within early tribal communities.

A survey released in May 2004 by the National Center for Complementary and Alternative Medicine focused on who used complementary and alternative medicines (CAM), what was used, and why it was used. The survey was limited to adults age 18 years and over during 2002 living in the United States. According to this survey, herbal therapy, or use of natural products other than vitamins and minerals, was the most commonly used CAM therapy (18.9%), when all use of prayer was excluded. Herbal remedies are most common in Europe. In Germany, the term apothecary (Apotheke) is still used, and next to prescription drugs one can order essential oils, herbal extracts, or herbal teas. It is even seen as a preferred treatment over the unnecessary overuse of industrialized production of chemical medication.

Herbs contain a vast spread of pharmacologically active ingredients and each herb has its own unique combination and properties. They are classified in modern herbal medicine according to their spheres of action. Many herbs contain ingredients, which provide the whole plant with several such actions, combined in the one medicine. Recognized actions include alterative, anodyne, anthelmintic, anticatarrhal, anti-emetic, anti-inflammatory, antilithic, antibacterial, antifungal, antispasmodic, aperient/laxative, aromatic, astringent, bitter, cardiac, carminative, cathartic/purgative, cholagogue and anticholagogue, demulcent, diaphoretic, diuretic, emetic, emollient, expectorant, febrifuge, galactagogue,

hepatic, hypnotic, rubefacient, sedative, sialogogue, soporific, stimulant, styptic, tonic, vesicant and vulnerary.

1.8. Scientific evidence and herbal medicine[20]

A substance that has a physiological effect on the body is by definition: a drug. Therefore those herbal remedies that have an effect, do so because of the drugs that they contain; and drugs can be beneficial or harmful. Although some very useful drugs are obtained from plant sources, it should be noted that some of the most deadly poisons are also obtained from plants: the alkaloid poisons for example. There is some scientific evidence to support some herbal remedies. The evidence is not strongly in favour of the remedies that do show up positive results, although it does show up the need for more quality research.

1.9. Various bioactive compounds from herbs[23-26]

The plant materials contain thousands of chemicals, which act against diseases, and infections of humans and animals when properly used. Plants contain different types of compounds such as resins, rubbers, gums, waxes, dyes, flavors, fragrances, Proteins, Amino acids, bioactive peptides, phytohormones, sugar, flavonoids and bio pesticides.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. That the medicinal actions of plants are unique to particular plant species or groups is consistent with this concept as the combinations of secondary products in a particular plant are often taxonomically distinct. This is in contrast to primary products, such as carbohydrates, lipids, proteins, chlorophyll, and nucleic acids, which are common to all plants and are involved in the primary metabolic processes of building and maintaining plant cells. India has an ancient heritage of traditional medicine. 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 and Unani.

In recent years, there has been a tremendous growth of interest in plant-based drugs, pharmaceuticals, perfumery products, cosmetics and aroma compounds used in food flavors and fragrances and natural colors in the world. There is a definite trend to adopt

plant based products due to the cumulative derogatory effects resulting from the use of antibiotic and synthetics and except for a few cultivated crops, the availability of plant based material is mainly from the natural sources like forests and wastelands. There is a need to introduce these crops into the cropping system of the county, which, besides meeting the demands of the industry, will also help to maintain the standards on quality, potency and chemical composition. India is sitting on a gold mine of well-recorded and well-practiced knowledge of traditional herbal medicine. But, unlike China, India has not been able to capitalize on this herbal wealth by promoting its use in the developed world despite their renewed interest in herbal medicines. This can be achieved by judicious product identification based on diseases found in the developed world for which no medicine or only palliative therapy is available; such herbal medicines will find speedy access into those countries.

Indians have believed from time immemorial that nature provides cures for all illnesses and the curatives are derived from plants. Over a million practitioners of the traditional system of medicines in India, use around 7500 species of plants in the preventive and curative applications, in the codified and oral streams. It is estimated that about 2 million people are engaged in activities like gathering medicinal plants (mostly from their natural habitats, processing them, manufacturing medicines out of them and marketing the same; which includes about 0.6 million licensed, registered medical practitioners of the codified systems of Indian medicine. A cumulative effect of these trends has been a quantum jump in volumes of plant materials extracted and traded within the country and exported outside. Conservative estimates place the economic value of medicinal plant related trade in India to be of the order of Rs.100 billion a year. Apprehensions are being expressed that trends are pointing towards an inexorable monetisation and commercialization of medicinal plants economy .The demand on medicinal plants, on the one hand, is increasing sharply and supplies on the other hand, are dwindling rapidly. Two serious consequences of the widening gap between demand and supply are:

(a) Suppliers taking advantage of gaps in understanding correct identity of plants and making available incorrect plants.

(b) Suppliers resorting to adulteration of medicinal plant parts by similar looking plant parts which are not known to be of medicinal value.

It is therefore essential to provide scientific background to establish correct identity of plants and to document diagnostic features to identify and sort out spurious plant parts.

1.10. Risks, benefits and effectiveness of herbal medicine[27-29]

A common misconception about herbalism and the use of "natural" products in general, is that "natural" equals safe. However many plants have chemical defense mechanisms against predators that can have adverse or lethal effects on humans. Examples are poison hemlock and nightshade, which can be deadly, although they are not sold as herbs. Herbs can also have undesirable side effects just as pharmaceutical products can. These problems are exacerbated by lack of control over dosage and purity. Furthermore, if given in conjunction with drugs, there is danger of 'summation', where the herb and the drug have similar actions and add together to make an 'overdose'. In animals, there are other dangers. There may be residues in food from farm animals (e.g. eggs, milk, and meat) or danger of 'doping' in competition animals. The latter may also apply to human athletes.

There is a danger that herbal remedies will be used in place of other medical treatments, which have been scientifically tested for safety and efficacy, resulting in the development or worsening of a medical condition, which could have been better, prevented or treated. There is also a danger that an herbal remedy may itself cause harm, which is unanticipated due to a lack of a full understanding of its composition and biochemical effects.

Herbalists tend to use parts of plants, such as the roots or leave but not isolate particular phytochemicals. They argue that the synergy of the combined substances enhances the efficacy and dilutes toxicity. Western medicine on the other hand prefers single ingredients on the grounds that dosage can be more easily quantified. Dosage is in general an outstanding issue for herbal treatments: while most conventional medicines are heavily tested to determine the most effective and safest dosages (especially in relation to things like body weight, drug interactions, etc.), there are few established dosage standards for various herbal treatments on the market. Furthermore, herbal medicines taken in whole form cannot generally guarantee a consistent dosage or drug quality (since certain samples may contain more or less of a given active ingredient).

1.11. Standards, quality control and medical interaction of herbal medicines[30]

The legal status of herbal ingredients varies by country. For example, Ayurvedic herbal products may contain levels of heavy metals that are considered unsafe in the U.S., but heavy metals are considered therapeutic in Ayurvedic medicine. Heavy metals may be processed in such a way as to inactivate negative aspects, which are not recognized in US or European statutes. In the United States, most herbal remedies are regulated as dietary supplements. Reports in the media have spread the idea that the herbal medicine industry is not well regulated. In fact, the Food and Drug Administration (FDA) regulates it very closely. The FDA reviews an herbal product's labels, manufacturing standards, and contents. It collects reports of adverse effects, issues warnings, and pulls products off the shelves if problems are reported. In addition, the National Nutritional Foods Association (the industry's largest trade association), has developed a program to examine the herbal products and factory conditions of its member companies and give them the right to display GMP (Good Manufacturing Practices) seals of approval on their products. This is a fairly comprehensive process, which resembles the certification process used to accredit hospitals. The program has been in wide operation since 2002.

In consultation with a physician, usage of herbal remedies should be clarified, as some herbal remedies have the potential to cause adverse drug interactions when used in combination with various prescription and over-the-counter pharmaceuticals. Dangerously low blood pressure may result from the combination of an herbal remedy that lowers blood pressure together with prescription medicine that has the same effect. In particular, many herbs should be avoided during pregnancy.

1.12. Importance and scope of herbs[31]

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs had been priced for their medicinal, flavoring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. Over three-quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant

species, at one time or other was used for medicinal purposes. It is estimated that world market for plant derived drugs may account for about Rs.2,00,000 cores. Presently, Indian contribution is less than Rs.2000 cores. Indian export of raw drugs has steadily grown at 26% to Rs.165 cores in 1994-95 from Rs.130 cores in 1991-92. The annual production of medicinal and aromatic plant's raw material is worth about Rs.200 cores. This is likely to touch US $1150 by the year 2000 and US $5 trillion by 2050. Thus, the economic importance of medicinal plants is much more to countries such as India than to rest of the world. In India, drugs of herbal origin have been used in traditional systems of medicines such as *Unani* and *Ayurveda* since ancient times. The *Ayurveda* system of medicine uses about 700 species, *Unani* 700, *Siddha* 600, *Amchi* 600 and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. Even the Allopathic system of medicine has adopted a number of plant-derived drugs, which form an important segment of the modern pharmacopoeia. Some important chemical intermediates needed for manufacturing the modern drugs are also obtained from plants (e.g. diosgenin, solasodine). Not only, that plant-derived drug offers a stable market world wide, but also plants continue to be an important source for new drugs.

Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capsaicin, allicin, curcumin, artemesinin and ephedrine among others. In some cases, the crude extract of medicinal plants may be used as medicaments. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, works in both mixture of traditional medicine and single active compounds are very important. Where the active molecule cannot be synthesized economically, the product must be obtained from the cultivation of plant material.

1.13. Action of herbs[32-34]

A great deal of pharmaceutical research has gone into analyzing the active ingredients of herbs to find out how and why they work. This effect is referred to as the herb's action. Herbal actions describe the ways in which the remedy affects human physiology. In some cases the action is due to a specific chemical present in the herb (as in the antiasthmatic effects of ma-huang) or it may be due to a complex synergistic interaction between various constituents of the plant (the sedative valerian is an example). A much older and far more relevant approach is to categorize herbs by looking at what kinds of problems can be treated with their help. Plants have a direct impact on physiological activity and by knowing what body process one wants to help or heal, the appropriate action can be selected. The qualities of herbs which make them beneficial in treating the human body include:

- *Adaptogenic*: Herbs increase resistance and resilience to stress, enabling the body to adapt around the problem and avoid reaching collapse.
- *Alterative*: Herbs that gradually restore proper functioning of the body, increasing health and vitality.
- *Anthelminitic*: Herbs that destroy or expel intestinal worms.
- *Anti-inflammatory*: Herbs that soothe inflammations or reduce the inflammatory response of the tissue directly.
- *Antimicrobial*: Antimicrobials help the body to destroy or resist pathogenic (disease-causing) microorganisms. Herbs help the body strengthen its own resistance to infective organisms and throw off illness. While some contain chemicals that are antiseptic or poisonous to certain organisms, in general they aid the body's natural immunity.
- *Antispasmodic*: Antispasmodics ease cramps in smooth and skeletal muscles. They alleviate muscular tension and can ease psychological tension as well.
- *Astringent*: Astringents have a binding action on mucous membranes, skin, and other tissue. They have the effect of reducing irritation and inflammation, and creating a barrier against infection that is helpful to wounds and burns.
- *Bitter*: Herbs with a bitter taste have a special role in preventative medicine. The taste triggers a sensory response in the central nervous system leading to a range

of responses, including, stimulating appetite and the flow of digestive juices; aiding the liver's detoxification work; increasing bile flow, and motivating gut self-repair mechanisms.

- *Carminative*: Plants that are rich in aromatic volatile oils stimulate the digestive system to work properly and with ease. They soothe the gut wall; reduce any inflammation that might be present; and ease griping pains and help with the removal of gas from the digestive tract.
- *Demulcent*: Demulcent herbs are rich in mucilage and soothe and protect irritated or inflamed tissue. They reduce irritation down the whole length of the bowel, reduce sensitivity to potentially corrosive gastric acids, help prevent diarrhea, and reduce the muscle spasms that cause colic.
- *Diuretic*: Diuretics increase the production and elimination of urine. They help the body to eliminate waste and support the whole process of inner cleansing.
- *Emmenagogue*: Emmenagogues stimulate menstrual flow and activity. With most herbs, however, the term is used in the wider sense for a remedy that affects the female reproductive system.
- *Expectorants*: Herbs that stimulate removal of mucous from the lungs Stimulating expectorants "irritate" the bronchioles (a subdivision of the bronchial tubes) causing expulsion of material. Relaxing expectorants soothe bronchial spasm and loosen mucous secretions, helping in dry, irritating coughs.
- *Hepatics*: Hepatics air the liver. They tone and strengthen the liver and in some cases increase the flow of bile. In a broad holistic approach to health they are of great importance because of the fundamental role of the liver in maintaining health by not only facilitating digestion but by removing toxins from the body.
- *Hypotensive*: Hypotensives are plant remedies that lower abnormally elevated blood pressure.
- *Laxative*: These are plants that promote bowel movement. They are divided into those that work by providing bulk, those that stimulate the production of bile in the liver and its release from the gallbladder, and those that directly trigger peristalsis (wavelike contractions of the smooth muscles of the digestive tract).

- *Nervine*: Nervines help the nervous system and can be subdivided into three groups. Nervine tonics strengthen and restore the nervous system. Nervine relaxants ease anxiety and tension by soothing both body and mind. Nervine stimulants directly stimulate nerve activity.
- *Stimulating*: Stimulants quicken and invigorate the physiological and metabolic activity of the body.
- *Tonic*: Tonics nurture and enliven. They are used frequently in Traditional Chinese Medicine and Ayurvedic medicine, often as a preventative measure. Tonic herbs like ginseng build vital energy.

1.14. Herbal medicine Today[35]

Herbal medicines are still in use today. In some respects they have gained a new momentum in the medical field. As many people seek alternative treatments and begin to check out Traditional and Eastern Medicine, herbs are becoming more popular. As physicians seek new treatments for many common illnesses they are beginning to revisit the traditional remedies, using herbal medicines.

Pharmaceutical medications, with their potential for harmful side effects and addiction, are becoming less popular. People are seeking alternatives to the modern medical interventions. Improving and maintaining health naturally is a very popular approach to overall wellness.

The herbs used today are generally cultivated for those purposes. Very few herbs are harvested from the wild, with the exception of a few still found in the rainforests and higher elevations. The cultivation of herbs for medicinal uses is a large field and more people are beginning to plant their own herb gardens. Many monasteries continue to grow large herbal gardens within their walls.

Modern herbal medicine takes a syncretic approach, trying to cross-reference the benefits of various herbs and treatments from different traditions, and find the best combination of herbal remedies. It is estimated that upwards of three quarters of the people on the planet use herbal medicine as part of their primary health care regimen.

Many traditional herbs are aromatics the compounds we use to treat illness are an effect of plants conducting chemical warfare on each other and to fend off herbivores. The same

compounds that make many herbs bitter or smell strongly are the ones used in herbal medicine and clinical trials. While there are countless claims that herbal remedies have "no side effect", this is not the case; it's more than the side effects cannot be attributed to one compound in the herbal repertoire, and sometimes this leads to dangers for example, cochineal was used to treat gout (rheumatoid arthritis, and a number of related symptoms), but also has severe side effects on the function of the kidneys and liver, as it has a slow, cumulative toxin.

Herbal medicines are now lining in shelves in most health food stores. As the current health care system becomes overwhelmed to give everyone access to the medical care that they may need, more and more people are turning to the ancient practice of using herbal medicines to self-treat. Despite claims that herbal medicines are absolutely safe and effective, because they come from natural sources, it is important to know that not all herbal medicines are safe and/or effective.

Herbal medicine, or phytotherapy, indicates the use of herbs for remedies. It therefore covers everything from medicinal plants with powerful actions, such as Digitalis and Belladonna, to those with very gentle action, such as chamomile, mint and many others. It should be noted that 'very gentle' action, when referring to chamomile or mint, does not mean they are more or less ineffective, but rather that one would not expect these plants to produce instant and powerful effects like those seen, for instance, after an injection of digitalis or strophanthin. Gentle action also means that these simple medicinal plants do not as a rule have any appreciable toxic effects, and may therefore be safely taken over an extended period of time.

1.15. Future view of herbal medicine[35]

The number of patients seeking alternate and herbal therapy is growing exponentially. Herbal medicines are the synthesis of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years. Herbal medicines are now in great demand in the developing world for primary health care not because they are inexpensive but also for better cultural acceptability, better compatibility with the human body and minimal side effects. Thousand years of traditional use can provide us with valuable guidelines to the selection, preparation and application of herbal

formulation. To be accepted as viable alternative to modern medicine, the same vigorous method of scientific and clinical validation must be applied to prove the safety and effectiveness of a therapeutical product.

1.16. Scenario of herbal medicine in Sikkim Himalaya region[36,37]

The use of plants as a means to cure/abate certain ailments and disease is an age-old practice throughout the world and the hills of Sikkim are not an exception. Sikkim is a small state of India that falls in the eastern Himalaya (27°3¢47² to 28°7¢34²N lat and 88°3¢40² to 88°57¢19²E long). The total area of the state is 7096 km^2, which forms just 0.02% of the total geographical area of the country.

It is criss-crossed by green valleys, high peaks, rippling rivers and is home to exotic species of flora and fauna. Covering 7,096 square kilometers, the State is 113 kilometers long and some 64 kilometers wide. Hills ranging from 300metres above sea level to 7,000metres result in a climate that varies from sub-tropical to alpine.

The unique geographical position and wide range of topography, high fertile soil, sufficient rainfall and presence of large number of perennial stream makes the state of Sikkim one of the treasure houses of bio-diversity in the country. Sikkim has vast reserve of medicinal plants and rich culture of folk medicine. The rich flora of Sikkim has a number of raw drugs described in Ayurvedic texts. There are about 420 plants used by the tribal people for various diseases in Sikkim Himalayas region, out of which few are in utilized on commercial basis. . It is necessary for the establishment of scientifically medical plant farm and bio-chemical laboratories for the identification of active ingredients / molecule from the plant species.

The Sikkim Himalayan people have a close relationship with nature. They use both psychosomatic treatments, which depends on propitiating the gods/spirits and the herbal and mineral medicine. The Himalayan Medicine System (HMS) is not at all systematized and depends upon an oral tradition. The persons, prescribing these medicines, use the traditional knowledge. Some of the Himalayan medicines were known widely and were even exported. For example, Kuth (*Saussurea costus*) was exported to east as is mentioned in *Atharvaveda*. It was also exported to China. Thus HMS is a vast treasure of herbal medicine, which needs to be exhaustively studied and used for the economic

regeneration of the local people, as also for the medical benefit of the world at large. We feel that the *Materia medica* of Ayurveda and even the Chinese medicine system may have borrowed heavily from the Himalayan Medicine System. There are effective medicines in Himalayan Medicine System even for incurable diseases.

Sikkim displays extraordinary biological diversity. There are more than 5,000 species of angiosperms, 4,000 species of flowering plants, 450/500 species of orchids and 300 species of ferns and allies.

1.17. Living status of local people and medicinal plants in Sikkim[38]

Sikkim had traditionally been a major supplier of medicinal herbs to markets in Kolkata and New Delhi. Species like Chirayata (*Swertia chirayata*), Jatamansi (*Nardostachys grandiflora*), Pipla (*Piper longum*), Kutki (*Picrorhiza kurrooa*) and Aconites (*Aconitum* spp.) have been the major produces. Local people have traditionally been collecting herbs from the wild for local as well as market consumption. If the medicinal plants farming is implemented systematically and a scientific approach are adopted, this will pave way for development of the state in a big way in the near future. Herbs are extracted by the local people for ethnic medicinal use, for use in Ayurveda and Tibetan medicines and also for use in modern bio-pharmaceuticals. The state can attain high value addition resulting in higher employment generation and increased income to the people, if propagation of these high value medicinal and aromatic plants is adopted. The preservation, cultivation and adoption of scientific approach in the field of medicinal plants of Sikkim are highly necessary.

In the present study two unexplored plants *Kaempferia rotunda* Linn. and *Eupatorium cannabinum* Linn. of Sikkim Himalayan region have been investigated for their medicinal values, since they are known by the indigenous people of Sikkim for therapeutic properties and are not systematically researched and documented. The aim of this study is to focus on the two species found predominantly, by means of isolation and identification of the plant constituents, be able to relate the therapeutic activity on the basis of literature precedents, to the compounds extracted.

1.18. References

1. Holt GA, Chandra A. Herbs in the modern healthcare environment: An overview of uses, legalities, and the role of the healthcare professional. *Clin. Res. Regulatory Affairs*. 2002, **19**, 83–107.
2. Gijtenbeek JMM, Vanden Bent MJ and Vecht CJ. Cyclosporine neurotoxicity. *J. Neurol.*, 1999, **246**, 339-346.
3. Johnson WC and William OW. Warfarin toxicity. *J. Vasc. Surg.*, 2002, **35**, 413-421.
4. Rajshekharan PE. Herbal medicine in World Science, *Employment News*, 21, Nov-2002, p 3.
5. WHO, General guidelines for Methodology on research and evaluation of traditional medicine, World Health Organization, Geneva, 2000.
6. WHO, in Progress Report by the Director General, Document No. A44/20, 22 March, World Health Organization, Geneva, 1991.
7. Capasso LL. 5300 years ago the Ice Man used natural laxatives and antibiotics. *Lancet*, 1998, **352**, 1864.
8. Ang-Lee MK, Moss J and Yuan CS. Herbal medicines and perioperative care. *J. Am. Med. Ass.*, 2001, **286**, 208-216.
9. Barrett B, Kiefer D and Rabago D. Assessing the risks and benefits of herbal medicine: An overview of scientific evidence. *Altern. Ther. Health Med.*, 1999, **5(4)**, 40-49.
10. Chevalier A. *Encyclopedia of Medicinal Plants*. DK publishing, Dorling Kindersley, London, 2001.
11. http://www.herbalmedicine holisticonline.com.
12. Farnsworth NR, Akerele O, Bingel AS, Soejarto DD and Guo Z. Medicinal plants in therapy. *WHO Bulletin*, 1985, **63(6)**, 965-981.
13. Winston D. *Cherokee Medicine and Ethnobotany*. In: Tierra M, Ed., American Herbalism, The Crossing Press, Freedom, CA, 1992.

14. Winston D and Dattner A. The American System of Medicine. *Clinics in Dermatology*, 1999, **17(1)**, 53-56.

15. Collins SB. Learning about herbs. *Beginnings*. 2007, **27(3)**, 20-21.

16. American Chemical Society. *Folk Medicine*, Washington DC, USA, 1986.

17. British Herbal Medicine Association, *British Herbal Compendium*, Vol. 1. Published by the Great Britain, 1992.

18. Chang HK. *The Pharmacology of Chinese Herbs*, Kentucky, CRC Press, 1993.

19. Chang HM and Pui-Hay B. *Pharmacology and applications of Chinese Materia Medica*, Vols. 1 & 2, Philadelphia, World Scientific Publishing Co. Ltd., 1986.

20. Jull A and Mhurchu CN. Review: Herbal remedies, where's the evidence? *J. Royal New Zealand Coll. Gen. Pract.*, 2004, **31(3)**, 193.

21. Beri RM. Phytosterol in some plant materials. *Indian Oil Soap J.*, 1970, **35**, 274.

22. Lietava J. Medicinal plants in a Middle Paleolithic grave. *J. Ethnopharmacol.*, 1992, **35(3)**, 263-66.

23. Shinwari MI and Khan MA. Indigenous use of medicinal trees and shrubs of Margalla Hills National Park, Islamabad. *J. Forest*, 1998, **48(4)**, 63-90.

24. Wink M and Schimmer O. Modes of action of defensive secondary metabolites. In: M Wink, Ed., Functions of Plant Secondary Metabolites and Their Exploitation in Biotechnology. CRC Press, Boca Raton, FL, 1999, p 17-112.

25. Kaufman PB, Cseke LJ, Warber S, Duke JA and Brielmann HL. Natural Products from Plants. In: Bioseparation of compounds, Kane H, ed. CRC Press, Boca Raton, FL, 1999, p 343.

26. Warner PK, Nambiar VPK and Ganapathy PM. Some Important Medicinal Plants of the Western Ghats, India: A Profile. Medicinal and Aromatic Plants Program in Asia (MAPPA), South Asia Regional Office (SARO), New Delhi, 2001, p 1-2.

27. Bensky D, Steven C, Erich S and Gamble A. Chinese Herbal Medicine: Materia Medica, 3rd edn., 2004, p 1054-1055.

28. Vanherweghem JL, Depierreux M, and Tielemans C. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet*, 1993, **341(8842)**, 387-91.

29. Vanhaelen M, Vanhaelen-Fastre R, But P and Vanherweghem JL. Identification of aristolochic acid in Chinese herbs. *Lancet*. 1994, **343(8890)**, 174.

30. http://www.oneearthherbs.squarespace.com/safety-regulation

31. Joy PP, Thomas J, Mathew S and Skaria BP. Medicinal Plants. Tropical Horticulture. eds. Bose TK, Kabir J, Das P and Joy PP, Naya Prokash, Calcutta, Vol. 2, 2001, p 449-632.

32. Goldman P. Herbal medicines today and the roots of modern pharmacology. *Ann. Intern. Med.*, 2001, **135(8)**, 594-600.

33. Ernst E. Harmless herbs: A review of the recent literature. *Am. J. Med.*, 1998, **104(2)**, 170-178.

34. Miller LG. Herbal medicinal: Selected clinical considerations focusing on known or potential drug-herb interactions. *Arch. Intern. Med.*, 1998, **158(20)**, 2200-2211.

35. Pal SK and Shukla Y. Herbal medicine: Current status and the future. *Asian Pac. J. Cancer Prev.*, 2003, **4(4)**, 281-288.

36. Bennet SSR. Ethnobotanical studies in Sikkim. *Ind. For.*, 1983, **109(7)**, 477-481.

37. Bennet SSR. Ethnobotanical studies in West Sikkim. *J. Econ. Tax. Bot.*, 1985, **7(2)**, 317-321.

38. Mudaiya RK, Tiwari RN and Majundar R. Threatened and rare medicinal plants of Sikkim, Bulletin on Medico ethno Botany Research , CCRAS, New Delhi, Vol-xv, 1987, p 24-26.

CHAPTER - 2

REVIEW OF LITERATURE

2.1. Kaempferia species

Kaempferia rotunda belongs to the family Zingiberaceae. Members of the Zingiberaceae are famous for their use as spices or as medicinal herbs; well-known examples include the rhizomes of *Zingiber officinale* (ginger) or *Curcuma longa* (turmeric)[1,2]. Rhizomes of *C. longa* or of *Kaempferia pandurata* are important in the folk medicine of South East Asia as antiseptics for wounds or as expectorants[3-7]. Rhizomes of several species from the Zingiberaeeae, however, also contain insecticidal constituents. Dried and powdered rhizomes of *C. longa,* for example, have been reported to act against storage-pest insects, such as *Tribolium castaneum*[8]. Recently, we could show that the sesquiterpene, xanthorrhizol, as well as other sesquiterpenes that are present in rhizomes of *Curcuma xanthorrhiza* or C. *zedoaria,* which are insecticidal towards larvae of the vigorous pest insect, *Spodoptera littoralis,* when applied topically via the larval integument[9]. The 50 species of the genus *Kaempferia* are nearly stemless herbs with thick, aromatic rhizomes. Most *Kaempferia* have silver to purple feather pattern in the middle of the upper side of the leaf radiating outwards with various green shades. Many, of the *Kaempferia* spp. produce small white, pink or orange flowers. *Kaempferia rotunda* has proven to be one of the *Kaempferia* for landscape planting[10].

2.1.1. *Kaempferia rotunda* Linn.

2.1.1.1. Synonyms of the plant

Hindi- Bhuichampa; Beng.- Bhuichampa; Mar- Bhuichampa; Oriya-Bhuinchampa; Tel.- Bhuchampakamu; Nep.- Bhuichampa; Lepcha– Ribrip; Eng- Black horm; Guj- Bhuichampo.

2.1.1.2. Distribution

The plant is found to be distributed throughout India in the hilly regions, at elevation ranging from 3000 to 5000 ft[11,12].

2.1.1.3. Description of different parts of plant

Kaempferia rotunda Linn. belonging to the family Zingiberaceae is an aromatic herb with tuberous root-stalk and very short stem. Leaves are simple, few, erect, oblong or ovate-lanceolate, acuminate, 30cm long, 10cm wide, variegated green above and tinged with

Fig.1.1. *Kaempferia rotunda* Linn.

Fig.1.2. Rhizomes with flower of *K. rotunda* Linn.

purple below. Flowers are fragrant, white, tip purple or lilac arranged in crowded spikes opening successively. The plant produces a subglobose tuberous rhizome from which many roots bearing small oblong or rounded tubers arise[13]. The description of the plant is also undertaken by Gamble, Kirtikar and Basu[14,15].

2.1.1.4. Pharmacological and phytochemical aspects of *K. rotunda* and allied species

The ethnopharmacological studies showed that the rhizomes of *K.rotunda* are widely used as a local application for tumours, swellings and wounds. The roots have a hot ginger-like taste. They are also given in gastric complaints[16]. They help to remove blood clots and other purulent matter in the body. The juice of the rhizomes is given in dropsical affections of hands, feet, and of effusions in joints. In Ayurveda, the improvement formulations using the herb are *Chyavanaprasam, Asokarishtam, Baladthatryaditailam, Kalyanakaghritham* etc. It also improves complexion and cures burning sensation, mental disorders and insomnia[17]. The rhizomes are useful in vitiated conditions of vata and kapha, gastropathy, dropsy, inflammations, wound, ulcers, blood clots, tumours and cancerous swelling[18,19]. The decoction is applied with much benefit to wounds with coagulated blood and with any purulent matter. The rhizomes contain crotepoxide and β-sitosterol[20]. Rastogi and Asolkar *et. al.*[21,22] also reported crotepoxide in rhizomes. Rhizome contains essential oil, which gives a compound with melting point 149°C, which yielded benzoic acid on hydrolysis.

Chang *et.al.* reported that the extracts of Chinese medicinal herb belongstoZingeberaceae family having an effect of anti inflammatory and antioxidant[23].

Habsah *et. al.* screened dichloromethane and methanol extracts of 13 Zingiberaceae species from the Alpinia, Costus and Zingiber genera for antimicrobial and antioxidant activities. The antimicrobial activity of most of the extracts was antibacterial with only the methanol extract of *Costus discolor* showing very potent antifungal activity against only *Aspergillus ochraceous* (MID, 15.6μg/disc). All the extracts showed strong antioxidant activity comparable with or higher that of alpha-tocopherol[24].

Somchit *et. al.* evaluated the anti- pyretic activities (25, 50 and 100mg/kg) by using Brewers yeast induced pyrexia in rats and analgesic activities (25, 50 and 100mg/kg) by using acetic acid-induced writhing in mice of aqueous and ethanol extracts of *Zingiber zerumbet* rhizomes. Both the extracts showed significant anti-pyretic activities in Brewers

yeast induced pyrexia in rats through out the observation period of 8h. The ethanol extracts of the rhizomes how ever significantly decreased the writhing movements in mice in acetic acid induced writhing test[25].

Suvara *et. al.* showed the rhizomes of *Kaempferia parviflora* (Zingiberaceae) have been used in Thai traditional medicine for health promotion and for the treatment of digestive disorders and gastric ulcer. The data demonstrated that KP has a great potential for a supplemental use in vascular endothelial health promotion[26].

Mohsen *et. al.* studied the effects of hydroalcoholic extract of ginger on an acute model of duodenal ulcer induced by cysteamine orally (350 & 700mg/kg) and intraperitoneally (350mg/kg). It has concluded that ginger hydroalcoholic extract was effective to protect against duodenal ulceration and for i.p. injection as well as chronic administration, the efficacy was comparable with ranitidine[27].

Mahmood *et. al.* studied the effects of methanolic extract of the rhizomes of *Zingiber officinale* in rats for their ability to inhibit gastric lesions induced by ethanol. Animals pre-treated with ginger root extract significantly inhibited gastric lesions compared to control rats .The root extract at a dose of 1000mg/kg orally exert highly significant cytoprotection against ethanol-induced gastric lesions compared to 500mg/kg.This cytoprotection was accompanied with increase in mucus synthesis by gastric mucosa grossly when compared with control rats .These observation strongly suggested the cytoprotective effect of the ginger extract against ethanol-induced gastric ulcer in rats[28].

Shanbhag *et. al.* studied the wound healing effect of alcoholic extract of *Kaempferia galanga* and its effect in dexamethasone suppressed wound healing in Wistar rats. Three wound models viz. incision, excision and dead space wounds were used in this study. The parameters studied were breaking strength in case of incision wounds, epithelialization and wound contraction in case of excision wound and granulation tissue dry weight, breaking strength and hydroxyproline content in case of dead space wound. The dexamethasone treated group showed a significant ($P<0.001$) reduction in the wound breaking strength when compared to control group in incision type of wound model. Coadministration of *K. galanga* with dexamethasone had significantly ($P<0.001$) increased the breaking strength of dexamethasone treated group.In excision wound model, the percentage of the wound contraction was significantly ($P<0.05$) increased by

K. galanga only on 16th day and also it reversed the dexamethasone suppressed wound contraction on the 16 day. *K. galanga* significantly ($P<0.001$) reduced the time required for epithelialization and reversed the epithelialization delaying effect of dexamethasone significantly ($P<0.001$)[29].

Indrayan *et. al.* compared 'Kasthuri' and 'Rajani' varieties of medicinal and ornamental plant *Kaempferia galanga* differ morphologically. The essential oils from their rhizomes have remarkably different specific gravities, refractive indices, saponification and iodine values and in their chemical compositions. A total no. of 58 and 56 compounds have been identified in 'Kasthuri' and 'Rajani', respectively. 13 compounds are identified in the rhizome oil of 'Kasthuri' that are not present in the rhizome oil of 'Rajani' and another 11 compounds identified in 'Rajani' oil are not present in 'Kasthuri' oil. 45 compounds have been found common in both oils but their percentages differ in the two varieties[30].

Sirirugsa *et. al.* reported tuber and rhizome of *Kaempferia galanga* are used as a remedy for toothache or a wash for dandruff or scabson the head. It is stimulant, stomachic, and carminative. The rhizome is externally used to treat abdominal pain, swelling and muscular rheumatism. The tuber of *Kaempferia rotunda* is used to treat abdominal illness, gastric complaints. The rhizome is used to treat stomachache and is also used for cosmetics. The leaves are used as body lotion[31].

Nicolo *et. al.* studied plants of 27 families including Zingiberaceae, encompassing 75 species, have been selected on the basis of medicinal folklore in a broad screening programme for their anti-inflammatory activity, using carrageen in foot oedema in rats. Only 4 species including Zingiberace were very active, inhibiting carrageen in foot oedema by 42 to 74%, but overall 72% exhibited some anti-inflammatory activity[32].

Decoction of *K. galanga* used in the inflammed parts to reduce swelling. The leaves are used in sore eyes, sore throat, swellings, rheumatism and fever[33]. The essential oils of *K. galanga* root and rhizome showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*[34].

2.2. Eupatorium species

Eupatorium cannabinum belongs to the family Compositae (Asteraceae) is one of the largest and most diverse families of flowering plants, comprising one-tenth of all known

angiosperm species. It is characterized by the compound inflorescence that has the appearance of a single "composite" flower from which it derives its name. The Compositae is divided into two major subfamilies and one minor subfamily with 12 to 18 tribes, 1,100 to 2,000 genera and 20,000 or more species[35-38]. The family is a rich source of powerful insecticides and industrial chemicals, e.g., pyrethrum (*Chrysanthemum*) and rubber (guayule). Several species are grown as medicinal and culinary herbs. *Echinacea* and others may be sources of biologically active compounds with medical or nutritional benefits. The Compositae also includes several detrimental weeds (dandelion, ragweed, and thistle). Lettuce and sunflower are the best genetically characterized members of this family[39].

The genus Eupatorium is made up of more than 500 species Worldwide. They are mostly herbaceous perennials, some shrubs and rarely annuals. Various species of Eupatorium are reputed to be of medicinal value in the United States and other parts of the World. *Eupatorium purpureum* used for its tonic, astrigents and diuretic properties. *E. agerrratoides* is also used as an antispasmodic, diurectic and diaphoretic. *E. aromaticum* and *E. incarnatum* which have gained much reputation in diseases connected with inflammation and irritability of the bladder. The leaves of *Eupatorium glutinosum* employed as styptics. *E. cannabinum* used as purgative and for other purposes. *E. ayapana* is used as an antidote to the bites of venomous reptiles. *E. nervosum* is very efficacious in cholera, typhoid fevers, small pox[40,41].

Fig.1.2. *Eupatorium cannabinum* Linn.

2.2.1. *Eupatorium cannabinum* Linn.

2.2.1.1. Synonyms of the Plant

Nepali – Banamara; Lepcha - Nam nong; English - Hemp agrimony.

2.2.1.2. Distribution

The plant is found to be distributed throughout India. It is abundant in temperate Himalayas at altitudes of 3, 000-11,000 ft.[42].

2.2.1.3. Description of dfferent parts of plant

The Hemp Agrimony, *Eupatorium cannabinum*, belongs to the great composite order of handsome, tall-growing perennial herb. The root-stock is woody and from it raises the erect round stems, growing from 2-5 feet high with short branches springing from the axils of the leaves, which are placed on it in pairs. The stems are reddish in colour, covered with downy hair and are woody below. They have a pleasant aromatic smell when cut. The root-leaves are on long stalks, but the stem-leaves have only very short root-stalks. They are divided to their base into three, more rarely five, lance-shaped toothed lobes, the middle lobe much larger than the others, the general form of the leaf being similar to that of the Hemp (hence both the English name and the Latin specific name, derived from *cannabis*, hemp). In small plants the leaves are sometimes undivided. They have a bitter taste, and their pungent smell is reminiscent of an umbelliferous rather than of a composite plant. All the leaves bear distinct, short hairs, and are sparingly sprinkled with small inconspicuous, resinous dots. Recently the plant has been found of use as an immune system stimulant, helping to maintain resistance to acute viral and other infections[43].

2.2.1.4. Pharmacological and phytochemical aspects of *E. cannabinum* and allied species

Traditionally the tribals of Sikkim Himalayan used juice of *Eupatorium cannabinum* leaves as an antiseptic and curing of wounds. *Eupatorium cannabinum* traditionally reported that it will stop the bleeding both externally and internally making it excellent for use against ulcers[44,45]. Leaves are kept with children suffering from smallpox probably to reduce virulence of infection. Chloroform extracts of leaves of plant exhibited *in vitro* antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*,

Staphylococcus aureus and *Aspergillius niger*[46]. The leaves contain a volatile oil (with α-terpinene, p-cymene, thymol and anazulene), which acts on the kidneys, and likewise some tannin and a bitter chemical principle which cut short the chill of intermittent fever. The Hemp agrimony contains flavonoids, pyrrolizidine alkaloids; polysachharides and p-cymene which is antiviral. It also contains eupatoriopicrin (sesquiterpene lactones) having anti cancer properties & inhibits cellular growth. The polysachharides stimulate the immune system[47].

It is used as cathartic, diuretic and anti-scorbutic and blood purifier. A homoeopathic tincture is prepared, given in frequent small well-diluted doses with water, for influenza, or for a similar feverish chill, and a tea made with boiling water poured on the dry leaves give prompt relief if taken hot at the onset of a bilious catarrh or of influenza[48].

Hemp agrimony has been employed chiefly as a detoxifying herb for fevers, colds, flu and other viral conditions. It also stimulates the removal of waste products via the kidneys. Due to its content of alkaloids; the plant should only be used under professional supervision[49]. The roots are diaphoretic, laxative and tonic. A homeopathic remedy is made from the leave is used in the treatment of influenza and feverish chills and also for disorders of the liver, spleen and gall bladder[50]. The leaves and flowering tops are, cholagogue, diuretic, emetic, expectorant, febrifuge, purgative and tonic[51-53]. The plant has a long history of use as a gentle laxative that does not provoke irritation though excessive doses cause purging and vomiting. Recent research has shown that the plant might have anti-tumour activity[54].

Rao *et. al.* reported the ethanolic extracts of *E. capillifolium* showed activity against *Bacillus subtilis* grown in a chemically defined medium but not in a complex natural medium[55].

Woerdenbag *et.al.* showed Eupatoriopicrin (EUP), a sesquiterpene lactone from *Eupatorium cannabinum* L. possesses cytostatic activity. This was demonstrated for FIO 26 cells *in vitro* with the aid of a clonogenic assay and *in vivo* by tumour growth delay in FIO 26 and Lewis lung tumour-bearing mice. *In vitro* the IC50 for 1 h exposure to EUP was 1.5 microgram/ml (4.1 nmol/ml)[56].

Phan *et. al. reported E. odoratum* showed significant inhibition of collagen gel contraction. Synergistic properties may contribute to wound-healing mechanisms[57].

Habtemariam *et.al* isolated. 5-acetyl-6-hydroxy-2,3-dihydro-cis-2-isopropenyl-3-tiglinoyloxy benzofuran from *E. purpureum*, which dose dependently inhibited inflammation in rat paw by carrageenan and is a potent inhibitor of some beta 1 and beta 2 integrin-mediated cell adhesions[58].

Urzua *et. al.* showed the antimicrobial testing of resinous exudate of *E. salvia* against five gram-negative and five gram-positive bacteria and found that the acetate of 7-hydroxy-8(17)-labden-15-oic acid to be the major active component[59].

Clavin *et. al.* reported that infusions of *E. laevigatum* produced a 46.6% reduction of stretches in acetic acid induced writhing test and showed no antinociceptive effects in hot plate test suggesting analgesic activity mechanism is unrelated to interaction with opioid systems[60].

Miraldi *et. al.* investigated the traditional ethnobotany and ethnomedicine of West Azerbaijan (Iran) including compositae family. In this region medicinal plants are often the only easily accessible health care alternative for most of the population in rural areas and in fact folk herbal medicine is the most used remedy to cure common diseases. They presented the most frequently used native species and the most common preparations made from them, in order to preserve the plant popular knowledge, which has traditionally been only an oral one[61].

Gupta *et. al.* reported petroleum ether and methanolic extracts of leaves of *Eupatorium ayapana* were tested for their antimicrobial activity. The petroleum ether extract showed higher antibacterial and antifungal activity than the methanolic extract[62].

Hesham *et. al.* isolated new diterpene glucoside 3,15-dihydroxy-*ent*-labd-7-en-17-oic acid 3-*O*-β-D-glucoside (**1**) and its aglycone (**2**) from *Eupatorium glutinosum*. The structures were determined by IR, one- and two-dimensional NMR, high-resolution MS, chemical transformations, and comparison of spectroscopic data with closely related diterpenes. Crude extracts showed antimicrobial and cytotoxic activities, but compounds 1 and 2 showed only antimicrobial activity. These results support the vernacular medicinal use of the plant as an antimicrobial[63].

Rios *et. al.* isolated two new benzofurane compounds, in addition to espeletone encecalinol beta-sitosterol and stigmasterol were isolated from *Eupatorium*

aschenbornianum which showed antimicrobial activities against *T. mentagrophytes* and *T. rubrum*[64].

Baccharis teindalensis (compositae) is a herbal plant which is widely used in folk medicine in Ecuador as an antiinflammatory, analgesic and antimicrobial remedy. Vidari *et. al.* evaluated the antidiarrhoeic and antiulcer activities of this extract in different mouse models. The ethanol extract of *B. teindalensis* showed antidiarrhoeic activity against the castor oil induced diarrhoea, at all doses tested. Furthermore, the ethanol extract induced a significant increase in myeloperoxidase activity as an index of the neutrophilic infiltration ($p<0.05$ vs control) and the higher dose of this extract (100 mg/kg) inhibited it in a remarkable way ($p<0.001$). These results confirm the gastrointestinal protection afforded by *B. teindalensis* and suggest that the antiulcer effect could be partially due to its antiinflammatory properties[65].

Calendula officinalis L. belongs to the Asteraceae family has long been used in topical applications, to treat skin ulcers, infected wounds, diaper rash, eczema, varicose veins, hemorrhoids, periodontitis and conjunctivitis. Reepithelizing and wound healing activityis one of the most extensively used actions of calendula. Creams containing calendula floral extract 5% in combination with alantoin, promoted remarkable epithelization in rat experimental models, with especial intensity on the metabolism of glycoproteins and collagen fibres during tissue regeneration. Alonso *et. al.* suggested that the water extracts of calendula flowers, applied on skin wounds, play a role as microvascularization inducing agents, thus contributing to speed up healing[66].

Albuquerque *et. al.* identified 12 compounds when the essential oils from leaves and roots of *Eupatorium betonicaeforme* were analyzed by GC-MS. The essential oil from roots and 2,2-dimethyl-6-vinylchroman-4-one (10.3-25.5%) can considered as natural larvicidal agents[67].

Lactuca sativa is a member of Compositae family. In folk medicine of Iran, the seeds of this plant were used for relieving of inflammation and osteodynia. Sayyah *et. al.* evaluated the anti-nociceptive and anti-inflammatory activities of crude methanol/petroleum ether (70/30, v/v) extract of the seeds. The extract exhibited a time- and dose-dependent analgesic effect in formalin test and also a dose-dependent anti-inflammatory activity in a carrageenan model of inflammation[68].

Suksamram *et. al.* isolated & identified flavanones, 2 chalcones & 2 flavones from flowers of *Eupatorium odoratum*. Isosakuranetin exhibited antimicobacterial activity, acacetin showed moderate cytotoxicity against NCI-H187 cells whereas luteolin exhibited moderate toxicity[69].

Maher *et. al.* proved that the ethyl acetate extract of the whole aerial parts of *Varthemia iphionoides* (Compositae) have a pronounced antibacterial activity. The compound sesquiterpene, selina-4,11(13)-dien-3-on-12-oic acid isolated from ethyl acetate extract exhibited potent antimicrobial activity against six bacterial species (*Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Bacillus cereus* and *Salmonella enteritides*). The minimum inhibitory concentrations (MICs) of this compound which was determined by the agar dilution method ranged between 250 and 500 μg/ml[70].

Chomnawang *et.al.* studied 13 medicinal plants, amongst them *Eupatorium odoratum*, *Garcinia mangostana*, and *Barleria lupulina* had strong inhibitory effects against growth of *Propionibacterium* a pus-forming bacteria triggering an inflammation in acne[71].

Dabaghi *et. al.* tested flavonoids of the leaves of *Eupatorium litoralle* for oxidative metabolism of isolated rat liver mitochondria. It revealed that hispidulin as an uncoupler of oxidative phosphorylation with distinct prooxidant and antioxidant properties when compared to eupafolin[72].

Muschietti *et. al.* presented methanol extracts from 11 Argentine medicinal plants. When the extracts assayed *in vitro* for antifungal activity against yeasts, hialohyphomycetes & dermatophytes, strongest effect was presented by *Eupatorium buniifolium* and *Terminalia triflora*[73].

Sasikumar *et. al.* studied antibacterial screening of petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of *Eupatorium glandulosum* leaves which exhibited a broad spectrum of inhibitory activity against Gram (+) and Gram (-) pathogenic bacteria[74].

Shen *et. al.* studied Eupaheliangolide A, 3-epi-heliangin and heliangin of *Eupatorium kiirunense* exhibited cytotoxicity against human oral epidermoid (KB), cervical epitheloid (Hela) and liver (hepa59T/VGH) carcinoma cells[75].

Dubey *et. al.* screened essential oils extracted from 17 higher plants belonging to different families against *Botryodiplodia theobromae* and *Colletotrichum gloeosporioides* causing stem end rot disease and anthracnose disease in mango respectively. The essential oil of *Eupatorium cannabinum* was found to be fungitoxic in nature against both the mango-rotting fungi. *Eupatorium* oil was standardized through physico-chemical and fungitoxic properties. The oil showed a broad fungitoxic spectrum and was recorded to be more efficient than some synthetic fungicides. The oil also showed an inhibitory effect on pectinase and cellulase enzymes. The LD_{50} of *Eupatorium* oil was found to be 22.01 ml/kg body weight on mammalian mice[76].

Judzentiene A isolated Germacrene D, Neryl acetate, neryl isobutyrate and β-Bisabolene from the essential oils of *Eupatorium cannabinum* L.[77].

Mullika *et. al.* investigated the activity of Thai medicinal plants on inflammation caused by *Propionibacterium acnes* in terms of free radical scavenging and cytokine reducing properties. *P. acnes* have been recognized as pus-forming bacteria triggering an inflammation in acne. Antioxidant activity was determined by DPPH scavenging and NBT reduction assay. The result showed that *Garcinia mangostana* possessed the most significant antioxidant activity and reduced reactive oxygen species production. *Eupatorium odoratum*, and *Senna alata* had a moderate antioxidant effect[78].

Clavin *et. al.* isolated and identified three anti-inflammatory compounds: nepetin, jaceosidin and hispidulin from *Eupatorium arnottianum* Griseb. dichloromethane extract. Nepetin reduced the TPA mouse ear edema by 46.9%; and jaceosidin by 23.2%. All these compounds are reported for the first time in this species. The finding of topical antiinflammatory activity exerted by *Eupatorium arnottianum* extract and the identification of active principles could support the use of this plant for the treatment of inflammatory affections[79].

Several species of the genus Tanacetum are traditionally used in a variety of health conditions including pain, inflammation, respiratory and gastrointestinal disorders. In the current investigation, Ishfaq *et. al.* evaluated the plant extract of *T. artemisioides* and some of its pure compounds (flavonoids) for analgesic, anti-inflammatory and calcium antagonist effects in various *in-vivo* and *in vitro* studies. Moreover the findings support

the traditional reputation of the genus Tanacetum for its therapeutic benefits in pain and inflammatory conditions[80].

Maria *et. al.* collected the information to cover the most recent developments in the ethnopharmacology, pharmacology and phytochemistry of this genus *Baccharis* genus (Compositae) which is an important source of natural medicinal products.. This review describes its traditional and folkloric uses, phyto-constituents and pharmacological of the prominent species of the genus *Baccharis*. Flavonoids and other phenolic compounds, diterpenoids and volatile constituents have been reported as the major phyto-constituents of the *Baccharis* species. Pharmacological studies are mainly based on the anti-inflammatory, antioxidant, antimicrobial, the treatment of wounds and ulcers, fever, gastrointestinal illnesses, as spasmolytics, diuretics and analgesics, and in the treatment of diabetes and bacterial/fungal infections[81].

2.3 Antioxidants from herbal sources

Reactive oxygen species (ROS), which consist of free radicals such as superoxide anion ($O_2^{\cdot}$) and hydroxyl ($HO^{\cdot}$) radicals and non-free radical species such as H_2O_2 and singled oxygen ($^1O_2$), are different forms of activated oxygen[82]. ROS are produced by all aerobic organisms and can easily react with most biological molecules including proteins, lipids, lipoproteins and DNA. *In vivo*, some of these ROS play positive roles in cell physiology; however, they may also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations leading to cancer, degenerative, and other diseases. Thus ample generation of ROS proceed to a variety of athophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity[83-87]. Free radicals due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress, cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins. Oxidation process is one of the most important routes for producing free radicals in food, drugs and even living systems. Catalase and hydroperoxidase enzymes convert hydrogen peroxide and hydroperoxides to non radical forms and function as natural antioxidants in human body. Due to depletion of immune system natural antioxidants in different maladies, consuming antioxidants as free radical

scavengers may be necessary[88]. Therefore, living organisms possess a number of protective mechanisms against the oxidative stress and toxic effects of ROS.

Antioxidants regulate various oxidative reactions naturally occurring in tissues and are evaluated as potential anti-aging agents. Hence, antioxidants can terminate or retard the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as electron donors. Antioxidants have been widely used as food additives to provide protection from oxidative degradation of foods and oils. Hence, antioxidants are used to protect food quality mainly by the prevention of oxidative deterioration of constituents of lipids. The most extensively used synthetic antioxidants are propylgallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ). However BHT and BHA have been suspected of being responsible for liver damage and carcinogenesis[89,90]. Natural antioxidants are able to protect from ROS as well as other free radicals and retard the progress of many chronic diseases and lipid oxidative rancidity in foods[91]. Polyphenols are widely distributed in plants and phenolic antioxidants have been found to act as free radical scavengers as well as metal chelators[92]. It has also been reported that some types of polyphenols such as catechin, epicatechin, epigallocatechin, catechin gallate, epicatechin gallate and epigallocatechin gallate are present in the seaweeds like *Halimeda* algae[93].

Naturally occurring antioxidants in leafy vegetables and seeds, such as ascorbic acid, vitamin E and phenolic compounds, possess the ability to reduce the oxidative damage associated with many diseases, including cancer, cardiovascular disease, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and aging[94]. Antioxidants are important in the prevention of human diseases. Antioxidant compounds may function as free radical scavengers, complexers of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation[95]. Antioxidants are often used in oils and fatty foods to retard their autoxidation[96].

Antioxidants are also important to the food industry. Manufacturers have strived to produce high quality food with superior texture, color, flavor and nutritional values in the shelf life period. However, many foods are subject to many factors that lead to the quality deterioration.

Among these undesirable factors, lipid autooxidation is one of the most concerned. The need of protecting food against oxidative degradation has prompted the wide usage of food additives.

Many studies have been shown that the presence of natural antioxidants from various aromatic and medicinal plants is closely related to the reduction of chronic diseases such as DNA damage, mutagenesis, and carcinogenesis[97]. Therefore, there has been a growing interest in research concerning alternative antioxidant active compounds, including plant extracts and essential oils that are relatively less damaging to the mammalian health and environment.

The antioxidative potential of methanol extract of *Ecklonia cava* was evaluated by using 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, ferrous ion chelating, reducing power and lipid peroxidation inhibition assays. The methanol extract showed significant ($p<0.05$) activities in all antioxidant assays and contained a high level of total phenolic conten[98].

Antioxidant activities of the extracts of *Teucrium* species were evaluated using three complementary *in vitro* assays: inhibition of DPPH radical, inhibition of hydroxyl radicals and protection of carotene-linoleic acid model system. It has seen that *Teucrium* species possess free radical and hydroxyl radical scavenging activity as well as antioxidant activity *in vitro*[99].

Potential antioxidative activities of enzymatic extracts from seven species of brown seaweeds were evaluated using four different reactive oxygen species (ROS) scavenging assays containing DPPH free radical, superoxide anion, hydroxyl radical and hydrogen peroxide scavenging assay. The enzymatic extracts exhibited more prominent effects in hydrogen peroxide scavenging activity compared to the other scavenging activities[100].

Total phenol contents and radical scavenging activity of the water extract of *Acanthopanax senticosus* extracts was determined for antioxidant activity. *A. senticosus* showed significant antioxidant activity and protective effect against oxidative DNA damage[101].

By using DPPH radical scavenging assay the antioxidant activity of crude methanol extracts from the leaves, flowers and pods of *Cassia alata* L has been investigated and it

has been found that the leaf extract exhibited a stronger antioxidant activity than the extracts from the flowers and pods[102].

The antioxidant potency of cultivated fruit-bodies of *Cordyceps militaris* L. was investigated by employing DPPH free radical scavenging, hydroxyl radical eliminating, iron chelating, inhibition of linoleic acid, lipid peroxidation and reducing power. The aqueous extract of *C. militaris* fruit-bodies shows a significant scavenging effect on DPPH, eliminating the capability on hydroxyl radicals and the chelating effect on ferrous iron and also shows positive results of inhibiting linoleic acid lipid peroxidation and reducing power[103].

The antioxidant effects, the levels of total phenol and contents of volatile oils and plant extracts *Rosmarinus officinalis* were determined in eight various Rosemary clones. Antioxidant acitvities and the total phenol contents were measured by spectophotometric method as well as the volatile oil content of the fresh plants with gas chromatograph it indicates that the antioxidant capacity of volatile oils and plant extracts closely related to the total phenol contents[104].

The methanolic crude extracts of 12 traditionally used Indian medicinal plants were screened for their antioxidant and free radical scavenging properties. The antioxidant activity of *Lawsonia inermis* was the strongest, followed in descending order by *Ocimum sanctum, Cichorium intybus, Piper cubeba, Punica granatum, Allium sativum, Delonix regia, Terminalia chebula, Terminalia ellerica, Mangifera indica, Camellia sinensis,* and *Trigonella foenum-graecum*[105].

The antioxidant properties of ethanol extract a *Ptychopetalum olacoides* was evaluated by using various *in vitro* systems. Extract acted as a scavenger of nitrogen oxides as well as superoxide generated by the xanthine-xanthine oxidase system. The extract also showed a high antioxidant capacity using a luminol chemiluminescence derived from a thermolabile diazocompound[108].

2.4. Wound healing agents from herbal source

Wounds occur as a result of physical injuries that break and expose the skin epidermal and dermal layers, causing damage to the blood vessels and leading eventually to bleeding. Wound healing is a complex sequence of events intiated by the stimulus of

injury to tissues. These events involve four main processes: coagulation, inflammation and debridement of wound, epithelia repairs and tissue remodeling and collagen deposition[107]. It is known that any agent that accelerates one or two of the above events is a promoter of wound healing[108]. Medicinal plant extracts, decoctions and concotions have been in use traditionally to treat various skin lesions (burns and wounds), and these plants have demonstrated wound healing properties in various animal models[109-111].

Wound healing consists of an orderly progression of events that establish the integrity of the damaged tissue. The process of wound healing is essential to prevent the invasion of damaged tissue by pathogens and to partially or completely reform the damaged tissue. The healing involves different phases, including inflammation, granulation, fibrogenesis, neo-vascularization, wound contraction and epithelialization[112]. The process of wound healing is promoted by several natural and plant products, which are composed of active principles like flavonoids, triterpenes, alkaloids, tannins and other biomolecules. These agents usually influence one or more phases of the healing process. The wound healing properties of *Aloe vera*[113], *Centella asiatica*[114], *Tridax procumbens*[115] and curcumin[116] have been reported and experimentally studied on various animal models.

Normal wound healing response begins the moment the tissue is injured. Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production. Later, the epithelial tissue is regenerated[117].

Ayurveda, the Indian traditional system of medicine, is based on empirical knowledge of the observations and the experience over millennia[118]. Healing of wounds is an important area of clinical medicine explained in most of the Ayurvedic texts since about 5000 BC under the heading "*Vranaropaka*." The wound as a medical problem was first discussed by Maharshi Agnibesha in *Agnibesha Samhita* (later known as Charaka *Samhita*) as Vrana[119] Maharshi Sushruta in *Sushruta Samhita*[120] elaborated on the subject.

Wound healing occupies an important field of research in modern biomedical sciences. The detailed pathophysiology of wounds is better understood following the establishment of the theory of a cell signal cascade system involved in the formation of new tissues repairing the wound. Modern biomedical scientists are now trying to develop suitable wound healing drug, corroborating the activity with cell signal triggering properties.

According to the Ayurveda, *Vrana* (wounds or ulcers) is the discontinuation of lining membrane that after healing leaves a scar for life[121] closely resembling the modern definition. Similarly, inflammation is considered to be an early phase in the pathogenesis of wounds termed *Vranashotha*. Different types of wounds as mentioned in Ayurveda may be endogenous in origin due to a defect in human functional units, such as *Vata* (nerve impulses), *Pitta* (enzymes and hormones), and *Kapha* (body fluids), or exogenous due to trauma, such as *Chinna* (cut wound), *Bhinna* (perforated wound), *Viddha* (punctured wound), *Kshata* (lacerated wound), *Picchita* (contusion), and *Ghrista* (abrasion wound[122]. These steps have striking similarities with wounds described in modern medicine[123,124]. In modern medicine, it is understood that there are certain essential polypeptides of the low concentration present in animal serum that control cell proliferation. These are called growth factors. Growth factors act by autocrine, paracrine and endocrine-signaling systems. There are several growth factors, which help in the healing of wounds in different ways. Platelet-derived growth factor is responsible for stimulation of connective tissue proliferation, epidermal growth factor mainly stimulates cutaneous tissue proliferation, and fibroblast growth factor stimulates many cell types with special references to fibroblast cells. Transforming growth factor, on the other hand, inhibits excess growth of some cell types[125]. However, a recent study reveals that some of these growth factors may have serious untoward effects such as carcinogenesis[126]. Classical management of wounds according to *Sushruta Samhita* follows 60 therapeutic steps, starting with an aseptic dressing of the affected part and ending with the rehabilitation of the normal structure and function[127]. These therapeutic measures were aimed not only to accelerate the healing process but also to maintain the quality and aesthetics of the healing. As described in different Ayurvedic classics like *Charaka Samhita* (ca. 5000 BC), *Sushruta Samhita* (ca. 1000 BC), *Astamga Hridaya* (ca. AD 600)[128] *Bhavaprakash Nighantu* (ca. AD 1500)[129], *Dhanwantari Nighantu* (ca. AD 1800)[130] and *Ayurveda Siksha* (AD 20th century)[131].

Table 1: Ayurvedic *Vranaropaka* (wound healing) drugs of plant origin[118-120, 128-131]

Sl. No	Botanical name	Family	Parts used for wound healing
01	*Abies webbiana* Linn.	Pinaceae	Leaves
02	*Acorus calamus* Linn.	Araceae	Rhizome
03	*Achyranthus aspera* Linn	Amaranthaceae	Whole plant
04	*Adiantum lunulatum* Burm.	Polypodiaceae	Leaves
05	*Albizzia lebbeck* Benth.	Leguminosae	Stem bark
06	*Alstonia scholaris*	Apocyanaceae	Leaves
07	*Andropogon muricantus* Retz.	Graminae	Root
08	*Anogeissus latifolia* Wall.	Combretaceae	Stem bark
09	*Boerhaavia diffusa* Linn.	Nyctaginaceae	Whole plant
10	*Balanites roxburghii* Planch.	Simaronbaceae	Stem bark
11	*Cedrus deodara* Roxb. Loud.	Anonaceae	Leaves
12	*Coleus vettiveroides* Benth.	Labiatae	Whole plant
13	*Crocus sativus* Linn.	Iridaceae	Flower
14	*Curcuma longa* Linn.	Zingiberaceae	Rhizome
15	*Curcuma zedoria* Rosc.	Zingiberaceae	Tuber
16	*Cynodon dactylon* Linn.	Gramineae	Whole plant
17	*Datura fastuosa* Linn.	Solanaceae	Leaves
18	*Desmodium gangeticum* D.C	Leguminosae	Whole plant
19	*Dolichos biflorus* Linn.	Leguminosae	Seed
20	*Eclipta alba* Hassk.2 Keshuta	Compositae	Root
21	*Emblica officinalis* Linn.	Euphorbiaceae	Fruit, leaves
22	*Euphorbia thymifolia* R.Br.	Euphorbiaceae	Whole plant
23	*Ficus racemosa* Linn.	Moraceae	Leaves

24	*Ficus bengalensis* Linn.	Moraceae	Stem bark
25	*Gymnema sylvestre* R.Br.	Asclepiadaceae	Leaves
26	*Heliotropium indicum* Linn.	Boraginaceae	Leaves
27	*Hordeum vulgare* Linn.	Gramineae	Grain
28	*Iris germanica* Linn.	Irideae	Stem
29	*Jasminum sambac* Ait.	Oleaceae	Leaves
30	*Lens culinaris* Linn.	Leguminosae	Leaves
31	*Mimosa pudica* Linn.	Mimosoidae	Whole plant
32	*Mertynia diandra* Glox.	Mertyneaceae	Fruit, flower
33	*Nerium indicum* Mill.	Apocyanaceae	Root
34	*Oledelandia biflora* Linn.	Rubiaceae	Whole plant
35	*Pisum sativum* Linn.	Verbenaceae	Leaves
36	*Psoralia corylifolia* Linn.	Leguminosae	Seeds
37	*Rannunculus scleratus* Linn.	Rannunculaceae	Whole plant
38	*Saraca indica* Linn.	Leguminoae	Stem bark
39	*Swertia chirata* Buch.Ham3 Chireta	Gentianaceae	Stems, leaves
40	*Terminalia chebula* Retz.	Combretaceae	Fruits
41	*Tinospora tomentosa* Colebr.	Menispermaceae	Stem
42	*Tricosanthes dioica* Roxb.	Cucurbitaceae	Leaves, stem bark
43	*Vitex negundo* Linn.	Verbenaceae	Leaves
44	*Vateria indica* Linn.	Dipterocarpaceae	Latex
45	*Wedelia calendulacea* Less.	Compositae	Leaves
46	*Woodfordia fruticosa* Kurz.	Lytheraceae	Flower
47	*Zingiber officinale* Rosc.	Zingiberaceae	Rhizome

Folk medicine practitioners in Peru employ plant preparations as wound-healing agents. The results of a scientific evaluation of the wound-healing activity of nine plants, *Peperomia galioides, Mentzelia cordifolia, Mutisia acuminata, Himatanthus sucuuba, Spondias mombin, Eleutherine bulbosa, Muehlenbeckia tamnifolia, Anredera diffusa and Jatropha curcas* were studied for wound healing activity on superficial and internal wounds (gastric ulcers). Significant wound-healing activity was detected in *Peperomia galioides, Anredera diffusa* and *Jatropha curcas*[132].

Aloe vera improves wound healing and inhibits inflammation. Since mannose-6-phosphate is the major sugar in the Aloe gel, Davis *et. al.* examined the possibility of its being an active growth substance and concluded that the mice receiving 300mg/kg of mannose-6-phosphate had improved wound healing.and anti-inflammatory activity[133].

Wound healing potential of different extracts of leaves of *Lawsonia alba* Lam. was evaluated by Mandawgade *et. al.* on the rat excision and incision wound models. The oral administration of ethanol extract of *Lawsonia alba* Lam. leaves exhibited significant healing response in both the wound models[134].

Chandanadi yamak was tested in male Wistar rats by two wound models. Incision wounds for tensile strength and excision wounds for wound contraction were employed along with histopathological evaluation. The application of the test formulation alone promoted wound contraction and reduced the time for wound closure showing healing potential comparable to marketed framycetin sulphate cream (1% W/W)[135].

A methanol extract of *L. lavandulaefolia* was examined for its wound healing activity both in the form of an ointment as well as an injection in two types of wound model in rats:

(i) the excision wound model
(ii) the incision wound model.

Both the injection and the ointment of the methanol extract of the plant material produced a significant response in both of the wound types tested. The results were also comparable to those of a standard drug, nitrofurazone, in terms of wound contracting ability, wound closure time, tensile strength and regeneration of tissues at the wound site[136].

2.5. Anti ulcer agents from herbal source

Gastric ulceration occurs due to imbalances between offensive and defensive factors of the gastric mucosa. The antiulcerogenic activity of many plant products is reported to an increase in mucosal defensive factors rather than decrease in the offensive factors[137].

A number of antiulcer drugs like gastric antisecretory drug H-2 receptor antagonists, antimuscarinic agents, proton pump inhibitors, mucosal protective agents–carbenoxolone sodium, sucralfate and prostaglandin analogues are available which are shown to have side effects and limitations[138]. There are several herbal ayurvedic preparations which have a protective effect against drug-induced gastric mucosal injury[139]. Constituents of ambrex are *Withania somnifera* roots (6%), *Orchis mascula* root (10%), *Cycas circinalis* male cone (25%), *Shorea robusta* resin (10%) and *Amber fossil* resin (15%). Individual components have been suggested to exhibit several properties like antistress' antidepressant, antioxidant, immunomodulatory[140], antitumor[141] (*Withania somnifera*), antihyperdipsia[142] (*Cycas circinalis*) antidiarrheal, antidysentery[143] (*Orchis mascula*), antibacterial[144], antiulcer[145] (*Shorea robusta*), antiseptic and antispasmodic activities[146].

Gastric ulceration is believed to be the result of constant confrontation in the stomach and upper small bowel between acid-pepsin aggression and mucosal defense. The regulation of mucosal microcirculation is intimately involved in the maintenance of gastric integrity and endogenous nitric oxide (NO) has been established to have a role in this regulation[147,148]. Reduced glutathione is also important for mucosal integrity since depletion of GSH from the gastric mucosa by electrophilic compounds induces macroscopic mucosal ulceration[149,150].

Peptic ulcer therapy has undergone many strides over the past few years and a number of drugs are now available for treatment. These drugs are broadly classified into two, those that decrease or counter acid pepsin secretion and those that afford cytoprotection by virtue of their effects on mucosal defensive factors. These drugs act by different mechanisms. Most of the commonly used drugs such as H2 blockers (ranitidine, famotidine *etc*), M-1 blockers (pirenzepine, telenzepine etc), proton pump inhibitors (omeprazole, lansaprazole *etc*), decrease secretion of acid while, drugs like sucralfate and carbenoxolone promote mucosal defenses. Of late the role of these drugs on the defensive factors is gaining importance. It is now assumed that these drugs ultimately balance the

aggressive factors (acid, pepsin, *H. pylori*, bile salts) and defensive factors (mucin secretion, cellular mucus, bicarbonate secretion, mucosal blood flow and cell turnover)[151]. Although these drugs have brought about remarkable changes in ulcer therapy, the efficacy of these drugs is still debatable. Reports on clinical evaluation of these drugs show that there are incidences of relapses and adverse effects and danger of drug interactions during ulcer therapy. Hence, the search for an ideal anti-ulcer drug continues and has also been extended to herbal drugs in search for new and novel molecules, which afford better protection and decrease the incidence of relapse.

Numerous plants and herbs are used to treat gastrointestinal disorders in traditional medicine. There has been renewed interest in identifying new antiulcer drugs from natural sources[152]. Before introduction of potent antiulcerogenic agents, i.e. H2 receptor antagonists, proton pump inhibitors, etc. plant remedies were widely employed for the treatment of various symptoms of peptic ulcer[153].

In Ayurveda, peptic ulcer mostly refers to *Amlapitta* or *Parinamasula. Amlapitta* is a disease of the gastrointestinal tract, especially of the stomach. It has not been described as an independent disease in major Ayurvedic texts, but has been mentioned in short in *Kashyapa* samhita. *Amlapitta* literally means, pitta leading to sour taste. Apart from the stress laid on food habits and personal hygiene, some herbal drugs have also been mentioned. Modern medicine has not adequately evaluated the usefulness of these drugs in ulcer therapy, although studies have been reported. Some active constituents have also been isolated from these potential anti- plantain banana (*Musa sapientum* var. paradisiaca), *Tamrabhasma* (an indigenous preparation of copper), ginger (*Zingiber officinale*) and satavari (*Asparagus racemosus*) and give an overview on other potential anti-ulcerogenic drugs[154].

Suba *et.al.* evaluated the gastric cytoprotective activity of the methanol extract of aerial parts of the plant *Barleria lupulina* Linn. in albino rats using various models of ulcers such as drug induced ulcers, restraint ulcers, duodenal ulcers and pylorus ligated ulcers. The study suggested that the methanol extract of aerial parts of *Barleria lupulina* Linn. showed protective effect against experimental gastric and duodenal ulcers[155].

Saha *et. al.* examined effects of the flavonoid rich fraction of the stem bark of *Manilkara hexandra* by indomethacin+pylorus ligated gastric ulcers in experimental animals. Oral

administration of the ethyl acetate extract inhibited the formation of gastric lesions induced by ethanol in a dose dependent manner[156].

Butanol fraction of *G. pentaphyllum was* evaluated for its anti-gastric ulcer activity using experimental models. Oral administration of the plant extract at 200 and 400 mg/kg body wt. significantly inhibited gastric ulcer formation induced by indomethacin. The findings indicate that the butanol fraction of *G. pentaphyllum* possesses gastroprotective potential related to the preservation of gastric mucus synthesis and secretion[157].

Anti-ulcer effect of *Salvia leriifolia* leaf extracts was studied in mice. Gastric mucosal lesions were induced by oral administration of HCl/ethanol to mice. The oral administration of aqueous and ethanolic extract significantly inhibited the development of ulcer. At repetitive doses, the oral administration of aqueous and maceration extracts before the nercotizing agents significantly inhibited the lesion. The results suggest that *S. leriifolia* leaf extracts have effective anti-ulcer activity in mice[158].

The antiulcer activity of a hydro-ethanolic extract prepared from the stems of *Kielmeyera coriacea* Mart. (Guttiferae) was evaluated in rats employing the ethanol-acid, acute stress and Indomethacin models to induce experimental gastric ulcers. Treatment with *K. coriacea* hydro-ethanolic extract provided significant antiulcer protection in the ethanol-acid and Indomethacin models, but not in the acute stress model. These results suggested that the *K. coriacea* hydro-ethanolic extract increased resistance to necrotizing agents, providing a direct, protective effect on the gastric mucosa[159].

The aqueous and ethanolic extracts of *Portulaca oleracea* were studied in mice for their ability to inhibit gastric lesions induced by HCl or absolute ethanol. In addition, their effects on gastric acid secretion were measured. Both extracts showed a dose-dependent reduction in severity of ulcers. The highest dose of extracts exerted similar activity to sucralfate. The oral and intraperitoneal administration of extracts reduced the gastric acidity in pylorus-ligated mice. These results suggest that *Portulaca oleracea* has gastroprotective action and validates its use in folk medicine for gastrointestinal diseases[160].

The anti-ulcerogenic potential of the methanol extract of *Ocimum suave* was investigated using four methods of gastric lesion induction in experimental Wistar rats; HCl/ethanol-induced gastric lesions, absolute ethanol-induced gastric lesions, indomethacin-

HCl/ethanol-induced gastric lesions and pylorus ligation-induced gastric lesions. Administration of the extract of *Ocimum suave* to the rats by oral route prevented the formation of acute gastric lesions induced using the four experimental techniques[161].

Evaluation of ethanolic extract of *Kaempferia parviflora* for its anti-gastric ulcer activity by experimental models has been observed. Oral administration of the extract at 30, 60 and 120 mg/kg significantly inhibited gastric ulcer formation induced by indomethacin, HCl/EtOH and water immersion restraint-stress in rats. In pylorus-ligated rats, pretreatment with the extract had no effect on gastric volume, pH and acidity output. The findings indicate that the ethanolic extract of *Kaempferia parviflora* possesses gastroprotective potential which is related partly to preservation of gastric mucus secretion and unrelated to the inhibition of gastric acid secretion[162].

The majority of *Ethiopian population* relies on traditional remedies and some of which may also have nutritional value. *Trigonella foenum-gracum* infusion and *Linum usitatissimum* water extract are used to manage peptic ulcer. Mequanente *et. al.* showed that both aqueous *T. foenum-gracum* and *L. usitatissimum* seed extracts reduced the ulcer index and ulcer number of ethanol induced lesions ($P<0.001$)[163].

A methanolic fraction from an extract of *Bryophyllum pinnatum* leaves was found to possess significant anti-ulcer activity in nine different experimental animal models. Premedication tests in rats revealed that the extract possessed significant protective action against the gastric lesions induced by aspirin, indomethacin, serotonin, reserpine, stress and ethanol. Significant protection with extract treatment was observed to occur for aspirin-induced ulcer in pylorus-ligated rats and for histamine-induced duodenal lesions in guinea pigs. Significant enhancement of the healing process was also found to occur in acetic acid-induced chronic gastric lesions in rats[164].

Ethanol extracts of *Terminaliapallida brandis* was evaluated for its anti-ulcer activity against drug-induced ulcers, histamine-induced ulcers in Swiss albino rats. The extracts at the doses of 250 and 500 mg/kg per os (p.o.) exhibited significant protection against ulcers produced by indomethacin, histamine and the effect was comparable to that of the reference drug famotidine orally[165].

2.6. Anti-inflammatory agents from herbal source

Inflammation is the defensive mechanism of tissue to any injury which may be caused by injection of chemical/physical agents but may lead to development of inflammatory bowl disease[166]. It involves pain, heat, redness, swelling and loss of function of effected parts. Various therapeutic approaches are available for reducing long term inflammatory response. These anti- inflammatory agents exert various effects that result in reduction in the number and activity of immune system cells. Several natural products are being used as good anti-inflammatory agents without the risk of side effects from the time immemorial.

Inflammation is generally considered as an essentially protective response to tissue injury caused by noxious physical, chemical or microbiological stimulus. It is a complex process involving various mediators, such as prostaglandins, leukotrienes and platelet activating factor[167]. The major macrophage derived inflammatory mediators such as proinflammatory cytokines, tumour necrosis factor-a (TNF- a) and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS), contribute to the development of inflammatory diseases[168], thus, inhibition of the excessive production of TNF-a and/or NO could be employed as criteria to evaluate potential anti-inflammatory compounds. The current management of inflammatory diseases is limited to the use of anti-inflammatory drugs whose chronic administration is associated with several adverse effects. Plant-derived products are slowly emerging as a viable alternative because they are cheap, abundantly available and relatively less toxic.

Medicinal plants with anti-inflammatory activity are considerably employed in the traditional treatment of several disorders of inflammation. The inflammatory response involves a complex array of enzyme activation mediator release; fluid extravasations, cell migration, tissue breakdown and repair[169], which are aimed at host defense and usually activated in most disease conditions. These different reactions in the inflammatory response cascade are therapeutic targets which anti-inflammatory agents including medicinal plants interfere with to suppress exacerbated inflammatory responses usually invoked in such disorders as rheumatoid arthritis, in infection or injury. Inhibition of the synthesis of pro-inflammatory prostaglandins is one of such therapeutic targets to which some of the potent anti-inflammatory agents of clinical relevance (e.g. NSAIDs) owe

their activity. Several anti-inflammatory medicinal plants have also demonstrated the ability to inhibit the synthesis[170-172].

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects[173,174]. The research for plants with alleged folkloric used as pain relievers, anti-inflammatory agents, should therefore be viewed as a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs[175].

The rhizome extract of *Zingiber officinale* was investigated for anti-inflammatory and analgesic properties in albino rats and Swiss mice respectively. The extract produced significantly ($P<0.05$) inhibition of the carrageenan induced rat paw edema and a reduction in the number of writhing induced by acetic acid in mice. It has shown that rhizome extract of *Z. officinale* possesses anti-inflammatory and analgesic agents[176].

Anti-inflammatory activity of the ethanolic extract of the leaves of *Morus indica* Linn. was studied in Wistar rats using the carrageenan induced left hind paw edema, carrageenan induced pleurisy model. The ethanolic extract inhibited carrageenan induced rat paw edema. It has indicated that the ethanolic extract produced significant ($p<0.05$) anti-inflammatory activity when compared with the standard and untreated control[177].

The hydroalcoholic extract of *Plumbago capensis* showed a maximum inhibitory action on carrageenan induced paw edema and inhibited the leukocyte migration in a dose dependent manner. The anti-inflammatory activity observed was compared to the standard non-steroidal anti-inflammatory drug indomethacin. *Plumbago capensis* has shown significant anti-inflammatory activity with potential constituents targeting different components of inflammatory process[178].

Anti-inflammatory activity of the ethanolic extract of the orange tubular calyx of *N. arbor-tristis* and petroleum ether extract of root bark of *O. echioides* was studied in albino rats of Wistar strain using the carrageenan induced paw edema model. The results indicated that all the extract produced significant ($p< 0.05$) anti-inflammatory activity when compared with the standard drug (diclofenac sodium) and untreated control[179].

The effect of alcoholic extracts of leaf from *Araucaria bidwillii* was evaluated in experimental models of pain and inflammation. The alcoholic extracts of *A. bidwillii* showed significant inhibition in carrageenan and serotonin induced hind paw oedema. It

has suggested that the anti-inflammatory and analgesic effect of the extracts as claimed in folklore medicine[180].

The anti-inflammatory and antinociceptive properties of total methanolic extracts of the flowering aerial parts of two *Stachys* species in rat were investigated by carrageenan-induced paw edema and formalin test. Methanolic extracts of *Stachys schtschegleevii* and *Stachys balansae* have analgesic and anti-inflammatory effects in formalin test and carrageenan-induced paw edema[181].

The methanol extract of *Alangium salvifolium* plant roots has been studied for analgesic and anti-inflammatory activities in animal models. The methanol extract produced significant dose-dependent inhibition of carrageenan induced rat paw edema and marked analgesic activity[182].

Teucrium stocksianum species was studied using carrageenan induced rat paw edema, cotton-pellet method, and by topical application of the extract on edema. *T. stocksianum* showed significant analgesic and anti-inflammatory activities in all the models studied. Topical application of the extract was also shown to be anti-inflammatory. Results support the traditional use of the plant in the treatment of painful, inflammatory conditions[183].

2.7. Analgesic and Anti pyretic agents from herbal source

Pain is an unpleasant sensory experience which is essential for survival considerably. It has two components i.e. sensory experience and emotional or psychological component. Pain sensitivity varies from person to person. Pain can be classified as superficial (cutaneous) or deep (visceral). The superficial pain is usually sharp pricking and has quick response of sudden onset, while deep pain is dull and lasting. Since pain is both sensory and emotional, drugs may act as painkillers by altering either of these two aspects. The peripheral pain reception at the nerve endings can be interrupted by salicylates, the neuronal conduction is suspectible to local anasthetics and both opoid and non opoids can interfere with central integration of sensory and emotional components of pain.

An analgesic may be defined as a drug bringing about insensibility to pain without loss of consciousness. They are broadly of two types: narcotics and non-narcotic. Non-narcotics, which has three important properties namely analgesic, antipyretic and anti-inflammtory. Among the non-narcotic analgesic salicylates and para-amino phenol derivatives are used

for analgesic and antipyretic purposes. Narcotics can modify pain perception at the CNS[184,185].

The management and treatment of pain is probably one of the most common and yet the most difficult aspects of medicinal practice. Analgesic therapy is currently dominated by two major classes of analgesic drugs; namely opioids and non steroidal anti-inflammatory drugs (NSAIDs). Both classes of analgesic drugs produce serious side effects, such as gastrointestinal disturbances, renal damages with NSAIDs, respiratory depression and possibly dependence with opioids[186,187]. It is obvious that the design of analgesic agents with fewer side effects is desirable. One of the ways to achive this aim is the use of medicinal plants which are a rich source of potentially effective novel compounds.

Medicinal herbs have been used as a form of therapy for the relief of pain throughout history. The treatment of rheumatic disorder is an area in which the practitioners of traditional medicine enjoy patronage and success. Natural products in general and medicinal plants in particular, are believed to be an important source of new chemical substances with potential therapeutic efficacy[188]. Taking into account the most important analgesic prototypes (e.g. salicylic acid and morphine) were originally derived from the plant sources, the study of plant species traditionally used as pain killers should still be seen as a fruitful research strategy in the search of new analgesic and anti-inflammatory drugs. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, though relatively little knowledge about their mode of action is available. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional systems of medicine[189].

Regulation of body temperature requires a delicate balance between the production and loss of heat and the hypothalamus regulate the set point at which body temperature is maintained[190]. In pyrexia (fever) the hypothalamic thermostat is disturbed and set for a high temperature[191]. Antipyretics are remedial agents that lower the elevated temperature of the body.They exert their action on the heat regulating centre in the hypothalamus[192]. Salicylates act centrally and reset this mechanism at the normal level and there by bring down the temperature; they donot show any demonstrable antipyretic activity in a normal individual.

Salicylates, paracetamol and other antipyretics act by inhibiting brain prostaglandin (PG) synthesis and release. They reduce heat production but increase dissipation of heat mainly by producing cutaneous vasodialation. Accompanying sweating assists the reduction of body temperature[193]. The screening of natural products has led to the discovery of somany potent antipyretic drugs.

Gupta *et. al.* investigated the methanol extract of *Caesalpinia bonducella* leaves for anti-inflammatory, analgesic and antipyretic activity by carrageenan induced edema, hot plate ,acetic acid induced writhing methods and Yeast-induced hyperpyrexia respectively. This study exhibited that the methanol extracts of leaves of *C. bonducella* possess anti-inflammatory, analgesic and antipyretic activities[194].

Amabeoku *et. al.* investigated analgesic and antipyretic activities of water extracts of *Dodonaea angustifolia* L. and *Salvia africana-lutea* L. by using acetic acid and hot plate induced writhing tests, and lipopolysaccharide (LP) induced pyrexia test in mice and rats. *D. angustifolia* and *S. africana-lutea* significantly inhibited acetic acid-induced writhing and also significantly delayed the time of reaction of mice to thermal stimulation produced by the hot plate. *D. angustifolia* and *S. africana-lutea* significantly reduced fever induced by LP[195].

Mutalik *et. al.* reported preliminary phytochemical screening of the dry residue of *S. melongena* which showed the presence of flavonoids, alkaloids, tannins and steroids produced significant analgesic and antipyretic effect in a dose dependent manner[196].

Oral administration of the aqueous extract of the stem of *Urtica macrorrhiza* reduced the number of writhings and stretchings induced by acetic acid and decreased licking activity of the late phase in formalin test and it suppressed Yeast-induced fever in rats at doses of 200 and 400 mg/kg[197].

2.8. Antimicrobial agents from herbal source

Antimicrobials are substances used in the treatment of infectious diseases that selectively suppress the infecting microorganism without significantly affecting the host. They affect microbial cells by interfering with one or more following process. Inhibition of cellwall synthesis leading to loss of viability and cell lysis, interfering with cell membrane function leading to leakage of intracellular compounds, inhibits protein synthesis, interfering with DNA-RNA synthesis, interfering with ignition complex and causing misleading of m-RNA, inhibition of viral DNA polymerase inhibiting the metabolism[198].

Antimicrobial agent include antibiotics which is defined as a chemical compound derived from or produced by living organisms which is capable, in small concentrations of inhibiting the growth of micro-organisms[199]. This definition limited antibiotics to substances produced by microorganisms but the definition could now be extended to include similar substances present in higher plants. Plants have many ways of generating antibacterial compounds to protect them against pathogens[200]. External plant surfaces are often protected by biopolymers e.g. waxes and fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, diterpenoids, steroid glycoalkaloids and other compounds, which inhibit the development of fungi and bacteria[201]. Cell walls of at least some monocotyledons also contain antimicrobial proteins, referred to as thionins[202].

The use of medicinal plants and their extracts for the cure of localized and specific human 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[203]. 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[204]. 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[205,206].

Herbal medicine is used to treat various infectious diseases, in most of the world's cultures, offering enormous prospects for discovering new drugs in popular medicine. Focusing attention on the plants medicinally used by indigenous people is the most efficient way to identify plants that may contain bioactive substances[207]. Considering the enormous variety of higher plant species, their potential as new drug sources has not been completely explored. Only 17% of this plant group has been systematically studied in the discovery of biologically active compounds[208]. Plants have been traditionally used for the treatment of diseases of different etiology. Plant extracts are used, for instance, as a source of medicinal agents to cure urinary tract infections, cervicitis, vaginitis,

gastrointestinal disorders and skin infections, such as herpes simplex virus[209]. Diseases caused by protozoa are responsible for considerable mortality in the tropical and subtropical countries. New drugs are now required for amoebiasis, leishmaniosis, malaria and trypanosomiasis treatment. The crisis of reemerging infectious diseases and the resistance of many pathogens for current drugs have been widely recognized as serious and of immediate concern. In addition, the compounds used in parasitic illness treatment, such as benznidazole, nifurtimox pentavalent antimonials, melarsoprol and pentamidine, are highly toxic, expensive and require long-term treatments[210-212]. The number of drugs available for human and animal trypanosomiases treatment is limited now a day. Effective drugs are urgently needed as therapeutical alternatives for antiprotozoa chemotherapy, and the higher plants are a potential source of new antiprotozoal drugs.

Even though pharmaceutical industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased. According to World Health Organization[213] medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficacy[214]. The use of both plant extracts and phytochemicals, with known antimicrobial properties, can be of great significance in therapeutic treatment. In the last few years, a no. of studies have been conducted in different countries to prove such efficiency[215-221].

Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds of plants have been investigated by a number of researchers world wide, especially in Latin America. In Argentina, a research tested 122 known plant species used for therapeutic treatments[222].

Ahmed *et. al.* studied the antibacterial activities of the chloroform and water extracts of *Ferula persica* Var. roots by the disc diffusion method.They reported that the chloroform extract of *F. persica* roots showed antibacterial activity; where as the water extract of the

roots at the concentrations that tested did not show any activity against *B. subtillis*, *B. cereus*, *E. coli*, *K. ponumoniae*, *S. typhi*, *S. aureus*, and *S. epidermilis*[223].

The water and ethanol extracts of the stem bark of two Nigerian medicinal plants, *Alstonia boonei* and *Morinda lucida*, were tested on clinical isolates of two Gram-positive and five Gram-negative bacteria. Ethanol extract of *A. boonei* was not active against any of the bacterial tested while the aqueous extracts of stem barks of *A. boonei, M. lucida and* ethanol extracts of stem bark of *M. lucida* showed antibacterial activity[224].

Bactericidal and antifungal assays were done using extracts derived from. *Zanthoxylum chalybeum* and *Warburgi ugandensis* by agar well diffusion, disc diffusion and colony count assays. *W. ugandensis* water extracts elicited antibacterial activity against both *Escherischia coli* and *Staphylococcus aureus* in the agar well assay. *Warburgia ugandensis* water extracts also showed antifungal activity against *Candida albicans*. However, *Z. chalybeum* extracts showed neither antifungal nor antibacterial activities[225].

Single or combined extracts of black thyme, fennel, sage, wild tea and wild mint were used to evaluate *in vitro* antibacterial activity against common pathogenic and lactic acid bacteria. The combined plant extracts (1:1 ratio) provided an entire antibacterial effect against pathogenic bacteria compared to the single plant extracts. The combined plant extracts with moderate inhibitory effects against both pathogenic and lactic acid bacteria could be sufficiently optimal when considering a natural feed additive to improve animal's gut health[226].

The acetone and alcoholic extracts of the leaves of *Cassia alata* showed significant *in vitro* antibacterial activity against *Staphylococcus aureus*, coagulase positive *Staphylococcus aureus, Bacillus 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 cholera*[227]. Alcoholic extract of dry nuts of *Semecarpus anacardium* 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, and green fruit) also possess antibacterial properties, especially the leaf extract. No dermatoxic effect (irritant property) was observed in the mouse skin irritant assay[228].

2.9. References

1. Ulbricht TLV. Evolution of Crop Plants. Simmonds NW, (Ed), Longman, London, 1976, p 339.
2. Purseglove JW. Tropical Crops, Monocotyledons. Riley ID, (Ed), Longman, London, 1972, p 719.
3. Rose MFS and Brain KR. An Introduction to Phytopharmacy, Pittman, Medical Tunbridge, Wells, UK, 1977, p 159.
4. Evans BK, James KC and Luscombe DK. Quantitative structure activity relationships and carminative activity. *J. Pharmacol Sci.*, 1978, **67**, 277.
5. Haginiwa J, Harada M, Morishita I. Properties of essential oil components of aromatics and their pharmacological effect on mouse intestine. *Pharmacological studies on crude drugs*. 1963, **83**, 624.
6. Bunyapraphatsara N. In: Economic and Medicinal Plant Research, Wagner H and Farnsworth NR, (Eds), Vol. 4, Academic Press, London, 1990, p 141.
7. Tuntiwachwuttikul P, Pancharoen O, Reutrakul V and Byrne LT. Panduratin A: A constituent of the red rhizomes of a variety of *Boesenbergia pandurata* Roxb. *Aust. J. Chem.*, 1984, **37**, 449.
8. Grainge M and Ahmed S .*Handbook of Plants with Pest-Control Properties*.Gunstone FD, (Eds), John Wiley, New York, 1988, p 175.
9. Pandji C, Grimm C, Wray V, Witte L and Proksch P. Insecticidal constituents from four species of the Zingiberaceae*Phytochemistry*.1993, **34**, 415.
10. Pilar MD and Kuehny JS. Ornamental Gingers as Flowering Potted Plants. *Lousiana Agriculture*. 2005, **21**, 20.
11. Gurung, B. *The medicinal plants of the Sikkim Himalaya*, 1st edn., Subash publication, Sikkim, 2002, p 224.
12. Nadkarni KM. *Indian Medicinal Plants and Drugs-with their Medicinal Properties and Uses*. Asiatic Publishing House, New Delhi, 1998, p 450.

13. Warrier PK, Nambiar VPK and Ramankutty C. *Indian Medicinal Plants*. vol. 1-5, Orient Longman Ltd., Madras, 1993-1998.

14. Gamble JS. *Flora of the Presidency of Madras*. Bishen Singh Mahendra Pal Singh, Dehra Dun, India. 3, 1987, p 1478-1490.

15. Kirtikar KR and Basu BD. *Indian Medicinal Plants*. International Book Distributors, 2nd edn., Dehra Dun, Vol 4, 1987, p 2444-2449.

16. Ambasta S.P. The useful plants of India. National Iinstitute of Science Communication and Information Resources (NISCAIR), New Delhi, 2006, p 307.

17. Sivarajan VV and Balachandran I. *Ayurvedic drugs and their Plant Sources*. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, 1994, p 570.

18. Chopra RN, Nayar SL and Chopra IC. *Glossary of Indian Medicinal Plants*. CSIR, New Delhi, 1980, p 7.

19. Nadkarni KM. *Indian Materia Medica*. Sangam Books Ltd., London, 1976, p 1319.

20. Husain A, Virmani OP, Popli SP, Misra LN, Gupta MM, Srivastava GN, Abraham Z and Singh AK. *Dict. Ind. Med. Plants*. CIMAP, Lucknow, India, 1992, p 546.

21. Rastogi RP and Mehrotra BN. *Compendium of Indian Medicinal Plants*. Central Drug Research Institute, Lucknow and Publications and Information Directorate, New Delhi. Vol. II, 1991, p 833.

22. Asolkar LV, Kakkar KK and Chakre OJ. *Second Supplement to Glossary of Indian Medicinal Plants with Active Principles*, Part I (A-K). Publications and Informations Directorate, CSIR, New Delhi. 1992, p 414.

23. Chang CP, Chang JY, Wang FY, Chang JG. The effect of Chinese medicinal herb Zingiberaceae rhizome extract on cytokine secretion by human peripheral blood mononuclear cells. *J. Ethnopharmacol.*, 1995, **48**, 13-19,

24. Habsah M, Amran M, Mackeen MM, Lajis NH, Kikuzaki H, Nakatani N, Rahman AA, Ghafar A. Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. *Ethnopharmacol.*, 2000, **72(3)**, 403-410.

25. Somchit MN, Sukhriya MHN, Bastamam AA, Zuraini A. Antipyretic and analgesic activities of *Zingiber zerumbet*. *International J. Pharmacology*, 2005, **1(3)**, 277-280.

26. Suvara K. Wattana P, Maneewan S, Linda C, Angkana H, Suphan C, Nuchattra C. *Kaempferia parviflora* ethanolic extract promoted nitric oxide production in human umbilical vein endothelial cells. *Journal of Ethnopharmacology*, 2007, **110(3)**, 559-562

27. Mohsen M, Alireza G, Alireza K. Anti-ulcerogenic effect of ginger (rhizome of *Zingiber officinale Roscoe*) on cystemine induced duodenal ulcer in rats. *DARU*, 2006, **14(3)**, 97-101.

28. Mahmood AA, Philip K, Salmha I. Anti ulceragenic effect of rhizomes of *Zingiber officinale* againist ethanol induced gastric ulcer in rats. *Journal of Animal and Veterinary Advances*, 2006, **5(2)**, 1222-1225.

29. Shanbhag TV, Sharma C, Adiga S, Laxminarayana K, Smita S, Ganesh S. Wound healing activity of alcoholic extract of *Kaempferia galanga in* Wistar Rats. Indian J. Physiol. Pharmacol., 2006, **50(4)**, 384–390

30. Indrayan AK, Kurian A, Tyagi PK, Shatru A, Rathi AK. Comparative chemical study of two varieties of attractive medicinal plant *Kaempferia galanga* Linn. *Natural Product Radiance*, 2007, **6**, 327-333.

31. Sirirugsa P. Taxonomy of the genus *Kaempferia (Zingiberaceae)* in Thailand. *Thai For. Bull.*, 1992, **(19)**, 1-15.

32. Nicolo M, Giuseppina A, Francesco C, Alessandro M, Maria P. Biological screening of Italian medicinal plants for anti-inflammatory activity. *Phytotherapy Research*, 2006, **1(1)**, 28-31.

33. Sudhir K. The medicinal plants of North East, Scientific publishers, Jodhpur, 2002, p 118.

34. Arambewela LSR, Perera A, Wijesundera RLC. Antibacterial activity of *Kaempferia galanga*. *Fitoterapia*, 1999, **70(4)**, 425-427.

35. Rastogi RP and Mehrotra BN. *Compendium of Indian Medicinal Plants*. Central Drug Research Institute, Lucknow and Publications and Information Directorate, New Delhi. Vol 1, 1990, p 233.

36. Stebbins GL. A new classification of the tribe Cichorieae, family Compositae. *Madrono*, 1953, **12**, 65-81.

37. Cronquist A. The compositae revisited. *Brittonia*, 1977, **29**, 137-153.

38. Jansen RK, Michaels HJ, Palmer JD. Phylogeny and character evolution in the Asteraceae based on chloroplast DNA restriction site mapping. *Syst. Bot*, 1991, **16**, 98-115.

39. Harbourne JB and Turner BL. An overview to the Compositae. In: Heywood VH, Harbourne JB, Turner BL, (Eds), The Biology and Chemistry of the Compositae, Academic Press, 1977, p 1-20.

40. Triska D. *Hamlyn Encyclopaedia of Plants*, 1975, Hamlyn ISBN 600-33545.

41. King RM and Robinson H. A Eupatorium, a composite genus of Arcto-Tertiary distribution. *Taxon*, 1970, **19**, 769-774.

42. Kirtikar KR and Basu BD. Indian medicinal plants. *International Book Distributors, Dehra Dun*. 2nd edn., Vol-2, 1988, p 1333.

43. Clapham AR, Tootin TG and Warburg EF. *Flora of the British Isles*. Cambridge University Press, 1962, p 688.

44. Gurung B. The medicinal plants of the Sikkim Himalaya, 1st edn., Subash Publication, 2002, p 171.

45. Rai LK, Sharma E. Medicinal plants of Sikkim Himalaya status, uses and potential. Bishen Singh Mahendra Pal Singh, Dehra Dun, India, 1994, p 39.

46. Iwu MM, Chiori C. Antimicrobial activity of *Eupatorium odoratum* extracts. *Fitoterapia*, 1986, **55(6)**, 354-356.

47. Huxley A. *The New RHS Dictionary of Gardening*. MacMillan Press, 1992, ISBN 333-474944-474945.

48. Chiej R. *Encyclopaedia of Medicinal Plants*. MacDonald, 1984, ISBN 356, 10541.

49. Chevallier A. *The Encyclopedia of Medicinal Plants* Dorling Kindersley. London, 1996, ISBN 9-303148.

50. Launert E. *Edible and Medicinal Plants*, 1981, Hamlyn ISBN 600-37216.

51. Lust.J.*The HerbBook* .Bantam Books, 1983, ISBN 553-23827.

52. Uphof JC. *The Dictionary of Economic Plants.* Engelmann HR, (Ed), Hafner Publishing Co., Weinheim, New York, 1959, p 400.

53. Bown D. *Encyclopaedia of Herbs and their Uses.* Dorling Kindersley, London, 1995, ISBN 751-32031.

54. Grieve M and Leyel, CF. *A Modern Herbal.* Tiger Books International, London, 1992, p169-172.

55. Rao KV, Alvarez FM. Antibiotic principle of *Eupatorium capillifolium. J. Nat. Prod.*, 1981, **44(3)**, 252-256.

56. Woerdenbag HJ, Lemstra W, Malingre TM, Konings AW. Enhanced cytostatic activity of the sesquiterpene lactone eupatoriopicrin byglutathione depletion. *Br. J. Cancer*, 1989, **59(1)**, 68-75.

57. Phan TT, Hughes MA, Cherry GW, Le TT, Pham HM. An aqueous extract of the leaves of *Chromolaena odorata* (formerly *Eupatorium odoratum*) (Eupolin) inhibits hydrated collagen lattice contraction by normal human dermal fibroblasts. *J. Altern. Complement. Med.*, 1996, **2(3)**, 335-343.

58. Habtemariam S. Cistifolin, an integrin-dependent cell adhesion blocker from the anti-rheumatic herbal drug, gravel root (rhizome of *Eupatorium purpureum*). *Planta Medica*, 1998, **64(8)**, 683-685.

59. Urzua A, Caroli M, Vasquez L, Mendoza L, Wilkens M, Tojo E. Antimicrobial study of the resinous exudate and of diterpenoids isolated from *Eupatorium salvia. J. Ethnopharmacol.*, 1998, **62(3)**, 251-254.

60. Clavin ML, Gorzalczany S, Mino J, Kadarian C, Martino V, Ferraro G, Acevedo C. Antinociceptive effect of some Argentine medicinal species of Eupatorium. *Phytother. Res.*, 2000, **14(4)**, 275-277.

61. Miraldi E, Ferri S, Mostaghimi V. Botanical drugs and preparations in the traditional medicine of West Azerbaijan (Iran). *Journal of Ethnopharmacology*, 2001, **75(2)**, 77-87.

62. Gupta M, Mazumder UK, Chaudhuri I, Chaudhuri RK, Bose P, Bhattacharya S, Manikandan L, Patra S. Antimicrobial activity of *Eupatorium ayapana*. *Fitoterapia*, 2002, **73(2)**, 168-170.

63. Hesham R, Noriko SK, Torssell BG, Shigeru N. New Labdene Diterpenes from *Eupatorium glutinosum*. *J. Nat. Prod.*, 2002, **65(5)**, 728–729.

64. Rios MY, Aguilar GAB, Navarro V. Two new benzofuranes from *Eupatorium aschenbornianum* and their antimicrobial activity. *Planta Medica*, 2003, **69(10)**, 967-970.

65. Vidari G, Vita FP, Zarzueloa GJ, Zafra C. Chiriboga X, Berenguer B, La CC, Alarcon DL, Lastra C. Motilva V, Martin MJ. Antiulcer and antidiarrhoeic effect of *Baccharis teindalensis*. *Pharmaceutical Biology*, 2003, **41(6)**, 405-411.

66. Alonso J. Tratado de Fitofarmacosy Nutraceuticos. *Barcelona Corpus*, 2004, p: 251-55.

67. Albuquerque MR, Silveira ER, De A Uchoa DE, Lemos TL, Souza EB, Santiago GM, Pessoa OD. Chemical composition and larvicidal activity of the essential oils from *Eupatorium betonicaeforme*. *J. Agric. Food Chem.*, 2004, **52(22)**, 6708-6711.

68. Sayyah M, Hadidi N, Kamalinejad M. Analgesic and anti-inflammatory activity of *Lactuca sativa* seed extract in rats. *J. Ethnopharmacol.*, 2004, **92(2)** 325-329.

69. Suksamram A, Chotipong A, Suavansri T, Boongird S, Timsuksai P, Vimuttipong S, Chuaynugul A. Antimicobacterial activity and cytotoxicity of flavonoids from the flowers of *Chromolaena odorata*. *Arch. Pharm. Res.*, 2004, **27(5)**, 507-511.

70. Maher M, Dabbas AL, Hashinaga F, Abdelgaleil SAM, Toshihiko S, Akiyama K, Hideo H. Antibacterial activity of an eudesmane sesquiterpene isolated from *Varthemia iphionoides*. *J. Ethnopharmacology*, 2005, **97(2)**, 237-240.

71. Chomnawang MT, Surassmo S, Nukoolkarn VS, Gritsanapan W. Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria. *J. Ethnopharmacol.*, 2005, **3**, 330-333.

72. Dabaghi BP, Mariante RA, Franco CLA, Heleno OB, Benigna MOM, Gunilla SCE, Cadena SM, Eliane MRM. Hispidulin: Antioxidant properties and effect on mitochondrial energy metabolism. *Free Radic. Res.*, 2005, **(12)**, 1305-1315.

73. Muschietti L, Derita M, Sülsen V, Munoz J, Ferraro G, Zacchino S, Martino V. *In vitro* antifungal assay of traditional Argentine medicinal plants. *J. Ethnopharmacol.*, 2005, **102(2)**, 233-238.

74. Sasikumar JM, Doss AP, Doss A. Antibacterial activity of *Eupatorium glandulosum* leaves. *Fitoterapia.* 2005, **76(2)**, 240-243.

75. Shen YC, Lo KL, Kuo YH, Khalil AT. Cytotoxic sesquiterpene lactones from *Eupatorium kiirunense*, a coastal plant of Taiwan. *J. Nat. Prod.*, 2005, 68(5), 745-750.

76. Dubey R, Rajesh K, Dubey N. Evaluation of *Eupatorium cannabinum* Linn. oil in enhancement of shelf life of mango fruits from fungal rotting. *World J. Microbiol. Biotechnol.*, 2007, **23(4)**, 467-477.

77. Judzentiene A. Chemical composition of leaf and inflorescence essential oils of *Eupatorium cannabinum* L. from eastern Lithuania. *The Journal of Essential Oil Research,* 2007, **19(5)**, 403-406.

78. Mullika T. Chomnawang MT, Surassmo S, Nukoolkarn VS, Gritsanapan W. Effect *of Garcinia mangostana* on inflammation caused by *Propionibacterium acnes. Fitoterapia*, 2007, **78(16)**, 401-408.

79. Clavin M, Gorzalczany S, Macho A, Muñoz E, Ferraro G, Acevedo C, Martino V. Anti-inflammatory activity of flavonoids from *Eupatorium arnottianum. Journal of Ethnopharmacology*, 2007, **112(3)**, 585-589.

80. Ishfaq AB, Rafeeq AK, Anwar-ul HG, Abdul JS, Javid H, Viqar UA. The Analgesic, anti-inflammatory and Calcium Antagonist Potential of *Tanacetum artemisioides. Arch. Pharm. Res.*, 2007, **30(3)**, 303-312.

81. Maria JA, Paulina B. *Baccharis* (Compositae): A review update. *ARKIVOC*, 2007, **7**, 76-96.

82. Halliwell B and Gutteridge JM. *Free Radicals in Biology and Medicine*. Oxford University Press, Kourounakis, Oxford, 1999.

83. Cerutti AA.Oxidant stress and carcinogenesis. *Eur. J. Clin. Inves.*, 1991, **21**, 1-11.

84. Harman D. Free radical theory of aging, increase the functional life span. *Annals of the New York Academy of Sciences*, 1994, **717**, 1-15.

85. Ames B. Micronutrients prevent cancer and delay aging. *Toxicol. Letters*, 1998, **102**, 5-18.

86. Finkel T and Holbrook NJ. Oxidant, oxidative stress and biology of ageing, *Nature*, 2000, **408**, 239-247.

87. Galanakis D and Tsiakitzis K. Synthesis and pharmacological evaluation of novel derivatives of anti-inflammatory drugs with increased antioxidant and anti-inflammatory activities. *Drug Development Research*, 1999, **47**, 9-16.

88. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence. *Lancet*, 1994, **344**, 721-724.

89. Sherwin ER. Antioxidants. *Food Additives*. Branen R. (Ed), Marcel Dekker, New York, 1990, p 139–193.

90. Grice HC. Safety evaluation of butylated hydroxytoluene (BHT) in the liver, lung and gastrointestinal tract. *Food and Chemical Toxicology*, 1986, **24**, 1127–1130.

91. Pryor WA. The antioxidant nutrient and disease prevention what do we know and what do we need to find out? *Am. J. Clin. Nutr.*, 1991, **53**, 391-393.

92. Shahidi F and Wanasundara PKJPD. Phenolic antioxidants: criteria review. *Food Science and Nutrition*, 1992, **32**, 67-103.

93. Yoshie Y, Wang W, Hsieh YP and Suzuki T. Compositional difference of phenolic compounds between two seaweeds. *Halimeda* spp., *J. Tokyo University of Fisheries*, 2002, **88**, 21-24.

94. Pietta P, Simonetti P and Mauri P. Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.*, 1998, **46**, 4487-4490.

95. Andlauer W and Furst P. Antioxidative power of phytochemicals with special reference to cereals. *Cereal Foods World*, 1998, **43**, 356-359.

96. Jayaprakasha GK, Selvi T and Sakariah KK. Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extract. *Food Res. Int.*, 2003, **36**, 117-122.

97. Briskin DP. Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiology*, 2000, **124**, 507-511.

98. Senevirathne M, Siriwardhana N and Ha Jin-Hwan. Antioxidant potential of *Ecklonia cava* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. *Food Science Tech. Int.*, 2006, **12**, 27-38.

99. Panovska TK, Kulevanova S and Stefova M. *In vitro* antioxidant activity of some Teucrium species (Lamiaceae). *Acta. Pharm.*, 2005, **55**, 207-214.

100. Heo SJ, Park EJ, Lee KW and Jeon YJ. Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology*, 2005, **96**, 1613-1623.

101. Park HR, Park E, Rim AR, Jeon KI, Hwang JH and Lee SC. Antioxidant activity of extracts from *Acanthopanax senticosus*. *African Journal of Biotechnology*, 2006, **5(23)**, 2388-2396.

102. Panichayupakaranant P and Kaewsuwan S. Bioassay-guided isolation of the antioxidant Constituent from *Cassia alata* L. leaves Songklanakarin. *J. Sci. Technol.*, 2004, **26**, 103-107.

103. Zhan Y, Dong CH and Yoa YJ. In Vitro Antioxidant Activities of Aqueous Extract from Cultivated Fruit-bodies of *Cordyceps militaris*. *Journal of Integrative Plant Biology*, 2006, **48(11)**, 1365-1370.

104. Banyai S, Tulok MH, Renner C and Varga S. Antioxidant effect of various rosemary (*Rosmarinus officinalis*). *Acta Biol. Szegediensis*, 2003, **47**, 111-113.

105. Aqil F, Ahmad I and Mehmood Z. Antioxidant and Free Radical Scavenging Properties of Twelve Traditionally Used Indian Medicinal Plants. *Turk J Biol.*, 2006, **30**, 177-183.

106. Siqueira1 IR, Cordova CAS, Nunes DS and Nettol CA. Antioxidant action of an ethanol extract of *Ptychopetalum olacoides*. *Pharmaceutical Biology*, 2002, **40**, 374-378.

107. Rashed AN, Afifi FU and Disi AM. Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L. in *Mus musculus*. *J. Ethnopharmacol.*, 2003, **88**, 131-136.

108. Orafidiya LO, Fakoya FA, Agbani EO and Iwalewa EO. Vascular permeability-increasing effect of the leaf essential oil of *Ocimum gratissimum* linn. as a mechanism for its wound healing property. *African Journal. Traditional, Complementary and Alternative Medicines*, 2005, **2(3)**, 253-258

109. Chitra P, Sajithlal GB and Chandrakasan G. Influence of *Aloe vera* on collagen characteristics in healing dermal wounds in rats. *Mol. Cellular Biochem.*, 1998, **181**, 71-76.

110. Suguna L, Sivakumar P and Chandrakasan G. Effects of *Centella asiatica* extract on dermal wound healing in rats. *Ind. J. Exp. Bio.*, 1996, **34**, 1208-1211.

111. Bale SH and Sheikh KA. The wound healing properties of *Channa striatus*-cetrimide cream-wound contraction and glycosaminoglycan measurement *J. Ethnopharml.*, 2000, **73**, 15-30.

112. Clark RAF. Wound repair: Overview and general considerations. *The Molecular and Cellular Biology of Wound Repair*. Clark RA and Henson PM. (Eds), Plenum Press, New York, 1996, p 3.

113. Chitra P, Sajithlal GB and Chandrakasan G: Influence of *Aloe vera* on the glycosamino- glycans in the matrix of healing dermal wounds in rats. *J. Ethnopharmacol.*, 1998, **59**, 179-186.

114. Shukla A, Rasik AM, Jain GK, Shanker R, Kulshrestha DK and Dawan BN. *In vito* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica*. *J. Ethnopharmacol.*, 1999, **65**, 1-11.

115. Udupa AL, Kulkurni DR and Udupa SL. Effect of *Tridex procumbens* extracts on wound healing. *Int. J. Pharmcognosy*, 1995, **33**, 37-40.

116. Sidhu GS, Singh AK, Thaloor D, Banaudha KK, Paitnaik GK, Srimal RC and Maheshwari RK. Enhancement of wound healing by curcumin in animals. *Wound Rep. Reg.*, 1998, **6**, 167-177.

117. Souba WW and Wilmore D. Diet and nutrition in case of the Patient with surgery, Trauma and sepsis. *Modern Nutrition in Health and Disease*. Shils M, Olson J, Shike M, (Eds), 9th edn., Baltimore, Williams and Wilkins, 1999, p 1589-1618.

118. Sharma S. Ayurvedic drug production, regulatory status in India, domestic and export market. Proc. 4th Int. Sem. Ayurvedic education, research and drug standardization: A global perspective, Gujarat Ayurveda University, Jamnagar, January 5-7, 2003, p 4-15.

119. Dutta C. In: Acharya VYT, Charaka Samhita (Sanskrit), Chikitsasthana, chap. 25, Vers.8, 26, 1941, Bombay, Satyabhamabhai Pandurang.

120. Sushruta M. Sushruta Samhita (Sanskrit). Sutrastahana, Vranaprasna, chap. 21, Vers.21.

121. Kar M. Madhavanidanam (Hindi), Shastri S and UpadhyayaY, (Eds), chap. 42, Vers 4, 1989, Chaukhamba Sanskrit Sansthan, Varanasi.

122. Sushruta Sadyyovrana. Sushruta Samhita (Sanskrit), Chikitsasthana, chapter 2, Vers. 6.

123. Shankaran P. Inflammation and repair. Sushruta's contribution to surgery, Prasad GC, Udupa KN, (Eds), Indological Book House, Varanasi, India, 1976, p 64-70.

124. Rubin E and Farber J. Repair, regeneration and fibrosis. Pathology, Lippincott JB, 2nd edn., Philadelphia, 1994, p 70-95.

125. Coltran R, Kumar V and Robins S. Cellular growth and differentiation. Pathologic basis of disease, Robins S, (Eds), 5th edn., 1994, p 35-50.

126. Ellis L *et. al.* Down regulation of vascular endothelial growth factor in a human colon carcinoma cell line transfected with an antisense expression vector specific for c-SRC. *J. Biol. Chem.*, 1998, **273**, 1052-1057.

127. Sushruta, Sushruta Samhita (Sanskrit), Chikitsasthanam, chap 21, Vers. 22.

128. Vagbhatta. Sothanidanam. Sen P, (Ed), Astanga Hridaya (Bengali), Nidanasthanam, 146, Lower Chitpur Road, Calcutta, 1931, p 71.

129. Mishra B. Bhavaprakash Nighantu (Hindi), Chunekar K and Pandey G, (Eds), 7th edn., Varanasi, India, Chaukhamba Bharati Academy, 1986, p 145.

130. Bhogika M. Dhanwantari Nighantu (Hindi), Ojha J and Mishra U, (Eds), 1st edn., Varanasi, India, Adarsh Vidya Niketan, 1985, p 45.

131. Biswas TK and Mukherjee B. Plant Medicines of Indian Origin for Wound Healing Activity: A Review. *Lower Extremity Wounds*, 2003, **2**, 25-39.

132. Khalil EA, Afifi FU and Al-Hussaini M. Evaluation of the wound healing effect of some Jordanian traditional medicinal plants formulated in Pluronic F127 using mice (Musmusculus). *J. Ethnopharmacol.*, 1997, **55**, 193-200.

133. Davis RH, Donato JJ, Hartman GM and Haas RC. Anti-inflammatory and wound healing activity of a growth substance in *Aloe vera*. *J. Am. Podiatr. Med. Assoc.*, 1994, **84**, 77-81.

134. Mandawgade SD and Patil KS. Wound healing potential of some active principle of *Lawsonia alba* Lam. leaves. *Indian J. of Pharm. Sci.*, 2003, **65(4)**, 390-394.

135. Fulzele SV, Satturwar PM, Jhoshi SB and Dorle AK. Wound healing activity of *Chandanadi yamak* in rats. *Indian J. of Pharm. Sci.*, 2003, **65(4)**, 301-304.

136. Saha K, Mukherjee PK, Das J, Pal M and Saha BP. Wound healing activity of *Leucas lavandulaefolia* Rees. *J. Ethnopharmacol*, 1997, **56(2)**, 139-144.

137. Goel RK, Chakrabarthy A and Sanyal AK. The effect of biological variables on the antiulcerogenic effect of vegeta. plant in banana. *Planta Medica*, 1985, **30**, 285-88.

138. Barrowman JA and Pfeiffer CJ. Carbenoxolone: A critical analysis of its clinicalvalue in peptic ulcer. In: Drugs and Peptic Ulcer, Pfeiffer CJ, (Eds), Boca Raton, CRL Press, 1982, p 123-132.

139. Shetty R, Kumar KV, Naidu MUR, Ratnakar KS. Effect of *Gingko biloba* extract on ethanol-induced gastric mucosal lesions in rats. *Indian J. Pharmacol.*, 2000, **32**, 313-317.

140. Bhattacharya SK, Satyan KS, Ghosal S. Antioxidant activity of glycowithanolides from *Withania somnifera. Indian J. Exp. Biol.*, 1997, **35**, 236-239.

141. Prakash J, Gupta SK, Kochupillai V, Singh N, Gupta YK and Joshi S. Chemopreventive activity of *Withania somnifera* in experimentallyinduced fibrosarcoma tumours in Swiss albino mice. *Phytother. Res.*, 2001, **15**, 240-244.

142. Vaidyaratnam PSV. Indian Medicinal Plants. Warrier PK, Nambiar VPK, Ramankutty C, (Eds), 1st edn., Orient Longman Ltd., Chennai, 1997, p 272.

143. Nadkarni KM. Indian plants and drugs with their medicinal properties and uses. 1st edn., Asiatic Publishing House, New Delhi, 1998, p 450.

144. Vaidyaratnam PSV. Indian Medicinal Plants. Warrier PK, Nambiar VPK, Ramankutty C, (Eds), 1st edn., Orient Longman Ltd., Chennai, 1997, p 124.

145. Parrotta JA. *Healing plants of peninsular India*. 1st edn., CABI publishing, New York, 2001.

146. Nadkarni AK. *Dr. K.M. Nadkarni's Indian Materia Medica*. Vol .I, Sangam Books, London, 1982.

147. Pique JM, Esplugues JV, Whittle BJR. Endogenous nitric oxide as a mediator of gastric mucosal vasodilatation during acid secretion. *Gastroenterology*, 1992, **102**, 168-174.

148. Tepperman BL, Whittle BJR. Endogenous nitric oxide and sensory neuropeptides interact in the modulation of the rat gastric microcirculation. *Br. J. Pharmacol.*, 1992, **105**, 171-175.

149. Boyd SC, Sesame HA and Boyd MR. Gastric glutathione depletion and acute ulcerogenesis by diehylmaleate given subcutaneously to rats. *Life Science*, 1981, **28**, 1987-1992.

150. Ghanayem BI, Boor PJ and Ahmed MR. Acrylonitrile induced gastric mucosal necrosis: Role of gastric glutathione. *J. Pharmacol Exp. Ther.*, 1985, **232**, 570-577.

151. Goel RK and Bhattacharya SK. Gastroduodenal mucosal defense and mucosal protective agents. *Indian J. Exp. Biol.*, 1991, **29**, 701-714.

152. Brito ARMS, Cota RHS and Nunes DS. Gastric antiulcerogenic effects of *Dalbergia monetaria* L in rats. *Phytother. Res.*, 1997, **11**, 314-316.

153. Gurbuz I, Ustun O, Yesilada E, Sezik E and Akyurek N. *In vivo* gastroprotective effects of five Turkish folk remedies against ethanol-induced lesions. *J. Ethnopharmacol*, 2002, **83**, 241-244.

154. Tewari PV, Kumar N, Sharma RD and Kumar A. Treatment of *Amlapitta* (Khila-Sthana). Kasyapa Samhita, Tewari PV, (Eds), Varanasi, Chaukhambha, Visva Bharati, 1996, p 630-635.

155. Suba V, Murugesan T, Pal M, Mandal SC and Saha BP. Antiulcer activity of methanol fraction of *Barleria lupulina* Lindl. in animal models. *Phytotherapy Res.*, 2004, **18(11)**, 925-929.

156. Shah MB, Goswami SS and Santani DD. Effect *of Manilkara hexandra (Roxb.) Dubard* against experimentally-induced gastric ulcers. *Phytotherapy Res.*, 2004, **18(10)**, 814-818.

157. Rujjanawate C, Kanjanapothi D and Amornlerdpison D. The anti-gastric ulcer effect of *Gynostemma pentaphyllum* Makino. *Phytomedicine*. 2004, **11(5)**, 431-35.

158. Hosseinzadeh H, Haddadkhodaparast MH and Hosseini E. Anti-ulcer effect of *Salvia leriifolia* Benth. leaf extracts in mice. *Pharmaceutical and Pharmacological Letters*, 2000, **10(2)**, 63-64.

159. Evaluation of gastric anti-ulcer activity in a hydroethanolic extract from *Kielmeyera coriacea. Brazilian Archives of Biology and Technology*, 2005, **48(1)**, 211-216.

160. Karimi G, Hosseinzadeh H and Ettehad N. Evaluation of the gastric antiulcerogenic effects of *Portulaca oleracea* L. extracts in mice. *Phytother Res.*, 2004, **18(6)**, 484-487.

161. Tan PV, Nyasse B, Dimo T and Mezui C. Gastric cytoprotective anti-ulcer effects of the leaf methanol extract of *Ocimum suave* (Lamiaceae) in rats. *J. Ethnopharmacol.*, 2002, **82**, 69-74.

162. Rujjanawate C, Kanjanapothi D and Amornlerdpison D. Anti-gastric ulcer effect of *Kaempferia parviflora* L. *J. Ethnopharmacol.*, 2005, **102(1)**, 120-122.

163. Solomon M, Eyasu M and Asfaw D. Gastroprotective effect of aqueous *Trigonella feonum gracum* and *Linum ussitatissimum* seed extracts in Mice. *Pharmacologyonline*, 2006, **2**, 324-334.

164. Pal S and Caudhuri AN. Studies on anti-ulcer activity of *Bryophyllum pinnatum* extract in experimental animals. *J. Ethnopharmacol.*, 1991, **33**, 97-102.

165. Gupta M, Mazumder UK, Manikandan L, Bhattacharya S, Senthilkumar GP and Suresh R. Anti-ulcer activity of ethanol extract of *Terminaliapallida brandis* in Swiss albino rats. *J. Ethnopharmacol.*, 2005, **97(2)**, 405-408.

166. Kuby J. *Immunology*. 3rd edn., W H Freeman and Company, NewYork, 1997, p 565-566.

167. Gryglewski RJ. Molecular mechanisms of inflammation. *Eur. J. Rheumatol. Inflammation*, 1981, **4**, 153-159.

168. Freeman BD and Natanson C. Anti-inflammatory therapies in sepsis and septic shock. *Expert Opin. Investigl. Drugs*, 2000, **9**, 1651-1663.

169. Vane JR and Bolting RM. New insights into the mode of action of anti-inflammatory drugs. *Inflamm. Res.*, 1995, **44(1)**, 1-10.

170. Flower RJ and Vane J R. Inhibition of prostaglandins synthesis. *Biochem. Pharmacol.*, 1974, **23**, 1439-1450.

171. Jager AK, Hutchings A and Van Staden J. Screening of Zulu medicinal plants for prostaglandin synthesis inhibition. *J. Ethnopharmacol.*, 1996, **52**, 95-100.

172. McGaw LJ, Anna KJ and Van Staden J. Prostaglandins synthesis inhibitory activity in Zulu, Xhosa and Sotho medicinal plants. *Phytother. Res.*, 1997, **11**, 113-117.

173. Farnsworth NR. Screening plants for new medicines. In: Biodiversity, Part II, Wilson EO, (Ed), National Academy Press, Washington, 1989, p 83-97.

174. Eisner T. Chemical prospecting: A call for action. In: Ecology Economic and Ethics, Edited by: Borman FH and Kellert SR, (Eds), The Broken Circle, Yale University Press, New Haven CT, 1990, p 196-202.

175. Elisabetsky E, Amodor TA, Albuquerque RR, Nunes DS and Carvalho ACT. Analgesic activity of *Psychotria colorata* Muell Arg. Alkaloids. *J. Ethnopharmacol.*, 1995, **48**, 77-83.

176. Raji Y, Udoh US, Oluwadara O. Akinsomisoye OS, Awobajo O and Adeshoga K. Anti-inflammatory and analgesic properties of the rhizome extract of *Zingiber officinale*. *Afr. J. Biomed. Res.*, 2002, **5**, 121-124.

177. Balasubramaniana A, Ramalingam K, Krishnan S and Christina A. Anti-inflammatory activity of *Morus indica* Linn. *Iranian journal of Pharmacology and Therapeutics*, 2005, **1**, 13-15.

178. Charles Domi AI, Vidyalakshmi KS, Hannah RV, Rajamanickam GV and Dubey GP. Anti-inflammatory activity of *Plumbago capensis*. *Pharmacognosy Magazine*, 2006, **2**, 239-243.

179. Omkar A, Jeeja T and Gadgoli C. Evaluation of Anti-inflammatory activity of *Nyctanthes arbor-tristis* and *Onosma echioides*. *Pharmacognosy Magazine*, 2006, **2**, 258-260.

180. Ahamed KN, Venkatesan K, Sundarrajan R, Mukherjee K and Mukherjee PK. Anti-nociceptive and anti-Inflammatory activity of *Araucaria bidwillii* Hook, *Iranian journal of pharmacology and Therapeutics*, 2005, **4**, 105-109.

181. Shamsali R, Kebryaeezadeh A, Hamedani M, Shafiee A, and Isfahani S. Anti-inflammatory and analgesic activity of methanolic extracts of aerial parts of *Stachys schischegleevii* Sosn and *Stachys balansae* Boiss and *K. otschyex* Boiss in rats. *DARU*, 2005, **13(4)**, 165-169.

182. Porchezhian E, Ansari SH and Ahmad S. Analgesic and anti-Inflammatory effects of *Alangium salvifolium*. *Pharmaceutical Biology*, 2001, **39(1)**, 65-66.

183. Radhakrishnan R, Analgesic and Anti-Inflammatory activities of *Teucrium stocksianum*. *Pharmaceutical Biology*, 2001, **39(6)**, 455-459.

184. Dandiya PC and Kulkarni SK. Analgesic, antipyretics and anti-inflammatory agents. In: Introduction to pharmacology. 6th edn., Vallabh prakashan, New Delhi, 2003, p 162.

185. Kar A. Antipyretics and analgesics. In: Medicinal chemistry, Rev. 3rd edn., New Age publisher, New Delhi, 2005, p 213.

186. Domaj MI, Glassco W, Aceto MD and Martin BR. Antinociceptive and pharmacological effects of metanicotina, a selective nicotine agonist. *J. Pharmacol. Exp. Ther.*, 1999, **291**, 390-398.

187. Dahl V and Reader JC. Non-opioid postoperative analgesia. *Acta Anaesthesiol Scand.*, 2000, **44**, 1191-1203.

188. Shanmugasundaram P and Venkataraman S. Anti-nocicepteve activity of *Hygrophila auriculata* Heine. *Afr. J. Trad.*, 2005, **2(1)**, 62-69.

189. Gupta M *et. al.* Evaluation of Anti-inflammatory activity of chloroform extract of *Bryonia laciniosa* in experimental animal models. *Biol. Pharm. Bull.*, 2003, **26(9)**, 1342-1344.

190. Mukerjee PK. Evaluation of analgesic and antinociceptive agents. In: Quality control of herbal drugs. 1st edn., Business Horizon, New Delhi, 2002, p 558- 559.

191. Dandiya PC and Kulkarni SK. Analgesic, antipyretics and anti-inflammatory agents. In: Introduction to pharmacology. 6th edn., Vallabh prakashan, New Delhi, 2003, p164.

192. Kar A. Antipyretics and analgesics. In: Medicinal chemistry, Rev. 3rd edn., New Age publisher, New Delhi, 2005, p 212.

193. Satoskar RS, Bhandarkar and Rege N. Analgesic, antipyretic and nonsteroidal anti-inflammatory Drugs. In: Pharmacology and Pharmacotherapeutics, Rev. edn., Popular prakashan, Mumbai, 2005, p 159-160.

194. Gupta M *et.al.* Studies on anti-inflammatory, analgesic and antipyretic properties of methanol extract of *Caesalpinia bonducella* leaves in experimental animal models. *Iraian J. Pharmacol. and Therap.*, 2003, **2**, 30-34.

195. Amabeoku GJ, Eagles P, Scott G, Mayeng I and Springfield E. Analgesic and antipyretic effects of *Dodonaea angustifolia* and *Salvia africana-lutea*. *J. Ethnopharmacology*, 2001, **75**, 117-124.

196. Mutalik S, Paridhavi K, Rao CM and Udupa N. Antipyretics and analgesic effect of leaves of *Solanum melongena* Linn. in rodents. *Indian Journal of Pharmacology*, 2003, **35,** 312-315.

197. Yongna Z, Wantana R, Pisit B, Zhongkun L and Rongping Z. Analgesic and antipyretic activities of the aqueous extract of *Urtica macrorrhiza* in experimental animals. *Fitoterapia,* 2005, **76(1),** 91-95.

198. Tripathi KD. Antimicrobial Drugs. In: Essentials of medical pharmacology. 5th edn., Jaypee Brothers Medical Publisher, 2003, p 627-630.

199. Evan WC and Trease GE. *Text book of Pharmacognosy.* 13th edn., Bailliere, Tindall London, 1989, p 26-35.

200. Kuc J. Compounds from plants that regulate or participate in disease resistance: Bioactive compounds from plant. Ciba foundation symphosium, Wiley, 1990, p 213-228.

201. Kuc J. Increasing crop productivity and value by increasing disease resistance through non- genetic techniques. Forest potential productivity and value. Ballard R, (Ed), Weyer Haeuser Company Press, Centralia, 1985, p 147-190.

202. Carn JD and Rogers CB. Chemosystematic studies of the genus *Combretum*. A convenient method of identifying species of this genus by a comparision of the polar constitutents extracted from leaf material. *South African J.of Botany*, 1987, **53,** 173-176.

203. Howard BJ, Klass JI, Rubin SJ, Weissfeld AS and Titton RC. *Clinical and Pathogenic Microbiology*. St. Louis Missouri, (Ed), C.V. Mosby Company, 1987, p 123-130.

204. Skinner FA. *Modern methods of plant analysis*. Springer-Verlag, Berlin, 1995, p 626.

205. Verpoorte R, Tjenatsoi A, Van-Doorne J and Svedsen AB. Medicinal plants of Suriname: Antimicrobial activity of some medicinal plants. *J. Ethnopharmacol.*, 1982, **5,** 221-226.

206. Ratnayake CK, Arambewela LSR, De Silva KTD, Atta-ur-Rahman and Alvi KA. Alkaloids of *Alstonia macrophylla. Phytochem.*, 1987, **26 (3)**, 868-870.

207. Schultes RE. *Amazonian ethnobotany and the search for new drugs*. Symposium Ciba Foudation, Amsterdam, Vol- 185, 1994, p 106-112.

208. Soerjato DD. Biodiversity prospects and benefits haring: perspectives from the field. *J. Ethnopharmacol*, 1996, **51**, 1-15.

209. Caceres A *et. al.* Plants used in Guatemala for treatment of gastrointestinal disorders. Screening of 84 plants against *Enterobacteria. J. Ethnopharmacol*, 1990, **30**, 55-73.

210. Wright CW and Phillipson JD. Natural products and the development of selective antiprotozoal drugs. *Phytother. Res.*, 1990, **4**, 127-139.

211. Croft SL. The current status of antiparasitic chemotherapy. *Parasitology*, 1997, **114**, 3-5.

212. Delorenzi JC *et. al.* Antileishmanial activity of an indole alkaloid from *Peschiera australis. Antimicrob. Agents Chemothe.*, 2001, **45(5)**, 1349-1354.

213. Santos PRV, Oliveira ACX and Tomassini TCB. Controle microbiogico de produtos fitoterapicos. *Rev. Farm. Bioquim.* 1995, **31**, 35-38.

214. Ellof JN. Which extract should be used for the screening and isolation of antimicrobial components from plants? *J. Ethnopharmacol*, 1998, **60**, 1-6.

215. Almagboul AZ, Bashir AK, Farouk A and Salih AKM. Screening for antibacterial activity: Antimicrobial activity of certain Sudanese plants used in folkloric medicine. *Fitoterapia*, 1985, **56**, 331-337.

216. Artizzu N, Bonsignore L, Cottiglia F and Loy G. Studies of the diuretic and antimicrobial activity of *Cynodon dactylon* essencial oil. *Fitoterapia*, 1995, **66**, 174-175.

217. Ikram M and Inamul H. Screening of medicinal plants for antimicrobial activities. *Fitoterapia*, 1984, **55**, 62-64.

218. Izzo AA, Di Carlo G, Biscardi D, Fusco R, Mascolo N, Borreli F, Capasso F, Fasulo MP and Autore G. Biological screening of Italian medicinal plants for antibacterial activity. *Phytother. Res.*, 1995, **9**, 281-286.

219. Kubo L, Muroi H and Himejima M. Structure-antibacterial activity relationships of anacardic acids. *J. Agri. Food Chem.*, 1993, **41**, 1016-1019.

220. Shapoval EES, Silveira SM, Miranda ML, Alice CB and Henriques AT. Evaluation of some pharmacological activities of *Eugenia uniflora*. *J. Ethnopharmacol.*, 1994, **44**, 136-142.

221. Sousa M, Pinheiro C, Matos MEO, Matos FJ, Lacerda MI and Craveiro AA. Constituintes Químicos de Plantas Medicinais Brasileiras. Universidade Federal do Ceara. *Fortaleza.*, 1991, **45**, 385-388.

222. Anesini E and Perez C. Screening of plants used in Argentine folk medicine for antimicrobial activity. *J. Ethnopharmacol.*, 1993, **39**, 119-128.

223. Ahmed SR, Iranshahi M, Mirjani R. Bioassy guided isolation and identification of antibacterial compounds from *Ferula persica* Var. roots. *DARU*, 2005, **13(1)**, 17.

224. Adomi O. Antibacterial activity of aqueous and ethanol extracts of the stem bark of *Alstonia boonei* and *Morinda lucida*. *Sientific Res. Essay*, 2006, **1(2)**, 50-53.

225. Olila D, Olwa O and Opuda J. Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis* Ugandan medicinal plants. *African Health Sciences*, 2001, **1(2)**, 66-72.

226. Yasarl S, Sagdic O and Kisioglu AN. *In vitro* antibacterial effects of single or combined plant extracts. *J. Food, Agriculture & Environment*, 2005, **3(1)**, 39-43.

227. Sakharkar PR and Patil AT. Antimicrobial activity of *Cassia alata*. *Ind. J. Pharma Sci.*, 1998, **60**, 311-312.

228. Nair A and Bhide SV. Antimicrobial properties of different parts of *Semecarpus anacardium*. *Indian Drugs*, 1996, **33**, 323-328.

CHAPTER - 3

PHYTOCHEMICAL STUDIES

3.1. Introduction

Phytochemical analysis is intended to serve as a major source of information on analytical and instrumental methodology in the plant drug research. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects[1]. Searching for new drugs in plants implies the screening of plant extracts for the presence of novel compounds and investigation of their biological activities. The path that leads from an intact plant to its pure constituent is long and the work may last from weeks to years. This work includes collection of the plant material, identification of the species by a botanist, extraction using different solvents, followed by the analysis of the extracts by different chromatographic methods, fractionation and isolation steps using different preparative chromatographic techniques, structure elucidation of the constituents by a combination of various spectroscopic and chemical methods, pharmacological and toxicological testing[2].

When the whole sample *e.g.* a plant extract, has proved to be biologically active it is necessary to isolate the pure constituents that are responsible for the activity. The isolated constituents can be used for structure elucidation, further bioassays and structural modifications for studying the mode of action of the compounds, their side effects and toxicology[3]. Tracking and isolating the process of obtaining bioactive compounds from natural products is a challenge which needs new innovations in order to become more rapid, more effective and less expensive[4].

The plants extracted and submitted to the screening are selected on the basis of their use in traditional medicine, the available literature data and chemotaxonomic criteria. The extracts presenting the most interesting activities are then selected for activity-guided phytochemical investigation in order to identify the active compounds. Moreover, the isolation and characterization of further molecules presenting original chemical structures and a potential therapeutic interest is performed. The extract is screened for the presence of various constituents employing standard screening test. There is increasing scientific interest in the extraction and isolation of secondary metabolites from plants as part of biosynthetic biochemical, chemotaxonomic, ecological, phytochemical, pharmacological, and plant tissue culture. The most common plant secondary metabolites occur in the form

of alkaloid, anthraquinones, coumarins, essential oils, flavonoids, steroids, terpenoids etc[5].

Plant parts in fresh or dried form are used for extraction. Plants may be dried before extraction. The drying operations should be performed under controlled conditions to avoid to occur too many chemical changes. It should be dried as quickly as possible without using high temperatures preferably in a good air draft once thoroughly dried; plants can be stored before analysis for long period of time. The effective extraction of a plant compound of interest from a natural source depends largely on solubility stability and functional group considerations. Extraction methods to be employed to obtain a crude plant extract as well as precautions that must be taken to avoid compound decomposition, side reactions or rearrangements during extraction process. Mode of extraction naturally depends on the texture and water content of the plant material being extracted and on the type of substance that is being isolated.

The classical chemical procedure for obtaining organic constituents from dried plant tissue is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents. The extract obtained is clarified by filtration through celite on a water pump and is then concentrated in vacuum. This is now usually carried out in a rotary evaporator, which will concentrate bulky solution down to small volume without bumping at temperature of between 30-40°C. When investigating the complete phytochemical profile of a given plant species, fractionation of a crude extract is desirable in order to separate the main constituent from each other prior to chromatographic analysis.

Many plant extracts owe their potency to the presence of substances such as tannins, phenolic compounds and so on. These substances are usually found in various parts of the plants like roots, leaves, shoots and bark. Many plants have therefore become sources of important drugs and the pharmaceutical industries have come to consider traditional medicine as a source of bioactive agents that can be used in the preparation of synthetic medicine[6].

Recently, chromatographic fingerprint technique, as a more meaningful quality control method of herbal samples, has been attracting more and more people's attention because the fingerprint technique emphasizes on the integral characterization of compositions of samples with a quantitative degree of reliability and focus on identifying and assessing

the stability of the plants. Chromatographic fingerprint is a kind of method to show chemical information of medicinal constituents with chromatograms, spectrograms and other graphs by analytical techniques. Chromatographic techniques are used to isolate and purify the natural products. Varieties of chromatographic techniques involving fingerprint include TLC, gas chromatography, high performance liquid chromatography (HPLC), MPLC (medium pressure liquid chromatography) which play an important role in the isolation processes[7-12].

Identification of compounds usually involves a combination of different techniques including Ultra Violet (UV), Infrared (IR), Mass (MS) and Nuclear Magnetic Resonance (NMR) Spectrometry. Other ways of confirming the identification of the compounds include calculation of the R_f values in different solvent systems and determination of melting points.

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Flavonoids are widely distributed in plants fulfilling many functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) means that many animals, including human, ingest significant quantities in their diet[13]. Over 4,000 flavonoids have been identified, many of which occur in fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks). The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, and antioxidant activities[14].

More than 1300 different flavonoid compounds have been isolated from plants. Individual flavonoids in a group differ from each other by the number and position of the hydroxy, methoxy, and sugar substituents.

Flavonoids, also referred to as bioflavonoids, are polyphenol antioxidants found naturally in plants. They are secondary metabolites, meaning thereby they are organic compounds that have no direct involvement with the growth or development of plants. More simply, flavonoids are plant nutrients that when consumed in the form of fruits and vegetables are

non-toxic as well as potentially beneficial to the human body. More importantly, the consumption of foods containing flavonoids has been linked to numerous health benefits. Though research shows flavonoids alone provide minimal antioxidant benefit due to slow absorption by the body, there is indication that they biologically trigger the production of natural enzymes that fight disease. Recent research indicates that flavonoids can be nutritionally helpful by triggering enzymes that reduce the risk of certain cancers, heart disease, and age-related degenerative diseases. Some research also indicates that flavonoids may help to prevent tooth decay and reduce the occurrence of common ailments such as the flu. These potential health benefits, many of which have been proven, have become of particular interest to consumers and food manufacturers. Foods that contain high amounts of flavonoids include blueberries, red beans, cranberries, and blackberries. Many other foods, including red and yellow fruits and vegetables and some nuts, also contain flavonoids. Red wine and certain teas also are rich in flavonoids.

Flavonoids are a large and important group of natural products derived from 'flavone'. Some flavonoids are intensely coloured, providing a spectrum of colours from red to blue in flowers, fruit and leaves. Other flavonoids are essentially colourless, producing the 'whiteness' of white flowers. Besides their contribution to plant colour, flavonoids have a variety of other roles in the growth and development of plants. Leaf flavonoids provide protection from the potential damage of UV radiation. Certain flavanones are formed as antifungal barriers in plant leaves in response to microbial infection and others play an important part in plant reproduction. Flavonoids also exhibit a wide range of biological properties including anti-microbial, insecticidal and oestrogenic activities[15]. 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 rhizome of the plants under investigation.

3.2. Materials and methods

3.2.1. Plant material

The rhizomes of *K. rotunda* Linn. and leaves of *E. cannabinum* Linn. were collected from different parts of Sikkim. They were authenticated at Botanical survey of India, Gangtok, Sikkim. A voucher specimen was retained in our laboratory for further reference. The rhizomes of *K. rotunda* and leaves of *E. cannabinum* were dried in shade, pulverized in a

mechanical grinder and passed through 40-mesh sieve to get the powder of the plant parts and kept under dry condition in the sterile container for further work.

3.2.2. Extraction procedure for *K. rotunda*

Coarsely powered dry rhizomes (1kg) were extracted separately by cold percolation with 4 liters of 80% methanol in a percolator for 72h at room temperature. The residue was removed by filtration and the methanol extract was concentrated and dried by using rotary flash evaporator, at 42-45°C to give a solid residue and kept in vacuum desiccators for complete removal of solvent and for use in different pharmacological experiments and to further fractionation with the help of different solvents.

3.2.3. Fractionation of the methanolic extract by different solvents

The above methanolic extract obtained was concentrated, subjected to fractionation by distilled water. The remaining hydro-alcoholic part was further fractionated with petroleum ether and ethyl acetate successively. The ethyl acetate soluble fraction was subjected to thin layer chromatographic analysis. The aqueous, petroleum ether fraction did not show any positive pharmacological activities of this investigation and was discarded. Flow chart of extraction has been shown in Fig 3.1.

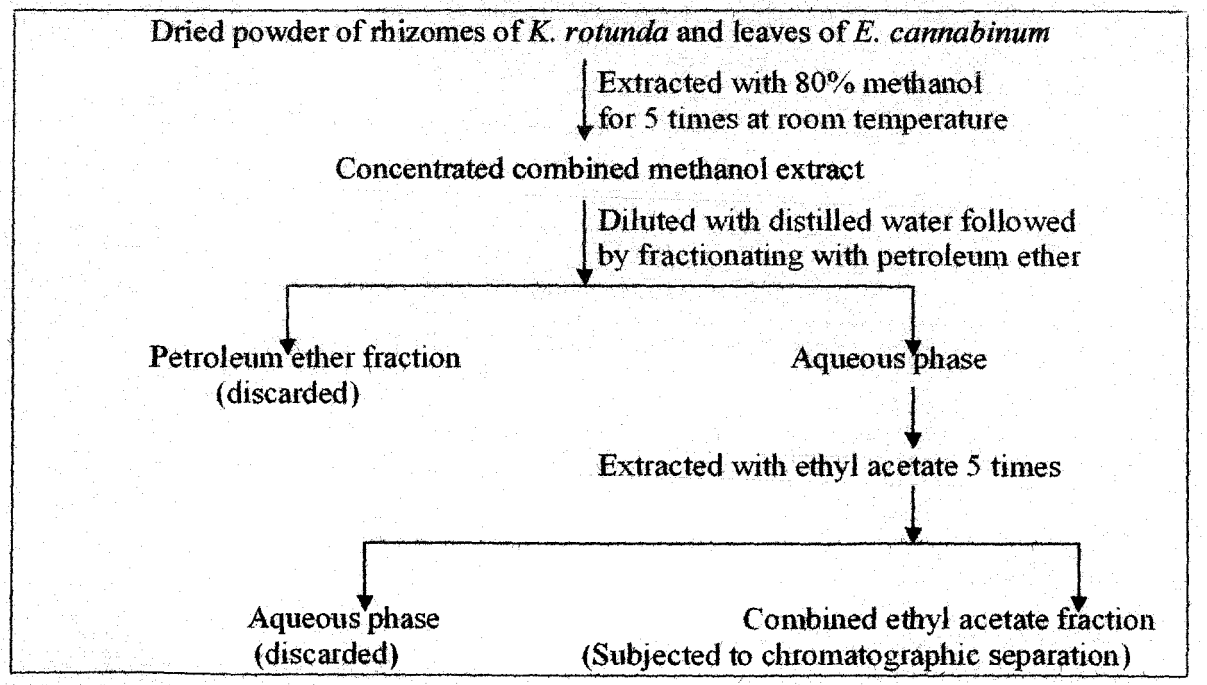


Figure 3.1. Flow chart showing the method of extraction of rhizomes of *K. rotunda* and leaves of *E. cannabinum*

3.2.4. Isolation and purification of phytoconstituent from the extract of *K. rotunda*

3.2.4.1. Preliminary phytochemical tests

The preliminary phytochemical group test of extract of *K. rotunda* rhizome was performed by the standard methods[16-18].

Tests for alkaloids

Small quantity of the extract was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent. There was absence of yellowish buff colored precipitate which indicated the negative test for alkaloids.

A small quantity of extract was treated with few drop of dilute hydrochloric acid and filtered and the filtrate was then treated with Dragendroff's reagent. There was no orange brown precipitate which indicated the negative test for alkaloids.

Small quantity extract treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. There was no reddish brown precipitate which indicated negative test for alkaloids.

Small quantity extract was treated with few drop of dilute hydrochloric acid and filtered. The filtrate was treated with Hager's reagent. There was no yellowish precipitate which indicated the negative test of alkaloid.

Test for steroids and triterponoids

Libermann-Buchard test: 10mg of extract 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 and dissolved in 1.0 ml of chloroform. A reddish blue colour exhibited by chloroform layer and green fluorescence by the acid layer indicated the presence of steroids.

Test for flavonoids

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

A small quantity of extract 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 solution 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.

Test for saponins

Small quantities of extract 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 extract 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 tannins

Small quantity of extract 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 extract of 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 extract 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 extract 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 gums and mucilage

Small quantity of extract 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), absence of red-violet ring at the junction of the two layers indicated the negative test for gums (Molish's test).

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 95% alcohol. Absence of precipitation indicated the absence of gums and mucilage.

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. The filtrate was treated with 0.008gm of ruthenium red in 10ml of 10% solution of lead acetate. Absence of red colour indicated the absence of gum and mucilage.

Test for reducing sugar

A small quantity of extract was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Benedict's reagent was added and heated for few minutes. No brick red precipitate was found which confirmed the absence of reducing sugars.

Small quantity of extract was dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Fehling's A and B solution were added and heated for few minutes. No development of brick red colour demonstrated the absence of reducing sugars.

Test for volatile oil

50gm of powdered material was taken in volatile oil estimation apparatus and subjected it to hydrodistillation, for the detection of volatile oil, the distillate collected in a graduated tube, which indicates the presence of volatile oil.

3.2.4.2. TLC of the methanolic extract and its ethylacetate fraction of *K. rotunda*

Thin layer chromatography (TLC) is a technique used to separate chemical compounds by following processes.

3.2.4.2.1. Preparation of TLC plates

Chromatographic plates having the size of 20cm x10cm were prepared by coating of the slurry of 20% suspension of Silicagel G with the help of conventional spreader to a layer thickness of 0.25mm. After spreading the resultant plates were allowed to dry at room

temperature and then activated by heating in an oven for 30 minutes at 110°C. After cooling the plates were kept in a dessicator until required for further use.

3.2.4.2.2. Sampling on plates and development of Chromatogram

With the help of microcapillary tubes the methanolic extract and its ethylacetate fraction of *K. rotunda* was spotted at 2cm from the edge of the plate. On the plates the chromatogram was developed in chromatographic chambers using selected solvent systems at a room temperature (28°C) and at an angle of 70°. The loaded TLC plate was carefully placed in the TLC chamber with the spot of solution containing the methanolic extract and ethylacetate fraction toward the bottom. The plate whose top was leaned against the jar wall was allowed to sit on the bottom of the chamber and was in contact with the developing solvent (solvent surface was well below the extract line). The TLC chamber was covered. The TLC plate was allowed to remain undisturbed. When the solvent front has reached three quarters of the length of the plate, the plate was removed from the developing chamber and the position of the solvent front was immediately marked. The solvent moved up the plate by capillary action and met the sample mixture, which was dissolved and was carried up the plate by the solvent. Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the stationary phase. 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. Alternatively the developed TLC plates were placed in iodine chamber. Further the R_f values of the spots were calculated.

3.2.4.2.3. Solvent systems used for *K. rotunda*

i) Hexane: Ethyacetate (1:1) ii) Chloroform: Methanol: Water (64:50:10)

iii) Tolune: Ethyacetate (93:7) iv) n-Butanol: Glacial acetic acid: Water (4:1:5)

v) Ethyacetate: Formic acid: Glacial acetic acid: Water (100:11:11:2)

3.2.4.2.4. Spray reagents used

i) NPPEG- Natural product polyethylene glycol reagent

ii) VS: Vaniline sulphuric acid reagent iii) AS: Anisaldehyde sulphuric acid reagent

iv) $SbCl_3$: Antimony trichloride reagent v) ADB: 2-aminoethyl diphenyl borinate

3.2.5. Column Chromatography of extract of *K. rotunda*

The isolation and purification of different components from ethyl acetate fractions of total methanolic extract of *K. rotunda* rhizome by column chromatography[22] involved the following steps.

3.2.5.1. Column

A glass column (25cm in length, 3.5cm dia.) fitted with a stop cock and the bottom of the column was plugged with glass wool was used for separation.

3.2.5.2. Adsorbent: Silica gel 60-120.

Silica gel slurry: Using a beaker of an appropriate size, silica gel was made into thin slurry with the eluting solvent and the slurry was poured into the glass column.

3.2.5.3. Eluting solvent (mobile phase): Hexane-Ethylacetate

Hexane and ethylacetate was used as eluting solvent with increasing polarity of ethylacetate.

3.2.5.4. Preparation of column and separation of the compound isolated from ethyl acetate fraction of extract of *K. rotunda*

The column 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 silica gel (60-120mesh) and solvent system. The solvent system was allowed to drip at the rate of 50 drops per minute and a layer of the solvent was maintained on the top of the silica gel surface.

The excess solvent on the top of the column was allowed to flow down and then the concentrated solution of ethyl acetate fraction of the methanolic extract with required quantity of silicagel (60-120mesh) was layered on the top of the column. A thin layer of cotton was placed over it. The eluting solvent was allowed to flow down slowly till the mixture was adsorbed on the top of the column. Gradient elution was carried out using

hexane and ethylacetate and with step increasing the polarity by increasing the ratio of ethylacetate from 10% to 100%. The rate of elution was adjusted at 50 drops per minute and fractions of 20 ml each were collected in 100 ml of serially numbered conical flasks. After collection of fractions from the column, TLC was done for each fraction with the same solvent system, which was used as the main eluent in the column chromatography.

The eluted fraction number 42-62 having identical hR_f values were pooled together and evaporated to dryness. It was re-chromatographed in a silicagel 60-120 column. Gradient elution was carried out using ethyl acetate and increasing the polarity with methanol in 10% stepwise elutions to 100% methanol. Fraction number 19-26 were combined and evaporated to dryness to provide a crystalline powder, which was crystallized to give fine, needle shaped yellowish crystals. The isolated crystalline material was further examined by different physico-chemical techniques for its structure elucidation.

3.2.6. Qualitative analysis of the compound isolated from ethyl acetate fraction of *K. rotunda*

The isolated compound was further examined by chemical tests to confirm its chemical nature.

3.2.6.1. Physical nature of the compound isolated from ethyl acetate fraction of *K. rotunda*

The isolated compound was examined by various physicochemical parameters i.e. physical appearance, solubility and melting point.

3.2.6.2. TLC study of the compound isolated from ethyl acetate fraction of *K. rotunda*

Thin layer chromatographic study of the isolated compound was carried out on silicagel G plates with different solvent systems. The plates were prepared as described in 3.2.4 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).

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 366nm before and after spraying the spray reagents i.e 5% solution of 2-aminoethyl diphenyl borinate in methanol.

3.2.7. UV absorption spectral analysis of the compound isolated from ethyl acetate fraction of *K. rotunda*

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. 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 purified 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 800nm.

3.2.8. Infrared spectrum of the compound isolated from ethyl acetate fraction of *K. rotunda*

The IR spectra of the isolated compound were taken with Perkin-Elmer FTIR spectrophotometer in KBr discs. The spectra were recorded in the region of 4000 cm^{-1} to 400 cm^{-1}, which is the typical region of absorption for isolated compound.

3.2.9. Nuclear Magnetic Resonance (NMR) spectra of the compound isolated from ethyl acetate fraction of *K. rotunda*

The ^1H NMR spectra of the isolated compound from crystallization process was undertaken in Brucker WM 400 Spectrophotometer in DMSO-d_6 (Dimethyl sulphoxide) solution. The NMR data chemical shifts are expressed in δ (ppm) from tetramethylsilane as an internal standard and coupling constants (J) are given in Hz. The spectra are recorded and tabulated.

3.2.10. Mass spectra of the compound isolated from ethyl acetate fraction of *K. rotunda*

Mass spectrum (FAB^+MS) of the isolated compound was recorded on a JEOL JMS 600 Spectrophotometer.

3.2.11. Isolation and purification of phytoconstituent from the leaf extract of *Eupatorium cannabinum*

The methods used for isolation and purification of phytoconstituents from the leaf of *E. cannabinum* were as per the procedure followed for the isolation and purification of phytoconstituents from extract of *K. rotunda* rhizome described in **3.2.3** to **3.2.10** except the following points. The phytochemical studies show the presence of alkaloids in both the methanol extract and ethylacetate fraction of methanolic extract *E. cannabinum*. Alkaloid showed the presence in the solvent system of tolune: thylacetate: di-ethylamine by spraying Dragendorff's reagent.

The identical eluted fractions number 49-94 in column chromatographic separation were re-chromatographed by using chloroform and methanol as eluent. Gradient elution was carried out by increasing the polarity with methanol in 10% stepwise to 100% methanol. The re-chromatographed fractions numbers 15 to 25 were combined and evaporated to dryness to result a pale yellow amorphous powder. The isolated material was further examined by different physico-chemical techniques for its structure elucidation.

3.3. Results

3.3.1. Phytochemical study of *Kaempferia rotunda*

The concentrated methanol extract obtained from the shade-dried rhizome was fractionated successively with distilled water, petroleum ether, and ethyl acetate. It was observed that methanolic extract and the ethyl acetate fraction exhibited significant pharmacological activities under this investigation, so they were subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of steroids, flavonoids, tannins, triterpenoid, volatile oil and saponins (Table 3.1). The thin layer chromatographic study of the methanol extract and ethyl acetate fraction showed the presence of components with selected mobile phase and spraying reagents (Table 3.2 and

3.3). Ethylacetate fraction was subjected to column chromatographic separation on Silicagel (60-120mesh) column in which sixty-eight fractions were collected. The fractions having identical results were mixed together (Table 3.4). They were purified, which yielded compound I. It was further subjected to chemical tests and TLC studies to confirm the chemical nature of the compound I, which are depicted in table 3.5 & 3.6. The chemical nature of the isolated compound was further characterized from its physical parameters and spectral (UV, IR, Mass, and ^{1}H NMR) data[19,20].

Compound I was obtained as yellow needle shaped crystal, soluble in methanol. The compound was melted at 138-139°C. The compound showed strong absorption at 242nm in its spectrum, which implied the presence of phenolic aromatic rings. The UV absorption spectrum of compound I is shown in figure 3.2. The IR spectrum of the compound is presented in figure 3.3 The IR spectrum shows the presence of absorption bands at 3424, 2852 and 1070 cm^{-1}. Other prominent peaks were 1272, 1373, 1452, 1178, 1070, 756, 712 cm^{-1}. The IR spectrum confirmed the presence of aromatic ring (2923 cm^{-1}) and hydroxyl group (3424 cm^{-1}) in compound I. The ^{1}H NMR spectrum of compound I is presented in figure 3.4. In the ^{1}H NMR spectra in DMSO-d_6, the signals for aromatic proton were observed at δ6 (J=2MHZ) δ 7.7 (J=4MHZ). The signals at δ 5.75 (J=1.8MHZ) and δ 5.79 (J=1.8MHZ) signified the presence of aromatic two protons. The signals at 2x6.96 (J=2MHZ) indicates the presence of two aromatic protons and δ 7.70 (J=3MHZ) signified the presence of another two aromatic protons. The signals at δ 3.73 (J=3MHZ) signify the presence of three methoxy groups. The signals at δ 8.17 (J=6MHZ) and δ 7.39 (J=3MHZ) indicated the presence chalcone i.e. HC=CH–C=O. The signal at δ 6.69 (J=3MHZ) indicates the presence of free hydroxyl groups. The mass spectrum in fig. 3.5. of the compound I is in agreement with the assigned structure. The molecular ion peak (base peak, M^+) at m/z 312.2670 (calculated for $C_{18}O_5H_{18}$, 314.1154) and the fragmentation peaks at m/z 153, m/z 165 revealed the empirical formula of $C_{18}O_5H_{18}$. The result is corresponding to the molecular formula $C_{18}O_5H_{18}$. It has been concluded that the structure of the isolated compound I was established according to combined spectral data i.e. 2- hydroxy, 4, 4′, 6- trimethoxy chalcone.

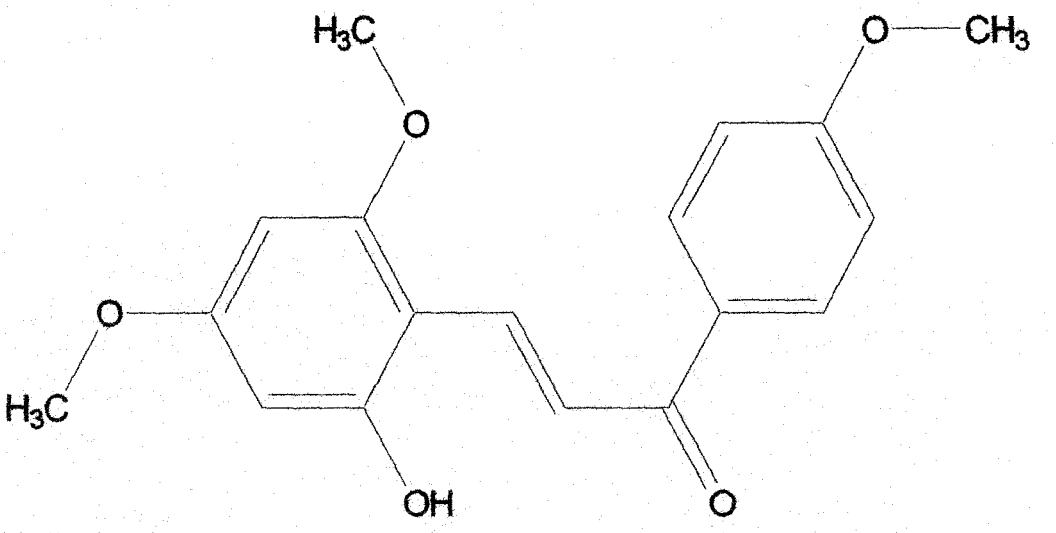
3.3.2. Phytochemical study of *Eupatorium cannabinum*

The concentrated methanol extract prepared from the shade-dried leaves of *E. cannabinum* was fractionated successively with distilled water, petroleum ether and

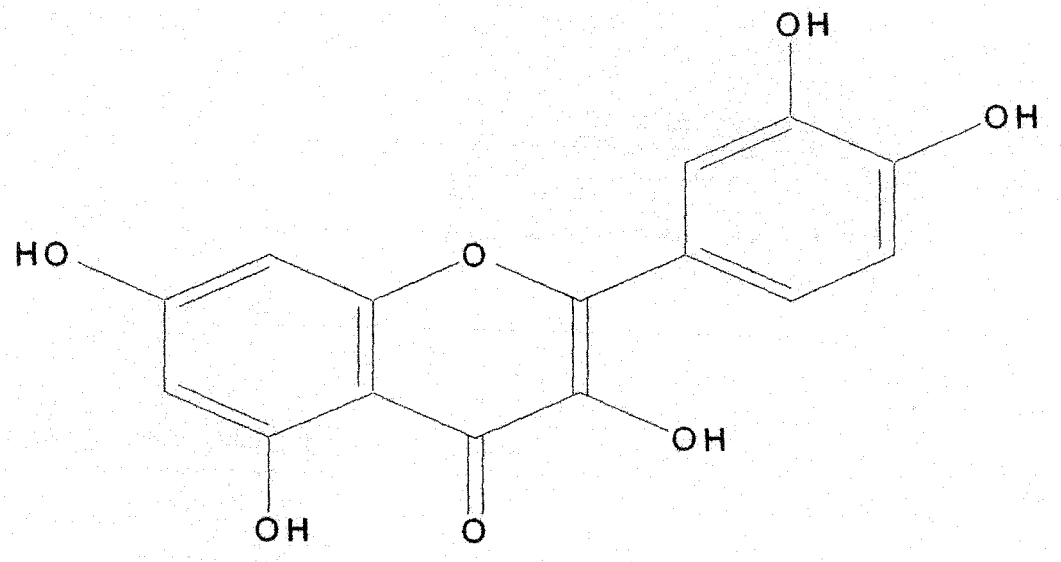
ethyl acetate. Different extract were undertaken for preliminary pharmacological studies. It was observed that the methanol extract and ethyl acetate fraction exhibited significant pharmacological activities. So they were subjected to phytochemical analysis.

The preliminary phytochemical group tests indicated the presence of flavonoids, tannins, saponins and alkaloids (Table 3.7). The TLC study of methanol extracts and ethyl acetate fraction showed the components with selected mobile phase and spraying reagents (Table 3.8 and 3.9). Ethylacetate fraction from methanolic extract was subjected to column chromatography, which yielded 103 fractions (Table 3.10). The fractions having identical hRf values were mixed together (Table 3.9). They were purified with a silica gel column, by using chloroform and methanol as mobile phase, which yielded (compound II). It was further subjected to chemical tests and TLC to confirm the chemical nature and was depicted in (Table 3.10 & 3.11). The chemical nature of the isolated compound was further characterized by physical parameters and spectral (UV, IR, Mass, ^{13}C and ^{1}H NMR) data[24, 25].

Compound II was obtained as pale-yellow amorphous powder, partially soluble in water & soluble in methanol. The compound was melted at 210-214°C. The UV spectrum showed two major absorption bands at 256 and 372nm which are typical for flavonols (Fig. 3.6). The IR spectrum shows the presence of absorption bands at 3648- 3611 cm^{-1} ν (OH), 3406 cm^{-1} ν (C=O) in flavone, 1610-1507 cm^{-1} ν (aromatic rings), 1382 cm^{-1}, 1263 cm^{-1}, 1132 cm^{-1}, 941 cm^{-1} and 700cm^{-1} (Fig. 3.7). In the ^{1}HNMR spectra (Fig. 3.8) in DMSO-d_6, showed two distinctive resonance groups. It displayed two doublets at δ7.78 (1H, J=1.8) and δ6.95 (1H, J=9HJ) and one doublet of doublets at δ at 7.65 (1H,J=9,1.8HZ) and δ, characteristics of a 1, 2, 4 trisubstituted benzene ring and two doublets at δ6.41 (1H, J=2HZ) and δ 6.26 (1H, J=2HZ) characteristics of a 1, 2, 3, 5-tetrasubstituted benzene ring. The isolated compound II showed significant peak in its mass spectra in fig. 3.9. The molecular ion peak (base peak, M^+) at m/z 302 and the fragmentation peaks at m/z 153, m/z 285 revealed the empirical formula of $C_{15}H_{10}O_7$. The UV, ^{1}H NMR and EI-MS data led to the identification of the compound II corresponds to be molecular formula $C_{15}H_{10}O_7$ and the structure of the isolated compound II was 3′,4′,5,7- tetrahydroxy flavonol or quercetin.



Structure of compound I



Structure of compound II

Table 3.1. Preliminary phytochemical tests of extract of *K. rotunda* rhizome

Kaempferia rotunda		
Phytoconstituents	**Methanolic extract**	**Ethyl acetate fraction**
Alkaloids	-	-
Amino acids	-	-
Proteins	-	-
Reducing sugars	-	-
Steroids and Triterpenoids	+	+
Flavonoids	+	+
Tannins	-	-
Saponins	+	-
Anthraquinones	-	-
Gums and Mucilages.	-	-
Volatile oil	+	-

'+ve' indicates presence and '-ve' indicates absence of the phytoconstituents.

Table 3.2. TLC study of methanolic extract of *K. rotunda* rhizome

Solvent system	Colour of the spot under UV light	Colour of the spot after spraying	hRf values	Constituent present
Ethyl acetate : Formic acid : Glacial-acetic acid : Water (100:11:11:2)	Yellow	Deep brown (NP-PEG reagent)	87	Flavonoids
Chloroform : Methanol : Water (64:50:10)	Nil	Blue (VS reagent)	65	Saponin
Toluene : Ethyl acetate (93:7)	Blue	Light green (AS reagent)	44	Essential oil
Hexane : Ethyl acetate (1:1)	Red	Violet ($SbCl_3$)	75	Triterpenoids
Butanol : Acetic acid : Water (4:1:5)	Dark purple	Pink (VS reagent)	52	Tannins

NP-PEG reagent : Natural product polyethylene glycol reagent

VS reagent : Vanillin- sulphuric acid reagent

AS reagent : Anisaldehyde- sulphuric acid reagent

$SbCl_3$: Antimony trichloride reagent

Table 3.3. TLC study of ethylacetate fraction of the methanolic extract of *K. rotunda*

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	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	87	89	90
2	Yellow	Yellow	Yellow	Yellow florescence	Yellow florescence	Yellow florescence	68	39	51
3	Brown	Brown	Brown	Brown	Yellow	Brown	59	78	45

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

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

TLC 3 : Benzene-ethyl acetate-formic acid (9:7:4)

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

Table 3.4. Column and Thin layer chromatographic separation of ethyl acetate fraction of *K. rotunda*.

Eluent Hexane:Ethyl acetate	Fraction number	Residue of selective fraction (gm)	Colour of TLC spots with hR_f values	Inference
100:0	1-10	4.2	NIL	Fatty solid
90:10	11-18	3.6	NIL	Fatty solid
80:20	19-26	3.1	NIL	Fatty solid
70:30	27-31	3.7	NIL	Fatty solid
60:40	32-35	10.1	NIL	Greenish semisolid
50:50	36-41	11.3	NIL	Dark green semisolid
40:60	42-46	5.5	Two yellowish spots (88, 82)	Mixture of compounds
30:70	47-52	6.0	Two bright yellow spots (77, 88)	Mixture of compounds
20:80	53-56	3.5	Two yellowish spots (79, 84)	Mixture of compounds
10:90	57-62	3.5	Three brown spots (87, 69)	Mixture of compounds
0:100	63-68	2.0	Nil	Colourless solids

Spray reagent : 1% Aluminium chloride in ethanol

Table 3.5. Qualitative analysis of the compound I isolated from ethyl acetate fraction of *K. rotunda*[21-23]

Sl. No.	Treatments	Observations	Inferences
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	Yellowish green colour	Presence of flavonoid
5	1 mg of the crystalline solid was treated with 1 ml of concentrated hydrochloric acid	Yellowish green colour	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

Table 3.6. TLC study of the compound I isolated from ethylacetate fraction of *K. rotunda*.

Solvent system	hR_f values	Colour of fluorescent produced		
		UV_{366nm}	UV_{366nm} +$AlCl_3$	UV_{366nm} +NH_3
TLC 1	88	Dark Brown	Dark Brown	Dark Brown
TLC 2	81	Yellow	Yellow	Yellow
TLC 3	69	Brown	Brown	Brown

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

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

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

$AlCl_3$: Aluminium chloride in ethanol

NH_3 : Ammonia

Table 3.7. Preliminary phytochemical tests of extract of of *E. cannabinum*

Eupatorium cannabinum		
Phytoconstituents	**Methanol Extract**	**Ethyl acetate fraction**
Alkaloids	+	-
Amino acids	+	-
Proteins	-	-
Reducing sugars	-	-
Steroids and Triterpenoids	+	+
Flavonoids	+	+
Tannins	+	-
Saponins	+	-
Anthraquinones	–	-
Gums and Mucilages.	–	-
Volatile oil	+	-

'+ve' indicates presence and '-ve' indicates absence of the phytoconstituents

Table 3.8. TLC study of methanolic leaf extract *E. cannabinum*

Solvent system	Colour of the spot under UV light	Colour of the spot after spraying	hRf values	Constituent present
Ethyl acetate : Formic acid : Glacial acetic acid : Water (100:11:11:2)	Yellow	Deep Brown (NP-PEG reagent)	85	Flavonoids
Chloroform : Methanol : Water (64:50:10)	Nil	Blue (VS reagent)	60	Saponin
Toluene : Ethyl acetate (93:7)	Blue	Light green (AS reagent)	44	Essential oil
Hexane : Ethyl acetate (1:1)	Red	Violet ($SbCl_3$)	73	Triterpenoids
Butanol : Acetic acid : Water (14:1:5)	Dark purple	Pink (VS reagent)	51	Tannins
Toluene: Ethyl acetate: Diethylamine (70:20:10)	Nil	Orange (DRG)	25	Alkaloid

NP-PEG reagent: Natural product polyethylene glycol reagent

VS reagent : Vanillin- sulphuric acid reagent

AS reagent : Anisaldehyde- sulphuric acid reagent

$SbCl_3$: Antimony trichloride reagent

DRG : Dragendorff reagent

Table 3.9. TLC study of ethyl acetate fraction of *E. cannabinum.*

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	Orange	Orange	Orange	Bright yellow	Bright yellow	Bright yellow	64	51	66
2	Orange	Orange	Orange	Orange florescence	Orange florescence	Orange florescence	58	31	39
3	Yellow	–	Yellowish green	Yellow	Yellow	Yellowish green	42	47	38

TLC 1 : Chloroform : Ethyl acetate (6:4)

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

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

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

Table 3.10. Column and Thin layer chromatographic separation of ethylacetate fraction of *E. cannabinum*

Eluent Hexane: Ethyl acetate	Fraction number	Residue of selected fractions (gm)	Colour of TLC spots with hR_f values	Inference
100:0	1-12	4.1	NIL	Fatty solid
90:10	14-20	3.2	NIL	Fatty solid
80:20	21-28	3.7	NIL	Fatty solid
70:30	29-32	7.3	NIL	Greenish semisolid
60:40	33-35	8.5	NIL	Greenish semisolid
50:50	36-40	9.3	NIL	Dark yellow semisolid
40:60	41-48	5.3	Two yellowish spots (70, 45)	Mixture of compounds
30:70	49-60	4.5	Two bright yellow spots (70, 44)	Mixture of compounds
20:80	61-65	3.6	Two yellowish spots (69, 43)	Mixture of compounds
10:90	65-94	4.2	Three red spots (67, 55, 29)	Mixture of compounds
0:100	94-103	2.0	Nil	Colourless solid

Spray reagent: 1% Aluminium chloride in ethanol

Table 3.11. Qualitative analysis of compound II from ethylacetate fraction of *E. cannabinum*

Sl.No	Treatment	Observation	Inference
1	1mg of the amorphous power 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 amorphous power 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 amorphous power 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 amorphous power was dissolved in 1 ml of methanol and allowed to react with 5% alcoholic ferric chloride solution	Yellowish green colour	Presence of flavonoid
5	1 mg of the amorphous power was treated with 1 ml of concentrated hydrochloric acid	Yellowish with green florescence	Presence of flavonoid
6	1 mg of the amorphous power was treated with magnesium hydrochloride solution (Shinoid's test)	Red to magenta red colour	Presence of flavonoid

Table 3.12. TLC of the compound II isolated from ethyl acetate fraction of *E. cannabinum*.

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	Orange	Orange	Orange	Orange
TLC 2	59	Yellow	Bright Yellow	Bright Yellow	Bright Yellow
TLC 3	41	Yellow	Yellow	Yellow	Yellow

TLC 1 : Chloroform: Ethyl acetate (6:4)

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

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

$AlCl_3$: Aluminium chloride in ethanol

NH_3 : Ammonia

ADB : 5% solution of 2- aminoethyl diphenyl borinate in methanol

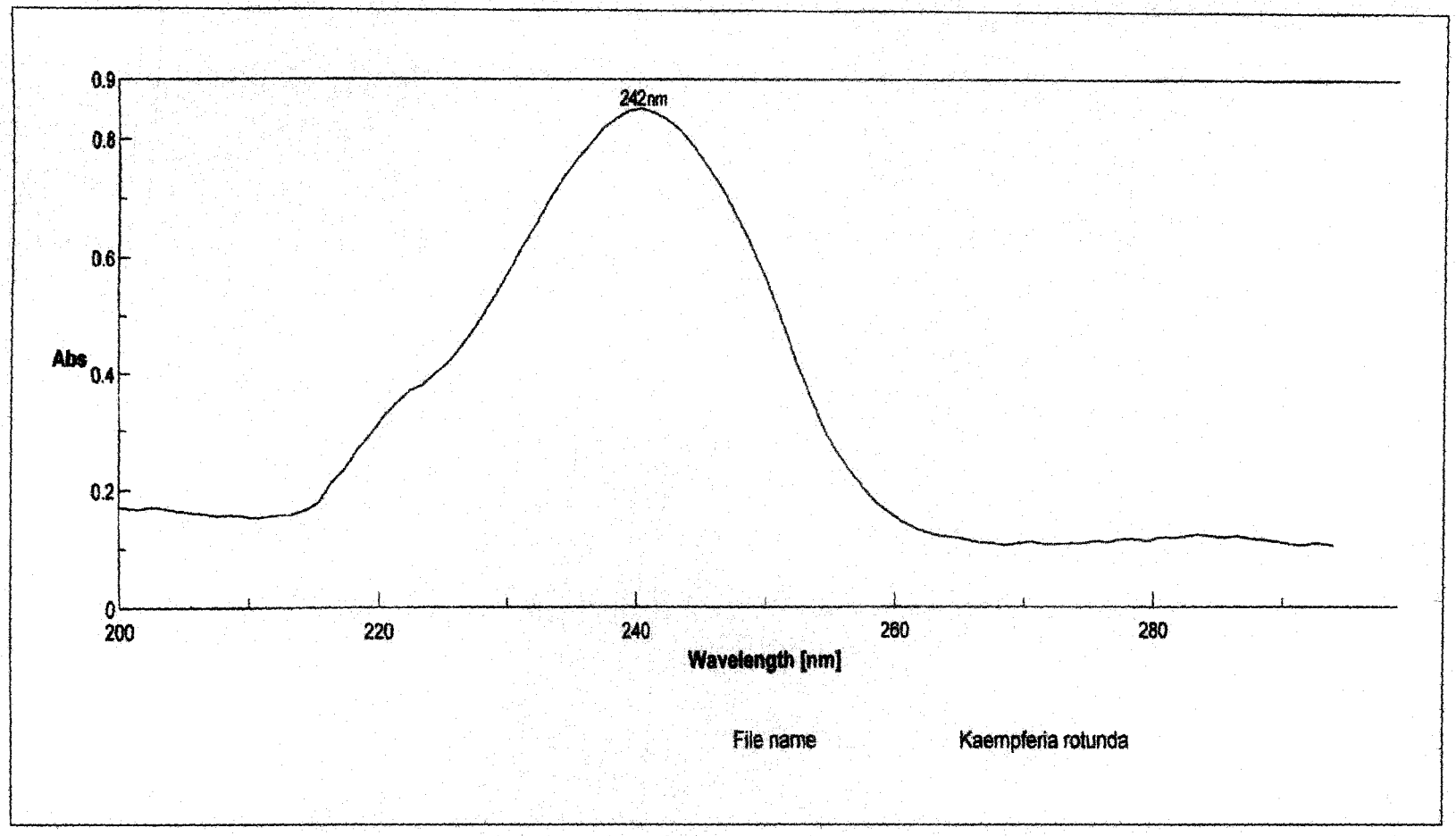


Figure 3.2. UV-Visible Spectro photometric analysis of compound I from *K. rotunda*.

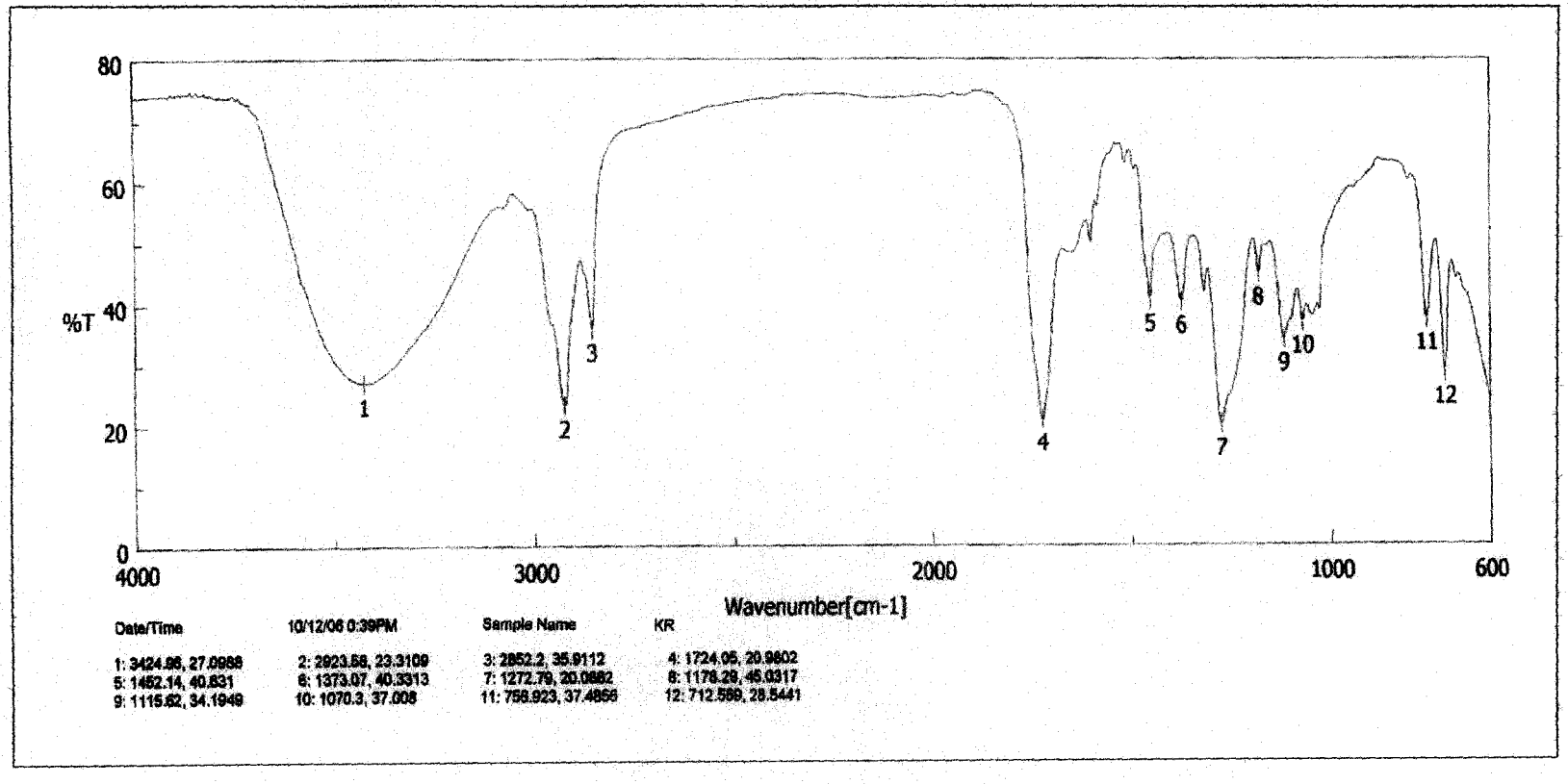


Figure 3.3. IR spectrum of compound I isolated from *K. rotunda*.

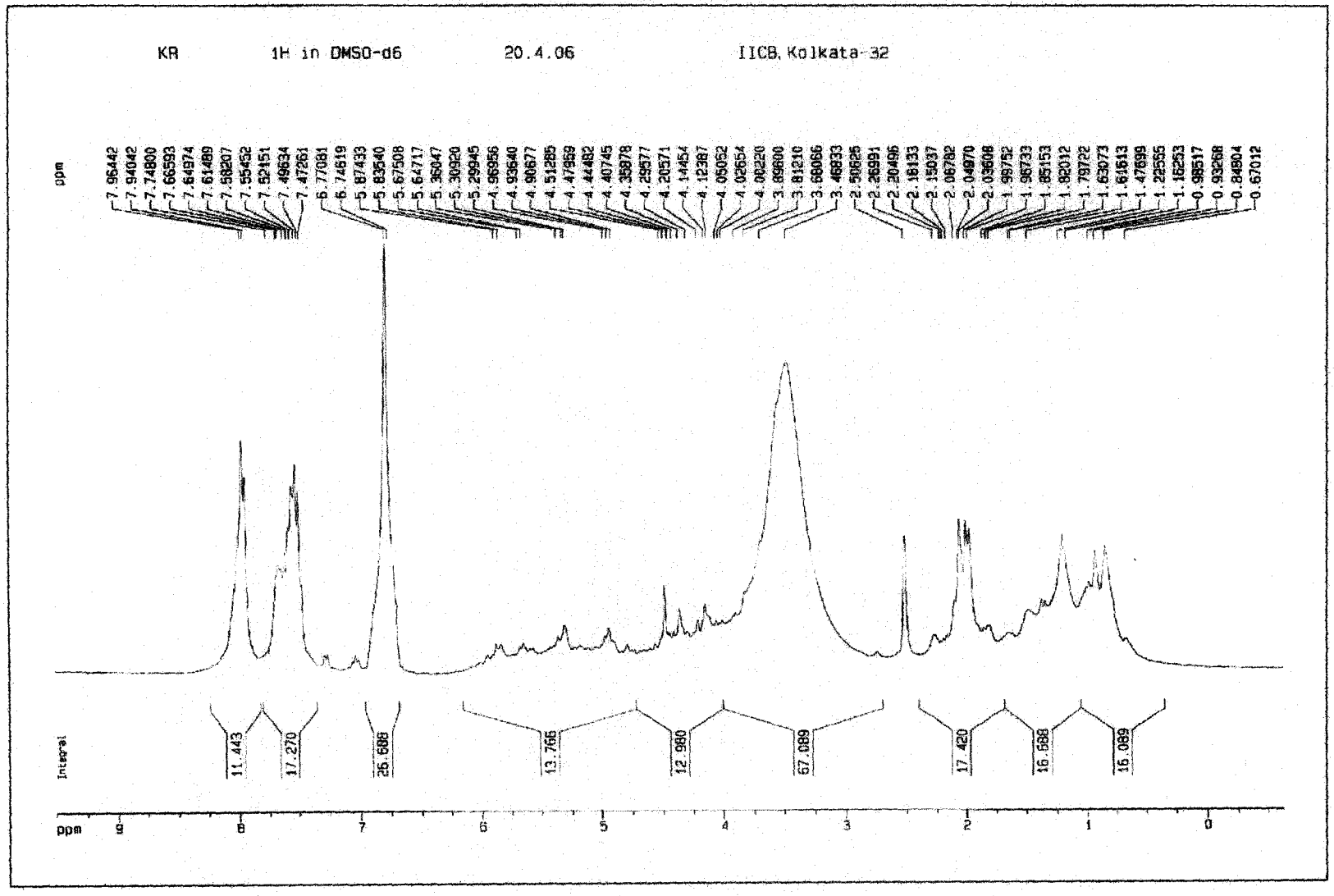


Figure 3.4. ^{1}HNMR spectrum of compound I isolated from *K. rotunda*.

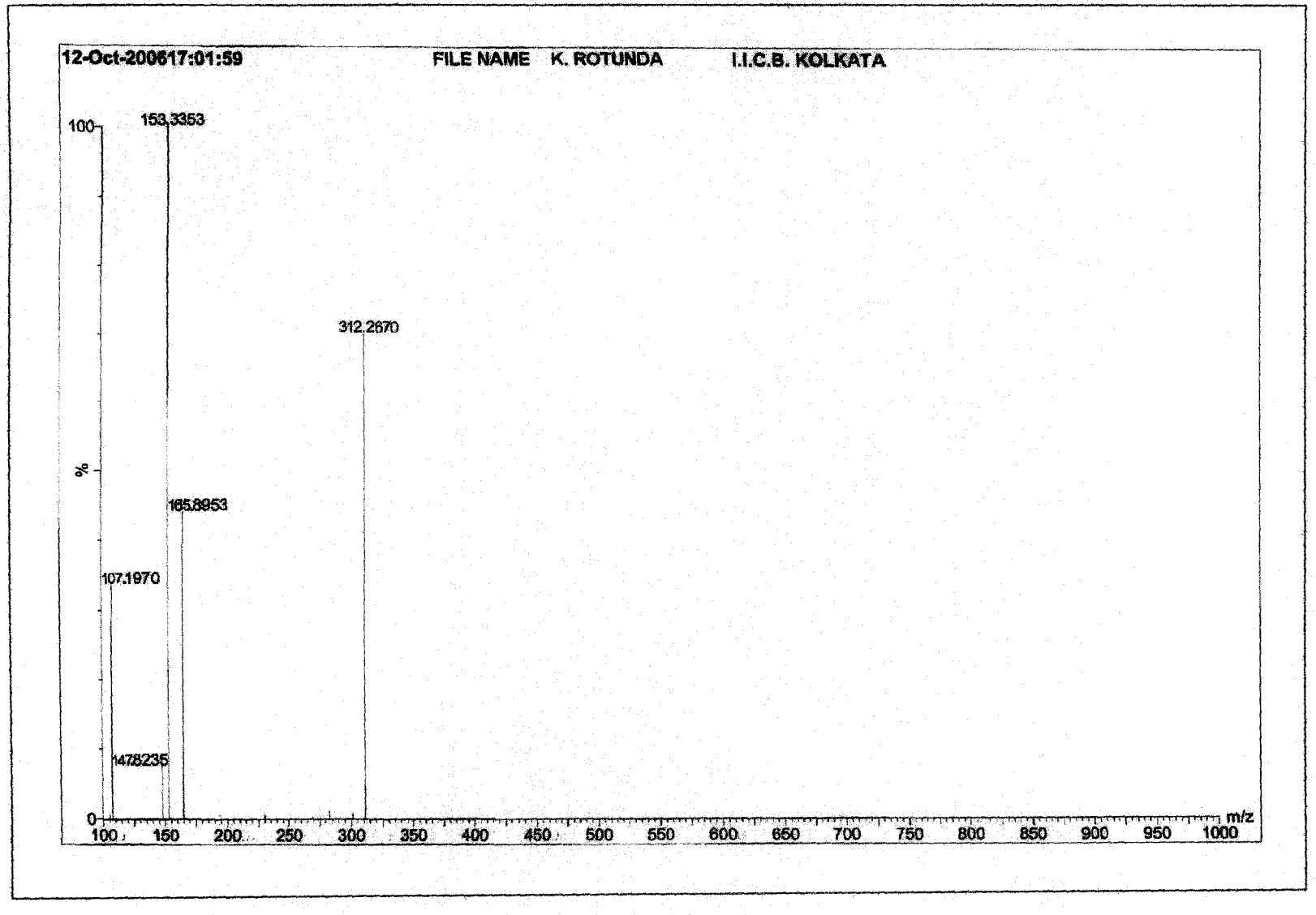


Figure 3.5. Mass spectrum of compound I isolated from *K. rotunda*.

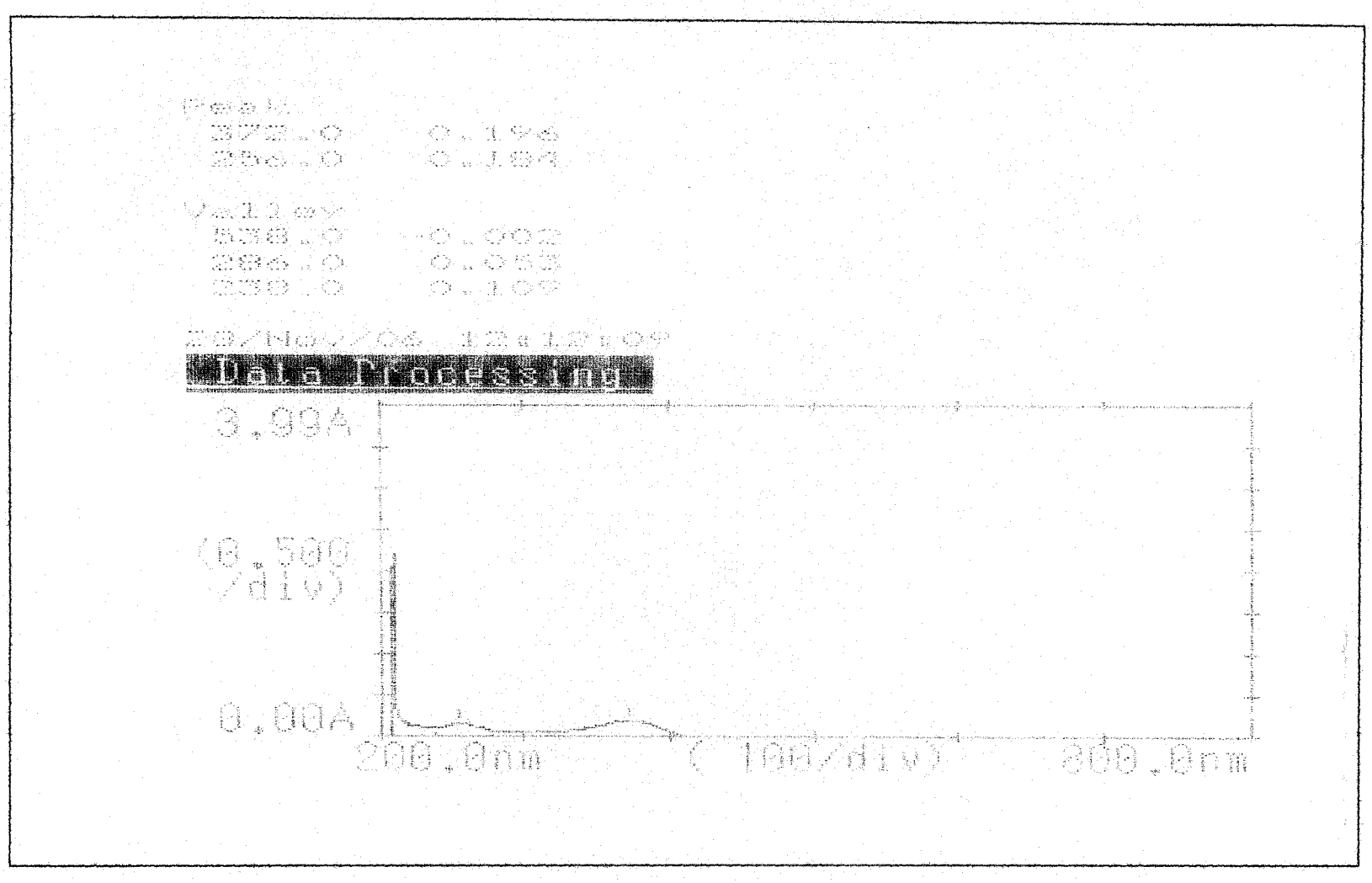


Figure 3.6. UV-Visible Spectro photometric analysis of compound II from *E. cannabinum*.

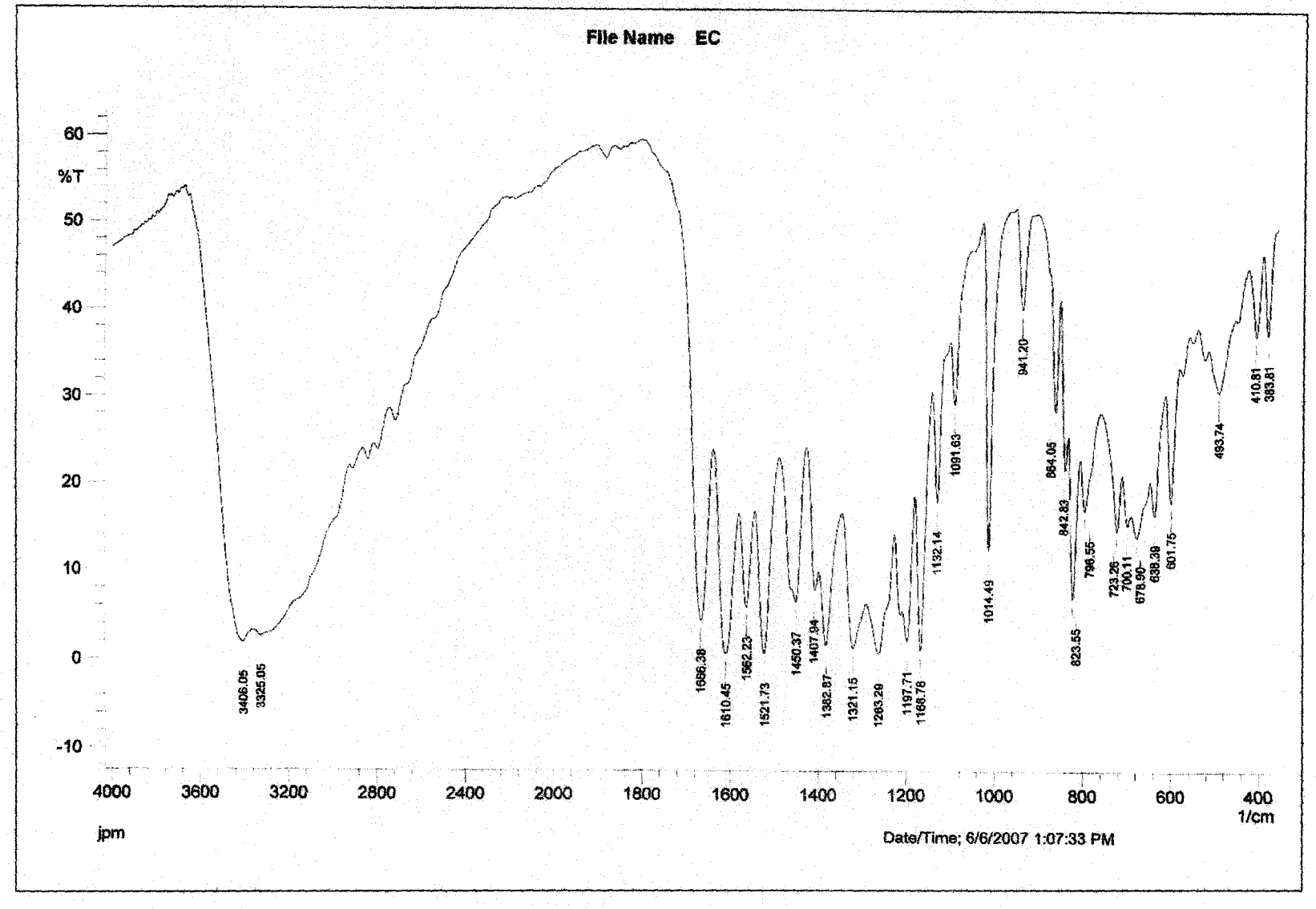


Figure 3.7. IR spectrum of compound II isolated from *E. cannabinum*.

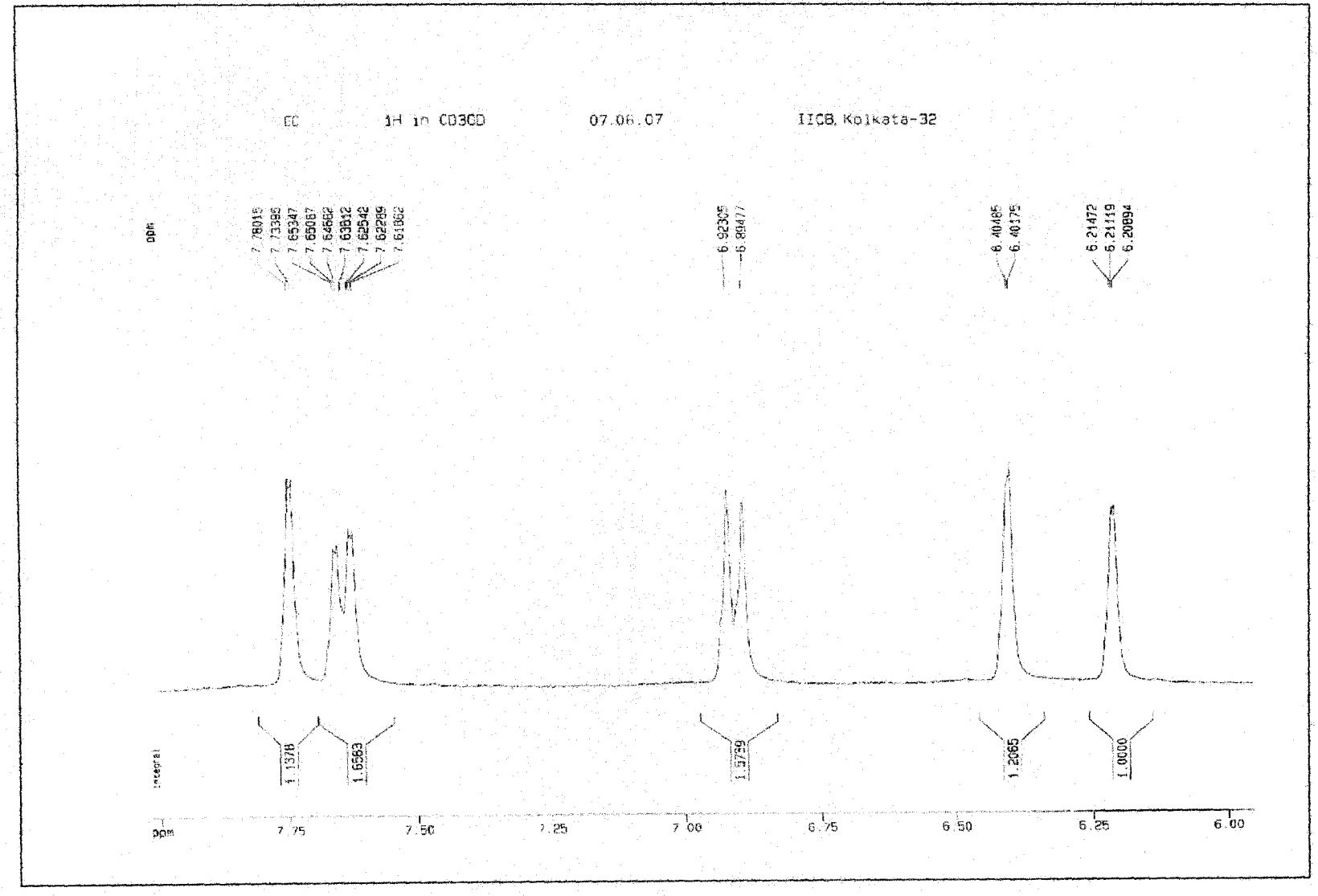


Figure 3.8. ^{1}H NMR spectrum of compound II isolated from *E. cannabinum*.

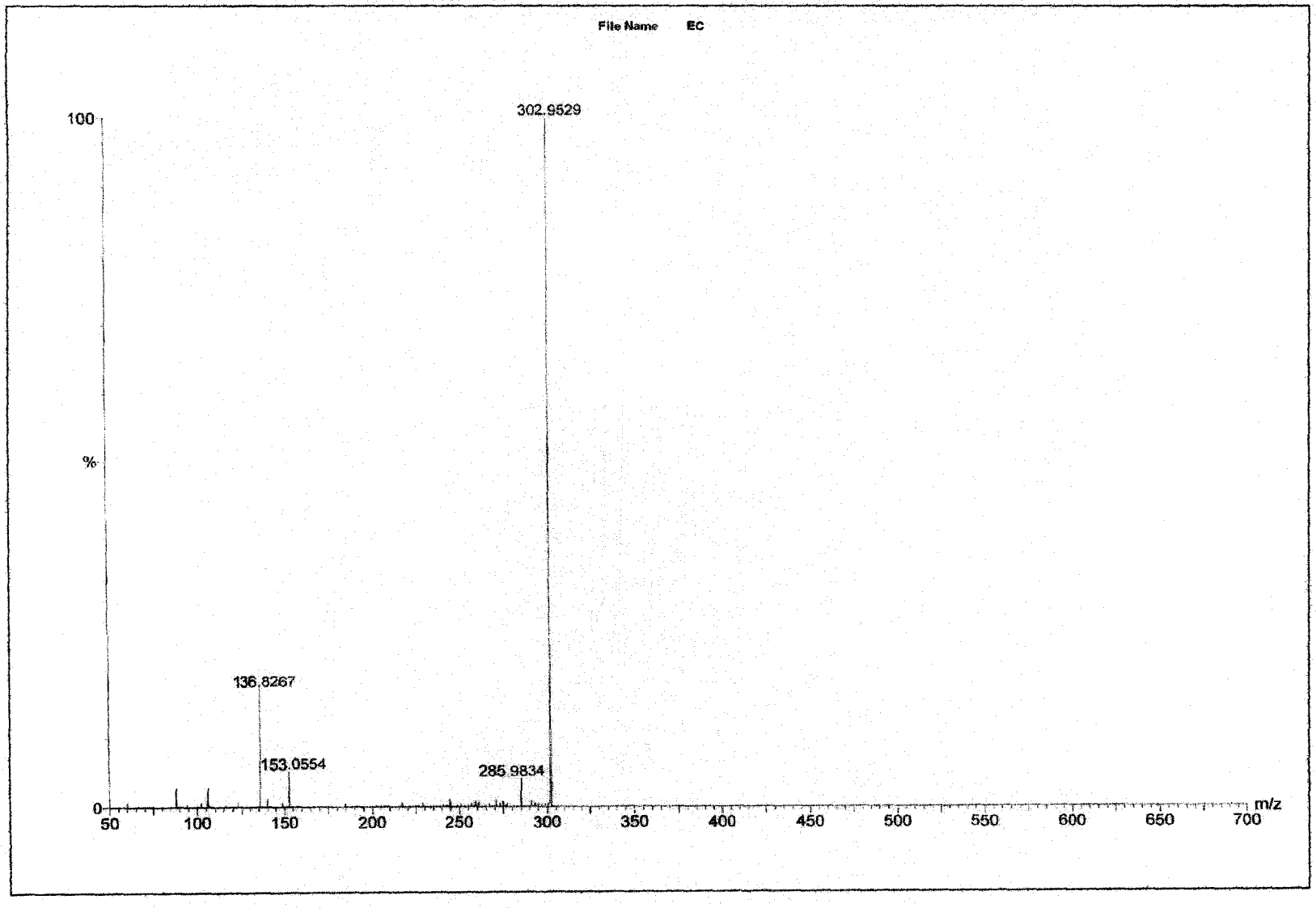


Figure 3.9. Mass spectrum of compound II isolated from *E. cannabinum*.

3.4. References

1. Farnsworth NR. Screening plants for new medicines. In: Biodiversity, Part II Edited by Wilson EO, National Academy Press, Washington, 1989, p 83-97.
2. Hostettmann K, Wolfender JL, Terreaux C. Modern screening techniques for plant extracts. *Pharm. Biol.*, 2001, **39**, 18-32.
3. Hostettmann K, Hamburger M, Hostettmann M and Marston A. New developments in the separation of natural products. *Modern Phytochem. Methods*, 1991, **25**, 1-32.
4. Hamburger M. Tracking bioactivity in plant extracts- New concepts and approaches. In: Society for Medicinal Plant Research 50 Years 1953–2003, A jubilee edition. Sprecher, E Eds. Wissenschaftliche Verlagsgesellschaft, Stuttgart, Germany, 2003, p 109-122.
5. Trease GE, Evans MC. Text book of Pharmacognosy. 12th edition. Balliere, Tindall, London, 1983, p 343-383.
6. Mukherjee PK. Quality control of herbal drugs, Ed, New Horizon, New Delhi, 2002, p 247-260.
7. Gan F and Ye R. New approach on similarity analysis of chromatographic fingerprint of herbal medicine. *J. Chromatogr.*, 2006, **1104**, 100-105.
8. Hasler A, Sitcher O and Meier B. 1992. Identification and determination of the flavonoids from *Ginkgo biloba* by high-performance liquid chromatography. *J. Chromatogr.*, 1992, **605**, 41-48.
9. Pietta P, Mauri P, Bruno A, Rava A, Manera E and Ceva P. Identification of flavonoids from *Ginkgo biloba* L., *Anthemis nobilis* L. and *Equisetum arvense* L. by high-performance liquid chromatography with diode-array UV detection. *J. Chromatogr.*, 1991, **553**, 223-231.
10. Vierling W, Brand N, Gaedcke F, Sensch KH, Schneider E and Scholz M. 2003. Investigation of the pharmaceutical and pharmacological equivalence of different Hawthorn extracts. *Phytomedicine*, 2003, **10**, 8-16.
11. Hostettmann K, Marston A and Hostettmann M. Preparative chromatography techniques: Applications in natural product isolation. Springer-Verlag, Berlin, Heidelberg, Germany, 1997, p 659.
12. Harsteen B. Flavonoids: A class of natural products of high pharmacological potency. *Biochem Pharmacol.*, 1983, **32**, 1141-1148.

13. David S. Studies force new view on biology of flavonoids. Adapted from a news release issued by Oregon State University, 07-03-2006.
14. Yamamoto C and Gaynor S. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. *Journal of Clinical Investigation*. 2001, **107(2)**, 135.
15. Middleton E. The Flavonoids. *Trends Pharmacol Sci* 1984, **5**, 335-338
16. Pollock JRA and Stevense R .Dictionary of organic compounds, vol-5, 4th ed., Eyre and Spottish woode, London. 1965, p 221.
17. Trease GE and Evans WC. Pharmacognosy, 12th ed., ELBS Publication, Baillier Tindall, East Bourne, 1996, p 344-352.
18. Plummer DI. An Introduction to Practical Biochemistry, 2nd ed., Tata Magraw-Hill Publishing Co. Ltd., New Delhi, 1985, p 136-143
19. Sherif EA, Gupta RK and Krishnamurti M. synthesis of chalcones, flavanones isolated from *Popowia cauliflora* and their analogues. *Agric. Biol. Chem.*, 1981, **45(2)**, 531-533.
20. Maheswar M, Rao K and Rao V. Isolation of new chalcone from the leaves of *Bauhinia variegata*. *Asian journal of chemistry*. 2006, **18**, 419-422.
21. Harborne, JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 2nd edn., Chapman and Hall, London, 1983, p 1-80, 210, 256
22. Geissman TA. The chemistry of Flavonoid compounds. Pergamon press, New York, 1962, p 32.
23. Markham KR. Techniques of Flavonoid identification, Academic Press, London, 1982, p 37-42
24. Moskuag JO, Carlson H, Myhrstad M. Molecular imaging of the biological effects of Quercetin and Quercetin-rich foods. *Mechanism of Ageing and Development.* 2004, **125**, 315.
25. Slavica B, Sandra S, Konstantinovic Z and Todorovic B. Flavonoids from flower of *Linum capitatum* kit. *Physics, Chemistry and Technology*, 2004, **3(1)**, 67-71.

CHAPTER - 4

ACUTE TOXICITY STUDIES

4.1. Introduction

Toxicology is the science that deals with the study of potential harmful effects of chemicals and drugs on living organisms. The word toxicology is derived from two words toxicon (poison) and logos (discourse) mean, study of poisons. The toxicologist, a specialist attained pharmacologist involved in the study of poisons, adverse or toxic effects of chemicals and drugs on animals and human beings. The scope of toxicology encompasses the qualitative determination of poison/chemicals, their deleterious (injurious) effects on the living organism, their incidence, mechanism, factors modifying them and reversibility (treatment) of such adverse effect[1].

Toxicology studies using animals and *in vitro* cellular or tissue preparations have been used to study the toxic effects and mechanism of action of drugs and chemicals and to determine the effective and safe dose of drugs in humans and the risk of toxicity from chemical exposures. Special attention should be paid to the solvent or dispersing agent of the drug as the toxic effect may arise from the solvent and animal care during the period of the toxicity tests is of paramount importance. Drugs that survive the initial screening and profiling procedure must be carefully evaluated for potential risks before clinical testing is begun[2].

The purpose of National Toxicology Program (NTP) is to identify toxic effects resulting from exposure to a particular chemical or agent and to characterize dose-response relationship[3]. Although chemicals and drugs can be evaluated for their toxic potential by using *in vivo* animal studies and *in vitro* models, it should be recognized that these testing procedures are only component in the process of evaluating the potential toxic risk of drugs and chemicals. The evaluation of the toxicity of drugs and chemicals should include, when possible, data obtained from a number of investigative approaches[4,5], i.e. secular trend or ecological trend analysis[6], animal studies[7], pharmacokinetic, toxicokinetic, pharmacodynamic, and toxic dynamic studies[8], mechanism of action (MOA) studies[9], and basic science studies that pertain specifically to the studies, which include receptor affinity, cytotoxicity, genotoxicity, organ toxicity, neurotoxicity[10,11] etc.

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[12]. 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[13]. Therefore, the median lethal dose is now estimated from the smallest number of animals possible[14].

In screening drugs determination of LD_{50} (the dose which has proved to be lethal to 50% of the tested group of animals) is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance. It is an initial assessment of toxic manifestations (provides information on health hazards likely to arise from short term exposure to drugs), and is one of the initial screening experiments performed with all compounds. Data from the acute study may

(a) serve as the basis for classification and labeling.

(b) provide initial information on the mode of the toxic action of a substance.

(c) help to arrive at a dose of a new compound

(d) help to determine LD_{50} values that provide many indices of potential types of drug activity.

Aim of acute toxicity test is to determine the therapeutic index i.e. ratio between the lethal dose and the pharmacologically effective dose in the same strain and species (LD_{50}/ED_{50}).

Greater the index, safer is the compound. LD_{50} with confidence limits is to be established on one common laboratory species such as mouse/rat using the standard method. The LD_{50} dose thus found was administered to guinea pigs, rabbits, cats or dogs on weight basis (on basis of relative surface area gives better results).To determine the absolute dose for a species in the column, the absolute dose given to the species in a row was multiplied by the factor given at intersection of the relevant row and column. Because of species variation, several species of animals (one rodent and one non rodent) were used to determine LD_{50}. When a clearly different response was observed in any of these species, a larger number of that species needs to be tested to establish the approximate LD_{50} value. Thus we suggest a method of acute toxicity testing and calculation of LD_{50}

that has to go through the entire process of validation with different categories of substances before its final acceptance by the regulatory bodies[15].

4.1.1. The median lethal dose (LD_{50})

The median lethal dose or LD_{50} is the dose which would be expected to kill one-half of a group of test animals of same species and strain. The LD_{50} dose is usually expressed as milligrams or grams of material per kilogram of animal body weight (mg/kg or g/kg). The material may be administered by mouth or applied to the skin. The median effective dose (ED_{50}) is the dose (mg/kg body weight), which produces a desired therapeutic action in 50 percent of the test population[16]. In this present study lethal dose (LD_{50}) of the methanol extracts of *K. rotunda* rhizome and *E. cannabinum* leaves has been determined by oral route of administration on mice.

4.2. Materials and methods

4.2.1. Plant materials

Methanol extracts of rhizomes of *Kaempferia rotunda* Linn. and leaves of *Eupatorium cannabinum* were used as test drug in these experiments and sodium CMC solution was used as control vehicle.

4.2.2. Experimental animals

Swiss albino mice of either sex weighing between 20-25 g were used for the study. They were housed in polypropylene cages maintained under standardized environmental conditions and had free access to food and water. Ethical clearance was obtained from Institution Animals Ethical Committee for using animals in the present study method. Experiment was conducted in accordance to the guidelines provided by committee for the control and supervision of experiments on animals.

4.2.3. Methods of evaluation

Ten groups of animals were used taking ten animals in each group. Different doses of methanol extract suspended in1% w/v sodium CMC solution was administered orally to nine groups of animals. One group of animal was treated as control and was fed with 1% solution of sodium CMC. The method of Lorke[17] was followed to determine the acute toxicity of the extracts. The animals were kept under observation in open field condition

for 72 hrs after the administration of extracts of rhizome and leaves in oral route as mentioned earlier, and the number of deaths and signs of clinical toxicity were recorded. The toxicological effect was assayed on the basis of mortality, which was expressed as an LD_{50} value, which was calculated by the graphical method of Miller and Tainter[18].

In the groups with no dead animals and the groups with only dead animals, the obtained percentages were corrected using the following formula:

Correction formula for 0% dead group = 100 (0.25/n)
Correction formula for 100% dead group = 100 [(n-0.25)/n]
where 'n' represents the number of animals in the group.
After correction, the percentages were converted into probits referring to the probit table. The values thus obtained were plotted against log dose. The dose corresponding to 50% or probit 5 was taken as LD_{50}. Probit 5 on the Y-axis is interpolated to the X-axis to get log LD_{50}, the antilogarithm of which gives LD_{50} (Fig. 4.1. and 4.2.).

Similarly acute toxicity studies were carried out for isolated compounds of *K. rotunda* and *E. cannabinum* with six groups of animals (four animals in each group) by using UP down or Stair case method[19]. Different doses of isolated compound I (150-325 mg/kg b.w) and compound II (150-250 mg/kg b.w) suspended in1% w/v sodium CMC solution were administered orally to five groups of animals. One group of animal was treated as control. The numbers of death in each group within 24h were recorded. The LD_{50} was estimated by the graphical method of Miller and Tainter[18].

4.3. Results

The results of the acute toxicity studies of methanolic extracts of rhizomes of *Kaempferia rotunda* and leaves of *Eupatorium cannabinum* have been presented in table & fig. 4.1, and 4.2 respectively. The LD_{50} of methanol extract of rhizomes of *K. rotunda* was found to be 5g/kg body weight where as the LD_{50} of the methanol extract of *E. cannbinum* leaf was found to be 4.5g/kg body weight by oral route. The LD_{50} of isolated compound I was found to be 213.7mg/kg body weight (Table & Fig. 4.3) where as the LD_{50} of the isolated compound II was found to be 196.3mg/kg body weight (Table & Fig. 4.4) by oral route. The doses were arbitrarily chosen which, was less than $1/10^{th}$ of the median lethal dose[20,21].

Table 4.1. Determination of LD_{50} of methanol extract of *K. rotunda* rhizome after oral administration in mice

No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	500	2.69	10	0	0	2.5	3.04
2	1500	3.17	10	1	10	10	3.72
3	2500	3.39	10	2	20	20	4.16
4	3000	3.47	10	3	30	30	4.48
5	4000	3.60	10	4	40	40	4.75
6	5000	3.69	10	5	50	50	5.00
7	5500	3.74	10	7	70	70	5.52
8	6000	3.77	10	8	80	80	5.84
9	6500	3.81	10	10	100	97.5	6.96

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 4.2. Determination of LD_{50} of methanol extract of *E. cannabinum* leaves after oral administration in mice

No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	500	2.69	10	0	0	2.5	3.04
2	1000	3.00	10	1	10	10	3.72
3	2000	3.30	10	2	20	20	4.16
4	3000	3.47	10	3	30	30	4.48
5	4000	3.60	10	4	40	40	4.75
6	4500	3.65	10	5	50	50	5.00
7	5000	3.69	10	7	70	70	5.52
8	5500	3.74	10	9	90	90	6.28
9	6000	3.77	10	10	100	97.5	6.96

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 Isolated Compound I after oral administration in mice

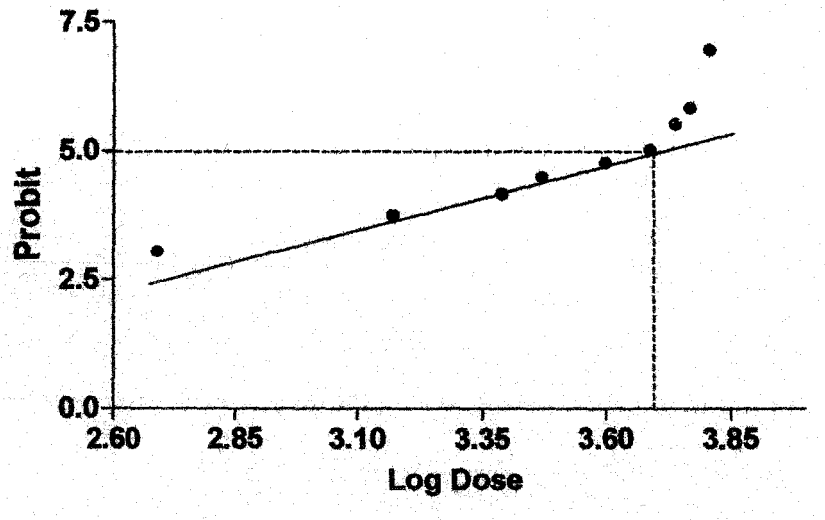
No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	150	2.17	4	0	0	6.25	3.45
2	175	2.24	4	1	25	25	4.33
3	225	2.35	4	2	50	50	5.00
4	275	2.43	4	3	75	75	5.67
5	325	2.51	4	4	100	93.75	6.55

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 4.4. Determination of LD_{50} of Isolated Compound II after oral administration in mice

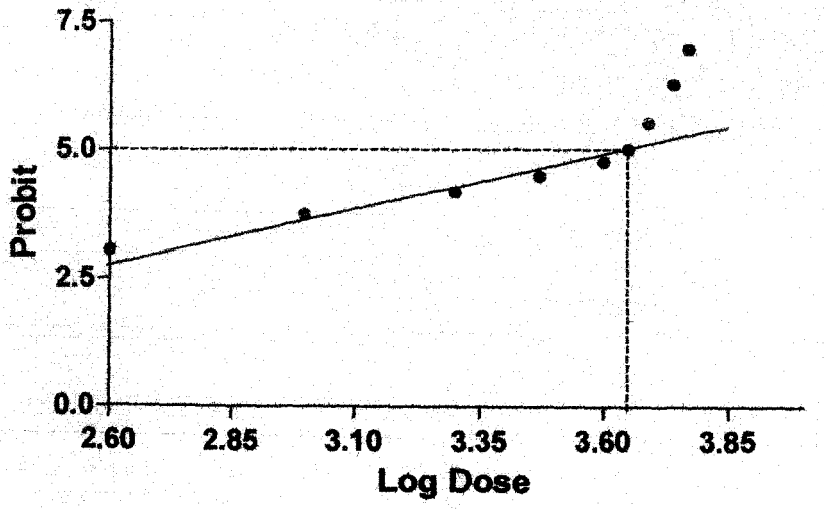
No. of Group	Dose (mg/kg body wt.)	Log dose (X)	No of animals used	No of animals dead	Dead (%)	Corrected dead (%)	Probit (y)
1	150	2.17	4	0	0	6.25	3.45
2	175	2.24	4	1	25	25	4.33
3	200	2.30	4	2	50	50	5.00
4	225	2.35	4	3	75	75	5.67
5	250	2.39	4	4	100	93.75	6.55

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.



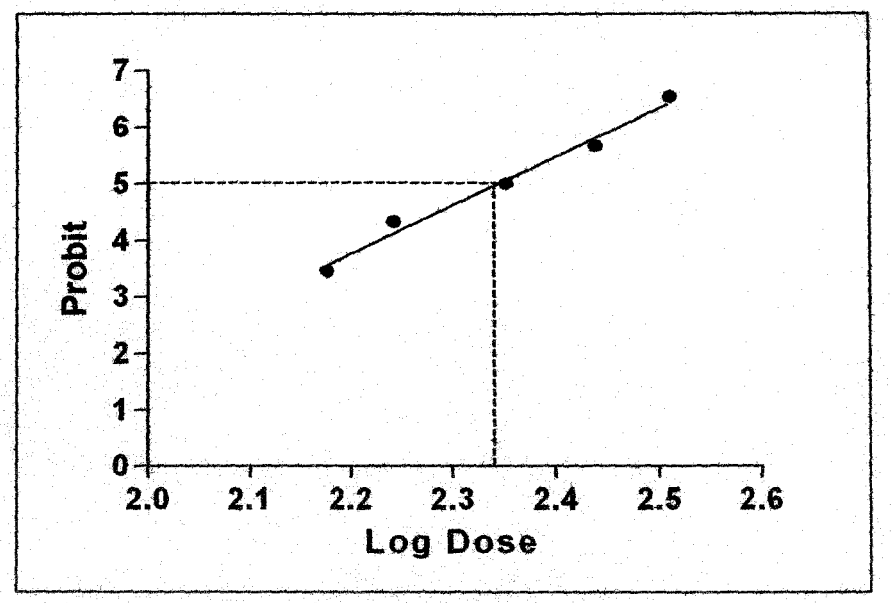
LD_{50} = Log 3.699 = 5000 mg/kg

Fig. 4.1. Probit vs Log dose plot for methanolic extract of *K. rotunda*



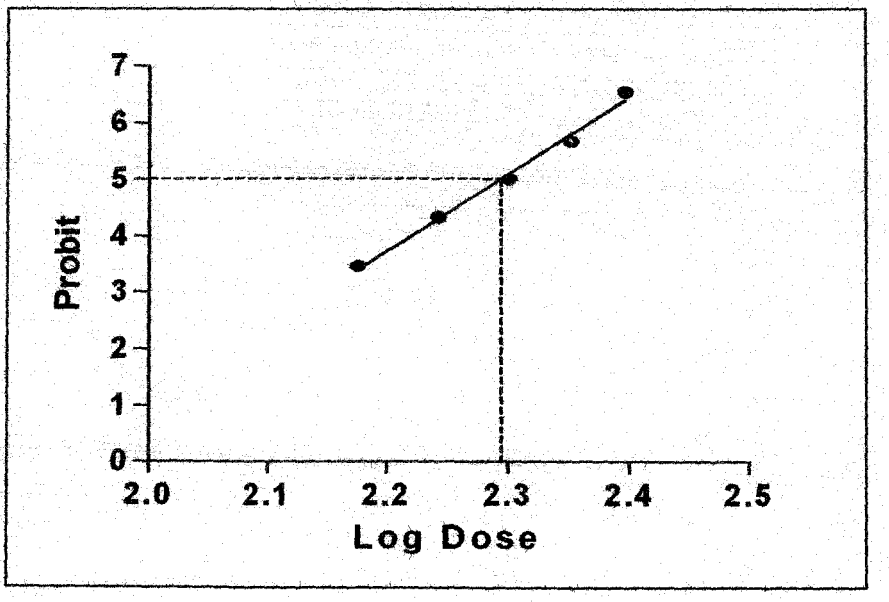
LD_{50} = Log 3.653 = 4497.79 mg/kg

Fig. 4.2. Probit vs Log dose plot for methanolic extract of *E. cannabinum*



LD_{50} = Log 2.33 = 213.7 mg/kg

Fig. 4.3. Probit vs Log dose plot for Compound I



LD_{50} = Log 2.29 = 196.3 mg/kg

Fig. 4.4. Probit vs Log dose plot for Compound II

4.4. References

1. Kulkarni SK. *Hand book of Experimental Pharmacology*. 3rd edn., Vallav Prakashan, New Delhi, 2005, p 169.
2. Robert L and Brent MD. Utilization of animal studies to determine the effects and human risks of environmental toxicants (Drugs, Chemicals, and Physical Agents). *Pediatrics*. 2004, **113(4)**, 984-995.
3. Clewell HJ and Andersen ME. Risk assessment extrapolations and physiological modeling. In: Stara JF, Erdreich LS, (Eds), Advances in health risk assessment for systemic toxicants and chemical mixtures, Toxicology and industrial health. Princeton Scientific Publishers, Princeton, NJ, 1985, p 111-113.
4. Brent RL. Evaluating the alleged teratogenicity of environmental agents. In: Brent RL, Beckman DA, (Eds), Clinics in Perinatology. WB Saunders, Philadelphia, PA, 1986, p 609.
5. Brent RL. Protecting the public from teratogenic and mutagenic hazards. *J. Clin. Pharmacol.*, 1972, **12**, 61-70.
6. Brent RL. Drug testing in animals for teratogenic effects: thalidomide in the pregnant rat. *J. Pediatr.*, 1964, **64**, 762-770.
7. Brent RL. The prediction of human diseases from laboratory and animal tests for teratogenicity, carcinogenicity and mutagenicity. In: Lasagna L, (Eds), Controversies in Therapeutics, WB Saunders, Philadelphia, PA, 1980, p 134–150.
8. Brent RL. Predicting teratogenic and reproductive risks in humans from exposure to various environmental agents using *in vitro* techniques and *in vivo* animal studies. *Congenit. Anom. Kyoto.*, 1988, **28**, 41-55.
9. Christian MS, Brent RL. Teratogen update: Evaluation of the reproductive and developmental risks of caffeine. *Teratology*, 2001, **64**, 51-78.
10. Brent RL. Book review: Scientific Frontiers in Developmental Toxicology and Risk. Assessment by the National Research Council, National Academy of Sciences, National Academy Press, Washington, DC, 2002, p 88-96.

11. Brent RL. Teratogen update: reproductive risks of leflunomide (Avara); a pyrimidine synthesis inhibitor: counseling women taking leflunomide before or during pregnancy and men taking leflunomide who are contemplating fathering a child. *Teratology*. 2001, **63**, 106-112.

12. Kingk W. Commission of the European communities, Industrial Health and safety; quality assurance of toxicological data; Proceeding of the international colloquium Luxembourg. *Report Eur.*, 1979, **89**, 7270.

13. Malmford T and Teiling A. LD_{50} its value for the pharmaceutical industry in safety evaluation of drugs. *Acta. Pharmacol. and Toxicol.*, 1983, **52(2)**, 229.

14. Katzung BG. Basic and Clinical Pharmacology, 7th edn., Appleton & Lange, Stamford, Connecticut, U.S.A., 1998, p 62-67.

15. Ghosh MN. Toxicity studies. In: Fundamentals of Experimental pharmacology, Scientific Book Agency, Hilton & Company, Calcutta, 1984, p 153-158.

16. Satoskar RS and Bhandakar SD. Pharmacology and pharmacotherapeutics. In: Dose response relationship, Satoshker RS, (Ed), 16th edn., Popular Prakashana, 1978, Bombay, India, p 30-34.

17. Lorke D. A new approach to practical acute toxicity testing. *Arch. Toxicol.*, 1983, **54**, 275-284.

18. Miller LC and Tainter ML. Estimation of the ED50 and its error by means of logarithmic-probit graph paper. *Proc. Soc. Expt. Biol. Med.*, 1944, **57**, 261.

19. Ghosh MN. Fundamentals of Experimental Pharmacology, 2nd edn., Scientific Book Agency, Kolkata, 1984, p 156-157.

20. Paget GE, Barnes JM. Evaluation of drug activity. In: Laurence DA and Bacharach AL, Eds., Pharmacometrics, Academic Press, New York, 1983, p 1-115.

21. Patro CP, Sahu PK, Barik BB. Antipyretic and wound healing activity of aqueous extract of leaves of *Vitex pinata*. *Indian Drugs*, 2007, **44(7)**, 532-534.

CHAPTER - 5

STUDIES ON ANTIOXIDANT ACTIVITY

5.1. Introduction

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxy nitrite radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system; however, oxidative stress is generated when equilibrium favors free radical generation as a result of a depletion of antioxidant levels. Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, such as cancer, cardiovascular diseases, neurodegenerative disorders and ageing[1]. Nitric oxide (NO) a diatomic free radical, is synthesized in biological systems by constitutive and inducible nitric oxide synthase (cNOS) and (iNOS)[2]. Excess generation of NO by NOS has been found to contribute too many diseases, such as carcinogenesis, septic shock, cerebral injury, atherosclerosis, rheumatoid arthritis, cell apoptosis and necrosis[3]. Moreover, under pathological conditions, macrophages can greatly increase their production of both NO and superoxide anion simultaneously to form a peroxy nitrite anion ($ONOO^-$), which is more toxic than O^{-2} or NO to biological systems, by causing modification of proteins[4] or nucleic acid[5]. Thus, reducing the NO generation in excess amounts is now accepted widely as an important goal for the chemoprevention of various diseases, e.g., cancer. Search for natural antioxidants, especially from plant sources, as a potential preventive intervention for free-radical mediated diseases has already turned into an attractive research field and a very important social issue for improvement of the quality of human life. Polyphenols, including phenolic acids, flavonoids, tannins, lignins and others are widespread in plant foods and in different medicinal plants. Plant phenolics may function as potent free radical scavengers, reducing agents, quenchers of ROS and protect against lipid peroxidation[6].

Free radicals and oxygen derivatives are constantly generated *in vitro* both by accident of chemistry and specific metabolic purposes. The reactivity of free radicals varies with many to cause inflammation or even severe damage to biological molecule, especially to DNA, lipids and proteins. The generation of free radicals during the metabolic process is now observed to be responsible for wide range of human condition such as ageing, cancer, atherosclerosis, arthritis, viral infection, stroke, myocardial infraction, pulmonary

condition, inflammatory bowel disease, neurodegenerative disease and others may be produced by reactive oxygen species (e.g., oxygen free radicals, hydrogen peroxide). The action of free radicals is counteracted by free radicals either endogenous or exogenous[7].

The term oxygen free radical refers to the form of oxygen exhibiting high reactivity and having at least one unpaired electron. However, other reactive forms of O_2 are also known as non-free radical. Both these forms are collectively referred to as active Oxygen Species (ROS) and include singlet oxygen, hydrogen peroxide, hydroxyl radical[8]. They are produced continuously by ionizing radiation as well as by the byproduct of cellular metabolism, which are toxic and are important factor for several pathological conditions such as diabetes, inflammation and cancer[9,10].

Free radicals are a normal product of metabolism; the body produces its own antioxidants (e.g., the enzyme superoxide dismutase) to keep them in balance. However, stress, ageing, and environmental sources such as polluted air and cigarette smoke can add to the number of free radicals in the body, creating an imbalance. The highly reactive free radicals can damage healthy DNA and have been linked to changes that accompany ageing (such as age-related muscular degeneration, a leading cause of blindness in older people) and with disease processes that lead to cancer, heart disease and stroke[11].

Antioxidants act as a major defense against radical mediated toxicity by protecting the damage caused by free radicals. Potential antioxidant therapy should therefore include either natural antioxidant enzymes or agents, which are capable of augmenting the function of this oxidative free radical scavenging enzymes[12].

Antioxidants are molecules that slow or prevent the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols[13].

In biological systems, the normal processes of oxidation produce highly reactive free radicals. Antioxidants work by binding to the free radicals, they transforms them into non-damaging compounds or repairs cellular damage. They are able to easily donate electrons to molecules that need of an electron, such as free radicals, before they steal one

from some place else, thus stabilize and prevent a damaging chain reaction. The antioxidant molecules interact with the oxygen free radicals and halt the spread of cancer causing cells with damaged DNA before other vital molecules are damaged and they neutralize the production of free radicals which are chemical complexes that cause harm to our cells and play a major role in the disease process.[14]

Antioxidants help alleviate the symptoms and side effects of many of these diseases. According to the free radical theory, radicals damage cells in an organism, causing ageing. Antioxidants break the free radical chain reaction by sacrificing electrons, and then humbly existing without stealing more. The body naturally circulates many nutrients for their antioxidant properties, and creates antioxidant enzymes just for the purpose of controlling free radicals and their chain reactions. Antioxidants are thought to thwart heart disease by preventing oxidation. Antioxidants combat chronic inflammation. Vitamin E suppresses platelet stickness, acting as an anticoagulant to discourage the formation of clots that lead to heart attacks. Vitamin C decreases a blood factor needed to build clots. Antioxidants fight cancer by neutralizing DNA-damaging free radicals. Antioxidants neutralize free radicals as the natural by-product of normal cell processes and prevent injury to blood vessel membranes, helping to optimize blood flow to the heart and brain, defend against cancer-causing DNA damage, and help to lower the risk of cardiovascular disease and dementia, including Alzheimer's disease. Antioxidants can cancel out the cell-damaging effects of free radicals[15] and people who eat fruits and vegetables rich in polyphenols and anthocyanins have a lower risk of cancer, heart disease and some neurological diseases[16]. This observation suggested that these compounds might prevent conditions such as macular degeneration[17], suppressed immunity due to poor nutrition[18] and neurodegeneration, which are caused by oxidative stress[19].

Antioxidants are found in varying amounts in foods such as vegetables, fruits, grain cereals, legumes and nuts. Some antioxidants such as lycopene and ascorbic acid can be destroyed by long-term storage or prolonged cooking[20,21]. Other antioxidant compounds are more stable, such as the polyphenolic antioxidants in foods, whole-wheat cereals and tea[22,23]. In general, processed foods contain less antioxidant than fresh and uncooked foods, since the preparation processes may expose the food to oxygen[24].

Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxy nitrite[25,26]. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Several authors demonstrated that antioxidant intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks[27-29]. As well as antioxidant defense system of our body, antioxidants that are mainly supplied as dietary consumptions can also impede carcinogenesis by scavenging oxygen radicals or interfering with the binding of carcinogens to DNA which includes vitamin C, vitamin E (tocopherol), carotenoids (carotene, cryptoxanthin, lutein, zeaxanthin, lycopene) and several polyphenolic compounds including flavonoids (catechins, flavonols, flavones, isoflavonoids[30,31]). In particular, phenolic compounds have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals. The commercial development of plants as sources of antioxidants to enhance health and food preservation is of current interest[32].

Plant extract screening studies pointed out further investigations, as several promising plant materials with strong free radical scavenging properties showed up. Plants are the rich source of compounds, which may protect organism from free radical injury and disease[33].

The aim of the present study is to determine the antioxidant activity of the methanol extract of *K. rotunda* Linn. and *E. cannabinum* Linn. For this purpose, plant extracts were tested for antioxidant activity and their capacity to reduce lipid peroxidation by using malonaldehyde (MDA) and 4-hydroxy 2-nolnel (4-HNE) as the model molecule.

5.2. Materials and methods

5.2.1. Plant materials

Methanol extracts of *K. rotunda* rhizomes and *E. cannabinum* leaves were used as test drug in these experiments. The crude extracts were mixed with DMSO to prepare the stock solution (10µg/10ml). The test samples were prepared from stock solution by serial dilution to attain the concentrations of 100, 200, 500 and 1000µg/ml.

5.2.2. Drugs and chemicals

Thiobarbituric acid (TBA) was obtained from Loba Chemie, India. 2, 4 dinitrophenyl hydrazine (DNPH) and 1,1,3,3-tetramethoxy propane (TEP) were obtained from Sigma Chemicals, USA. Dimethyl sulphoxide (DMSO), ethylenediamine tetra acetic acid (EDTA), ferrous sulphate, trichloroacetic acid (TCA), hydrogen peroxide, ascorbic acid, potassium dihydrogen phosphate, potassium hydroxide, hexane, methanol and HCl were of analytical grade and obtained from Ranbaxy Fine Chemicals. 4-HNE (4 hydroxy- 2 noneal) was obtained from Ranbaxy Ltd. as gift sample. All other chemicals and reagents used were of analytical grade.

- **Preparation of 5 mM DNPH**

100 mg of DNPH was dissolved in 100 ml of 0.5 M HCl and stirred for approximately 1 hour at room temperature. It was filtered to remove undissolved DNPH and stored in an amber coloured bottle.

- **Preparation of 0.5 M HCl**

21ml conc. HCl was added to 479ml of water to produce 500ml of 0.5 M HCl.

- **Preparation of 4-HNE (4 hydroxy-2 noneal) primary standard**

1mg of 4-HNE (6.4 µmole) was dissolved in 6.4 ml of methanol to give a 1mM solution. Diluting 100µl of the solution to 5ml in methanol in a volumetric flask the solution was standardized and the absorbance was measured at 220nm using methanol as blank. The concentration of 4-HNE in the primary standard was calculated using the following equation.

4-HNE= (A_{220} / 13750) X 50

Where A_{220} is the absorbance at 220nm

'13750' is the molar extinction co-efficient for 4-HNE in methanol and 50 is the dilution factor.

5.2.3. Preparation of goat liver homogenate

The liver was chosen to estimate the markers of lipid peroxidation because the metabolites from liver may diffuse into various extra hepatic tissue causing lipid peroxidation and cellular damage[34]. The goat liver was purchased from local market and collected in phosphate buffer (pH-7.4). The liver was homogenized in 100ml of

phosphate buffer and filtered to get clear homogenate. This homogenate was used to estimate the lipid peroxidation markers. Necessary steps were taken as much as possible to simulate the conditions of *in vitro* system.

5.3. *In Vitro* antioxidant activity of rhizomes of *K. rotunda* Linn.

5.3.1. Lipid peroxidation model on MDA marker suppression activity

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[35]. Goat liver was purchased from local market. 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 10min. To study the comparative response, the experiment was performed in six conical flasks. Liver homogenate (3ml) was aliquoted to each of the flask. The flask number one and six were treated as control and standard where buffer and vitamin C were added respectively. To the no. two to five flasks different concentrations of methanol extract (100, 200, 500 and 1000µg/ml) were added respectively. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate in six flasks[36]. After 30min, 1.5ml of each reaction mixture was taken into centrifuge tubes containing 1.5ml of 10% TCA. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% TBA. The mixture was heated in a water bath at 85°C for 30min and in a boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 530nm in a spectrophotometer (Shimadzu 1601). The TBARS concentration was calculated by using standard graph[37,38].

5.3.2. Lipid peroxidation model on 4-HNE suppression activity

The liver supernatant was prepared as the procedure mentioned in 5.2.6. The liver homogenate was divided into six conical flasks containing 3ml in each. The flask number one and six were treated as control and standard where buffer and vitamin C were added respectively. To the no. two to five flasks, different concentrations of methanol extract (100, 200, 500, 1000µg/ml) were added. All the conical flasks were placed in mechanical shaker. After two hours the liver sample was transferred to the centrifuge tubes (3 for

each set i.e. control and so on). 2ml of TCA was added to all the centrifuge tubes and centrifuged for 30 minutes then the supernatant was filtered and 2ml of filtrate was taken in a 13x100mm glass tube with cap. 1ml of DNPH was added to all the tubes containing liver supernatant, mixed and set aside for 1h to react with 4-HNE. Then the formed adduct of 4-HNE and DNPH was extracted by hexane, which was evaporated under argon at 40°C. After cooling, 2ml of methanol was added to all the samples and the absorbance was measured at 350nm in the spectophotometer[39]. The quantity of 4-HNE was calculated by linear regression analysis[40].

5.3.3. Preparation of standard calibration curves

- **Standard curve for the estimation of MDA**

Different aliquots from TEP solutions were taken graduated stopper (10ml.) test tube at volume of each solution was made up to 5ml. TBA reagent (5ml) was added and the mixtures were heated in a steam bath for 30 min. to develop pink coloured solution. The solutions were cooled to room temperature and absorbances were noted at 530nm using TBA reagent as blank. A curve was obtained by taking conc. vs absorbance (Fig. **5.5**).

The quantity of MDA was calculated by using the following equation:
μM of MDA = 6.578×10^{-3}/A. where A is the absorbance at 530nm.

- **Standard curve for the estimation of 4-HNE**

2ml of 4-HNE sample (300, 400, 500 and 600μl) was pipette out and transferred into a 13x100 mm glass tube with cap. 3.1ml of DNPH reagent was added to all the samples and mixed by cyclomixer and allowed to react at room temperature for 1h. The samples were extracted with 2ml aliquots of hexane combining the extracts in a glass tube. Hexane portion was evaporated under argon at 40°C and reconstituted with methanol. The absorbance was measured at 350nm. The standard calibration curve was plotted between absorbance and concentration of 4-HNE, which is shown in fig. **5.6**. A linear regression analysis was performed to determine the slope and intercept of the calibration line.

The quantity of 4HNE is calculated based on the results of the linear regression analysis using the following equation.

nmole of 4-HNE = (4-HNE peak area – intercept) / slope

5.4. *In vitro* antioxidant activity of leaves of *E. cannabinum* Linn.

The antioxidant activity of methanol extract of *E. cannabinum* leaves was determined as per the methods described above in **5.3.1** and **5.3.2** for *K. rotunda* Linn.

5.5. Results

The experimental study was based on the estimation of MDA and 4-HNE and their suppression by the extracts of *K. rotunda* Linn. and *E. cannabinum* Linn. which is presented in table 5.1, 5.2, 5.3, 5.4 respectively. From the experimental results, it has been proved that the methanolic extract of both the plant has significant antioxidant activity. This study also confirmed that the lipid peroxidation was significantly controlled at low concentration of extracts of *K. rotunda* Linn. The quantification of MDA and 4-HNE can be directly correlated with the lipid peroxidation inhibition capacity of the extract. The toxic radicals quantification is also an indicator to monitor the overall progress of polyunsaturated fatty acid oxidation. The antioxidant activity of *K. rotunda* Linn. was studied by dose dependent manner. From the fig. **5.1** and **5.2**, it was concluded that the significance of antioxidant activity has inverse relationship with dose i.e. high at low dose and vice versa. The extract at 100µg/ml and 200µg/ml has significant and moderate antioxidant activity respectively but 500 and 1000µg/ml has insignificant activity. From the fig. **5.3** and **5.4** the antioxidant activity has been observed in higher doses in case of *E. cannabinum* Linn. The dose of 100µg/ml and 200µg/ml has insignificant activity whereas 500µg/ml and 1000µg/ml has moderate and significant antioxidant activity respectively. The antioxidant activity of extracts was compared with standard antioxidant (ascorbic acid). The results were analyzed statistically and found significant at $P<0.05$ levels.

From the clinical point of view, the free radicals and lipid derived metabolites have been strongly implicated in the pathology of various diseases such as myocardial infarction, diabetes mellitus, hepatic injury, atherosclerosis, rheumatoid arthritis and cancer. The free radicals i.e. MDA and 4-HNE have strong link with the above-mentioned diseases. The suppression of the radicals by the methanolic extract of rhizomes of *Kaempferia rotunda* and leaves of *Eupatorium cannabinum* in the above study has thus been correlated with the antioxidant potential of the plant and this information can be used to control the age dependent diseases mentioned above.

Table 5.1. *In vitro* antioxidant activity of *K. rotunda* Linn. on lipid peroxidation by MDA model

Flask no.	Concentration of extract (µg/ml)	Concentration of MDA (µM)	Average of MDA ($\bar{x}$ ± S.E)
1	Control (Phosphate buffer pH 7.4)	18.97 19.13 18.52	18.87±0.18
2	100	14.02 14.59 14.71	14.44±0.21
3	200	16.96 16.57 17.05	16.86±0.15
4	500	17.28 17.47 17.13	17.29±0.09
5	1000	17.97 17.81 18.08	17.95±0.08
6	Standard Ascorbic acid (250mg/kg body wt.)	13.95 13.17 13.59	13.57±0.22

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$

Table 5.2. *In vitro* antioxidant activity of *K. rotunda* Linn. by 4-HNE model

Flask no.	Concentration of extract (µg/ml)	Concentration of 4-HNE (nM)	Average of 4-HNE ($\bar{x} \pm$ S.E)
1	Control (Phosphate buffer pH 7.4)	97.29 97.92 97.56	97.59±0.18
2	100	82.45 83.09 83.17	82.90±0.23
3	200	89.18 88.72 89.05	88.98±0.13
4	500	93.12 94.09 93.53	93.58±0.28
5	1000	95.32 94.87 95.09	95.09±0.13
6	Standard Ascorbic acid (250mg/kg body wt.)	75.52 75.86 76.08	75.82±0.16

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$

Table-5.3. *In vitro* antioxidant activity of *E. cannabinum* Linn. on lipid peroxidation by MDA model

Concentration of extract (µg/ml)	Concentration of MDA(µM)	Average of MDA ($\bar{x}$ ± S.E)
Control (Phosphate buffer pH 7.4)	18.97 19.13 18.52	18.87±0.18
100	18.01 17.75 17.85	17.87±1.89
200	16.95 17.01 17.09	17.01±0.63
500	16.32 16.35 16.32	16.33±0.036
1000	15.20 15.21 15.22	15.21±0.56
Standard Ascorbic acid (250mg/kg body wt.)	13.95 13.17 13.59	13.57±0.22

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$.

Table-5.4. *In vitro* effect of *E. cannabinum* by 4-HNE model

Concentration of extract (µg/ml)	Concentration of 4-HNE(nM)	Average of 4-HNE ($\bar{x}$ ± S.E)
Control (Phosphate buffer pH 7.4)	97.29 97.92 97.56	97.59±0.18
100	81.04 81.66 82.27	81.65±0.35
200	79.82 80.33 80.74	81.04±0.35
500	79.20 79.82 80.43	79.81±0.35
1000	77.67 77.06 77.36	77.36±0.17
Standard Ascorbic acid (250mg/body wt.)	75.52 75.86 76.08	75.82±0.16

$\bar{x}$ = Mean concentration

S.E. = Standard error

The values are significant at $P < 0.05$.

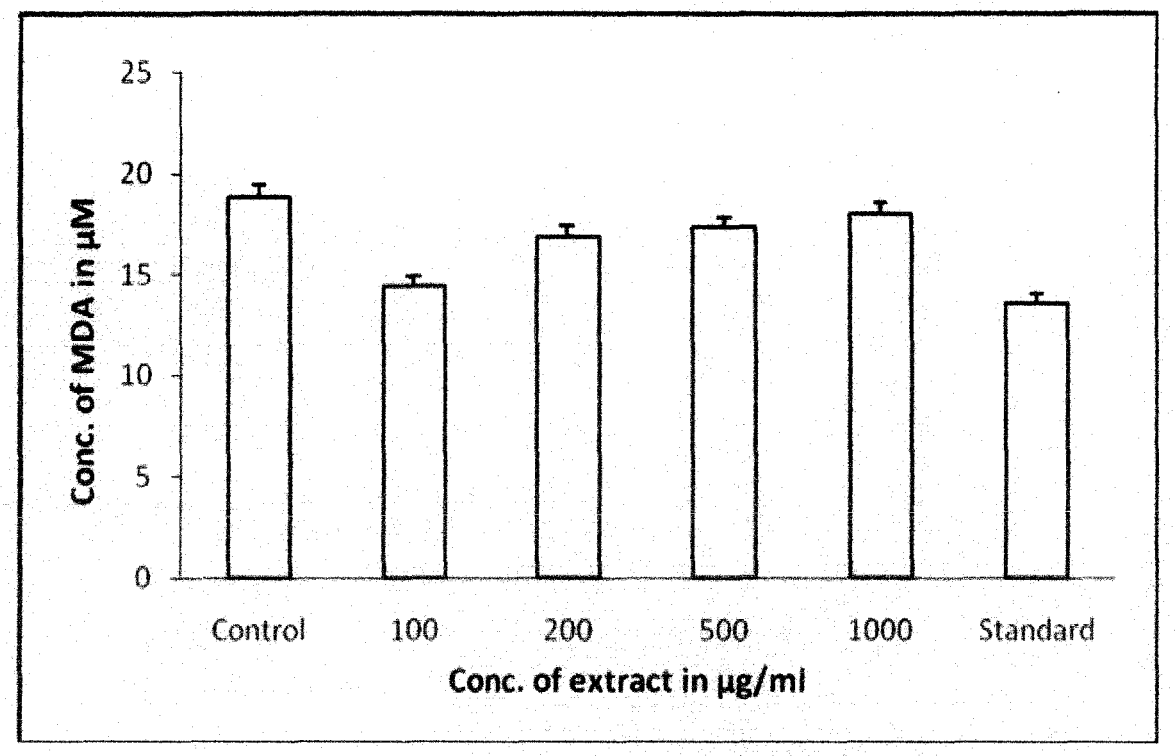


Fig.5.1. Antioxidant activity of *K. rotunda* Linn. by MDA suppression model

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

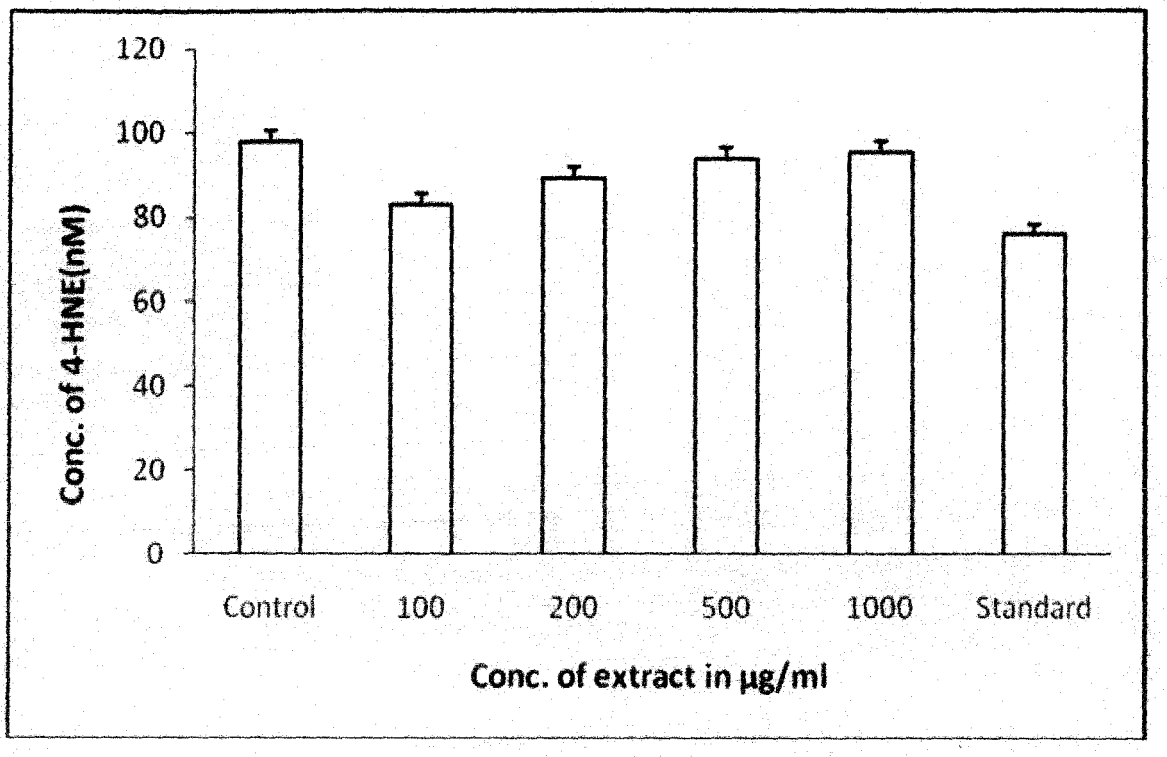


Fig. 5.2. Antioxidant activity of *K. rotunda* Linn. by 4-HNE suppression model

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

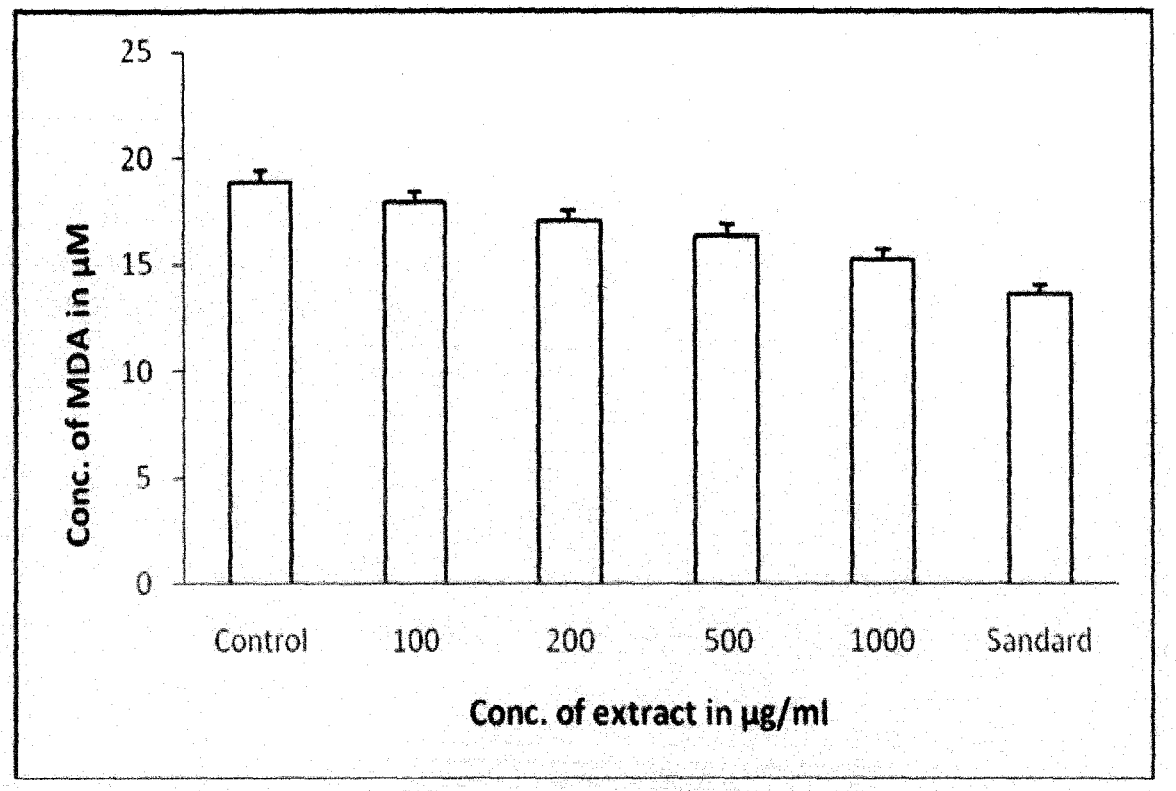


Fig.5.3. MDA suppression activity of *Eupatorium cannabinum*

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

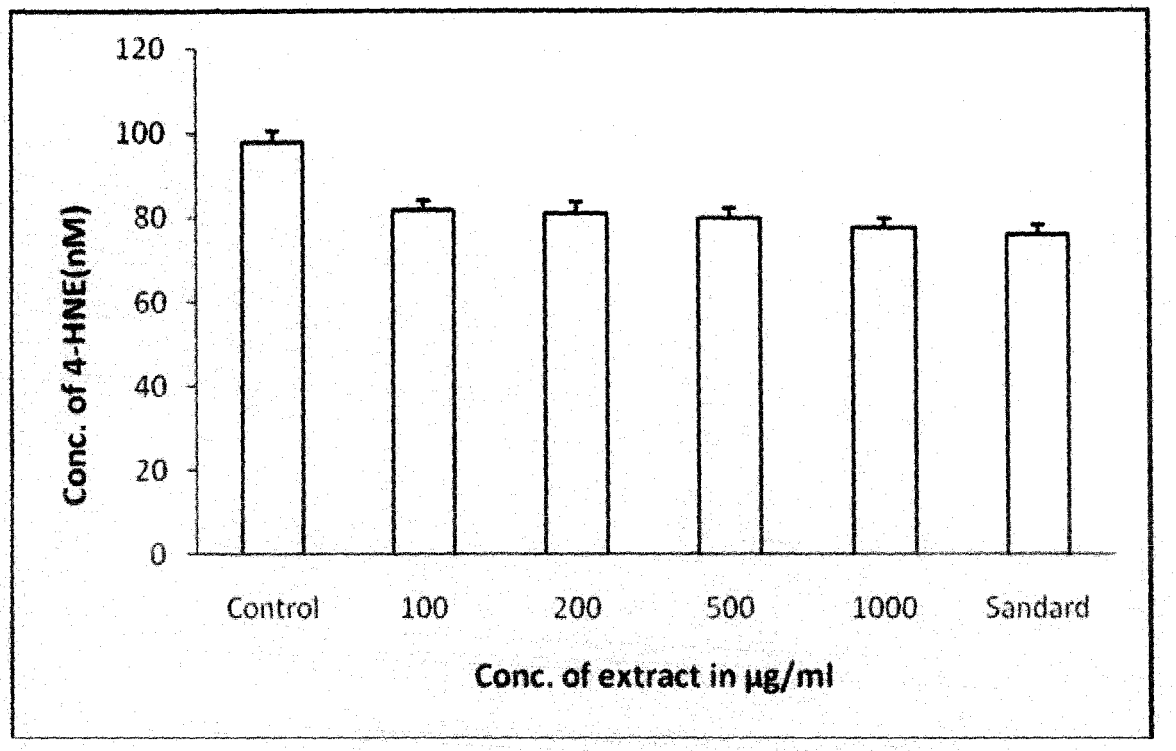


Fig.5.4. 4-HNE suppression activity of *Eupatorium cannabinum*

Each bar represents the average of three determinations with standard error

The probability levels of changes are significant at $P < 0.05$

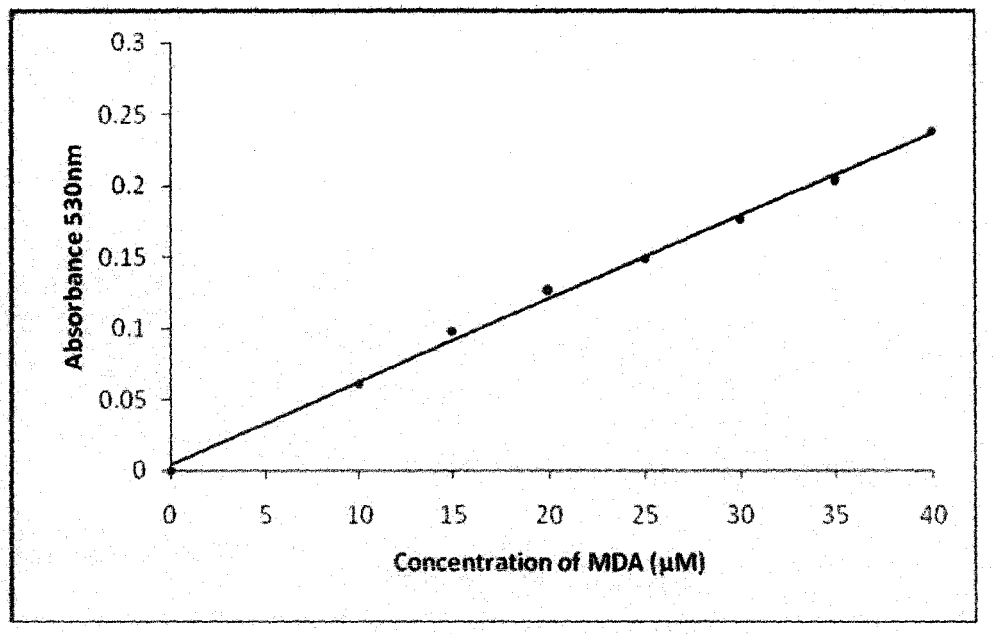


Fig.5.5. Standard calibration curve of MDA

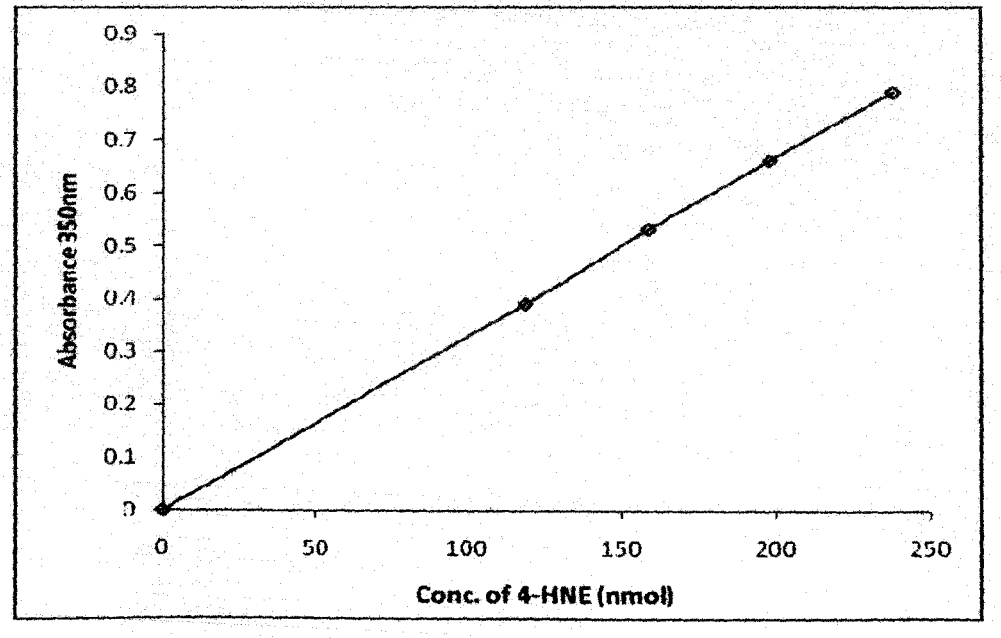


Fig.5.6. Standard calibration curve of 4-HNE

5.6. References

1. Finkel T and Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature*, 2000, **408**, 239-247.
2. Vanvaskas S and Schmidt HHW. Just say NO to cancer? *Journal of the National Cancer Institute*, 1997, **89**, 406-407.
3. Sheu F, Lai HH and Yen GC. Suppression effect of soy isoflavones on nitric oxide production in RAW 264.7 macrophages. *Journal of Agricultural and Food Chemistry*, 2001, **49**, 1767-1772.
4. Vander VA, Eiserich JP, O'Neil CA, Halliwell B and Cross CE. Tyrosine modification by reactive nitrogen species: acloser look. *Archives of Biochemistry and Biophysics*, 1995, **319**, 341-349.
5. Yermilov V, Rubio J, Becchi M, Freisen MD, Pignatelli B and Ohshima H. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. *Carcinogenesis*, 1995, **16**, 2045-2050.
6. Montoro P, Carbone V, De Simone F, Pizza C and De Tommasi N. Studies on the constituents of *Cyclanthera pedata* fruits: isolation and structure elucidation of new flavonoid glycosides and their antioxidant activity. *Journal of Agricultural and Food Chemistry*, 2001, **49**, 5156-5160.
7. Corner EM and Grisham MB. Inflammation, free radicals and antioxidants. *Nutrition*, 1996, **12**, 274.
8. Athar M. Oxidative stress and experimental carcinogenesis. *Ind. J. Exp. Biol.*, 2000, **40**, 656-667.
9. Jose N and Janarndhanan KK. Antioxidant and antitumour activity of *Pleurotusflorida*. *Current Science*, 2000, **72**, 941-943.
10. Gomes AJ, Lunard CN, Gonzalez S and Tedesco AC. Antioxidant action of *Polypodium leucotomos* extract and Koyic acid: Reactions with relative oxygen species. *Brazilian J. Med. Biol. Res.*, 2001, **34**, 1487-1494.
11. The Columbia Encyclopedia, Sixth Edition. Copyright 2007, University Press, Columbia.

12. Battacharya SK, Satyan KS and Ghosal S. Antioxidant activity of Glycowithanoloides from *Withania somnifera. Ind. J. Exp.Biol.*, 1997, **35**, 236-239.

13. E. Matuschek E and Svanberg U. Oxidation of Polyphenols and the Effect on *in vitro* Iron Accessibility in a Model Food System. *Journal of Food Science,* 2002, **67(1)**, 420–424.

14. Sies H and Hossain MA. Antioxidant Molecules. *Journal of Biological Chemistry,* 1985, **260(24)**, 12920-12926.

15. Sies H. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.,* 1997, **82**, 291-95

16. Stanner SA, Hughes J, Kelly CN and Buttriss J. A review of the epidemiological evidence for the antioxidant hypothesis. *Public Health Nutr.,* 2004, **7(3),** 407-22.

17. Bartlett H, Eperjesi F. Age-related macular degeneration and nutritional supplementation: a review of randomised controlled trials. *Ophthalmic Physiol. Opt.,* 2003, **23(5)**, 383-399.

18. Wintergerst E, Maggini S and Hornig D. Immune-enhancing role of vitamin C and zinc and effect on clinical conditions. *Ann. Nutr. Metab.,* 2006, **50(2)**, 85-94.

19. Wang J, Wen I Huang Y, Chen Y and Ku M. Dual effects of antioxidants in neurodegeneration: Direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation. *Curr. Pharm. Des.,* 2006, **12(27)**, 3521-33.

20. Xianquan S, Shi J, Kakuda Y and Yueming J. Stability of lycopene during food processing and storage. *J. Med. Food,* 2005, **8(4)**, 413-22.

21. Rodriguez-Amaya DB. Food carotenoids: Analysis, composition and alterations during storage and processing of foods. *Forum Nutr.*, 2003, **56**, 35-37.

22. Baublis A, Lu C, Clydesdale F and Decker E. Potential of wheat-based breakfast cereals as a source of dietary antioxidants. *J. Am. Coll. Nutr.*, 2000, **19(3)**, 308-11.

23. Rietveld A and Wiseman S. Antioxidant effects of tea: Evidence from human clinical trials. *J. Nutrition,* 2003, **133(10)**, 3285-3292.

24. Henry C and Heppell N. Nutritional losses and gains during processing: Future problems and issues. *Proc. Nutr. Soc.*, 2002, **61(1)**, 145-148.

25. David JM, Barreisors ALBS and David JP. Antioxidant phenyl propanoid esters of triterpenes from *Dioclea lasiophylla*. *Pharm. Biol.*, 2004, **42**, 36.

26. Dasgupta N and De B. Antioxidant activity of *Piper betle* Linn. leaf extract *in vitro*. *Food Chem.*, 2004, **88**, 219-214.

27. Hertog MG, Feskens EJ, Hollman PC, Katan MB and Kromhout D. Dietary antioxidiant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. Lancet, 1993, **342**, 1007-1011.

28. Kris-Etherton PM and Krummel D. Role of nutrition in the prevention and treatment of coronary heart disease in women. *J. Am. Diet Assoc.*, 1993, **93**, 987-93.

29. Anderson JV, Palombo RD and Earl R. The role of nutrition in health promotion and disease prevention programs. *J. Am. Diet Assoc.*, 1998, **98**, 205-208.

30. Elsayed NM and Bendich A. Dietary antioxidants: potential effects on oxidative products in cigarette smoke. *Nutr. Res.*, 2001, **21**, 551-567.

31. Stoner GD and Mukhtar H. Polyphenols as cancer chemopreventive agent. *J. Cell Biochem.*, 1995, **22**, 169-180.

32. Rice-Evans CA, Muller NJ, Bolwell PG, BramLey PM and Pridham JB. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.*, 1995, **22**, 375-383.

33. Hocman G. Prevention of Cancer: Vegetable and plants comp. *Biochem. Physiol.*, 1989, **93**, 201.

34. Esterbauer H, Schaur RJ and Zollner H. *Free Radic. Biol. and Med.*, 1991, **11**, 81.

35. Ohkawa H, Oshishi N and Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid. *Anal. Biochem.*, 1979, **95**, 351-358.

36. Lowery OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein estimation with Folin Ciaco phenol reagent. *Biol. Chem.*, 1951, **193**, 265-275.

37. Sreejayan N and Rao MNA. Free radical scavenging by curcuminoids. *J. Pharm. Pharmacol.*, 1997, **49**, 105-109.

38. Ramazan A, Mehmet C and Ensari F. Protective effects of vitamin E, selenium and allopurinol against stress-induced ulcer formation in rats. *Turk. J. Med. Sci.*, 2001, **31**, 199-203.

39. Punchard NA and Kelly FK. *Free Radical: A Practical Approach*, Oxford University Press, London, 1996, p 134.

40. Esterbauer H, Schaur RJ and Zollner H. Chemistry and biology of 4-hydroxynonenal, malonylaldehyde and related aldehydes. *Free Radic. Biol. and Med.*, 1991, **11**, 81-128

CHAPTER - 6

SYUDIES ON WOUND HEALING ACTIVITY

6.1. Introduction

Wound may be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue[1]. It may result from trauma or from a surgical incision. The capacity of a wound to heal depends on its depth, as well as on the overall health and nutritional status of the individual. Normal wound healing response begins the moment the tissue is injured. It is the process of repair that follows injury to the skin and other soft tissue. Following injury, an inflammatory response occurs and the cells below the dermis (the deepest skin layer) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin layer) is regenerated[2].

In other words it is a break in the structure of an organ or tissue caused by some external factor. Most wounds affect the skin, the first line of defense against infection. Common examples include bruises, grazes, tears, cuts, punctures (made by pointed objects), incisions (clean cuts), contusions (may not break the skin but can cause damage), lacerations (jagged, irregular cuts), and burns. While most minor wounds heal easily, some can worsen into chronic open sores that can become seriously infected[3].

Moreover wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. This is a product of the integrated response of several cell types to injury. Wound healing is a complex process that results in the contraction and closure of the wound and restoration of a functional barrier[4]. Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue[5].Repair of injured tissues includes inflammation, proliferation, and migration of different cell types[6]. Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site. Wound healing or wound repair, is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded, a set of events takes place in a predictable fashion to repair the damage. These events overlap in time[7,8] and must be artificially categorized into separate steps: the inflammatory, proliferative, and remodeling phases[9]. In the inflammatory phase, bacteria and debris are phagocytized and removed and factors are released that cause the migration and division of cells involved

in the proliferative phase. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction[10]. In angiogenesis, new blood vessels grow from endothelial cells[11]. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin.

In epithelialization, epithelial cells crawl across the wound bed to cover it. In contraction, the wound is made smaller by the action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. When the cells' role is close to complete, the un-needed cells undergo apoptosis. In the maturation and remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis[12].

Wound healing is a complex phenomenon involving a number of processes, including induction of an acute inflammatory process, regeneration of parenchymal inflammatory process[13] migration and proliferation of both parenchymal and connective tissue cells, synthesis of extracellular matrix (ECM) proteins, remodeling of connective tissue and parenchymal components, and acquisition of wound strength[14]. All these steps are orchestrated in a controlled manner by a variety of cytokines including growth factors[15]. Some of these growth factors like platelet-derived growth factor B (PDGF), transforming growth factor B (TGF-B), fibroblast growth factor (FGF) and epidermal growth factor (EGF) have been identified in self-healing wounds[16]. In chronic wounds, the normal healing process is disrupted due to some unknown reasons, and in such cases, exogenous application of certain growth-promoting agents or compounds, which can enhance the *in situ* generation of these growth factors, is required to augment the healing process. Several factors delay or reduce wound repairing, including bacterial infection, necrotic tissue, and interference with blood supply, lymphatic blockage and diabetes mellitus. Generally if the above factors could be inhibited/controlled by any agent, increasing healing rate could be achieved[17].

Wound healing is an important biological process involving tissue repair and regeneration. A wound is described as "a break in the continuity of tissue, from violence or trauma" and is regarded as healed if there is a restoration of the wounded or inflamed tissue to normal condition[18]. This can be classified into any of three types – healing by

first intention, healing by second and then by third. In wounds healed by the first intention, the edges are smoothly closed that no scar is left. On the other hand, wound healing by second intention involves formation of granulation tissues, which fill up the gaps between the wound edges and is associated with significant loss of tissue, leaving little scars. Wounds healed by third intention, are usually those left open for three to five days until granulation bed falls before they are sutured, generally resulting in extensive scar formation.

There are four distinct stages involved in wound healing namely, inflammatory stage, debridement stage, proliferation stage and maturation/remodeling stage. When an injury occurs, the vascular integrity of the injured area is disrupted leading to extravasations of blood into the surrounding tissue or plasma when the damage is minor. The inflammatory stage is directed at preventing further loss of blood by platelet adhesion/accumulation at the site leading to coagulation those results to the formation of thrombus. The debridement stage occurs from the third to the sixth day after injury and involves the appearance of neutrophils to clear contaminating organisms. The proliferation or repair stage is characterized by endothelial budding in the nearby blood vessels forming new capillaries that penetrate and nourish the injured tissue. The maturation stage commences from the tenth day to several months depending on wound severity[19].

Certain factors that influence wound healing include bacterial infection, nutritional deficiency, drugs, sterility, obesity, movement of wound edges, site of wound and wasting diseases[20]. Several classes of drug have been used in the management of wounds. Among these are the antibiotics; penicillin and streptomycin have been widely employed in combating post-operative infections in man and animals[21]. The antibiotics are chosen based on their ability to destroy or inhibit the growth of pathogenic organisms, while the tissue is left unharmed. Antibiotics used should be applied to achieve maximum concentration in the tissue as quickly as possible and continued until 48h after disappearance of symptoms unless signs of toxicity are shown[22]. The wound healing activities of plants have since been explored in folklore. The significant successes recorded have led to investigation into medicinal plants with a view to confirming these acclaimed properties. Records have revealed that different parts of plants used for healing of wound contain some active principles[23].

Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. It mainly depends on the repairing ability of the tissue, type and extent of damage and general state of the health of the tissue. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema, and small new blood vessels. The undifferentiated mesenchymal cells of the wound margin modulate themselves into fibroblast, which start migrating into the wound gap along with the fibrin strands. The collagen composed of amino acid (hydroxyproline), the major component of extra cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of the hydroxylproline could be used as an index for collagen turnover. Wound healing is the process of repair that follows injury to the skin and other soft tissues.

Dietary modifications and nutritional and herbal supplements may improve the quality of wound healing by influencing these reparative processes or by limiting the damaging effects of inflammation. Normal wound healing response begins the moment the tissue is injured. Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production. Later, the epithelial tissue is regenerated[2].

Tissue healing is an important process, which is the basis of various surgical manipulations. It can be enhanced by using several herbal drugs having antiseptic, anti-inflammatory, antimicrobial and biostimulator property. There are certain plants, which are proved helpful in wound healing, fracture healing and healing of nervous tissue in animals[24].

The process of wound healing is promoted by several natural products[25] (plant products), which are composed of active principles like triterpenes, alkaloids, flavonoids[26] and biomolecules[27]. India has a rich tradition of plant-based knowledge on healthcare. A large number of plants/plant extracts/decoctions or pastes are equally used by tribals and folklore traditions in India for treatment of cuts, wounds, and burns. The present review thus attempts to analyze the ethno botanical knowledge base for treatment of cuts and wounds which includes usage of plants, methods employed by tribals and folklore practices prevailing in India. Pharmacological reports available on Indian medicinal

plants employing various wound healing models and its underlying molecular mechanism, wherever available, has also been briefly reviewed. This pharmacological validation on Indian medicinal plants is very limited and a large number of plants used in tribal and folklore with enormous potential have not been validated for their wound healing activity. This review therefore attempts to bridge the lacunae in the existing literature and offers immense scope for researchers engaged in validation of the traditional claims and development of safe and effective and globally accepted herbal drugs for cuts and wounds[28].

Several drugs of plant, mineral, and animal origin are described in the Ayurveda for their wound healing properties. Most of them derived from plant origin. Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and patients, but the potential of most remains unexplored[29].

The majority of the world's population relies on traditional medicine for their health care in the treatment of wounds. In developing countries, remedies prepared from herbal plants have been widely used for the treatment of soft tissue wounds and burns by traditional practitioners.

The objectives of the pharmacology of wound healing are to study the influence of various measures in wound management programs on healing and to screen drugs that promote healing. So, search for natural remedies for healing has drawn attention to herbals. However, the intensive research in wound healing has not yielded, until now, a safe, economic, and efficacious prohealing agent that could obviate the long hospitalization of patients following surgery and wound infliction. In this context, screening of our natural resources as one of the strategies for identification of such agents would seem highly beneficial[30].

A review of literature revealed that the wound healing property of these plants have not been subjected to scientific evaluation. Hence the present work was undertaken to evaluate the effect of methanol extracts and the isolated compounds on excision, incision and dead space wound models on rats.

6.2. Materials and Methods

6.2.1. Plant materials

Methanol extracts of rhizome of *Kaempferia rotunda* and leaves of *Eupatorium cannabinum* as well as their isolated compounds were used as test drug in these experiments.

6.2.2. Drugs and chemicals

All the chemicals used in this study were procured from an authorized dealer and were of analytical grade. Methanol was obtained from LOBA chemicals Kolkata, sodium alginate, carboxymethyl cellulose and Framycetin sulphate cream obtained from Zydus-Cadila. Methanolic extract of rhizomes of *Kaempferia rotunda* Linn. and leaves of *Eupatorium cannbinum* Linn. used in this study were extracted in our laboratory.

6.2.3. Test compound formulations

Two types of drug formulations were prepared from each of the extracts and their isolated compound-I and II for topical administration. Different concentrations of ointment were prepared in 2% sodium alginate base. Oral suspensions of the rhizome and leaf extracts and isolated compounds were prepared by suspending them separately in 1% solution of sodium carboxy methylcellulose to obtain suitable dosage forms.

6.2.4. Experimental animals

Swiss Wistar strain rats of either sex 150-200g were used for the study. They were procured from the laboratory animal resource section of the Himalayan pharmacy institute. The rats were housed in polypropylene cages on normal food and water *ad. libitum*. Animals were periodically weighed before and after experiments. The rats were anaesthetized prior to infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using light ether anesthesia. Animals were closely observed for any infection; those, which showed signs of infection, were separated and excluded from the study. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee (IAEC) prior to the initiation of the experiment and as per Indian norms laid down by the committee for the purpose of control and supervision of experimental animals (CPCSEA), New Delhi. After wounds

were created as described in the succeeding paragraph, animals were randomly assigned for treatment.

6.3. Wound healing activity of the extract and the isolated compound of *Kaempferia rotunda.*

Excision, incision and dead space wound models were used to evaluate the wound healing activity of rhizome and isolated compound of *Kaempferia rotunda.*

6.3.1. Excision Wound model

The rats were inflicted with excision wounds as described by Morton and Malon[31]. Under light ether anesthesia a circular wound of about 500sq.mm was made on depilated ethanol sterilized dorsal thoracic region of the rats. The animals were divided into six groups of six each. The animals of group I was left untreated and considered as the control. Group II served as reference standard and treated with 1% w/w framycetin sulphate cream. Animals of group III and IV were treated with ointment containing 200 and 400mg of methanol extracts of *Kaempferia rotunda* rhizome respectively. Group V and VI were treated with ointments containing 10mg and 20mg of isolated compound-I from the same plant respectively for 14 days. The ointment was topically applied once a day, starting from the day of the operation until complete epithelisation. The parameters studied were wound closure and epithelialization time. The wound were traced on mm2 graph paper on days 3, 6, 9, 12 and 15 and thereafter on alternate days until healing was complete. The percentage of wound closure was calculated. The period of epithelialization was calculated as the number of days required for filling of the dead tissue remnants of the wound without any residual wound.

6.3.2. Incision wound model

In Incision wound model, 6cm long paravertebral incisions were made through the full thickness of the skin on either side of the vertebral column of the rat as described by Ehrlich and Hunt[32]. After the incision, the parted skin was sutured 1cm apart, using surgical threads and curved needle. The animals were divided into six groups of six animals each. The animals of group I was left untreated and considered as the control. The group II served as reference standard and received 1% w/w framycetin sulphate cream ointment. Animals in group III and IV were treated with ointment containing 200 and 400mg of methanol extracts of *Kaempferia rotunda* rhizome. Animals of group V

and VI were treated with ointments 10 and 20mg of isolated compound-I of the same plant. The ointment was topically applied once in a day. The sutures were removed on the 8th post wound day. The skin breaking strength of the wounds was measured on the 10th day as described in the method of Lee *et. al.*[33].

6.3.3. Dead space wound model

Physical changes in the granuloma tissue were studied in this model. The animals were divided into six groups of six rats in each group. Group I served as the control, which received 1ml of 1% sodium CMC/kg b.w.p.o. The animals of group II and III received oral suspension of methanolic extract of *K. rotunda* rhizomes at doses 200 and 400mg/kg body weight respectively. Similarly the animal of group IV and V received oral suspension of isolated compound of the plant at 10 and 20mg/kg body weight respectively. Under light ether anesthesia, subcutaneous dead space wounds were inflicted in the region of the axilla and groin, by making a pouch through a small nick in the skin. Cylindrical grass piths measuring 2.5cm in length and 0.3cm in diameter were introduced into pouch to harvest the granulation tissue. Each animal received 2 grass piths and cotton pellets in different locations. The wounds were sutured and mopped with an alcoholic swab. Animals were placed into their individual cages after recovery from anesthesia. Excision of the granulomas from the surrounding tissue was performed on the 10th post wounding day under light ether anesthesia.

Granulation tissue surrounding the grass piths were excised and split open. The breaking strength of piece measuring about 15mm in length and 8mm in width (obtained by trimming the rectangular strip of granuloma tissue) was determined on 10th post wounding day by a continuous constant water flow technique of Lee[33]. The pieces obtained at the end of these measurements were preserved in 10% formalin solution for histopathological studies to evaluate the effects of the extract on collagen formation. Hydroxy proline estimation was carried out on 10th day old granulation tissue in control as well as test group by the method of Woessner[34].

6.4. Wound healing activity of the extract and the isolated compound of *Eupatorium cannabinum.*

The Wound healing activity of methanol extract of *E. cannabinum* leaves and its isolated compound-II were determined as per the methods described above in for *K. rotunda* Linn. in sections **6.3.1, 6.3.2** and **6.3.3**.

6.5. Statistical analysis

The results of all the assays were reported as (X±SEM). Statistical significant differences between the groups were calculated by means of one way ANOVA. All the results obtained in the study were compared with the control group and positive control group. P values < 0.05 were considered statistically significant.

6.6. Results

The effect of methanol extracts of *Kaempferia rotunda, Eupatorium cannabinum* and their isolated compounds (I and II) were screened on excision, incision and dead space wound models concurrently with the control and reference standard framycetin sulphate cream treated animals. Significant promotion of wound-healing activity was observed in isolated compound I and II and methanol extracts of both the plants in all the three wound models such as excision, incision and dead space wound.

In excision wound model, the mean percentage closure of wound area was calculated on the 3, 6, 9, 12 and 15 post wounding days. The percentage closure of wound area was significantly increased by the curative effect of methanol extracts of rhizome of *K. rotunda* (table 6.1) and leaves of *E. cannabinum* (table 6.4) and their isolated compounds in the respective animal groups. The data revealed that, *K. rotunda* extract treated animals showed significant reduction in the wound area and faster rate of epithialisation (18.50±0.43,18.22±0.07), as compared to *E. cannabinum* extract (23.00±0.47, 22.33 ±0.60) in both the doses. The isolated compound treated animals showed faster epithelisation of wounds than the animals treated with methanolic extracts of both the plants. The period of epithelisation is comparable with standard drug framycetin sulphate cream. The results of percentage wound contraction and period of complete epithelisation are shown in table 6.1 and 6.4 for the plant *K. rotunda* and *E. cannabinum* respectively.
In incision wound model the breaking strength of the 10th day old restored incision wound was significantly increased in animals treated with isolated compound I and II (10

and 20mg/kg body weight) and methanolic extracts of both the plants (200 and 400mg/kg body weight), when compared to control. The maximum breaking strength was observed in animals treated with isolated compound II in the dose of 20mg (393.51±2.66). Significant breaking strengths were also observed in the animals treated with isolated compound I followed by methanol extracts of rhizome of *K. rotunda* and leaves of *E. cannabinum* which are presented in table 6.2 and 6.5 respectively. The effects of oral administration of the suspensions of the extract of both the plants and their isolated compounds on **dead space wound** model were assessed by the increase in the weight of granuloma, increase in breaking strength and hydroxylproline content of the granuloma tissue. The data is depicted in the table 6.3 and 6.6 for *K. rotunda* and *E. cannabinum* respectively. This effect may be due to the enhancement of collagen maturation by increased cross linking of collagen fibers. The increased weight of the granuloma also revealed the presence of higher hydroxyl proline content. Among the methanolic extract and isolated compound treated groups the hydroxyl proline content was found to be more in isolated compound treated animals (*KR*: 2302.85±0.68, 2232.33±0.76 and *EC*: 2298.82±0.36, 2226.17±0.62) followed by methanolic extracts of *K. rotunda* and *E. cannabinum*.

Histological study of the granuloma tissue also evidenced the wound healing property of the extracts and isolated compounds. The sections of the granuloma tissue of the control animals showed more monocytes and fibroblasts in fig 6.1. The lesser epithelialization, poor collagen formation and lower concentration of hydroxylproline indicated the incomplete healing of wound in control animals. The sections of the granuloma tissue of the animals treated with methanol extract of both the plants showed complete epithelialization, increased fibrosis and collagen formation with lesser macrophages, whereas in the isolated compound treated animals the healing activity observed was comparatively more. From these findings it is concluded that isolated compound I and II exhibited significant wound healing activity (fig. 6.4 and 6.5) and (fig. 6.8 and 6.9) respectively followed by the methanolic extract in fig 6.2 and 6.3 for the plant *K. rotunda* and fig. 6.6 and 6.7 for the plant *E. cannabinum*. The present investigation offers scientific evidence to the folkloric accounts of the use for *K. rotunda* and *E. cannabinum* in treating cuts and wounds.

TABLE 6.1. Effects of topical application of methanolic extract of rhizomes of ***Kaempferia rotunda*** and its Isolated **compound I** in rats by **excision** wound model.

Group	Treatment	Percentage of Wound closure (original wound size 500sq.mm)						Period of epithelialization
		0-day (X ±SEM)	3rd day (X ±SEM)	6th day (X ±SEM)	9th day (X ±SEM)	12th day (X ±SEM)	15th day (X ±SEM)	
I	Control (Untreated)	512.78±0.48 0.00	482.11±0.41 5.98	388.58±0.74 24.22	301.64±0.11 41.17	227.12±0.31 55.70	183.28±0.42 64.25	25.29±0.23
II	1% w/w Framycetin Sulphate cream	511.40±0.52 0.00	427.23±0.51 16.45	345.14±0.54 32.51	230.12±0.21 55.00	130.88±0.38 74.40	9.26±0.48 98.18	16.24±0.07
III	Methanolic extract (200mg)	501.32±0.68 0.00	460.54±0.88 8.31	388.98±0.43 22.40	277.66±0.67 44.61	142.99±0.55 71.47	18.66±0.08 96.27	18.50±0.43
IV	Methanolic extract (400mg)	505.70±0.84 0.00	454.67±0.87 10.09	351.32±0.65 30.52	267.86±0.87 47.03	136.87±0.58 72.93	15.54±0.65 96.92	18.22±0.07
V	Isolated comp. I (10mg)	508.60±0.38 0.00	452.17±0.54 11.09	346.54±0.61 31.86	262.36±0.24 48.41	135.14±0.03 73.42	14.67±0.46 97.11	17.86±0.19
VI	Isolated comp. I (20mg)	509.28±0.31 0.00	450.58±0.47 11.52	333.23±0.24 34.56	248.81±0.14 51.14	133.44±0.39 73.79	10.85±0.41 97.86	17.67±0.42
One way ANOVA F	0.132	All values are statistically significant respect to control at 5% level of significance						

TABLE 6.2. Effects of topical application of methanolic extract of rhizomes of ***K. rotunda*** and its Isolated **compound I** in rats by **incision** wound model.

Group	**Treatment**	**Breaking strength (g)**
I	Control (Untreated)	245.63±4.12
II	Framycetin Sulphate cream (1% w/w)	386.24±2.12
III	Methanolic extract (200 mg)	370.37±3.65
IV	Methanolic extract (400 mg)	378.33±8.22
V	Isolated compound I (10 mg)	380.00±8.56
VI	Isolated compound I (20mg)	382.11±5.62

All the values are expressed in mean±SEM

$P<0.05$ when compared to control

n=6 animals in each group

TABLE 6.3. Effects of oral application of methanolic extract of rhizomes of ***K. rotunda*** and its Isolated **compound I** in rats by **dead space** wound model.

Group	**Treatment**	**Granulation tissue dry weight (mg/100g) (X ±SEM)**	**Breaking strength (g) (X ±SEM)**	**Hydroxyproline (µg/gm) (X ±SEM)**
I	Control (1ml of 1% sod CMC)	92.22±0.48	246.30±3.37	1397.27±1.04
II	Methanolic extract (200mg)	146.38±0.58	387.72±3.41	1972.68±0.92
III	Methanolic extract (400mg)	153.89±0.71	443.33±2.12	1982.00±0.58
IV	Isolated compound I (10mg)	159.83±0.54	467.53±1.85	2232.33±0.76
V	Isolated compound I (20mg)	197.58±0.69	489.29±3.28	2302.85±0.68
One way ANOVA F 0.079		All values are statistically significant respect to control at 5% level of significance		

TABLE 6.4. Effects of topical application of methanolic extract of leaves of ***Eupatorium cannabinum*** *and* its **compound II** in rats by **excision** wound model.

		Percentage of Wound closure (original wound size 500sq.mm)						
Group	**Treatment**	**0 day (X ±SEM)**	**3rd day (X ±SEM)**	**6th day (X ±SEM)**	**9 th day (X ±SEM)**	**12 th day (X ±SEM)**	**15 th day (X ±SEM)**	**Period of epithelialization**
I	Control (Untreated)	504.29±0.36 0.00	468.72±0.29 7.05	378.24±0.47 24.99	232.12±0.35 33.97	184.14±0.42 63.49	97.12±0.33 80.74	24.50±0.80
II	1% w/w Framycetin Sulphate cream	500.18±0.38 0.00	432.67±0.24 13.49	308.34±0.41 38.35	198.24±0.37 60.36	131.24±0.47 73.76	7.32±0.49 98.53	16.15±0.21
III	Methanolic extract (200mg)	498.29±0.26 0.00	461.22±0.28 7.43	340.24±0.39 31.71	228.24±0.29 54.19	153.36±0.54 69.22	20.82±0.41 95.82	23.00±0.47
IV	Methanolic extract (400mg)	499.22±0.41 0.00	458.88±0.21 8.08	336.18±0.46 32.65	220.37±0.42 55.85	144.70±0.21 71.01	17.58±0.38 96.47	22.33±0.60
V	Isolated comp. II (10mg)	501.32±0.68 0.00	450.54±0.88 10.12	329.98±0.43 34.17	213.66±0.67 57.38	142.99±0.55 71.47	12.66±0.08 97.47	20.32±0.62
VI	Isolated comp. II (20mg)	509.70±0.84 0.00	442.56±0.87 13.17	327.48±0.65 35.75	208.86±0.87 59.02	136.87±0.58 73.14	11.54±0.65 97.73	19.16±0.41
One way ANOVA F 0.047		All values are statistically significant respect to control at 5% level of significance						

TABLE 6.5. Effects of topical application of methanolic extract of leaves of ***E. cannabinum*** and its Isolated **compound II** in rats by **incision** wound model.

Group	**Treatment**	**Breaking strength (g) (X ±SEM)**
I	Control (Untreated)	235.63±2.55
II	Framycetin Sulphate cream (1% w/w)	396.24±4.89
III	Methanolic extract (200 mg)	346.0±6.51
IV	Methanolic extract (400mg)	364.0±7.57
V	Isolated compound II (10 mg)	389.7±6.32
VI	Isolated compound II (20mg)	393.51±2.66

All the values are expressed in mean±SEM

$P<0.05$ when compared to control

n=6 animals in each group

TABLE 6.6. Effect of oral application of methanol extracts of leaves of ***E. cannabinum*** and its Isolated **compound II** in rats by **dead space** wound model.

Group	**Treatment**	**Granulation tissue dry weight (mg/100g) (X ±SEM)**	**Breaking strength (g) (X ±SEM)**	**Hydroxyproline (µg/gm) (X ±SEM)**
I	Control (1% sodium CMC)	88.17±0.51	222.80±2.53	1387.76±1.21
II	Methanolic extract (200mg)	132.71±0.35	311.68±3.31	1967.28±0.41
III	Methanolic extract (400 mg)	146.34±0.61	347.12±3.53	1976.41±0.89
IV	Isolated compound II (10 mg)	184.46±0.46	387.72±3.23	2226.17±0.62
V	Isolated compound II (20 mg)	187.13±0.42	388.11±3.25	2298.82±0.36
One way ANOVA F 0.069		All values are statistically significant respect to control at 5% level of significance		

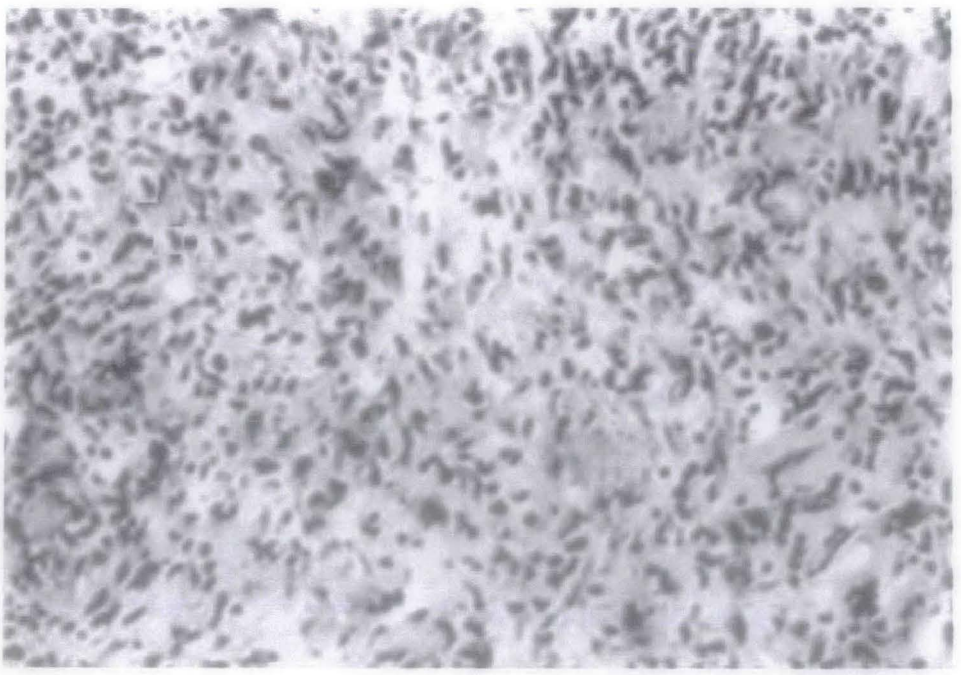


Figure 6.1. Histological section of granulation tissue of group I animal (control) showing with less collagen and more macrophages.

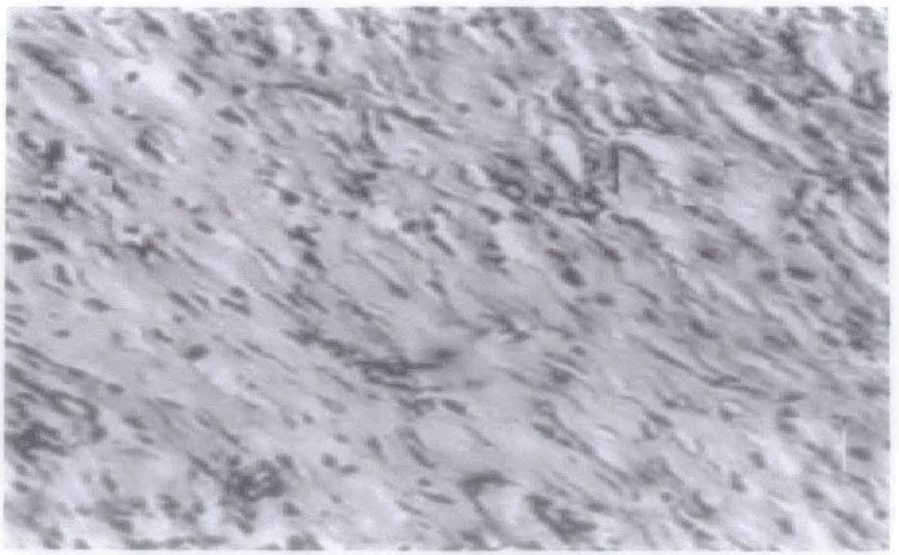


Figure 6.2. Histological section of granulation tissue of group II (methanolic extract of *K. rotunda*, 200mg/kg body wt.) treated animal showing moderate collagen and less macrophage.

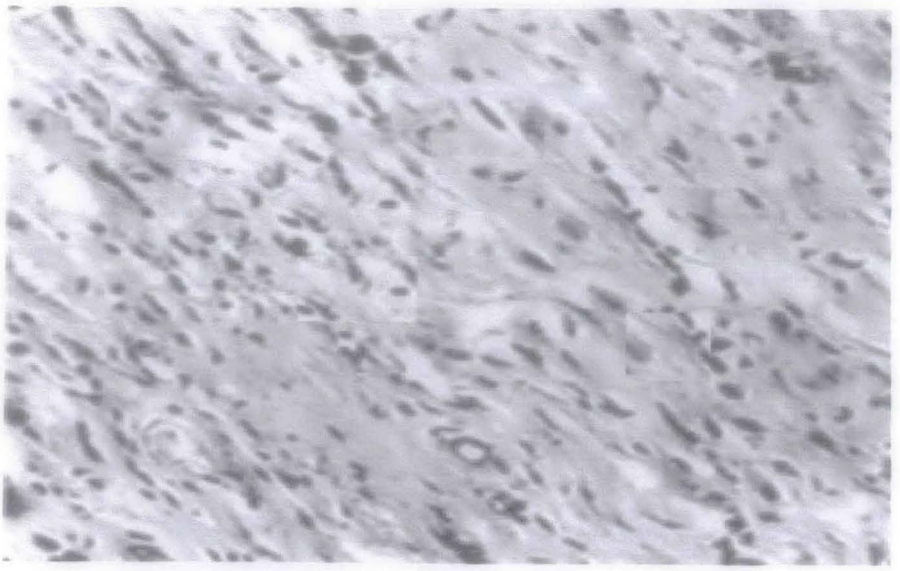


Figure 6.3. Histological section of Granulation tissue of group III (methanolic extract of *K. rotunda*, 400mg/kg body wt.) treated animal showing moderate deposition collagen.

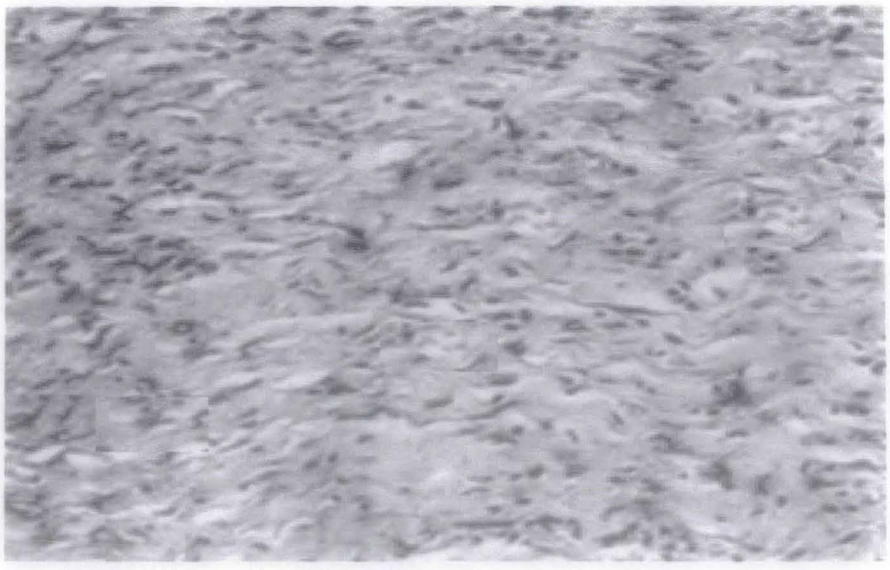


Figure 6.4. Histological section of Granulation tissue of group IV. (Isolated compound I, 10mg/kg body wt.) treated animal showing more deposition of collagen.

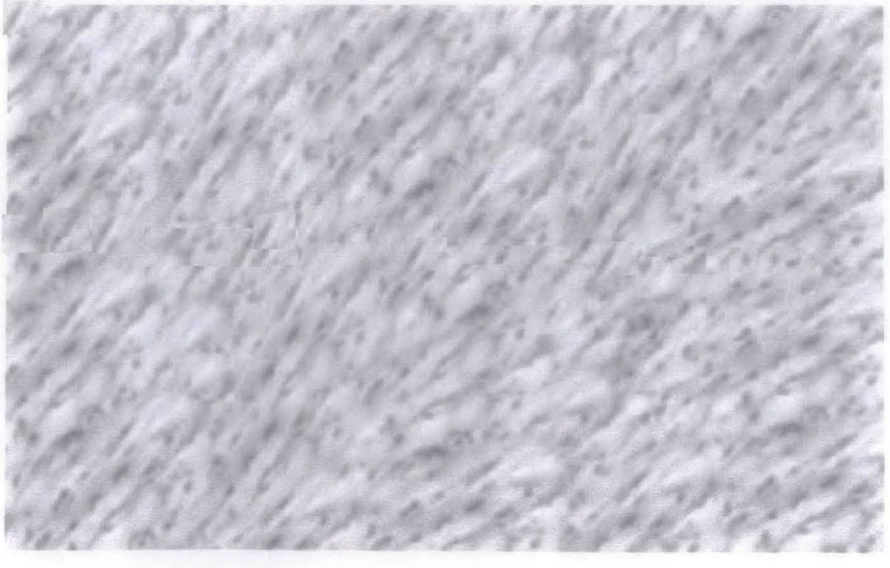


Figure 6.5. Histological section of Granulation tissue of group V (Isolated compound I, 20mg/kg) treated animal showing more deposition of collagen.

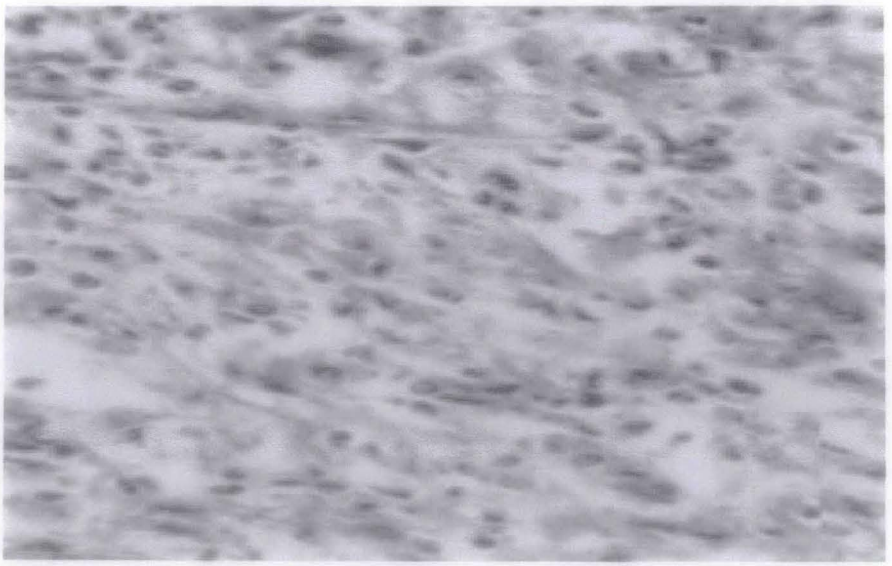


Figure 6.6. Histological section of granulation tissue of group II (methanolic extract of *E. cannabinum* 200mg/kg body wt.) treated animal showing moderate deposition collagen and less macrophage.

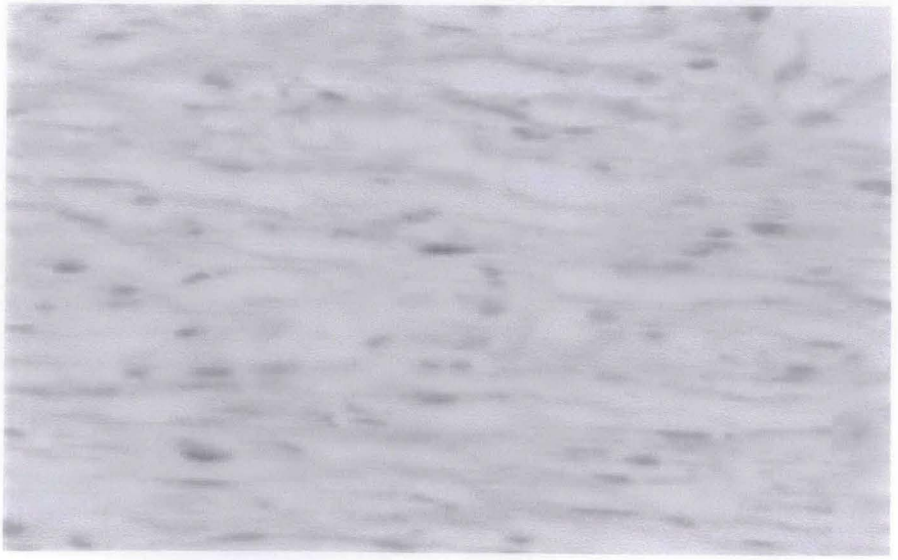


Figure 6.7. Histological section of granulation tissue of group III (methanolic extract of *E. cannabinum* 400mg/kg body wt.) treated animal showing moderate deposition collagen and less macrophage.

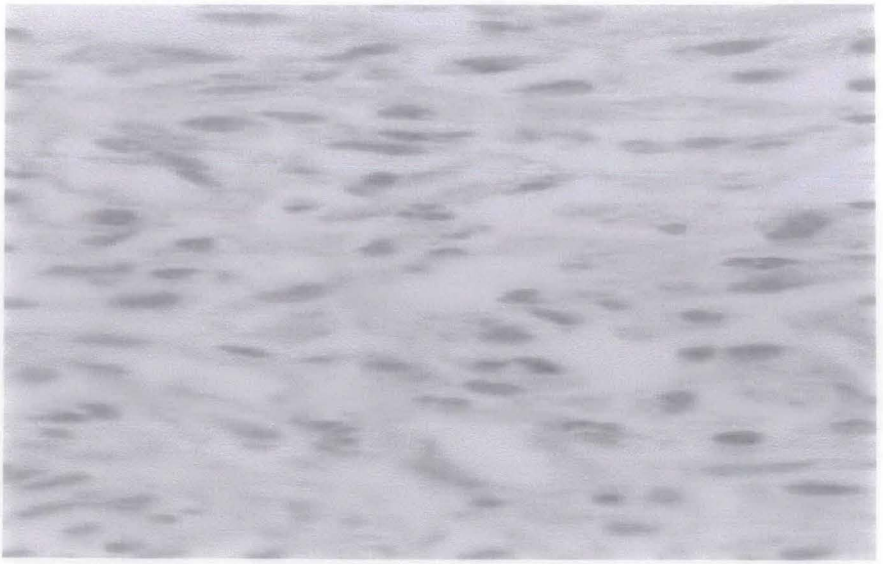


Figure 6.8. Histological section of granulation tissue of group IV (isolated compound II I0mg/kg body wt.) treated animal showing more collagen and less macrophages.

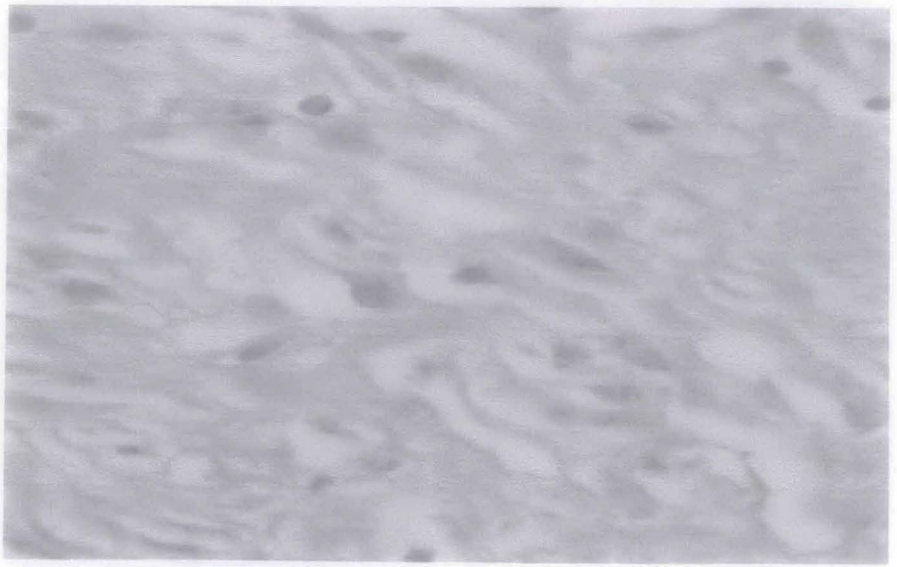


Figure 6.9. Histological section of granulation tissue of group V (isolated compound II 20mg/kg body wt.) treated animal showing more deposition collagen and less macrophages.

6.7. References

1. Patil MB, Jalalpure SS and Ashraf A. Wound healing activity of *Cyperus rotundus* linn. *Indian Drugs*, 2001, **38**, 288.

2. Souba WW and Wilmore D. Diet and nutrition in the care of the patient with surgery, trauma, and sepsis. In: Shils ME, Olson JA, Shike M *et. al.* (Eds), Modern Nutrition in Health and Disease, 9th edn., Baltimore, Williams & Wilkins, 1999, p 1589-1618.

3. Black JM and Matassarin E. Medical-Surgical Nursing: Clinical Management for Continuity of Care, 5th edn., Philadelphia, WB Saunders Co, 1997, p 254-256.

4. Chattopadhyay D, Arunachalam GAB, Mandal AB, Sur TK, Mandal SC and Bhattacharya SK. Antimicrobial and anti-inflammatory activity of folklore: Mellotus peltatus leaf extract, J. Ethno Pharmacol., 2002, 82, 229-237.

5. Phillips GD, Whitehe RA and Kinghton R. Initiation and pattern of angiogenesis in wound healing in the rat. Amer. J. Anat., 1991, 192, 257-262.

6. Sidhu GS, Mani H, Gaddipatti JP, Singh AK, Seth P, Banaudha KK, Patnaik GK and Maheshwari RK. Curcumin enhances wound healing in streptozotocin induced diabetic rats and genetically diabetic mice. Wound Repair Regen., 1999, 7, 362-74.

7. Stadelmann WK, Digenis AG and Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. The American Journal of Surgery, 1998, 176(2), 26-38.

8. Shibata A, Kato M and Masukawa T. Possible involvement of mast cells in collagen remodeling in the late phase of cutaneous wound healing in mice. International Immunopharmacology, 2004, 4(14), 1873-1880.

9. Quinn JV. Tissue Adhesives in Wound Care. Hamilton, Ont. B.C. Decker, Inc. Electronic Book, 1998, p 175-178.

10. Midwood KS, Williams LV and Schwarzbauer JE. Tissue repair and the dynamics of the extracellular matrix. *The International Journal of Biochemistry & Cell Biology*, 2004, **36(6)**, 1031-1037.

11. Chang HY and Sneddon JB. Gene Expression Signature of Fibroblast Serum Response Predicts Human Cancer Progression: Similarities between Tumors and Wounds. *Plos Biology*, 2004, **2(2)**, 204-214.

12. Nayak S. Effects of *Hibiscusrosa sinensis L.* on Wound Healing Activity: A Preclinical Study in a Sprague Dawley Rat. *The International Journal of Lower Extremity Wounds*, 2007, **6(2)**, 76-81.

13. Parmeshwaraiah S and Sivakumar HG. Wound healing activity of *Cyperus rotundus* Linn. *Indian J. Exp. Biol.*, 1998, **36**, 569.

14. Ramzi SC, Vinay K and Stanley R. *Pathologic Basis of Diseases*, Vol. V, WB Saunders Company, Philadelphia, 1994, p 86.

15. Pierce GF, Berg JV, Rudolph R, Tarpley J, Mustoe TA. Platelet-derived growth factor-BB and transforming growth factor beta 1 selectively modulate glycosaminoglycans, collagen, and myofibroblasts in excisional wounds. *Am. J. Pathol.*, 1991, **138**, 629-646.

16. Udupa SL, Udupa AL and Kulkarni DR. Studies on the anti-inflammatory and wound healing properties of *Moringao leifera* and *Aegle marmelos. Fitoterapia* 1994, **65**, 119-123.

17. Saipurana K, Reddy NP and Babu M. Wound healing activity of *Cyperus rotundus* linn. *Indian J. Exp. Biol.*, 1995, **33**, 673.

18. Taber CW. *Taber's Cyclopedic Medical Dictionary*, 10th edn., FA Davies Company, USA, 1965.

19. Thomas JC. Veterinary Pathology, 6th edn., William's & Wilkin, Maryland, USA, 1997, p 150-156.

20. Karl M, Lacrix, JV and Preston HH. Canine surgery, 4th edn., American Veterinary Publications, California.1995, p 42-45.

21. Esimone CO and Ibezim EC. The wound healing effect of herbal ointments formulated with *Napoleona imperialis, J. Pharma. Allied Sci.*, 2005, **3(1)**, 294-299.

22. Brander GC and Pugh DM. Veterinary Applied Pharmacology and Therapeutics, Bailliere Tindall, London, 1991, p 424-427.

23. Erasto P, Grierson DS and Afolayan AJ. Bioactive sesquiterpene lactones from the leaves of *Vernonia amygdalina. J. Ethnopharmacol.*, 2006, **106(1)**, 117-120.

24. Jaiswal S, Singh SV, Singh B and Singh HN. Plants used for tissue healing of animals. *Natural Product Radiance*, 2004, **3**, 284-329.

25. Suguna L, Chandrakasan G and Joseph KT. Influence of honey on biochemical and biophysical parameters of wounds in rats. *J. Clin. Biochem. Nutrit.*, 1999, **14**, 91.

26. Sharma SP, Aithal KS, Srinivasan KK, Udupa AL, Kulkarni DR and Rajagopal PK. Anti-inflammatory and wound-healing activities of the crude alcoholic extracts and flavanoids of *Vitex leucoxylon*. *Fitoterapia*, 1990, **61**, 263-265.

27. Chithra P, Suguna L and Chandrakasan G. Influence of arginine in wound healing in rats. *J. Clin. Biochem. & Nutrition*, 1995, **18**, 111-117.

28. Kumar B, Vijayakumar M, Govindarajan R and Pushpangadan P. Ethnopharmacological approaches to wound healing- Exploring medicinal plants of India, *J. Ethnopharmacol*, 2007, **114(2)**, 103-113.

29. Biswas TK and Mukherjee B. Plant Medicines of Indian origin for Wound Healing Activity: A review. *Int. J. Lower Extremity Wounds*, 2003, **2(1)**, 25-39.

30. Mustata MA, Mahamood AA, Sidik K and Noor SM. Evaluation of wound healing potential of *Ageratum conyzoides* leaf extract in combination with honey in rats as animal model. *Int. J Mol. Med. Adv. Sci.*, 2005, **1(4)**, 406-410.

31. Morton JJP and Malone MH. Evaluation of vulnerary activity by an open wound procedure in rats. *Arch. Int. Pharmacodyn.*, 1972, **196**, 117–126.

32. Ehrlich HP and Hunt TK. Effect of cortisone and Vitamin A on wound healing. *Annual Surgery*, 1968, **167**, 324-328.

33. Lee KH. Studies on mechanism of action of salicylates EII. Retardation of wound healing by aspirin. *J. Pharm. Sci.*, 1968, **57**, 1042-1043.

34. Wossner JF. The determination of hydroxy proline in tissue and protein samples containing small proportions of this imino acid. *Archi. Biochem. Biophys.*, 1961, **93**, 440-451.

CHAPTER - 7

STUDIES ON ANTIULCER ACTIVITY

7.1. Introduction

An ulcer is characterized by disruption of mucosal integrity leading to local defect or excavation due to active inflammation[1]. Pathophysiology of ulcer is due to an imbalance between aggressive factors (acid, pepsin, *H. pylori* and NSAID's) and local mucosal defensive factors (mucus, bicarbonate, blood flow and prostaglandins). Integrity of gastro duodenal mucosa is maintained through a homeostatic balance between these aggressive and defensive factors[2] Clinically, regulation of gastric acid secretion is considered as major therapeutic target in the management of disease[3]. Among clinically established drugs, H_2 blockers (ranitidine) and proton pump inhibitors (omeprazole) are most widely used as anti-ulcer drugs in addition to the cytoprotective agents like sucralfate and misoprostol. Ulcer healing consists of reconstruction of mucosal architecture and is a dynamic, active process of filling the mucosal defects with epithelial and connective tissue cells. It encompasses cell proliferation, division and migration[4-6]. Prostaglandins (PGs) and growth factors play an important role in healing of ulcers. Synthesis of PGs is governed by the expression of inducible cyclooxygenase-2 (COX-2) isozyme in gastric mucosa during healing process, further, COX-2 expression is enhanced in gastric epithelial cells after treatment with growth factors *in vitro* and *in vivo* during acetic acid induced gastric damage[7-9].

Peptic ulceration is one of the common disease affecting millions of people. Peptic ulcers are localized breaches of the gastric or duodenal mucosa with tissue destruction at least to the depth of the muscularis mucosa. Among different disorders of gastrointestinal system, peptic ulcer is the one which is more prevalent and have greatest clinical impact. It is now considered to be one of the symptom is pain. The most serious complications are bleeding and perforation. Modern age epidemics are affecting nearly 10% of world population. Research advances during last decade have offered new insights in the therapy and prevention of peptic ulceration[10].

Plants provide an alternative strategy in search for new drugs. There is a rich abundance of plants reputed in traditional medicine known to possess antiulcer properties. It is likely that plants will continue to be a valuable source of new molecules which may, after possible chemical manipulation, provide new and improved antiulcer drugs[11]. Herbal medicines are now used by up to 50% of the western population, in a number of instances

for the treatment or prevention of ulceration[12]. Although drug treatment for peptic ulceration has improved in the recent past, its complications still carry a significant mortality. Recently, widespread effort has been launched to identify novel anti-ulcer drugs from natural resources.

Herbal formulations are used by a majority of Indians from ancient times. They are used to treat a wide verity of conditions including hyperacidity and peptic ulcer. Nearly each and every herbal pharmaceutical company has its antacid and antiulcer products in market. These products are most of the times multidrug formulations[13].

There are various plant-originated "gastro protectors" with different composition that have been used in clinical and folk medicine for many centuries due to their beneficial effects on the mucosa of GIT. In China and Japan, polyphenol extracts such as Sopharadin extracts, containing flavonoids and its synthetic flavonoid derivative known as Solon are widely employed in peptic ulcer therapy and also as food additives and nutritional supplements, mainly because of their strong inhibition of prostaglandin (PG) metabolism and vasoconstrictive leukotriene inhibition[14].

The present study was carried out to assess the possible anti-gastric ulcer activity of methanolic extract of *K. rotunda*, *E. cannabinum* and their isolated products which are widely growing in Sikkim, to confirm and extend its uses in traditional medicine.

7.2. Materials and methods

7.2.1. Plant materials

Methanol extracts of rhizomes of *Kaempferia rotunda* Linn. and leaves of *Eupatorium cannabinum* Linn. as well as their isolated compounds were used as test drug in these experiments.

7.2.2. Drugs and chemicals

All the chemicals used in this study were procured from an authorized dealer and were of analytical grade. Methanol was obtained from LOBA chemicals Kolkata, carboxymethyl cellulose was obtained from Zydus-Cadila, India. Aspirin was collected from SD-Fine Chem, Ltd., Mumbai. Ranitidine was obtained from Cipla Ltd., Goa as a gift sample. Methanolic extract of rhizomes of *Kaempferia rotunda* Linn. and leaves of *Eupatorium*

cannbinum Linn. and their isolated compounds used in this study were extracted in our laboratory.

7.2.3. Test compound formulations

Oral suspensions of the rhizome and leaf extract of *K. rotunda* and *E. cannabinum* respectively and the isolated compounds of both the plants were prepared by suspending them separately in 1% solution of sodium carboxy methylcellulose to obtain suitable dosage forms.

7.2.4. Experimental animals

The study was conducted on Wistar albino rats (n=72) weighing 150-200g of either sex and maintained under standard environmental conditions as per a specific design (10% air exhaust in air conditioning unit was maintained along with a relative humidity of 60 ±5% and a temp of 25±3° with 12 h light and dark cycle. Amrut certified rodent diet and tap water (boiled water cooled to room temp) was provided *ad libitum* to the experimental animals. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee (IAEC) prior to the initiation of the experiment.

7.3. Anti ulcer activity of methanol extract of rhizome and isolated compound of *Kaempferia rotunda Linn.*

7.3.1. Ulcer induction procedure

Gastric ulcers were induced in the experimental animals by administration of Aspirin + pylorus ligation (300mg/kg by gavaging-Model A), Aspirin (300mg/kg by gavaging-Model B).

7.3.2. Model A: Aspirin + Pylorus ligation treatment induced ulcers

Both aspirin treatment as well as pylorus ligation procedure was used to induce peptic ulcers. Effects on gastric lesion and secretions were carried out by ligation of the pyloric end of the stomach by silk suture according to the method described by Shay *et.al.*[15]. Male Wistar rats between 150-200g were selected for pyloric ligation ulcer model. They were fasted in individual cages for 36 h. Care was being taken to avoid coprophagy. For antiulcer investigation animals were divided in to 6 groups (group-I to group-VI). In each group study, 36hrs fasted animals were used. The control animals (group I) were

administered with calculated dose of 1% sodium CMC (Sodium carboxy methyl cellulose) in distilled water. The positive control (group IV) was dosed with ranitidine (20mg/kg suspended in 1% sodium CMC in distilled water. The test group-II and III were dosed with test extract 200 mg/kg, and 400 mg/kg in 1% sodium CMC in distilled water and V, VI were dosed with isolated compound I at 10, 20 mg/kg respectively. All the animals received drug/ extract treatment along with 300 mg/kg of aspirin suspended in 1% sodium CMC once daily for five days. The sixth dose of drug was given 30 minutes prior to pylorus ligation. On the sixth day the 36 hrs fasted rats were subjected to pylorus ligation. They were sacrificed after 4 hrs post surgery and their intact stomachs were excised, observed and the contents were emptied in to a graduated centrifuge. The collected gastric juice was centrifuged at 3000rpm for 30min and the volume of gastric juice was measured. Total acidity in the supernatants was determined with 0.01 M NaOH and expressed as m.Eq/L gastric juice. The stomach was cut open along the greater curvature and pinned on a board for ulcer scoring[16-18].

7.3.3. Model B: Aspirin induced ulcers

In aspirin induced ulcer model[19] the rats were divided into six groups each consisting of three. The Group I served as control, the Group II served as positive control and the Group III, IV, V and VI served as test. Control animals received 1% sodium CMC in distilled water for 8 days and the group II treated with Ranitidine (20mg/kg). The Group III and IV were treated with methanolic extract of *K. rotunda* at 200mg/kg and 400mg/kg body weight respectively.

Group V and VI were dosed with isolated compound 1 at 10 and 20 mg/kg body weight respectively for 8 days orally. After 8 days of treatment animals were fasted for 36 hours. The animals were gavaged with aspirin suspended in 1% sodium CMC. The animals were then left as such for 4 hrs. After which they were sacrificed. The intact stomach was removed from each animal, washed in normal saline and cut along the greater curvature. The inner lining was observed for ulcer formation and ulcers were scored to obtain the ulcer index[20]. All the animal experimental protocols were approved by the Institutional Animal Experimental Committee.

7.4. Antiulcer activity of methanol extract of leaves and isolated compound of *Eupatorium cannabinum* Linn.

The antiulcer activity of methanol extract of *E. cannabinum* leaves and its isolated compound II were determined as per the methods described above in 7.3 for *K. rotunda* Linn.

7.5. Calculation of ulcer index

After sacrificing the rat, the stomach was removed and opened along the greater curvature. The mucosal layer of stomach was observed under a magnifying lens and was checked for ulcers, hemorrhagic areas or perforations. The ulcer index was determined as Ulcer Index =10/X (Where, X= Total area of stomach mucosa / total ulcerated area) [21].

7.6. Statistical analysis

The results of all the assays were reported as (X±SEM). Statistical significant differences between the groups were calculated by means of one way ANOVA. All the results obtained in the study were compared with the control group and positive control group treated with ranitidine. P values < 0.05 were considered statistically significant.

7.7. Results

Peptic ulceration was immensely induced with different intensities in each of the ulcer models. The ulcer index for *Kaempferia rotunda* extracts (200 and 400mg/kg) and isolated compound 1 (10, 20 mg/kg) in Model-A were calculated as 0.67±0.12, 0.45±0.018, 0.63±0.61 and 0.52±0.12 respectively which is summarized in table-7.1 and were found to be statistically significant when compared with control (1.16±0.082) as well as for positive control group (0.41±0.038) at 5% level of significance (P<0.05). The ulcer index for *Eupatorium cannabinum* extracts (200 and 400mg/kg) and isolated compound II (10mg and 20mg/kg) in Model-A were determined as 0.59±0.12, 0.38±0.041, 0.54±0.30 and 0.39±0.19 respectively which is summarized in table-7.3 and were found to be statistically significant when compared with control (1.02±0.082) and for positive control group (0.27±0.016) at 5% level of significance (P<0.05). Similarly the volume of gastric content, total acidity and total acidity output of the groups of animals treated with the extracts of *K. rotunda* and *E. cannabinum* at 200 and 400 mg/kg and their isolated compounds had significantly reduced the values when compared with

control. The group treated with 400 mg/kg for both the plant extract showed significant difference when compared with ranitidine. All the extract treated animal parametric values were comparable with that of standard group. In order to assess the statistical significance in between the groups one way ANOVA was carried out and it authenticates that almost all four parameter were significant. The extracts at the dose level of 200, 400 mg/kg body weight showed dose response antiulcer effect whereas the isolated compound I and II showed the dose response antiulcer effect at the dose level of 10, 20 mg/kg body weight. For the aspirin induced model (Model-B) the ulcer index values are epitomized in table7.2 and 7.4. Pretreatment with the methanolic extract of *Kaempferia rotunda, Eupatorium cannabinum* and their isolated compounds the results were comparable to control and ranitidine treated groups which are verified with one way ANOVA. The datas were found to be highly significant when compared with control and ranitidine group at 5% level of significance.

Table-7.1. Antiulcer activity of *K. rotunda* and its isolated compound-I in rats by Aspirin+pylorus ligation method (Model-A)

Acid secretary parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
	Control (X±SEM)	Plant extract 200mg/kg (X±SEM)	Plant extract 400mg/kg (X±SEM)	Ranitidine 20mg/kg (X±SEM)	Isolated product 1 (10mg/kg) (X±SEM)	Isolated product 1 (20mg/kg) (X±SEM)
Vol. of gastric content (ml/100g)	9.67±0.372	8.78±0.410	7.02±0.27	6.57±0.316	6.89±0.65	6.71±0.09
Total acidity m.Eq/L	72.89±2.17	59.63±3.10	48.53±2.02	47.88±4.71	48.40±0.11	48.01±0.10
Total acidity output m.Eq/100g	183.28±12.44	168.72±31.24	151.84±20.19	148.11±11.81	150.87±0.34	150.78±0.08
Ulcer index	1.16±0.082	0.67±0.12	0.45±0.018	0.41±0.038	0.63±0.61	0.52±0.12
One way ANOVA F 0.036	All values are statistically significant respect to control at 5% level of significance					

Table -7.2. Antiulcer activity of *K. rotunda and* its isolated compound-I in rats by Aspirin induced ulcer method (Model-B)

Group No.	Treatment	Ulcer index (X±SEM)
I	Control (1% sodium CMC)	0.93±0.218
II	Ranitidine(20mg/kg)	0.26±0.17
III	Plant extract (200mg/kg)	0.63±0.192
IV	Plant extract (400mg/kg)	0.32±0.23
V	Isolated compound-1(10mg/kg)	0.53±0.61
VI	Isolated compound- I (20mg/kg)	0.42±0.12

All the values were (X±SEM) significantly different from control and ranitidine at $p < 0.05$ level

Table-7.3. Effect of methanolic extract of *E. cannabinum* and its isolated compound II in rats by Aspirin + pylorus ligation method (Model-A)

Acid secretory parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
	Control (X±SEM)	Plant extract (200mg/kg) (X±SEM)	Plant extract (400mg/kg) (X±SEM)	Ranitidine (20mg/kg) (X±SEM)	Isolated Compound II (10 mg/kg) (X±SEM)	Isolated Compound II (20 mg/kg) (X±SEM)
Vol. of gastric content (ml/100g)	7.03± 0.096	5.64± 0.14	5.49 ± 0.32	5.38 ± 0.29	5.44±0.08	5.41±0.41
Total acidity (m.Eq/l)	66.82± 3.28	56.02± 4.88	41.25± 4.71	37.03 ± 5.62	42.87±0.10	42.03±0.25
Total acidity output (m.Eq/100g)	79.72±18.1	141.22±38.3	132.1±14.9	122.61±22.11	129.87±0.20	128.78±0.26
Ulcer Index	1.02± 0.082	0.59 ± 0.12	0.38± 0.041	0.27 ± 0.016	0.54±0.30	0.39±0.19
One way ANOVA F 0.020			All values are statistically significant respect to control at 5% level of significance			

Table-7.4. Effect of methanolic extract of *E. cannabinum* and its isolated compound II in rats by Aspirin induced ulcer method (Model-B)

Group No.	Treatment	Ulcer Index (X±SEM)
I	Control (1% sodium CMC)	0.81 ± 0.031
II	Ranitidine(20mg/kg)	0.26± 0.170
III	Plant extract (200mg/kg)	0.24 ± 0.033
IV	Plant extract (400mg/kg)	0.21± 0.015
V	Isolated compound II (10mg/kg)	0.20 ± 0.014
VI	Isolated compound II (20mg/kg)	0.18 ± 0.019

All the values were (X±SEM) significantly different from control and ranitidine at $p < 0.05$ level

7.8. References

1. Valle DL. Peptic ulcer diseases and related disorders. In: Harrison's Principles of Internal Medicine, Braunwald E, Fauci AS, Kasper DL, Hauser SL, Longo DL, Jameson JL. (Eds), 15th edn., New York, McGraw-Hill, 2002, p 1649-1665.
2. Hoogerwerf WA and Pasricha PJ. Agents used for control of gastric acidity and treatment of peptic ulcers and gastro esophageal reflux disease. In: Hardman JG, Limbird LE. (Eds), Goodmann's and Gilman's. The Pharmacological Basis of Therapeutics.10th edn., New York, Tata Mc Graw Hill, 2001, p 1005-1019.
3. Decktor DL, Pendleton RG, Kellner AT, *et. al.* Acute effects of ranitidine, famotidine and omeprazole on plasma gastrin in rats. *J. Pharma. Exp. Thera.*, 1989, **249(1)**, 1-5.
4. Tarnawaski A, Stachura J, Krause WJ, *et. al.* Quality of gastric ulcer healing. A new emerging concept. *J. Clin. Gastroenterol.*, 1991, **13**, 42-47.
5. Tarnawaski A. A cellular mechanism of gastric ulcer healing. In: The Stomach, Domschke W and SJ Konturex SJ, eds., Berlin, Springer-Verlag, 1993, p 177-192.
6. Tarnawaski A, Tanoue K, Santos AM, *et. al.* Cellular and molecular mechanism of gastric ulcer healing. Is the quality of mucosal scar affected by treatment? *J. Gastroenterol.*, 1995, **39**, 9-14.
7. Seibert K, Zhang Y, Leaky K, *et. al.* Distribution of COX-1 and COX-2 in normal and inflamed tissue. *Adv. Exp. Med. Biol.*, 1997, **400**, 167-170.
8. Tsuji S, Kawano S, Sawaoka H, *et. al.* Evidences for involvement of cyclooxygenase-2 in proliferation of two gastrointestinal cancer cell lines. *Prostaglandins Leukot Essential fatty Acids*. 1996, **55**, 179-183.
9. Sawaoka H, Tsuji S, Tsuji M, *et. al.* Expression of cyclooxygenase-2 gene in gastric epithelium. *J. Clin. Gastroenterol.*, 1997, **25**, 105-110.
10. Chan FK and Leung WK. Peptic-ulcer disease. *Lancet*. 2002, **360**, 933-941.
11. Shah JS, Shah MB, Goswami SS and Santani DD. Mechanism of action of antiulcer activity of bark extracts of *Manilkara hexandra* against experimentally induced gastric ulcers in rats. *Phcog. Mag.*, 2006, **2**, 5.
12. Langmead L and Rampton DS. Herbal treatment in gastrointestinal and liver disease benefits and dangers. *Aliment Pharmacol. Ther.*, 2001, **15**, 1239-1252.

13. Brito ARMS, Cota RHS and Nunes DS. Gastric antiulcerogenic effects of *Dalbergia monetaria* L. in rats. *Phytother. Res.*, 1997, **11**, 314-316.

14. Kyogoku K, Hatayama K, Yokomori S, *et. al.* Anti-ulcer effect of isoprenyl flavonoids and Synthesis and anti-ulcer activity of new chalcones related to sophoradin. *Chem. Pharm. Bull.*, 1979, **27**, 2943-2952.

15. Shay H, Komarov SA, Fels SS, Meranze D, Gruenstein M and Siplet H. A simple method for the uniform production of gastric ulceration. *Gastroenterology*. 1945, **5**, 43-61.

16. Parmar NS and Desai JK. A review of the current methodology for the evaluation of gastric and duodenal antiulcer gents. *Indian J. Pharmacol.*, 1993, **25**, 120-135.

17. Paul A, Patel S, Sevak R , Goswami S and Santani DD. Antiulcer and gastric acid inhibitory activity of ZD 7114, $A\beta_3$- adrenoceptor agonist in Rats. *Indian J. Pharma. Sci.*, 2001, **63(5)**, 413-418.

18. Singh S. Evaluation of gastric anti ulcer activity of fixed oil of *Ocimum basilieum* Linn. and its possible mechanism of action. *Indian J. Exp. Biol.*, 1999, **36**, 253-257.

19. Szelenyi I and Thiemer K. Distension ulcer as a model for testing of drugs for ulcerogenic side effects. *Arch. Toxicol.*, 1978, **41**, 99.

20. Hedge DA, Khosa RC and Goel RK. Antiulcer and cytoprotective action of *Wedelia calendlacea* Less. *Ancient science of life*, 1994, **14**, 77.

21. Rainsford KD. Side effect of anti-inflammatory and analgesic drugs, epidemiology and gastrointestinal tract. *Trends Pharmacol. Sci.*, 1984, **5**, 156-159.

CHAPTER - 8

STUDIES ON ANTI-INFLAMMATORY ACTIVITY

8.1. Introduction

In traditional practice, medicinal plants are used to control inflammation in many countries. This has caused an increase in the number of experimental and clinical investigations directed towards the validation of the anti-inflammatory properties which are putatively attributed to these remedies[1,2]. The inflammatory mechanisms in the body are very complicated and they cannot be attributed to a single mediator or factor. Inflammation mediators, such as histamine, serotonin, arachidonic acid metabolites and quinines, are known to have a major role in generation of the inflammatory reactions[3,4]. Inflammatory process has two phases: acute and chronic. Acute inflammation is characterized by fever, pain, and edema, while chronic inflammation is characterized by cellular proliferation[5]. Complement system, fibrinolytic system and hyaluronidase enzyme are activated in plasma during inflammation[6]. Hyaluronidase activity in blood is increased during inflammation and the decrease in inflammation parallels a decrease in hyaluronidase activity[7]. Models of acute inflammation, which induce inflammation by administration of formalin, dextran, histamine, serotonin, bradykinin, prostaglandin and carrageenan are used to investigate anti-inflammatory effects of drugs[8]. Carrageenan-induced inflammation model is a COX-dependent reaction and is used to determine COX inhibition[9]. Prolonged uses of both steroidal and non-steroidal anti-inflammatory drugs are well known to be associated with peptic ulcer formation[10]. Hence, search for new anti-inflammatory agents that retain therapeutic efficacy and yet are devoid of these adverse effects is justified. There is much hope of finding active anti-inflammatory compounds from indigenous plants as these are still used in therapeutics despite the progress in conventional chemistry and pharmacology in producing effective drugs. Herbal drugs are being proved as effective as synthetic drugs with lesser side effects. The enzyme, phospholipase A_2, is known to be responsible for the formation of mediators of inflammation such as prostaglandins and leukotrienes which by attracting poly-morphonuclear leucocytes to the site of inflammation would lead to tissue damage probably by the release of free radicals. Phospholipase A_2 converts phospholipids in the cell membrane into arachidonic acid, which is highly reactive and is rapidly metabolized by cycloxygenase (prostaglandin synthase) to prostaglandins, which are major components that induce pain and inflammation[11,12].

Rheumatoid arthritis is a highly variable, chronic inflammatory condition affecting mostly diarthrodial (hinge-like) joints but often with articular and systemic involvement. The available data indicate that 76% of patients with rheumatoid arthritis are taking NSAIDs[13,14]. Apart from treating the underlying disease, it is necessary to relieve patient's pain. This has led to major improvements in the treatment of acute and chronic pain. In the pharmacological treatment of acute pain, aspirin-like and morphine-like drugs still form the cornerstone of most therapies[15]. There was a controversy about the anti-inflammatory effect of *Harpagophytum procumbens* (devil's claw), an herbal product marketed in Canada and Europe, as a home remedy for relief of arthritic diseases.

Recent studies suggest that *Harpagophytum procumbens* has anti-inflammatory and analgesic effect. Extract of *Harpagophytum procumbens* have become the focus of research as a potential therapeutic agent in the treatment of rheumatic arthritis and pain due to its favorable side effects profile compared to synthetic alternatives[16]. *Harpagophytum procumbens* was effective in the treatment of osteoarthritis and reduced the need for analgesic and NSAIDs therapy[17]. Treatment of 800 mg of the extract, three times daily with total dose of not more than 2400mg per day has been accompanied by reduction of pain[18]. *Harpagophytum procumbens* can probably help many of those who suffer from low back pain with fewer side effects than NSAIDs treatment that are troublesome in the elderly, at a cost that is certainly not excessive[19]. In Indian system of medicine, a large number of drugs of either herbal or mineral origin have been advocated for various types of diseases and other different unwanted conditions in humans[20]. Ayurveda is one of the traditional systems of medicine practiced in India and Sri Lanka and can be traced back to 6000 BC[21]. Ayurvedic medicines are largely based upon herbal and herbomineral preparations and have specific diagnostic and therapeutic principles [22]. Inflammation is a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules[23]. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved in inflammation. Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer, colonic adenomas and Alzheimer's disease[24,25].

Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects[26,27]. The research into plants with alleged folkloric use, as

pain relievers, anti-inflammatory agents, should therefore be viewed as a fruitful and logical research strategy in the search for new analgesic and anti-inflammatory drugs[28]. Scientific interest in medicinal plants has burgeoned in recent times due to increased efficiency of new plant derived drugs and rising concerns about the side effects of conventional medicine. Inflammation is seen in conditions such as Alzheimer's disease, cancer, irritable bowel syndrome and hepatic diseases. It is believed that controlling inflammation may help to alleviate these conditions or even prevent them[29]. An attempt was made in this present study to assess the efficacy of two indigenous herbs for their anti-inflammatory activity in rats. Thus the present investigation was carried out to evaluate the anti-inflammatory potential of *K. rotunda* and *E. cannabinum.*

8.2. Materials and methods

8.2.1. Plant materials

Methanol extracts of rhizomes of *K. rotunda* and leaves of *E. cannabinum* were subjected as test drug in these experiments.

8.2.2. Drugs and chemicals

Carrageenan and Indomethacin was obtained from Sigma-Aldrich, Germany. All the solvents used were of analytical grade procured from E. Merck, Mumbai.

8.2.3. Experimental animals

Albino rats of Wistar strain (150-200g) of either sex were procured from the central animal house of the Institute. They were housed in standard polypropylene cages and kept under controlled room temperature (24±2^{0}C); relative humidity 60-70%) in a 12h light-dark cycle. The rats were given a standard laboratory diet and water *ad libitum.* Food was withdrawn 12h before and during the experimental hours. All experimental protocols were approved by the institutional animal ethics committee (IAEC).

8.3. Screening of Anti-inflammatory activity by using Carrageenan Induced Rat Paw Edema

8.3.1. Anti-inflammatory activity of methanol extract of rhizome of *K. rotunda*

Anti-inflammatory activity was assessed by the method described by Winter *et. al.*[30]. The rats were divided into four groups of six animals each. First group (negative control) received 0.2ml/100g b.w. of 0.5% w/v solution of sodium CMC in normal saline, second group (positive control) received 10mg/kg p.o. indomethacin. The third and fourth group received methanolic extract (200, 400mg/kg p.o.) of *K. rotunda* respectively. After 1h, the rats were challenged with subcutaneous injection of 0.1ml of 1% w/v solution of carrageenan into the plantar side of the left hind paw. The paw was marked with ink at the level of lateral malleolus and immersed in mercury cup up to the mark. The plethysmograph apparatus used for the measurement of rat paw volume was that of Singh and Ghosh[31]. The paw volume was measured immediately after injection (0h) and then every hour till 3h after injection of carrageenan to each group. The difference between the initial and subsequent reading gave the actual edema volume. Percent inhibition of inflammation was calculated using the formula,

% inhibition = 100 (1-Vt/Vc)

Where 'Vc' represents edema volume in control and 'Vt' edema volume in groups treated with test extracts.

8.3.2. Anti-inflammatory activity of methanol extract of leaves of *E. cannabinum*

The anti-inflammatory activity of methanol extract of *E. cannabinum* leaves was determined as per the methods described above in 8.3.1 for *K. rotunda*.

8.4. Statistical analysis

All the experimental data were expressed as mean±SEM. The statistical significance of the differences was accessed with one way ANOVA. Differences with P values less than 0.05 was considered as significant.

8.5. Results

The anti-inflammatory effect of *K. rotunda* is represented in table-8.1 and the effect of *E. cannabinum* is shown in table-8.2. The methanolic extract of both *K. rotunda* and *E. cannabinum* exhibited significant ($p<0.05$) anti-inflammatory activity at doses of 200 and 400mg/kg body weight orally. As shown in table-8.1 and table-8.2 methanolic extract of *K. rotunda* and methanolic extract of *E. cannabinum* exhibited inhibition in rat paw oedema by 21.36% and 18.50% for 200mg/kg body weight and 50.97% and 47.56% for 400mg/kg body weight respectively whereas standard drug indomethacin showed 53.77% and 50.62% respectively.

The present study indicates the potential of these herbal drugs as anti-inflammatory drugs. Such drugs can successfully be used in various inflammatory diseases. The activity may be attributed to the inhibition of the COX-2 enzyme or inhibition of the activation of transcription factors. It can be concluded that all the extracts have potential to act as anti-inflammatory agents due to presence of flavonoids as active constituent. It can be concluded that both the extracts have potential to be explored as anti-inflammatory agents.

Table-8.1. The effect of methanolic extract of *K. rotunda* on Carrageenan induced edema in rats

		Volume of mercury displaced in ml. (± SEM)				
Treatment	**Dose**	**0hr (X±SEM)**	**1hr (X±SEM)**	**2hr (X±SEM)**	**3hr (X±SEM)**	**% inhibition of paw of edema**
Control 0.5% w/v sodium CMC	0.2ml/100g	0.996±0.048	1.297±0.032	1.607±0.029	1.712±0.036	-
Indomethacin	10mg/kg	0.990±0.038	1.214±0.021	1.463±0.024	1.321±0.028	53.77
Plant extract	200mg/kg	0.997±0.037	1.262±0.017	1.523±0.011	1.560±0.031	21.36
Plant extract	400mg/kg	0.993±0.026	1.238±0.023	1.489±0.041	1.344±0.036	50.97
One way ANOVA F 0.315		All values are statistically significant respect to control at 5% level of significance				

Table-8.2. The effect of methanolic extract of *E. canabinum* on Carrageenan - induced edema in rats

		Volume of mercury displaced in ml. (± SEM)				
Treatment	**Dose**	**0hr (X±SEM)**	**1hr (X±SEM)**	**2hr (X±SEM)**	**3hr (X±SEM)**	**% inhibition of paw of edema**
Control (0.5% w/v sodium CMC)	0.2ml/100g	0.988±0.029	1.292±0.034	1.611±0.031	1.707±0.036	-
Indomethacin	10mg/kg	0.979±0.031	1.213±0.024	1.448±0.028	1.334±0.020	50.62
Plant extract 200mg/kg	200mg/kg	0.986±0.019	1.274±0.019	1.493±0.027	1.572±0.029	18.50
Plant extract 400mg/kg	400mg/kg	0.984±0.036	1.241±0.041	1.483±0.036	1.361±0.040	47.56
One way ANOVA F 0.238		All values are statistically significant respect to control at 5% level of significance				

8.6. References

1. Giron LM, Freire V, Alonzo A and Caceres A. Botanical survey of medicinal flora used by the Caribs. *J. Ethnopharmacol.*, 1991, **28**, 1956-1961.
2. Kumar VL and Basu N. Anti-inflammatory activity of the latex of *Calotropis procera*. *J. Ethnopharmacol.*, 1994, **44**, 123-125.
3. Maling HM, Webster ME, Williams MA, Saul W and Anderson W. Inflammation induced by histamine, serotonin, bradykinin and comp 48/80 in the rat: Aantagonists and mechanism of action. *J. Pharmacol. Exp. Ther.*, 1974, **191**, 300-310.
4. Portanova JP, Zhang Y, Anderson GD, Hauser SD, Masferrer JL, Seibert K and Gregory SA. Selective neutralization of prostaglandin E blocks inflammation, hyperalgesia and interleukin-6 production *in vivo*. *J. Exp. Med.*, 1996, **184**, 883-891.
5. Dunne MW. *Inflammation and repair.* In: Pathophysiology. Porth CM, (Ed), Lippincott, Philadelphia, 1990, p 165-176.
6. Collins T. *Acute and chronic inflammation.* In: Robbins Pathology Basic of Disease, Cortan RS, Kumar V and Collins T, (Eds), 6th edn., Saunders, Elsevier India, 2003, p 50-88.
7. Procida C, Montovani V and Bianchini P. Anti-hyaluronidase activity of some pyrazole derivatives. *Boll. Soc. Ital. Biol. Sper.*, 1971, **47**, 159-163.
8. Suleyman H and Buyukokuroolu ME. The effects of newly synthesized pyrazole derivatives on formaldehyde-carrageenan and dextran-induced acute paw edema in rats. *Biol. Pharm. Bull.*, 2001, **24**, 1133-1136.
9. Gamache DA, Povlishock JT and Ellis EF. Carrageenan induced brain inflammation and characterization of the model. *J. Neurosurg.*, 1986, **65**, 675-685.
10. Ewart A. *Remington's Pharmaceutical Sciences.* eds., Gennaro AF, (Ed), 16th edn., Mack Publishing Company, Easton, Pensyllvenia, 1980, p 873-874.
11. Higgs GA, Moncada S and Vane JR. Eicosanoids in inflammation. *Ann. Clin. Res.*, 1984, **16**, 287-99.

12. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New. Biol.*, 1971, **231**, 232-235.

13. Lanhers *et. al.* How recent findings affect thinking about nonsteroidal anti-inflammatory drugs? *Drugs*, 1997, **53(4)**, 563-582.

14. Schiff M. Emerging treatments for rheumatoid arthritis. *Am. J. Med.*, 1997, **102(1)**, 11-15.

15. Besson JM. The neurobiology of pain. *Lancet*, 1999, **353**, 1610-1615.

16. Fiebich BL, Kammerer N, Heinrich M and Hiller O. Inhibition of TNF-alpha synthesis LPS-stimulated primary human monocytes by Harpagophytum extract Stei Hap 69. *Phytomedicin*, 2001, **8(1)**, 28-30.

17. Leblan D, Chantre P and Fournie B. *Harpagophytum procumbens* in the treatment of knee and hip osteo-arthritis. Four-month results of a prospective, multicenter, double-blind trial versus diacerhein. *Joint Bone Spine.*, 2000, **67(5)**, 462-467.

18. Chrubasik S, Zimpfer Ch, Schutz U and Ziegler R. Effectiveness of *Harpagophytum procumbens* in treatment of acute low back pain. *Phytomedicine*, 1996, **3(1)**, 1-10.

19. Chrubasik S, Junck H, Breitschwerdt H, Conradt C and Zappe H. Effectiveness of *Harpagophytum* extract in the treatment of exacerbation of low back pain: A randomized, placebo controlled, double-blined study. *Eur. J. Anaesthesiology*, 1999, **16**, 118-129.

20. Brekhman C and Dardimov I. New substances of plant origin, which increase non-specific resistance. *Annu. Rev. Pharmacol.*, 1969, **21**, 219-226.

21. Charak Samhita (Trans): Shree Gulab Kunverba Ayurvedic Society, Jamnagar, India, 1949.

22. Patwardhan B and Hopper B. Ayurvedic and future drug development. *J. Alter. Coplement Med.*, 1992, **19(12)**, 9-10.

23. Mantri P and Witiak DT. Inhibition of cyclooxygenase and 5-lipoxygenase. *Curr. Med. Chem.*, 1994, **1**, 328-355.

24. Smith WL and De Witt DL. Biochemistry of prostaglandins endoperoxide H synthase-1 and synthase-2 and their differential susceptibility to NSAIDs. *Sem. Nephrol.*, 1995, **15**, 179-194.

25. Lipsky P. The clinical potential of COX-2 specific inhibitors. *Amer. J. Med.*, 1999, **106**, 51-57.

26. Farnsworth NR. *Screening plants for new medicines*. In: Biodiversity, Part II, Wilson EO, (Ed), National Academy Press, Washington, 1989, p 83-97.

27. Eisner T. *Chemical prospecting: A call for action*. In: Ecology, Economic and Ethics, Borman FH and Kellert SR, (Eds), The Broken Circle, Yale University Press, New Haven, 1990, p 258.

28. Elisabetsky E, Amodor TA, Albuquerque RR, Nunes DS and Carvalho ACT. Analgesic activity of *Psychotria colorata* (Wildex R. & S.) Muell Arg. Alkaloids. *J. Ethnopharmacol.*, 1995, **48**, 77-83.

29. Omkar TJ and Gadgoli C. Evaluation of Anti-inflammatory activity of *Nyctanthes arbor-tristis* and *Onosma echioides*. *Pharmacognosy Magazine*, 2006, **2(8)**, 258-60.

30. Winter CA, Risley EA and Silber RH. Anti-inflammatory activity of indomethacin and plasma corticosterone in rats. *J. Pharmacol. Exp. Ther.*, 1968, **162**, 196-201.

31. Singh H and Ghosh MN. Modified plethysmometer for measuring foot volume of unanesthetized rats. *J. Pharm. Pharmacol.*, 1968, **20**, 316-317.

CHAPTER - 9

STUDIES ON ANALGESIC AND ANTIPYRETIC ACTIVITY

9.1. Introduction

Acute pain, for the most part, results from disease, inflammation, or injury to tissues. It is immediate and usually of a short duration. Acute pain is a normal response to injury and may be accompanied by anxiety or emotional distress. The cause of acute pain can usually be diagnosed and treated. Chronic pain is continuous pain that persists for more than 3 months, and beyond the time of normal healing. It ranges from mild to severe and can last weeks, months, or years to a lifetime. The cause of chronic pain is not always evident, although it can be brought on by chronic conditions such as arthritis and fibromyalgia. Chronic pain can often interfere with a patient's quality of life, sleep, pain and the diseases of the bones, muscles, joints, and skin, which affect millions of peoples. Most of these diseases are chronic and may cause lifelong pain. In certain cases, such as with some rheumatic diseases, the sources of pain may include inflammation of the synovial membrane (tissue that lines the joints), the tendons, or the ligaments; muscle strain; and muscle fatigue. A combination of these factors contributes to the intensity of the pain. Muscle inflammation characterizes other painful disorders such as polymyositis (characterized by inflamed and tender muscles throughout the body, particularly those of the shoulder and hip) and dermatomyositis (characterized by patchy red rashes around the knuckles, eyes, and other parts of the body, along with chronic inflammation of the muscles[1].

An analgesic (colloquially known as painkiller) is any member of the diverse group of drugs used to relieve pain (achieve *analgesia*). This derives from Greek *an-*, "without", and *-algia*, "pain". Analgesic drugs act in various ways on the peripheral and central nervous system; they include paracetamol (acetaminophen), the nonsteroidal anti-inflammatory drugs (NSAIDs) such as the salicylates, narcotic drugs such as morphine, synthetic drugs with narcotic properties such as tramadol, and various others. Some other classes of drugs not normally considered analgesics are used to treat neuropathic pain syndromes; these include tricyclic antidepressants and anticonvulsants. Analgesics are frequently used in combination, such as the paracetamol and codeine preparations found in many non-prescription pain relievers. They can also be found in combination with vasoconstrictor drugs such as pseudoephedrine for sinus-related preparations, or with antihistamine drugs for allergy sufferers. The use of paracetamol, as well as aspirin,

ibuprofen, naproxen, and other NSAIDS concurrently with weak to mid-range opiates (up to about the hydrocodone level) has been shown to have beneficial synergistic effects by combating pain at multiple sites of action. NSAIDs reduce inflammation which, in some cases, is the cause of the pain itself while opiates dull the perception of pain thus, in cases of mild to moderate pain caused in part by inflammation[1]. The plant-derived secondary metabolites have, over the years, greatly contributed to our current understanding of the important mechanisms related to the process of pain transmission and treatment. Furthermore, they have permitted us to characterize receptor types and identify endogenous ligands involved in the mechanism of nociception. In this review, we discuss the recent advances that have occurred regarding plant-derived substances in the process of development of new analgesic drugs. Plants, such as *Papaver somniferum*, *Cannabis sativa* and those of the Capsicum and Salix species, have greatly accounted for the development of clinically relevant drugs, which are useful for the management of pain disorders. The recent advances in our understanding of the mechanisms of action of the above plant-derived substances, together with use of molecular biology techniques, have greatly accelerated attempts to identify promising targets for the discovery of new, safe and efficient analgesic drugs. Despite the great progress that has occurred in the elucidation of pain transmission and despite decades of use, leaving aside its known undesirable side effects, morphine continues to be one of the most used drugs in clinical practice for the treatment of pain disorders. Thus, safer and more efficacious analgesic drugs are urgently needed. A search through the literature reveals that many potentially active antinociceptive plant-derived compounds have been identified. However, studies aiming to investigate their cellular and molecular mechanisms of action and well-controlled clinical trials to prove their efficacy in humans are still lacking. Nevertheless, natural or synthetic substances that bind to vanilloid or cannabinoid receptors, or even those that are capable of modulating the endogenous ligands which bind to these receptors, are expected soon to appear to assist in the treatment of several pain disorders, including those of neuropathic or neurogenic origin[2]. Regulation of body temperature requires a delicate balance between the production and loss of heat and the hypothalamus regulates the set point at which body temperature is maintained. In fever the set point is elevated and paracetamol like drugs promote its return to normal. These drugs do not influence body temperature when it is elevated by such factors as exercise or increase in

the ambient temperature[3]. Fever (also known as pyrexia, or a febrile response, and archaically known as ague) is a medical symptom that describes an increase in internal body temperature to levels that are above normal (37°C, 98.6°F). Fever should not be confused with hyperthermia, which is an increase in body temperature over the body's thermoregulatory set-point (normally approximately 37°C, but increased during a fever). A fever is most accurately characterized as a temporary elevation in the body's thermoregulatory set-point, which is usually by about 1-2°C. This elevation in thermoregulatory set-point means that the previous "normal body temperature" would be considered hypothermic. Effector mechanisms, such as increased blood pressure, increased heart rate, activation of brown adipose tissue and muscular shivering attempt to counteract the perceived hypothermia, thereby reaching the new thermoregulatory set-point. It is the most common symptom of many diseases. Most people take medication against fever because the symptoms cause discomfort. Fever increases heart rate and metabolism, thus potentially putting an additional strain on elderly patients, patients with heart disease, *etc*. This may even cause delirium. Therefore, potential benefits must be weighed against risks in these patients. In any case, fever must be brought under control in instances when fever escalates to hyperpyrexia and tissue damage is imminent. An adaptive mechanism, fever is the body's reaction to pathogens; it attempts to raise core body temperature to levels that will speed up the actions of the immune system, and may also directly denature, debilitate, or kill the pathogen. Most fevers are caused by infections, and almost all infectious diseases can cause fever. However, there are instances when fever escalates to temperatures where the body is at risk of destroying its own cells and must be brought under control with suppressive medication[4-13].

Antipyretic drugs are drugs that prevent or reduce fever by lowering the body temperature from a raised state. However, they will not affect the normal body temperature if one does not have fever. Antipyretics cause the hypothalamus to override an interleukin-induced increase in temperature. The body will then work to lower the temperature and the result is a reduction in fever[14]. Medicinal plants are an important source of new chemical substances with potential therapeutic effects. The research into plants with alleged folklore use as pain relievers should therefore be viewed as a fruitful and logical strategy in the search of new analgesic drugs. In addition, although there is a wide availability of clinically useful anti-inflammatory and analgesic drugs, a continuing

search for new effective agents with less unwanted side-effects remains vital. The screening of Natural products has led to the discovery of so many potent antipyretic drugs[15]. This present study was carried out to assess the validity of the folkloric uses of the two plants i.e. *K. rotunda* and *E. cannabinum* in the management of pain and treatment of fever and establish the possible mechanisms of pharmacological action.

9.2. Materials and methods

9.2.1. Plant materials

Methanol extracts of rhizomes of *K. rotunda* and leaves of *E. cannabinum* were subjected as test drug in these experiments.

9.2.2. Drugs and chemicals

All the chemicals used in this study were of analytical grade. Sodium CMC and methanol were obtained from LOBA chemicals, Kolkata. Pentazocine and paracetamol were obtained from Zydus-Cadila Ltd. Sikkim as a gift sample. Brewer's yeast (AR grade) was purchased from Sigma Chemicals. Methanolic extract of rhizomes of *K.rotunda* and leaves of *E. cannabinum* used in this study was extracted in our laboratory.

9.2.3. Test compound formulations

Oral suspensions of the leaves and rhizome extract were prepared by suspending them separately in 0.5% solution of sodium carboxy methylcellulose to obtain suitable dosage forms.

9.2.4. Experimental animals

Albino rats of Wistar strain (150-200 g) and Swiss albino mice (25-30 g) of either sex were procured from the central animal house of the Institute. They were housed in standard polypropylene cages and kept under controlled room temperature (24±2°C) and relative humidity (60-70%) in a 12h light-dark cycle. The animals were given a standard laboratory diet and water *ad libitum*. Food was withdrawn 12h before and during the experimental hours. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC).

9.3. Screening of Analgesic activity using Tail immersion method[16]

9.3.1. Analgesic activity of methanol extract of rhizome of *K. rotunda*

Tail immersion method was used to determine the analgesic activity. Swiss albino mice (25-30 g) were randomly divided into 4 groups having 6 animals each and they were fasted overnight but during the experiment had free access to water. Methanolic extract of the plant *K. rotunda* was administered orally (200 and 400mg/kg) 60 minutes prior to the commencement of the estimation of reaction time. Control group of mice (n = 6) received 0.5% w/v solution of sodium CMC in normal saline (0.1ml/100g), and the mean reaction time (in seconds) was determined. Test groups of mice were treated with the methanol extracts of rhizomes and leaves at doses 200 and 400mg/kg orally. Standard group of mice received 10mg/kg pentazocine. The temperature of water in the organ bath was set at 55±0.5°C with the help of a thermostat. Mice were held in position in a suitable restrainer with the tail extending out. The reaction time was determined by immersing the tail in hot water and the time taken by the mice to withdraw its tail clearly out of water was noted. Observations were repeated at an interval of 15 minutes up to 60 minutes.

9.3.2. Analgesic activity of methanol extract of leaves of *E. cannabinum*

The analgesic activity of methanol extract of *E. cannabinum* leaves was also determined as per the methods described above in 9.3.1 for *K. rotunda*.

9.4. Screening of Antipyretic activity using yeast-induced hyperpyrexia method[17]

9.4.1. Antipyretic activity of methanol extract of rhizome of *K. rotunda*

The antipyretic activity of methanolic extracts of rhizomes of *K. rotunda* was screened by using yeast-induced hyperpyrexia method. Briefly, pyrexia was induced in rats by injecting 10 ml/kg of 20% (w/v) suspension of Brewer's yeast intramuscularly. After 18h, the animals developed 0.5°C or more rise in the rectal temperature. They were distributed into 4 groups of 6 each and the extract at the doses of 200 and 400mg/kg was administered orally to the test groups (two groups). Standard group was administered with Paracetamol (33mg/kg) orally. Control group was given 0.5% w/v solution of sodium CMC in normal saline (0.1ml/100g). At different time intervals (1hr, 2hr and 3hr) rectal temperature was noted. Similarly, over night fasted normal animals were divided

into four different groups of 6 each and the experiment was carried out in the same manner as described above. Percentage reduction in rectal temperature was calculated by considering the total fall in temperature to normal level as 100%.

9.4.2. Antipyretic activity of methanol extract of leaves of *E. cannabinum*

The antipyretic activity of methanol extract of *E. cannabinum leaves* was determined as per the methods described above in 9.4.1 for *K. rotunda.*

9.5. Statistical methods

Statistical significance was analyzed using one way ANOVA. P value less than 0.05 was considered as significant.

9.6. Results

The effects of methanolic extracts of rhizomes of *K. rotunda* rhizomes and leaves of *E. cannabinum* on tail immersion method using mice are epitomized in table- 9.1 and table-9.2 respectively. The tail-immersion test is reported to be specific for agents producing central antinociceptive activity. Both the extracts show a good degree of analgesic activity at doses 200 and 400mg/kg in comparison with pentazocine (10mg/kg). The analgesic activity exhibited by the methanolic extracts of both the plants may be due to inhibitory effect on histamine, 5-HT and kinin like substance.

The outcomes of effects of methanolic extracts of rhizomes of *K. rotunda* and leaves of *E. cannabinum* on yeast-induced pyrexia in rats are depicted in table- 9.3 and table- 9.4 respectively. *K. rotunda* produced significant antipyretic effects $p<0.05$ in a dose dependent manner. A significant effect was observed at 200 and 400mg/kg dose of *K. rotunda* and *E. cannabinum* with compared to the standard drug paracetamol (33mg/kg).

Table 9.1. Effect of methanolic extract of rhizomes of *K. rotunda* on tail-immersion test in mice.

			Reaction time (sec) ± SEM			
Treatment	**Dose**	**Basal reaction time (sec) (X±SEM)**	**15min (X±SEM)**	**30min (X±SEM)**	**45min (X±SEM)**	**60 min (X±SEM)**
Control 0.5% w/v sodium CMC	0.1ml/100g	2.32±0.33	2.33±0.192	2.54±0.204	2.66±0.192	2.71±0.345
Pentazocine	10mg/kg	2.21±0.28	4.00±0.408	5.50±0.390	6.83±0.723	8.16±0.597
Plant extract	200mg/kg	2.66±0.24	2.50±0.204	2.83±0.281	3.17±0.366	3.33±0.192
Plant extract	400mg/kg	2.43±0.25	3.50±0.204	4.66±0.500	5.88±0.549	6.75±0.710
One way ANOVA F 4.278			All values are statistically significant respect to control at 5% level of significance			

Table 9.2. Effect of methanolic extract of leaves of *E.cannabinum* on tail-immersion test in mice.

			Reaction time (sec) ± SEM			
Treatment	**Dose**	**Basal reaction time (sec) (X±SEM)**	**15min (X±SEM)**	**30min (X±SEM)**	**45min (X±SEM)**	**60 min (X±SEM)**
Control 0.5% w/v sodium CMC	0.1ml/100g	2.40±0.20	2.49±0.28	2.57±0.19	2.68±0.20	2.73±0.19
Pentazocine	10mg/kg	2.33±0.192	4.167±0.37	5.50±0.311	7.0±0.235	8.50±0.204
Plant extract	200mg/kg	2.67±0.192	3.170±0.152	3.33±0.192	3.5±0.312	3.63±0.208
Plant extract	400mg/kg	2.50±0.204	3.50±0.204	4.66±0.385	6.66±0.451	8.33±0.304
One way ANOVA F 3.116			All values are statistically significant respect to control at 5% level of significance			

Table 9.3. Effect of methanolic extract of rhizomes of *K. rotunda* on Yeast induced pyrexia model in rats.

		Rectal temperature (°C)		Rectal temperature after administration of drug (°C)		
Treatment	**Dose (mg/kg)**	**(A) Normal animals (X±SEM)**	**(B) 18h after yeast administration (X±SEM)**	**(C1) 1h (X±SEM)**	**(C2) 2h (X±SEM)**	**(C3) 3h (X±SEM)**
Control (Normal saline)	0.5 ml	37.97±0.17	38.70±0.16	38.68±0.21 (2.73±3.21)	38.67±0.18 (4.10±4.06)	38.65±0.04 (6.84±7.01)
Paracetamol	33	37.72±0.14	38.40±0.12	38.05±0.27 (51.47±5.31)	37.95±0.21 (66.17±6.87)	37.80±0.14 (88.23±9.68)
Plant extract	200	37.47±0.17	38.45±0.20	38.15±0.17 (30.61±4.31)	38.02±0.32 (43.57±5.37)	37.97±0.22 (48.97±7.68)
Plant extract	400	37.43±0.20	38.42±0.19	38.10±0.16 (32.32±4.56)	37.96±0.17 (46.46±4.96)	37.87±0.20 (55.55±6.75)
One way ANOVA F 3.567		All values are statistically significant respect to control at 5% level of significance				

% reduction = B- Cn / B-A x 100, where n= 1, 2, 3 represented in the table in parenthesis.

All the values are mean±SEM (n=6)

Table 9.4. Effect of methanolic extract of leaves of *E. cannabinum* on Yeast induced pyrexia model in rats.

		Rectal temperature (°C)		Rectal temperature after administration of drug (°C)		
Treatment	**Dose (mg/kg)**	**(A) Normal animals (X±SEM)**	**(B) 18h after yeast administration (X±SEM)**	**(C1) 1h (X±SEM)**	**(C2) 2h (X±SEM)**	**(C3) 3h (X±SEM)**
Control (Normal saline)	0.5 ml	37.89±0.23	38.58±0.19	38.56±0.25 (2.89±2.98)	38.53±0.21 (7.24±8.21)	38.50±0.16 (11.59±12.21)
Paracetamol	33	37.78±0.24	38.51±0.21	38.15±0.26 (49.31±5.48)	38.08±0.16 (58.90±6.54)	37.89±0.18 (84.93±9.08)
Plant extract	200	37.56±0.28	38.55±0.29	38.21±0.23 (34.34±4.31)	38.07±0.27 (48.48±4.87)	37.93±0.14 (58.58±6.32)
Plant extract	400	37.54±0.23	38.52±0.22	38.13±0.24 (39.79±4.89)	38.00±0.19 (53.06±5.35)	37.87±0.26 (66.32±7.25)
One way ANOVA F 1.556		All values are statistically significant respect to control at 5% level of significance				

% reduction = B- Cn / B-A x 100 where n= 1, 2, 3 represented in the table in parenthesis

9.7. References

1. *Cancer pain relief and palliative care*. Report of a WHO expert committee [World Health Organization Technical Report Series, 804] Geneva, Switzerland, World Health Organization, 1990, p 1-75.

2. Joao BC, Cristiano S, Michel O and Adair RS. Biological activity of plant extracts: Novel analgesic drugs. *Expert Opinion on Emerging Drugs*, 2001, **6(2)**, 261-279.

3. Gilman AG and Rau TW. *The pharmacological basis of Therapeutics*. 8th edn., Vol-1 Pargamon press, New York, 1990, p 935.

4. Cao C, Matsumura K, Yamagata K and Watanabe Y. Endothelial cells of the rat brain vasculature express cyclooxygenase-2 mRNA in response to systemic interleukin-1B: A possible site of prostaglandin synthesis responsible for fever. *Brain Res.*, 1996, **733**, 263-272.

5. Castellani JW, Young AJ, Sawka MN and Pandolf KB. Human thermoregulatory responses during serial cold-water immersions. *J. Appl. Physiol.*, 1998, **85**, 204-09.

6. Feng JD, Price M, Cohen J and Satinoff E. Prostaglandin fevers in rats: regulated change in body temperature or change in regulated body temperature? *Am. J. Physiol. Regulatory Integrative Comp. Physiol.*, 1989, **257**, 695-699.

7. Jakobsson PJ, Thoren S, Morgenstern R and Samuelsson B. Identification of human prostaglandin E synthase: A microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc. Natl. Acad. Sci.*, 1999, **96**, 7220-7225.

8. Milton AS and Wendlandt S. A possible role for prostaglandin E1 as a modulator for temperature regulation in the CNS. *J. Physiol.*, 1970, **207**, 76-77.

9. Milton AS and Wendlandt S. Effects on body temperature of prostaglandins of the A, E and F series on injection into the third ventricle of anesthetized cats and rabbits. *J. Physiol.*, 1971, **218**, 325-336.

10. Krueger JM. Interleukin 1-receptor antagonist blocks interleukin 1-induced sleep and fever. *Am. J. Physiol.*, 1991, **260(2)**, 453-457.

11. Scammell TE, Elmquist JK, Griffin JD and Saper CB. Ventromedial preoptic prostaglandin E2 activates fever-producing autonomic pathways. *J. Neur Sci.*, 1996, **16(19)**, 6246-6254.

12. Scammel TE, Griffin JD, Elquist JK and Saper CB. Microinjection of a cyclooxygenase inhibitor into the anteroventral preoptic region attenuate LPS fever. *Am. J. Physiol. Regul. Integrative Comp. Physiol.*, 1998, **274(3)**, 783-789.

13. Vane JR. and Flower RJ. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol. *Nature*, 1972, **240**, 410-411.

14. http://en.wikipedia.org/wiki/Antipyretic.

15. Bercy U, Dinorah A, Heberto S, Luis Q, Jose O and Beatriz G. Anti-inflammatory, anti-nociceptive, and antipyretic effects of *Lantana trifoliainnae* in experimental animals. *Invest. Clin.*, 2004, **45(4)**, 201.

16. Parimaladevi B, Boominathan R and Mandal SC. Studies on analgesic activity of *Cleome viscosa* in mice. *Fitoterapia*, 2003, **74**, 262-266.

17. Rao RR, Babu RM, Rao MRV and Babu MGV. Studies on antipyretic, analgesic and hypoglycemic activities of root of *Gynandropsis gynandra* Linn. *Indian Drugs*, 1997, **34**, 690-693.

CHAPTER - 10

STUDIES ON ANTIMICROBIAL ACTIVITY

10.1. Introduction

Infectious diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality world wide[1]. During the past several years there has been an increasing incidence of bacterial and fungal infections. This fact coupled with the resistance to antibiotics and with the toxicity during prolonged treatment with several antimicrobial drugs has been the reason for an extended search for newer drugs to treat opportunistic microbial infection[2,3].

For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of plant compounds for pharmaceutical purposes has gradually increased in India. According to World Health Organization[4] medicinal plants would be the best source to obtain a variety of drugs. About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency[5]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency[6-12]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example the phenolic compounds which are part of the essential oils[13].

Historically, plants have provided a good source of anti-infective agents and many of them remain highly effective in the fight against microbial infections. Besides, they are cost effective and have fewer side effects[14,15]. The antiseptic properties of aromatic and medicinal plants and their extracts have been recognized since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s[16,17]. Natural product contributes to a great extent to fight against pathogenic microorganisms. Many plants or their parts are used in food as spices and are thought to provide a natural preservation by inhibiting the microbial growth[18]. Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made

large contributions to human health and well-being. Their role is two fold in the development of new drugs, viz- they may become the base for the development of a medicine, and a natural blueprint for the development of new drugs or a phytomedicine to be used for the treatment of disease. The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many years as and amoebicidal drug as well as for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs naturally in the bark of *Cinchona* tree. Apart from its continued usefulness in the treatment of malaria, it can be also used to relieve nocturnal leg cramps. Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. Some strains of malarial parasites have become resistant to the quinines; therefore antimalarial drugs with novel mode of action are required[19].

Similarly, higher plants have made important contributions in the areas beyond anti-infective, such as cancer therapies. Early examples include the antileukaemic alkaloids, vinblastine and vincristine, which were both obtained from the Madagascan periwinkle (*Catharanthus roseus* syn. *Vinca roseus*)[20]. Other cancer therapeutic agents include taxol, homoharringtonine and several derivatives of camptothein. For example, a well-known benzyl isoquinoline alkaliod, papaverine, has been shown to have a potent inhibitory effect on the replication of several viruses including cytomegalovirus, measles and HIV[21]. Most recently, three new atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B, and C were isolated from a newly described species of tropical liana *Ancistrocladus korupensis* from the rainforest of Cameroon. The three compounds showed potential anti-HIV with michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effects of HIV-1 and HIV-2 on human lymphoblastoid target cell *in vitro*[22].

Much of the exploration and utilization of natural products as antimicrobials arise from microbial sources. It was the discovery of penicillin that led to later discoveries of antibiotics such as streptomycin, aureomycin and chloromycetin. Though most of the clinically used antibiotics are produced by soil micro-organisms or fungi, higher plants have also been a source of antibiotics Examples of these are the bacteriostatic and fugicidal properties of *Lichens*, the antibiotic action of allinine in *Allium sativum* (garlic),

or the antimicrobial action of berberines in goldenseal *Hydrastis canadensis*[23]. Plant based antimicrobials represent a vast untapped source for medicines. Continued and further exploration of plant antimicrobials needs to occur. Plants based antimicrobials have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Many plants have tropisms to specific organs or systems in the body. Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of disease. An example of this is *Hydrastis canadensis*. Hydrastis not only has antimicrobial activity, but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds[24].

Plants have supplied over 25% of prescription drugs used in human medicine and such pharmacologically active plants have also provided leads to natural pesticides. Himalayas has an extraordinarily rich flora and wide knowledge of indigenous medicinal plants is well documented. Accordingly, we are investigating the potential of Himalayan medicinal plants as a resource for new antimicrobial agent[25].

Critical review of literature revealed that the potency of the plant inhibiting the growth of pathogenic bacteria remained unexplored. So the present study was designed to investigate the antimicrobial activity of the methanol extract of rhizomes of *K. rotunda* and leaves of *E. cannabinum* on different pathogenic bacterial strains by using *in vitro* model system.

10.2. Materials and methods

10.2.1. Plant materials

Methanol extracts of rhizomes of *K. rotunda* and leaves of *E. cannabinum* were subjected as test drug in these experiments.

10.2.2. Drugs and chemicals

The standard antibiotic ciprofloxacin used in this study was obtained as a gift sample from Ankur Drugs, Himachal Pradesh. Dimethyl sulphoxide (DMSO) and Mueller Hinton Agar were obtained from Ranbaxy Fine Chemicals.

10.2.3. Microorganisms

A total of 80 strains belonging to seven different genera (*Staphylococcus Species, Salmonella Species, Shigella Species, Klebsiella Species, Pseudomonas, Vibrios, E. coli Species*) comprising both Gram positive and Gram negative were used as test organisms which were obtained from Dept. of Pharmaceutical Technology, Jadavpur University, Kolkata, India. They were used to evaluate the antibacterial activities of the methanolic extract of both the plants. All the strains were clinically isolated from human beings.

10.2.4. Media

10.2.4.1. Liquid media

- **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.

- **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

- **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

10.2.4.2. Solid media

- **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

- **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.

10.2.5. Preservation of bacterial cultures

All the strains of *Staphylococci, E. coli, Klebsiellae, Salmonellae, Shigellae, Pseudomonas* spp. and *Vibrios* were preserved as stab slant cultures at a temperature of 4°C. 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 bromothymol blue lactose agar[26].

10.2.6. Preparation of impregnated discs of extract and standard antibiotics

The filter paper discs of 6mm diameter were prepared by using Whatman filter paper No.1 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 *K. rotunda* rhizome and *E. cannabinum* leaves were weighed and dissolved in 0.5ml of (DMSO), as the extracts are not completely 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 solution of the standard antibiotic was prepared by dissolving the required amount of ciprofloxacin. All the stock solutions were then kept at 4°C and used for three months. For preparation of antibiotic impregnated discs 1.0ml of the stock solution of the antibiotic was added separately to each bottle of 100 discs. Each discs adsorbed 0.01ml of the solution, so the entire 1.0ml volume was adsorbed by the 100 discs, each giving the required two fold concentrations of 0-500µg/ml. The procedure was repeated for preparation of impregnated discs of the plant extracts. 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.

10.2.7. Antimicrobial activity of extract of *K. rotunda* rhizome (*In vitro* model)

Microbial sensitivity tests were performed by disc diffusion method[27]. Petriplates were prepared aseptically by pouring 20ml of Mueller Hinton Agar and allowed to solidify. Plates were dried and 0.1ml of standardized inoculums suspension was poured and uniformly spread. The excess inoculum was drained and the inoculum was allowed to dry for 5 minutes, then the discs were placed in the inoculated agar. Ciprofloxacin (5µg/disc) was used as positive control and 5% DMSO was used as negative control in these assays. The inoculated plates were incubated at 37^0C for 24 hours. The zones of inhibitions observed were measured in millimeters after 24h. Each assay in this experiment was repeated three times. The sensitivity was recorded for a zone of clearance around the discs. The MICs were determined by the standard agar dilution method[28].

The crude methanol extract was dissolved in 0.5ml of DMSO, as they are not completely 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 0-500µg/ml. The contents of the tubes were mixed thoroughly, pH adjusted to 7.2 to 7.4 and poured into sterile Petri dishes. The cultures were diluted to 10^6 colony forming units (CFU), spot inoculated using a bacterial planter (10µl) on the nutrient agar sterile Petri discs and incubated at 37°C±2°C for 24h to determine the MIC of the extracts. 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 ciprofloxacin was served as negative and positive control respectively[29,30].

10.2.8. Antimicrobial activity of extract of *E. cannabinum* leaves (*In vitro* model)

The antimicrobial activity of methanol extract of *E. cannabinum* leaves was determined as per the methods described above in 10.2.7 for *K. rotunda.*

10.3. Results

Out of seven different pathogenic Gram-positive and Gram-negative strains, the significant results were obtained with one Gram-positive i.e. *S. aureus* NCTC 6571 and two Gram-negative bacteria i.e. *Shigella dysentariae-2* and *Klebsiella pneumoniae 14.* Table 10.1 and 10.2 represents that the MIC of ciprofloxacin is 10µg/ml whereas the MIC of *K. rotunda* and *E. cannabinum* varies from 100 to 500µg/ml. From table 10.3, it is revealed that the diameters of the individual zone of inhibition of *K. rotunda* and ciprofloxacin against *S. aureus* NCTC 6571 were 6.1mm and 13.2mm respectively, which have been enhanced to 6.3 and 13.7mm respectively when both the disc was placed in the same culture. In the test of combined effect of crude extract and antibiotic, the surface areas of zones of inhibition were increased by 6.66% for *K. rotunda* and 7.72% for ciprofloxacin. It is also seen that in case of *Shigella dysentariae-2*, the diameters of zone of inhibition of *K. rotunda* and Ciprofloxacin were 9.4 and 15.0mm. These diameters were increased to 9.7 and 15.6mm with the percentage increase of 6.48 and 8.16 respectively when the discs were placed on the same culture. Similarly, when the discs of *K. rotunda* and ciprofloxacin were placed on plates flooded with *Klebsiella pneumoniae 14*, a notable increase in diameter of zones of inhibition were observed independently. These on combination showed an increase in diameter and the percentage increase data were found to be 6.35 for *K. rotunda.* and 8.10 for ciprofloxacin. From table

10.4, it is shown that the percentages of zone of inhibition were increased to 3.42 for *E. cannabinum* and 4.60 for ciprofloxacin against *S. aureus* NCTC 8530. Similarly, for *E. cannabinum* and ciprofloxacin the increased percentage of zones of inhibition were noted as 3.88 in former and 4.04 in latter case for *Shigella dysentariae-2* and were found to be 3.96 % and 5.36 % respectively for *Klebsiella pneumoniae 14*. In fig. 10.1 it was observed that the crude methanol extract of *K. rotunda* exhibits better antibacterial potency against selected pathogenic Gram-positive and Gram-negative strains as compared to the extract of *E. cannabinum*.

10.4. Synergistic effect

Synergism between the compounds is said to occur when more inhibition results from the combination form than that of the individuals. This implies that one drug is affecting the microorganism in such a way that it becomes more sensitive to the inhibitory effects of the other. A total of seven bacterial strains belonging to both Gram-positive and Gram-negative were screened for synergistic effect by the combination of plant extracts with standard antibiotic ciprofloxacin. The combined effect of two agents was performed as described by Miles and Amyes[31] following the procedure adopted by Lamanna and Shapiro[32]. The discs were placed at suitable distances apart so that the respective agents would not diffuse into one another to produce a continuous range of concentrations in the initial period of inhibition. In synergism, the ridge of growth disappeared and the two circles merged to one forming a single asymmetric ellipse[33]. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

The sensitivity tests of the microorganisms with respect to particular concentration (plant extract and antibiotic) were determined by measuring the diameters of zone of inhibition.

Table 10.1. Antibacterial spectra for MIC of Ciprofloxacin

Sl. No	Bacterial Species	No. of microorganism tested	MIC of CF ($\mu g.ml^{-1}$)			
			02	05	10	25
1.	*Staphylococcus Species*	14	-	01	10	03
2.	*Salmonella Species*	10	-	02	04	04
3.	*Shigella Species*	15	01	04	07	03
4.	*Klebsiella Species*	05	-	01	03	01
5.	*Pseudomonas*	06	01	01	04	-
6.	*Vibrios*	20	02	07	10	01
7.	*E. coli Species*	10	-	02	06	02

CF = Ciprofloxacin

Table 10.2. Antibacterial spectra for MIC of methanol extract of *Kaempferia rotunda* and *Eupatorium cannabinum*

Sl. No	Bacterial Species	No. of bacteria tested	MIC of KR ($\mu g.ml^{-1}$)					MIC of EC ($\mu g.ml^{-1}$)				
			50	100	200	500	750	50	100	200	500	750
1.	*Staphylococcus* Species	14	-	02	08	03	01	-	01	10	03	-
2.	*Salmonella* Species	10	01	02	05	02	-	01	01	04	03	01
3.	*Shigella* Species	15	01	03	08	02	01	01	02	07	04	01
4.	*Klebsiella* Species	05	01	01	02	01	-	-	01	02	01	01
5.	*Pseudomonas*	06	-	02	04	-	-	01	-	04	01	-
6.	*Vibrios*	20	03	06	08	02	01	02	05	07	05	01
7.	*E. coli Species*	10	-	02	02	06	-	01	-	05	03	01

KR = *Kaempferia rotunda*

EC = *Eupatorium cannabinum*

Table 10.3. Synergism between *Kaempferia rotunda* extract and ciprofloxacin by disc diffusion method

Sl. No	Bacterial Strains	Diameter of the inhibition zone(mm)				% of increase of zone of inhibition	
		Single effects (A)		Combined effects (B)			
		KR* (X±SEM)	CF** (X±SEM)	KR* (X±SEM)	CF** (X±SEM)	KR* (X±SEM)	CF** (X±SEM)
1.	*S. aureus* NCTC 6571	6.1±0.062	13.2±0.056	6.3±0.014	13.7±0.031	6.66±0.021	7.72±0.028
2.	*S. aureus* NCTC 8530	6.3±0.021	14.2±0.045	6.5±0.015	14.7±0.036	6.45±0.019	7.16±0.056
3.	*S. typhi 59*	8.2±0.047	13.5±0.089	8.4±0.018	13.9±0.032	4.94±0.016	6.01±0.065
4.	*S. dysenteriae 2*	9.4±0.032	15.0±0.029	9.7±0.019	15.6±0.039	6.48±0.022	8.16±0.057
5.	*Klebsiella pneumoniae 14*	9.6±0.051	15.1±0.012	9.9±0.017	15.7±0.041	6.35±0.025	8.10±0.048
6.	*Vibrio cholera 14033*	6.8±0.032	10.9±0.061	7.0±0.021	11.3±0.029	5.97±0.018	7.47±0.053
7.	*E. coli-10*	6.6±0.035	13.5±0.045	6.8±0.016	13.9±0.033	6.15±0.024	6.01±0.049

SEM=standard error mean (n=3)

KR= *Kaempferia rotunda*, CF = Ciprofloxacin

(*) = 200 $\mu g.ml^{-1}$ in disc, (**) = 10 $\mu g.ml^{-1}$ in disc

Mean surface area of the inhibition was calculated as πr^2 on basis of their mean diameter (2r) and % increase was calculated as (B-A/A) x 100

Table 10.4. Synergism between *Eupatorium cannabinum* extract and ciprofloxacin by disc diffusion method

Sl. No	Bacterial Strains	Diameter of the inhibition zone (mm)				% of increase of zone of inhibition	
		Single effects (A)		Combined effects (B)			
		EC* (X±SEM)	CF** (X±SEM)	EC* (X±SEM)	CF** (X±SEM)	EC* (X±SEM)	CF** (X±SEM)
1.	*S. aureus* NCTC 6571	5.4±0.038	14.2±0.056	5.5±0.014	14.6±0.029	3.73±0.021	5.71±0.028
2.	*S. aureus* NCTC 8530	5.9±0.025	13.2±0.045	6.0±0.021	13.5±0.031	3.42±0.019	4.60±0.056
3.	*S. typhi 59*	4.9±0.056	13.5±0.089	5.0±0.074	13.8±0.039	4.12±0.016	4.49±0.065
4.	*S. dysenteriae 2*	5.2±0.024	15.0±0.029	5.3±0.025	15.3±0.041	3.88±0.022	4.04±0.057
5.	*Klebsiella pneumoniae 14*	5.1±0.042	15.1±0.012	5.2±0.031	15.5±0.052	3.96±0.025	5.36±0.048
6.	*Vibrio cholerae 14033*	5.5±0.56	10.9±0.061	5.6±0.033	11.1±0.036	3.66±0.018	3.70±0.053
7.	*E. coli-10*	5.3±0.084	13.5±0.045	5.4±0.041	13.8±0.054	3.80±0.024	4.49±0.049

SEM=standard error mean (n=3)

EC= *Eupatorium cannabinum*, CF = Ciprofloxacin

(*) = 200 $\mu g.ml^{-1}$ in disc, (**) = 10 $\mu g.ml^{-1}$ in disc

Mean surface area of the inhibition was calculated as πr^2 on basis of their mean diameter (2r) and % increase was calculated as (B-A/A) x 100

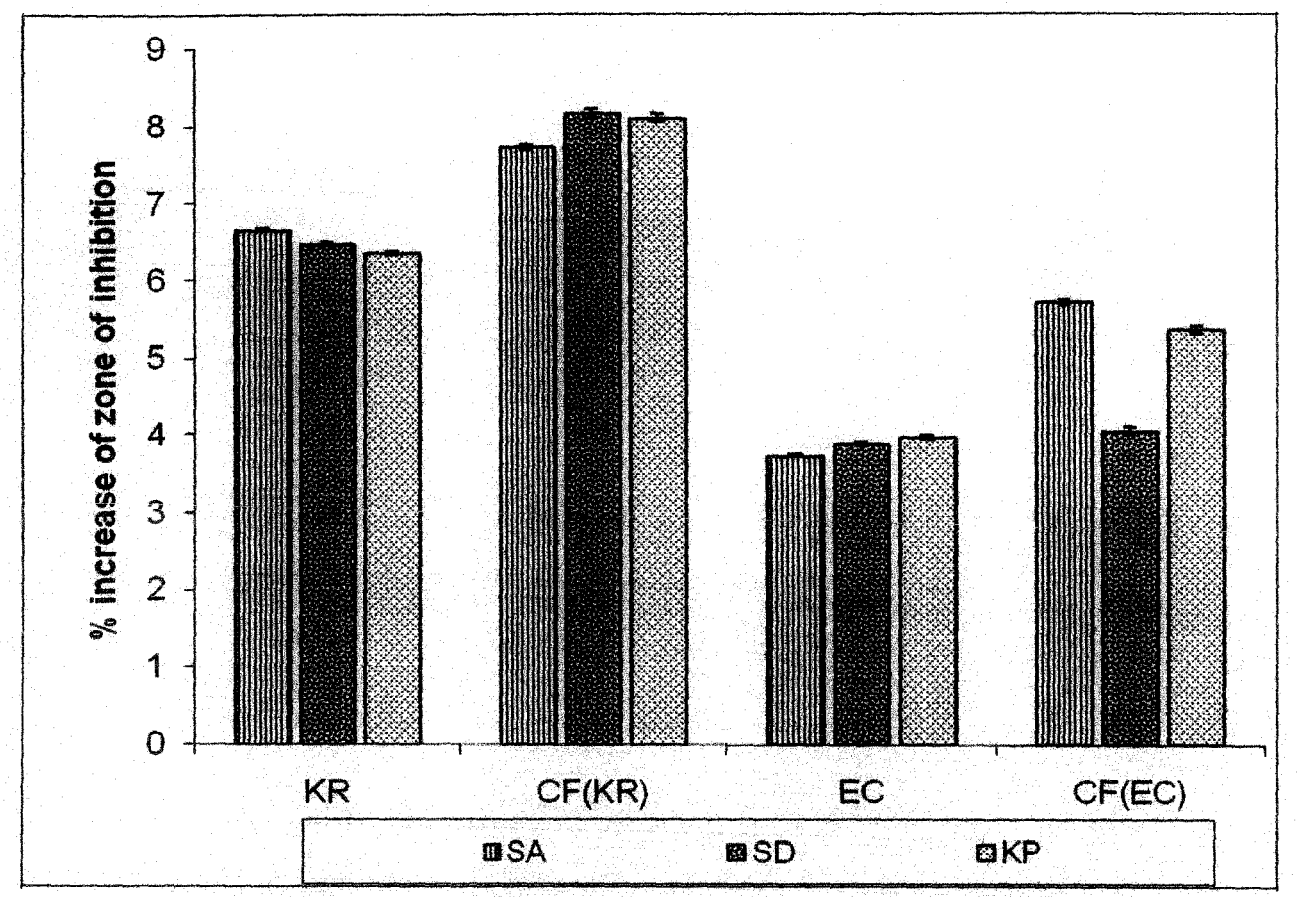


Fig 1: Synergism profile of plant extracts with Ciprofloxacin against SA, SD and KP

SEM = Standard error mean (n=3)

KR = *Kaempferia rotunda*, EC = *Eupatorium cannabinum*, CF = Ciprofloxacin

CF (KR) = % increase of ciprofloxacin with respect to *Kaempferia rotunda*

CF (EC) = % increase of ciprofloxacin with respect to *Eupatorium cannabinum*

SA = *S. aureus* NCTC 6571, SD = *S.dysenteriae-2*, KP = *Klebsiella pneumoniae 14*

10.5. References

1. World health organization. The world report life in the 21st century: A vision for all. Geneva, Switzerland, 1998, p 39-60.
2. Giordani R, Trebauz J and MandRegli P. Enhance antifungal activity of ketoconazole by Euphor chalacious latex against *Candida albica*. *J. Ethnopharmacol.*, 2001, **78**, 1-5.
3. Fostel J and Lartey P. Emerging novel antifungal agent. *Drug discovery today*. 2000, **75**, 25-32.
4. Saxena G, McCutcheon AR, Farmer S, Towers GHN and Hancock REW. Antimicrobial constituents of *Rhus glabra*. *J. Ethnopharmacol.*, 1994, **42**, 95-99.
5. Ellof JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *J. Ethnopharmacol.*, 1998, **60**, 1-6.
6. Almagboul AZ, Bashir, AK, Farouk A and Salih AKM. Antimicrobial activity of certain Sudanese plants used in folkloric medicine, Screening for antibacterial activity. *Fitoterapia*. 1985, **56**, 331-337.
7. Artizzu N, Bonsignore L, Cottiglia F and Loy G. Studies of the diuretic and antimicrobial activity of Cynodon dactylon essencial oil. *Fitoterapia*. 1995, **66**, 174-175.
8. Ikram M and Inamul H. Screening of medicinal plants for antimicrobial activities. *Fitoterapia*. 1984, **55**, 62-64.
9. Izzo AA, Di Carlo G, Biscardi D, Fusco R, Mascolo N, Borreli F, Capasso F, Fasulo MP and Autore G. Biological screening of Italian medicinal plants for antibacterial activity. *Phytother Res.*, 1995, **9**, 281-286.
10. Kubo L, Muroi H and Himejima M. Structure-antibacterial activity relationships of anacardic acids. *J. Agri. Food Chem.*, 1993, **41**, 1016-1019.
11. Shapoval EES, Silveira SM, Miranda ML, Alice CB and Henriques AT. Evaluation of some pharmacological activities of *Eugenia uniflora*. *J. Ethnopharmacol.*, 1994, **44**, 136-142.
12. Sousa M, Pinheiro C, Matos MEO, Matos FJ, Lacerda MI and Craveiro AA. Constituintes Químicos de Plantas Medicinais Brasileiras. Universidade Federal do Ceara, Fortaleza, 1991, p 385-388.

13. Jansen AM, Cheffer JJC and Svendsen AB. Antimicrobial activity of essential oils: literature review. Aspects of test methods. *Planta Med.*, 1987, **40**, 395-398.
14. Samsam SH and Moatar F. Natural medicines and plants. Mashal Publications, Tehran, 1991, p 123-130.
15. Remington's Pharmaceutical Sciences. 17th edn., Gennaro AR, eds., Mack Publishing Co., Eastan, Pennsylvania, 1991, p 1158.
16. Hoffman C and Evans AC. The uses of spices as preservatives. *J. Indian Engineering and Chemistry,* 1911, **3**, 835–838.
17. Martindale WH. Essential oils in relation to their antiseptic powers as determined by their carbolic coefficients. *Perfumery and Essential Oil Research,* 1910, **1**, 266–296.
18. Walker JRL. *Natural antibacterial system and food preservation*. Dillon V and Mandboard RG, eds., Wallingford, UK, CAB Int., 1994, p 181-204.
19. Iwu MW, Duncan AR and Okunji CO. New antimicrobials of plant origin. In: Janick J (eds), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA, 1999, p 457-462.
20. Nelson R. The comparative clinical pharmacology and pharmacokinetics of vindesine, vincristine and vinblastine in human patients with cancer. *Med. Pediatr. Oncol.*, 1982, **10**, 115-127.
21. Turano A, Scura G, Caruso A, Bonfanti C, Luzzati R, Basetti D and Manca N. Inhibitory effect of papaverine on HIV replication *in vitro*. *AIDS Res. Hum. Retrovir.*, 1989, **5**, 183-191.
22. Boyd M, Hallock Y, Cardellina J, Manfredi K, Blunt J, McMahon J, Buckheit R, Bringmann G, Schaffer M, Cragg G, Thomas D and Jato J. Anti-HIV michellamines from *Ancistrocladus korupensis*. *Med. Chem.*, 1994, **37**, 1740-1745.
23. Trease G and Evans W. Pharmacognosy. Univ. Press, Aberdeen, Great Britain. 1972, p 161-163.
24. Murray M. The healing power of herbs. Prima Publishing. Rocklin, CA, 1995, p 162-171.
25. Sener B, Bingol F, Erdogan I, Bowers WS and Evans PH. Biological activity of some Turkish medicinal plants. *Pure Appl. Chem.*, 1998, **70**, 403-406.

26. Barrow GI and Feltham RKA. *Cowan and steel's manual for the identification of medical bacteria*, Cambridge University Press, Cambridge, 1993, p 12.

27. Dash SK, Dastidar SG and Chakraborty AN. Antimicrobial property of Promazine hydrochloride. *Ind. J. Exp. Biol.*, 1977, **15**, 197-199.

28. Dastidar SG, Chaudhary A, Annadurai S, Roy S, Mukharjee M. and Chakraborty AN. *In vitro* and *in vivo* antimicrobial action of Fluphenazine. *J. Chemother.*, 1995, **7**, 201-207.

29. Abbott JD and Parry HE. Dysentery treated with tetracycline: A comparison with Phthalyl sulphathiazole and streptomycin. *Lancet*, 1955, **I**, 118.

30. Fischler E and Wallis K. Investigation of drug sensitivity in Shigella and its correlation with *in vivo* sensitivity. *Ann. Paedia.*, 1963, **201**, 49-63.

31. Amyes SGB and Miles RS. Medical Microbiology. Livingstone, Edinburgh, New York. 1996, p 151-177.

32. Lamanna C and Shapiro IM. Western blot analysis indicated migration of cytochrome *J. Bacteriol.*, 1943, **45**, 385-394.

33. Garrod LP, Lambert HP, Grady F and Worth PM. Antibiotics and chemotherapy Livingstone, Edinburgh, New York, 1981, p 405-408.

CHAPTER - 11

DISCUSSION

An herbal medicine can be defined as a medicinal product consisting of a substance produced by subjecting a plant or plants to drying, crushing or any other processes or of a mixture whose sole ingredients are two or more substances so produced, and water or some inert substances[1]. Herbs are an integral part of nature and play a vital role in humans by preventing diseases. Herbal medicine based upon the premise that plants contain natural substance that can promote health and alleviate diseases. Their increasing use in recent years is clear evidence of public interest in having alternatives to conventional medicines. Scientists have proven to humanity the effective use of some herbs. The development of modern chemistry has permitted the isolation of chemicals from medicinal herbs that have served as drugs or starting materials for the synthesis of many important drugs today[2]. The history of herbal medicine is a great help to our current state where many new diseases are being discovered. We need all the knowledge which can get from natural and herbal medicine in order to be able to find cures and alternatives to otherwise harmful medicines.

Medicinal plants are plants whose extracts can be used directly or indirectly for the treatment of different ailments. Therefore, the use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed[3]. Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering humanity. Furthermore, in the world more than 30% of the pharmaceutical preparations are based on plants[4]. However, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants. The use of medicines from plants in the form of local medicine dates back to 4000-5000 B.C. While the medicinal values of these plants are due to the presence of small doses of active compounds which produces physiological actions in the human and animal body .Some of the important bioactive compounds found in medicinal plants are alkaloids, glycosides, resins, gums, mucilages etc[5]. It was observed that developed countries mostly imports raw materials of valuable medicinal plants from developing countries. Where they are screened, analyzed and used in drug preparations, and returned as high priced medicines to developing countries[6].

Medicinal plants are valuable for modern medicine in four basic ways

a) They are used as sources of direct medicinal agent.
b) They serve as a raw material base for elaboration more complex semi-synthetic compounds.
c) The chemical structure derived from phytoconstituents can be used as models for new synthetic compounds.
d) Plants can be used as taxonomic markers for discovery of new therapeutic compounds[7].

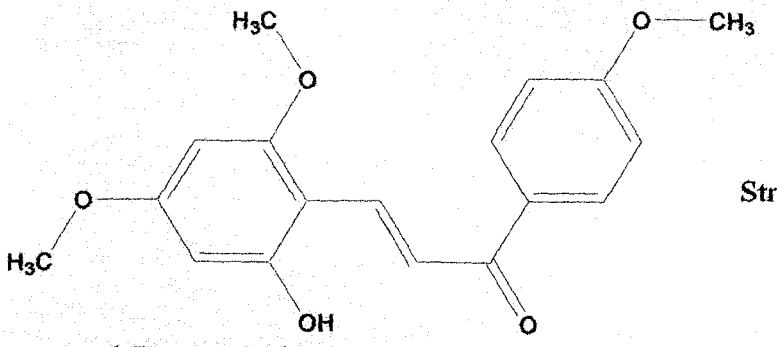
Plants show enormous versatility in synthesizing complex materials which have no immediate obvious growth or metabolic functions. These complex materials are referred to as secondary metabolites. Plants secondary metabolites have recently been referred to as phytochemicals. Phytochemicals are naturally occurring and biologically active plant compounds that have potential disease inhibiting capabilities. It is believed that phytochemicals may be effective in combating or preventing disease due to their antioxidant effect[8,9]. Antioxidants protect other molecules from oxidation when they are exposed to free radicals and reactive oxygen species which have been implicated in the etiology of many diseases and in food deterioration and spoilage[10,11]. Medicinal plants have been used for centuries before the advent of orthodox medicine. Leaves, flowers, stems, roots, seeds, fruit, and bark can all be constituents of herbal medicines. The medicinal values of these plants lie in their component phytochemicals, which produce definite physiological actions on the human body. Most important of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds[12].

Present study investigated for the phytochemical, their toxicities and pharmacological activities of two well-known plant used in traditional medicines of the family *Zingiberaceae* and *Asteraceae* from Sikkim Himalayan region. Both of these plants were extensively used by the traditional healer of Sikkim for their primary health care. The present study on the plants was undertaken to evaluate the bioactivity of two plants *K. rotunda* and *E. cannabinum* both *in vitro* and *in vivo* for justification of their use as ethnomedicine and 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 study of the plants was explained in **chapter 3**. The extractions of the plant parts were made at room temperature using methanol as solvent and the collected extracts were concentrated under reduced pressure. The extracts were then concentrated and extracted with distilled water. The water-soluble component was fractionated by extracting it successively with petroleum ether, ethyl acetate. The ethyl acetate soluble fraction was subjected to chromatographic analysis[13]. By using column chromatography technique with various developing phases compound I and compound II were isolated from ethyl acetate fractions respectively. Their structures were determined with the help of spectral analysis like Ultraviolet (UV), Infrared (IR), Proton Nuclear magnetic resonance (^{1}H NMR) and Mass (MS) spectra.

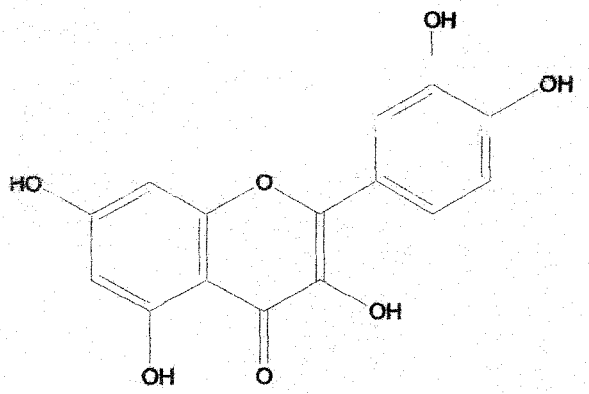
The crystalline material isolated from the *K. rotunda* yielded compound I (chalcone) a flavonoid soluble in water and methanol. The UV absorption spectrum confirmed that presence of phenolic aromatic rings in compound I. The IR spectrum confirmed the presence of hydroxyl group and aromatic ring in the compound. ^{1}H NMR spectrum confirmed that compound I was indicated the presence chalcone i.e. HC=CH–C=O. The molecular ion peak (base peak, M^+) at m/z 312.2670 (calculated for $C_{18}O_5H_{18}$, 314.1154) and the fragmentation peaks at m/z 153, m/z 165 revealed the empirical formula of $C_{18}O_5H_{18}$. The result is corresponding to the molecular formula $C_{18}O_5H_{18}$. It has been concluded that the structure of the isolated compound I was established according to combined spectral data i.e. 2- hydroxy, 4, 4′, 6- trimethoxy chalcone.



Structure of compound I

Compound II was obtained as yellow amorphous powder, partially soluble in water and methanol. The UV absorption spectrum showed two major absorption bands which are

typical for flavonols that confirmed presence of phenolic aromatic rings in compound II. The IR spectrum showed the presence of hydroxyl group and aromatic ring. The ^{1}HNMR spectrum indicated the presence of 1, 2, 3, 5- tetrasubstituted benzene ring. The molecular ion peak (base peak, M^+) at m/z 302 and the fragmentation peaks at m/z 153, m/z 285 revealed the empirical formula of $C_{15}H_{10}O_7$. The UV, ^{1}H NMR and EI-MS data led to the identification of the compound II corresponds to be molecular formula $C_{15}H_{10}O_7$ and the structure of the isolated compound II was 3′,4′,5,7- tetrahydroxy flavonol or quercetin.



Structure of compound II

All the recorded spectral data discussed in **chapter 3** conclusively prove the identity of the isolated compound I as 2-hydroxy 4,4′,6- trimethoxy chalcone and compound II as 3′,4′,5,7-tetrahydroxy flavonol confirmed one of the major bio constituent of the plant. A large number of natural flavonoids with biological activity have been identified in recent decades. One group of these products, the polyhydroxylated chalcones, exhibit antimicrobial antiviral, antitumoral and antiinflamatory activities, and applications of therapeutic effects have been reported. The increase of the bacteriostatic action due to free hydroxyl groups on the aromatic A- and B- rings which showed that the introduction of hydroxyl groups, especially in the 4- and 4′- positions enhance the bioactivity of 2-hydroxychalcone[14-21]. Chalcones are yellow phenolic pigments present in plants. Chalcone is an aromatic ketone that forms the central core for a variety of important biological compounds, which are known collectively as chalcones. They show antibacterial, antifungal, antitumor and anti-inflammatory properties. They are also intermediates in the biosynthesis of flavonoids, which are substances widespread in plants and with an array of biological activities. Chalcones are also intermediates in the

synthesis of flavones[22]. The flavones are characterized by a planar structure because of a double bond in the central aromatic ring. One of the best-described flavonoids, quercetin, is a member of this group. Quercetin is found in abundance in onions, apples, broccoli, and berries. The second group is the flavanones, which are mainly found in citrus fruit. An example of a flavonoid of this group is narigin. Flavonoids are mainly found in green and black tea and in red wine[23] whereas anthocyanins are found in strawberries and other berries, grapes, wine and tea. An important effect of flavonoids is the scavenging of oxygen-derived free radicals. *In vitro* experimental systems also showed that flavonoids possess antiinflammatory, antiallergic, antiviral, and anticarcinogenic properties[24]. The aim of this review was to give an overview of the research in the field of flavonoids. The potential valuable working mechanisms of flavonoids are discussed, followed by present knowledge on the absorption, conjugation, and toxicity of these substances. Quercetin 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, cataract prevention, antimicrobial activity, antioxidant activity and anti-inflammatory effects[25,26]. It is reported to inhibit metabolic enzyme systems and P-glycoprotein[27]. Recent reports indicate that quercetin and its glycoside stimulate human peripheral blood leukocyte proliferation and significantly increase the helper T-cells[28].

Determination of acute toxicity is usually an initial screening step in the assessment and evaluation of the toxic characteristics of unknown compound which has been included in **chapter 4** of the present studies. Fifty percent lethal dose or LD_{50} (acute oral toxicity) is performed in Swiss albino mice following standard protocol[29]. The median lethal dose (MLD) of the methanol extract of *K. rotunda* rhizome was found to be 5g/kg body weight in oral route. While the MLD of the methanol extract of *E. cannabinum* was to be 4.5g/kg body weight in oral route. The MLD of isolated compound I was found to be 213.7mg/kg body weight, where as the MLD of the isolated compound II was found to be 196.3mg/kg body weight by oral route. Therefore, the doses of 200mg/kg, 400mg/kg p.o. for extracts and 10mg/kg, 20mg/kg for isolated compounds were fixed 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 *K. rotunda* or *E. cannabinum*. The study included several pharmacological activities of the methanol extracts and isolated compounds of *K. rotunda* rhizome and *E. cannabinum* leaf on the basis of their ethnomedicinal uses. The toxicity study indicates that the extract is not toxic at the tested doses.

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. In humans, oxidative stress is involved in many diseases, such as Atherosclerosis, Parkinson's disease and Alzheimer's disease and it may also be important in ageing. In chemical terms, oxidative stress is a large increase (becoming less negative) in the cellular reduction potential, or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione[30]. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis[31]. A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds including quinones into more aggressive radical species that can cause extensive cellular damage[32]. Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism and the damage they cause to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart[33,34]. During lipid peroxidation, the end products like MDA and 4-HNE are formed by oxidation of polyunsaturated fatty acids. The MDA can be reacted with two molecules of thiobarbituric acid to give a pinkish red colour complex which is measured at 530nm. Likewise 4-HNE is treated with DNPH solution and the absorbance was taken at 350nm. Increase in MDA and 4-HNE levels of the drug-treated group suggests the occurrence of lipid peroxidation. This process of lipid- peroxidation especially occurs in the presence of some metal ions like Fe^{2+} and other prooxidants[35]. So the decrease in MDA and 4-HNE content of tissue homogenates implies the free radical scavenging property of ascorbic

acid. The increase in MDA content with respect to control when the tissue homogenates were treated with ascorbic acid alone indicates the prooxidant effect of ascorbic acid[36]. The quantification of MDA and 4-HNE can be directly correlated with the lipid peroxidation inhibition capacity of the extracts. The TBARS formation inhibitory effect of the methanolic extract of the plants of *K. rotunda* and *E. cannabinum* were significantly higher than that of control which was discussed in **chapter 5**. The lipid peroxidation inhibition capacity of *K. rotunda* was studied by dose dependent manner and it was concluded that the antioxidant activity has inverse relationship with dose i.e. high at low dose and vice versa. The extract at 100µg/ml and 200µg/ml has significant lipid peroxidation inhibition activity in respect to 500 and 1000µg/ml. The lipid peroxidation inhibition activity of extract of *E. cannabinum* has observed in higher doses. The dose of 100µg/ml and 200µg/ml has insignificant activity whereas 500µg/ml and 1000µg/ml has moderate and significant lipid peroxidation activity respectively.

Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. It mainly depends on the repairing ability of the tissue, type and extent of damage and general state of the health of the tissue. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema, and small new blood vessels. The undifferentiated mesenchymal cells of the wound margin modulate themselves into fibroblast, which start migrating into the wound gap along with the fibrin strands. The collagen composed of amino acid (hydroxyproline) is the major component of extra cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of the hydroxyproline could be used as an index for collagen turnover. In **chapter 6** the data obtained revealed that the hydroxyproline content of the granulation tissue of the animals treated with methanol extract was significantly increased when compared to the control group, indicating increased collagen turnover. Increase in breaking strength of granulation tissue of methanol extract treated animals indicated the enhanced collagen maturation by increased crosslinking. In addition, increase in dry granulation tissue weight also indicated the presence of higher protein content. The effect of methanol extracts of *K. rotunda*, *E. cannabinum* and their isolated compounds were screened on excision, incision and dead space wound models concurrently with the control and reference standard framycetin

sulphate cream treated animals. The preliminary phytochemical analysis of methanol extracts of *K. rotunda* and *E. cannabinum* revealed the presence of flavanoids and triterpenoids. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis. Flavonoids, triterpenoid are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation[37-39]. Thus, wound-healing property of *K. rotunda* and *E. cannabinum* may be attributed to the phytoconstituents present in it, which may be either due to their individual or additive effect that fastens the process of wound healing. The results of present study indicated that the isolated compound I and II i.e. chalcone and quercetin from *K. rotunda* and *E. cannabinum* respectively promotes significant wound healing activity. This was demonstrated by a significant increase observed in the rate of wound contraction, enhanced epithelialization, increase in hydroxyproline and hexosamine content. Hydroxyproline, the main constituent of collagen serves as a marker of collagen biosynthesis at the wound site. Collagen not only confers strength and integrity to the tissue matrix but also plays an important role in homeostasis and in epithelialization at the later phase of healing. Increased hexosamine content reflects the stabilization of collagen molecules by enhancing electrostatic and ionic interactions[40]. In the present study enhanced levels of collagen and hexosamine in flavones treated rats probably provided the strength to regenerate tissue and aided healing pattern. Histological evidences further supported these results greater degree of epithelialization and fibroblastic deposition observed in flavonoid treated wounds signifies pro-healing effect of the plant material. Significant promotion of wound-healing activity was observed in methanol extracts of both the plants and its isolated compound-I and II in all the three wound models such as excision, incision and dead space wound. In excision model the percentage closure of wound area was significantly increased by the curative effect of methanol extract of tuber of *K. rotunda* and *E. cannabinum* leaf and their isolated compound in the animal group. The data revealed that the rate of wound contraction was significantly high in the animals treated with isolated compound I and II. In incision

wound model the maximum breaking strength was observed in animals treated with isolated compound-I and II followed by methanol extracts of both the plants. In dead space wound model the hydroxyl proline content was found to be more in isolated compound treated animals in case of both the plants. Histological study of the sections of the granuloma tissue of the animals treated with methanol extract and isolated product showed complete epithelialization, increased fibrosis and collagen formation with lesser macrophages, whereas in the isolated compound treated animals the healing activity was comparatively more. From these findings it was concluded that isolated compound-I and II exhibited significant wound healing activity. The extracts as well as isolated compound I and II may be suggested for treating various types of wounds in human beings.

Uncontrolled acid secretion and ulceration of stomach mucosa due to several reasons have posed serious problems to the human health all over the globe[41]. Gastric mucus plays an important role in gastric defensive mechanisms by acting as a protective barrier, mainly because of its glycoprotein content. The water stored by this glycoprotein prevents hydrogen ions from reaching the cell surface[42]. Prostaglandins play an important role in modulating the integrity of the gastric mucosa in the presence of gastric acid secretion[43]. Non steroidal anti-inflammatory drugs can damage the gastrointestinal mucosa by local injury, when surface cells are damaged and allow acid diffusion into the sub mucosa, and by systemic injury, when systemic inhibition of prostaglandin synthesis occurs, thereby reducing gastric mucus production, bicarbonate secretion, and mucosal blood flow[44,45]. NSAIDs also delay the healing of peptic ulcers, interfere with the action of growth factors, decrease epithelial cell proliferation at the ulcer margin, decrease angiogenesis in the ulcer bed, and slow the maturation of granulation tissue[46]. Phytochemical analysis revealed the presence of flavonoids in the methanolic extract. These compounds, which are important for the normal growth, development, and defense of plants[47]. also exert a gastro protective action in mammals by increasing endogenous prostaglandin levels, decreasing histamine secretion, inhibiting *Helicobacter pylori*, and scavenging oxygen-derived free radicals[48]. This gastro protection has been reported for various flavonoids including rutin, naringin, quercetin, kaempferol, sophoradin and luteolin. Rutin reduces the levels of lipoperoxides and increases the activity of the anti oxidant enzyme GSH-PX[49-53]. Then the protective action of flavonoids may be assessed by the stimulation of mucus and bicarbonate secretion and by their

inhibitory effect on the proton pump of parietal cells[54]. Recent studies found that different substances from plant sources not only afford gastro protection but also accelerate ulcer healing[55-57]. Peptic ulceration was immensely induced with different intensities in each of the ulcer models. The ulcer index for *K. rotunda* extracts (200 and 400 mg/kg) and isolated compound I (10, 20 mg/kg) in model-A were found to be statistically significant when compared with control for positive control group at 5 % level of significance ($P<0.05$). The ulcer index for *E. cannabinum* extract (200 and 400 mg/kg) and isolated compound II (10mg, 20mg/kg) in model-A were found to be statistically significant when compared with control for positive control group. Similarly the volume of gastric content, total acidity and total acidity output/100 ml of extract treated groups for *K. rotunda* and *E. cannabinum* 200 and 400 mg/kg and their isolated compound had significantly reduced the values when compared with control. All the extract treated animal parametric values were comparable with that of standard group The extracts at the dose level of 200,400 mg/kg and the isolated compound I and II at the dose level of 10, 20 mg/kg showed dose response antiulcer effect. The group 400 mg/kg for both the plant extract showed significant difference when compared with standard drug ranitidine. For another model aspirin induced (model-B) the ulcer index values of methanolic extract of *Kaempferia rotunda* and *Eupatorium cannabinum* and their isolated compounds results compared to control and ranitidine treated groups and clarified then verified and found to be highly significant when compared with control and ranitidine group at 5% level of significance. The results of the present investigation in **chapter 7** showed antigastric ulcer properties of a methanolic extracts of *K. rotunda* and *E. cannabinum* as demonstrated by all experimental gastric ulcer models. Many factors and mechanisms are implicated in the ulcerogenesis and gastric mucosal damage induced by the different models used in the present study involving the increase of gastric acid output, vascular injury, depletion of gastric wall mucin, mucosal damage induced by nonsteroidal anti-inflammatory drugs. In effort to search curative and safe agents for the treatment of peptic ulcers in our indigenous medicinal plants for this purpose *Kaempferia rotunda* and *Eupatorium cannabinum* were selected for preliminary screening of their antiulcer in rats.

Inflammation is generally considered as an essentially protective response to tissue injury caused by noxious physical, chemical or microbiological stimulus. It is a complex

process involving various mediators, such as prostaglandins, leukotrienes and platelet activating factor[58]. The major macrophage derived inflammatory mediators such as pro-inflammatory cytokines, tumour necrosis factor-a (TNF- a) and the reactive free radical nitric oxide (NO) synthesized by inducible NO synthase (iNOS), contribute to the development of inflammatory diseases[59]. Thus, inhibition of the excessive production of TNF-a and/or NO could be employed as criteria to evaluate potential anti-inflammatory compounds Due to the increasing frequency of intake of NSAID's and their reported common side effects, there is need to focus on the scientific exploration of herbal drugs having fewer side effects. So, there is a continuous search for indigenous drugs, which can provide relief to inflammation. To give a scientific validation to this plant, an attempt was made to study the anti-inflammatory activity.

Carrageenan injection into the rat paw provokes a local, acute inflammatory reaction that is a suitable criterion for evaluation of anti-inflammatory agents[60]. Inhibition of carrageenan induced inflammation in rats is one of the most suitable test procedures to screen anti-inflammatory agents[61-64]. The development of edema in the paw of the rat has been described[65] as a biphasic event. The initial phase is attributed to the release of histamine and serotonine[66]. The second, accelerating, phase of swelling is due to release of prostaglandin like substance. The presence of prostaglandin E2 in inflammatory exudates from the injected foot can be demonstrated at three hours time period and thereafter[67]. Indomethacin is used as standard reference drug as it is reported to inhibit inflammation by its effect upon plasma exudation associated with carrageenan mediated inflammation[68]. It has been reported that the second phase of edema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents and they are releated to COX inhibition, especially COX-2[69]. Intraperitoneal injection of carrageenan leads to inflammation of the peritoneum resulting from carrageenan induced release of interleukin-1 from macrophages in the carrageenan insulated tissue. Interleukin-1, a pro-inflammatory cytokine, induces accumulation of polymorpho nuclear cells by a variety of processes including adhesion and cell mobility[70]. Leukocyte aggregation is a fundamental event during inflammation. Cell migration occurs as a result of much different process including adhesion and cell mobility. Indigenous drug systems can be source of variety of new drugs which can provide relief in inflammation. The anti-inflammatory activity of the methanolic extract of *K. rotunda* and *E. cannabinum* against oedema showed

significant anti-inflammatory activity and the results were comparable to that of standard steroidal anti-inflammatory drug. Carrageeenan induced odema is mediated by release of histamine and 5HT followed by the prostaglandin, kinin[71] and has been frequently used to assess the anti-inflammatory effects of natural products[72]. Phytochemical analysis revealed the presence of steroids, flavonoids, tannins and saponnins. Flavonoids which have been shown to exhibit useful anti-inflammatory activity[73] .Flavonoids may act by reducing the effect of inflammatory mediators. They have been shown to inhibit the migration of leucocytes in experimental models. Hence anti-inflammatory activity of the extract might be due to the presence of flavonoids. In **chapter 8** it was revealed that the methanolic extract of *K. rotunda* and *E. cannabinum* exhibited significant ($p<0.05$) anti-inflammatory activity at doses of 200 and 400 (mg/Kg b.w.p.o.). Both the extracts exhibited inhibition in rat paw odema. The present study indicates the potential of these herbal drugs as anti-inflammatory drugs. Such drugs can be explored in various inflammatory diseases. The activity may be attributed to the inhibition of the COX-2 enzyme or inhibition of the activation of transcription factors. It can be concluded that both the extracts have potential to be explored as anti-inflammatory agents due to presence of flavonoids as active constituent.

Pain is a complex phenomenon that is modified by experience, emotion. Pain begins with the activation of receptors known as nociceptors. Once these nerve endings are stimulated, their signals are transmitted to the spinal cord, and then relayed to higher brain centers where the impulse is interpreted. it is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage[74-76]. The primary purpose of an analgesic is to relieve pain. For many people, at the first sign of pain headache, migraine, muscle pain, backache, cramps, joint pains, premenstrual syndrome. Herbal pain medicine in the form of an herbal pain killer becoming a popular alternative to traditional pain-relief medication. Many people choose a type of herbal pain killer to relieve them of their pain[77]. Fever may be a result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease states. Antipyretic are drugs, which reduce elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained. In fever this set point is elevated and a drug like paracetamol do not influence body

temperature when it is elevated by factors such as exercise or increases in ambient temperature[78]. Paracetamol has been shown to suppress fever by inhibiting prostaglandin synthetase resulting in the blockade of synthesis of prostaglandin in the brain[79]. This study, intended to investigate the analgesic and antipyretic activities of *K. rotunda* and *E. cannabinum* by studying the effects of methanol extracts of the plants on nociception induced by immersing the tail in a cup of freshly filled water of exactly 55°C, and on fever induced by yeast. Phytochemical screening of the methanolic extract of the plant under investigation shows that it contains triterpenoid[80]. The plant-derived secondary metabolites have, over the years, greatly contributed to our current understanding of the important mechanisms related to the process of pain transmission and treatment[81]. In **chapter 9** the methanolic extracts of *K. rotunda* rhizomes and leaves of *E. cannabinum* on tail immersion method using mice was investigated. The methanolic extract of *K. rotunda* and *E. cannabinum* shows a good degree of analgesic activity at (200 and 400 mg/kg) in comparison with pentazocine (10mg/kg). The analgesic activity exhibited by the methanolic extract may be significant ($p < 0.05$) due to inhibitory effect on histamine, 5-HT and kinin like substance. On preliminary phytochemical screening the methanolic extract of *K. rotunda* and *E. cannabinum* was found to contain flavonoid compounds. Flavonoids are known to target prostaglandins which are involved in the late phase of acute inflammation and pain perception[82] .Hence, the presence of flavonoids in both the extracts may be contributory to the anti-inflammatory and analgesic activities. The plant extract, at doses of 200 and 400 mg/kg body weight, showed significant central as well as peripheral analgesic activity by oral route. Therefore, the current study indicated that the methanol extract *K. rotunda* and *E. cannabinum* have significant central and peripheral analgesic activity. Thus, the extracts could have a good analgesic activity in pain. The effect of methanol extract of *K. rotunda* and *E. cannabinum* on yeast induced pyrexia has been shown in chapter 9. Treatment with the extracts of both the plants at the doses of 200, 400mg/kg body weight and paracetamol decreased yeast induced elevation of body temperature of rats. The results thus obtained from both the standard drug treated group and extracts treated groups were compared with the control group and a significant reduction in the yeast elevated rectal temperature was observed in a dose dependent manner.

Antimicrobial therapy aims to treat infection with a drug to which the causative organism is sensitive. Antimicrobial agents can be administered on a 'best guess' basis, with a sound knowledge of the infectious disease, the most probable pathogen and the usual antibiotic sensitivity pattern of the pathogen. This is called empirical therapy, and contrasts with rational therapy when antimicrobial agents are administered after the sensitivity of the pathogen is established by culture and *in vitro* testing in the laboratory. In general, empirical therapy is undertaken in a majority of situations encountered in dentistry. Antimicrobial agents can either inhibit the growth of microbes (bacteriostatic agents) or kill micro-organisms (bactericidal agents) by a variety of mechanisms. Depending on the concentration of the antibiotic the same drug could act as a bacteriostatic or a bactericidal agent. In general, however, one or more of the following target sites are involved in the process: the cell wall, the cytoplasmic membrane, ribosomes, and nucleic acid replication sites. Large quantities of antimicrobial agents are produced and consumed globally with the simultaneous potential for exerting selective pressure for resistant bacteria. Mechanisms of antimicrobial resistance are inactivation of the drug and altered cell wall permeability: so that the drug is unable to enter the organism. Modification of the active site of the drug: modification of the enzyme or substrate with which the antimicrobial agent reacts enables the organism to function normally despite the presence of the drug[83]. 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[84]. The antimicrobial compounds mainly isolated from microbes are structurally different from the compounds isolated from plant sources. Many investigators have demonstrated the antimicrobial activity of the constituents of some higher plants and quite a number of chemical compounds of plant origin have been shown to possess antimicrobial activities. In diseases of microbial origin, the plants function as a result of antimicrobial activity against the causative agents[85-91]. 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. Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinines). The addition of a 3-hydroxyl group yields flavonoids. Flavonoids are also hydroxylated phenolic substances

but occur as a C_6-C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes[92,93]. The calculated minimum inhibitory concentration also proved to be effective. The antimicrobial activities of various plants have been reported by many researchers. Phytoconstituents present in plants namely flavonoids, alkaloids, tannins and triterpenoids are producing exciting opportunity for the expansion of modern chemotherapies against wide range of microorganisms. In present study a variety of Gram-positive, Gram-negative bacteria were selected for the screening of antimicrobial effect of three selected plant extracts to perceive the antimicrobial spectrum as well to authenticate ethnomedicinal claims[94,95]. The sensitivity tests of the microorganisms with respect to particular concentration (plant extract and antibiotic) were determined by measuring the diameters of zone of inhibition. The methanol extracts of *K. rotunda* and *E. cannabinum* demonstrated significant *in vitro* antimicrobial activity against seven different Gram-positive and Gram-negative bacteria. The disc diffusion test also demonstrated significant degree of antibacterial activity as compared with standard drug ciprofloxacin. It was also observed in **chapter 10** that the crude methanol extract of *K. rotunda* exhibits better antibacterial potency against selected pathogenic Gram-positive and Gram-negative strains as compared to the extract of *E. cannabinum*. A total of seven bacterial strains belonging to both Gram-positive and Gram-negative were screened for synergism effect by the combination of plant extracts with standard antibiotic ciprofloxacin. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment. The present investigation therefore revealed that the methanol extract of *K. rotunda* and *E. cannabinum* have a significant degree of antimicrobial activity, which may be due to the presence of flavonoids in both the plants[96-98].

References

1. Chaudhri RD. *Herbal drug industry*. Eastern publishers, New Delhi, 1996, p 1-5.
2. Edwin E, Sheeja E, Neeraj M, Sudha V and Vinod T. Herbal medicines, *The Indian Pharmacist*, 2005, **4(38)**, 38-41.
3. Edward A. *Pathogenesis Justicia adhatoda: New, Old and Forgotten remedies*. 2001, p 210-220.
4. Shinwari MI and Khan MA. Indigenous use of medicinal trees and shrubs of Margalla Hills National Park, Islamabad. *Pak. J. Forest.*, 1998. **48**, 63-90.
5. Sack RB and Froehlich JL. Berberine inhibits intestinal secretory response of *Vibrio cholerae* and *Escherichia coli* enterotoxins. *Infect Immune.*, 1982, **35**, 471-475.
6. Singh RP and Padmavathi P. Modulatory influence of *Adhatodava sica* leaf extract on the enzymes of xenobiotic metabolism, antioxidant status and lipid per oxidation in mice. *Mol. Cell Biochem.*, 2000, **213**, 99-109.
7. Raman N. *Phytochemical techniques*. New India publishing agency. New Delhi, 2006, p 2.
8. Halliwell B and Gutteridge JMC. Free radicals, antioxidants and human diseases: where are we now? *J. Lab. Clin. Med.*, 1992, **119**, 598-620.
9. Farombi EO, Nwamkwo JO and Emerole GO. Effect of methanolic extract of browned yam flour diet on 7,12-Dimetylbenzanthracene (DMBA) and 3-methylcholanthrene (3-MC) induced toxicity in the rat. *Proc. Fed. Afr. Soc. Biochem.*, 1998, **1**, 5-10.
10. Farombi EO. Mechanisms for the hepatoprotective action of kolaviron: studies on hepatic enzymes, microsomal lipids and lipid peroxidation in carbon tetrachloride-treated rats. *Pharmacol. Res.*, 2000, **42**, 75-80.
11. Koleva II, Niederlander HAG and Van Beek TA. An online HPLC method for detection of radical scavenging compounds in complex mixtures. *Anal. Chem.*, 2000, **72**, 2323-2328.
12. Hill AF. *Economic Botany*. A textbook of useful plants and plant products. 2nd edn., McGraw-Hill Book Company Inc, New York, 1952.
13. Litchfield JT and Wilcoxon F. A simplified method of evaluating dose effect experiments. *J. Pharmacol. Therpeu.*, 1949, **96**, 99-113.

14. Lorimer SD and Perry N.B. Antifungal hydroxyacetophenones from New Zeland Liverwort *Plagiochila fasciculta. Planta Medica*, 1994, **60**, 386-387.

15. Pappano NB, Centorbi OP and Ferretti FH. Determination of the responsible molecular zone for the chalcones bacteriostatic activity. *Rev. Microbiol.*, 1994, **25**, 168-174.

16. Hu C, Chen K, Shi Q, Kilkus RE, Cheng Y and Hsiung K. Anti-acids agents, Acacetin-7-O-a-D-Galactopyranoside, an anti-HIV principle from *Chrysanthemum morifolium* and a structure-activity correlation with some related flavonoids. *J. Nat. Prod.*, 1994, **57**, 42-51.

17. Mahmood N, Piacente S, Burke A, Khan A and Pizza C. Constituents of *Cuscuta reflexa* are anti-HIV agents. *Antiviral Chem. Chemother.*, 1997, **8**, 70-74.

18. Iwata S, Nishino T, Nagata N, Satomi Y, Nishino H and Shibata S. Anti-tumorigenic activities of chalcones. I. Inhibitory effects of chalcone derivatives on Pi-incorporation into phospholipids of HeLa cells promoted by 12-O-tetradecanoyl-phorbol 13-acetate (TPA). *Biol. Pharm. Bull.*, 1995, **18**, 1710-1713.

19. Makita H, Tanaka T, Fujitsuka H, Tatematsu N, Satoh K, Hara A and Mori H. Chemoprevention of 4-nitroquinoline 1-oxide-induced rat oral carcinogenesis by the dietary flavonoids chalcone, 2-hydroxychalcone and quercetin. *Cancer Res.*, 1996, **56**, 4904-4909.

20. Min B, Ahn B and Bae K. Synthesis and structure-activity relationship of cytotoxic 5,2,5′-trihydroxy-7,8-dimethoxyflavanone analogues. *Arch. Pharm.*, 1996, **19**, 543-550.

21. Williams CA, Hoult JRS, Harborne JB, Greeham J and Eagler J. A biologically active lipophilic flavonols from *Fanacetum parthenium. Phytochemistry*, 1995, **38**, 267-270.

22. O'Neil MJ. The Merck Index: An Encyclopedia of chemicals,drugs and biological.14th edn., Merk & Co., Whitehouse station, NJ, USA, 2006, p 336.

23. Geraets L, Moonen HJJ, Brauers K, Wouters KEFM, Bast A, and Hageman GJ. Dietary flavones and flavonoles are inhibitors of Poly (ADP-ribose) polymerase-1 in pulmonary epithelial cells. *J. Nutr.*, 2007, **137(10)**, 2190 – 2195.

24. Reddivari L, Vanamala J, Chintharlapalli S, Safe SH, and Miller JC. Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase dependent and caspase independent pathways, *Carcinogenesis*, 2007, **28(10)**, 2227-2235.

25. Anonymous. Monograph of quercetin. *Altern Med. Rev.*, 1998, **3(2)**, 140-143.

26. Dhanawat M, Singh GK and Paul A. Pharmacology and potential therapeutic uses of quercetin: A plant flavonoid. *Ind. J. Nat. Prod.*, 2005, **21(2)**, 3-11.

27. Zhang H, Wong CW, Coville PF and Wanwimolruk S. Effects of the grapefruit flavonoid on pharmacokinetics of quinine in rats. *Drug Metabol. Drug Interact.*, 2000, **17**, 351-363.

28. Bhattacharya R, Tulsawani R and Vijayaraghavan R. Effect of trolox and quercetin on sulfur mustard induced cytotoxicity in human peripheral blood lymphocytes. *Ind. J. Pharmacol.*, 2006, **38(1)**, 38-42.

29. Ghosh MN. Toxicity studies. In: Fundamentals of Experimental pharmacology, Scientific Book Agency, Calcutta, 1984, p 153-158.

30. Schafer F and Buettner G. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic. Biol. Med.*, 2001, **30 (11)**, 1191-212.

31. Lennon S, Martin S and Cotter T. Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Prolif.*, 1991, **24 (2)**, 203-214.

32. Valko M, Morris H and Cronin M. Metals, toxicity and oxidative stress. *Curr. Med. Chem.*, 2005, **12(10)**, 1161-208.

33. Lelli J, Becks L, Dabrowska M and Hinshaw D. ATP convert necrosis to apoptosis in oxidant-injured endothelial cells. *Free Radic. Biol. Med.*, 1998, **25(6)**, 694-702.

34. Lee Y and Shacter E. Oxidative stress inhibits apoptosis in human lymphoma cells. *J. Biol. Chem.*, 1999, **274(28)**, 19792-8.

35. Gutteridge JMC and Halliwell B. *Antioxidants in Nutrition, Health and Disease*. Oxford University Press, Oxford, 1994, p 12.

36. Feller DR, Hagerman LM, Newman HAI and Witiak DT. *Principles of Medicinal Chemistry*. In: Foye WO, Lemke TL, Williams DA, Eds., BI Waverly Pvt. Ltd., New Delhi, 1995, p 523.

37. Getie M, Gebre T, Reitz R and Neubert RH. Evaluation of the release profiles of flavonoids from topical formulations of the crude extract of the leaves of *Dodonea viscose*. *Pharmazie*, 2002, **57**, 320-322.

38. Tsuchiya H, Sato M, Miyazaki T, Fujiwara S, Tanigaki S, Ohyama M, Tanaka T and Iinuma M. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin resistant *Staphylococcus aureus*. *J Ethnopharmacol.*, 1996, **50**, 27-34.

39. Scortichini M and Pia Rossi M. Preliminary *in vitro* evaluation of the antimicrobial activity of terpenes and terpenoids towards *Erwinia amylovora* (Burrill). *J. Appl. Bacteriol.*, 1991, **71**, 109-112.

40. Clark RAF. Wound repair: Overview and general considerations. In: Clark RA and P.M. Henson PM, Eds., The Molecular and Cellular Biology of Wound Repair. Plenum Press, New York, 1996, p 3.

41. Bandopadhyay D, Biswas K, Bandopadhyay U, Reiter RJ and Banerjee RK. Melatonin protects against stress- induced gastric lesions by scavenging the hydroxy radical. *J. Pineal Res.*, 2000, **29(3)**, 143-151.

42. Motilva V, Lopez A, Martin MJ & Arcon DL. Cytoprotective activity ofcisapride on experimental gastric mucosal lesions induced by ethanol. Role of endogenous prostaglandins. *Prostaglandins*, 1996, **52**, 63-74

43. Curtis GH, Macnaughton WK, Gall DG and Wallace JL. Intra luminal pH modulates gastric prostaglandin synthesis. *Can. J. Physiol. Pharmacol.*, 1995, **73**, 130-134.

44. Wallace JL. Prostaglandins, NSAIDs, and cytoprotection. *Gastroenterol. Clin. J. North Am.*, 1992, **21**, 631-641.

45. Bjorkman DJ. 1996. Nonsteroidal anti-inflammatory drug-induced gastrointestinal injury. *Am. J. Med.*, 1996, **101**, 25S-32S.

46. Schmassmann A. 1998. Mechanisms of ulcer healing and effects of nonsteroidal anti-inflammatory drugs. *Am. J. Med.*, 1998, **104**, 43S-51S.

47. Cody V, Middleton E and Harborne JB. 1986. Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationships, Alan R (Eds.), New York, 1986, p 158.

48. Park S, Hahm KB, Oh TY, Jin JH and Choue R. Preventive effect of the flavonoid, wogonin, against ethanol-induced gastric mucosal damage in rats. *Dig. Dis. Sci.*, 2004, **49**, 384-394.

49. Lewis DA. Antiulcer Drugs from Plants. *Chemistry in Britain*, 1992, **28**, 141-144.

50. Di Carlo G, Mascolo N, Izzo AA and Capasso F. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. *Life Sci.*, 1999, **65**, 337-353.

51. Harborne JB and Williams CA. Advances in flavonoid research since 1992. *Phytochemistry*, 2000, **55**, 481-504.

52. Borrelli F and Izzo AA. The plant kingdom asa source of anti-ulcer remedies. *Phytother. Res.*, 2000, **14**, 581-591.

53. La Casa CC, Villegas I, Arcon DL, Motilva V and Calero MJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. *J. Ethnopharmacol*, 2000, **71**, 45-53.

54. Cristoni A, Malandrino S and Magistretti MJ. Effect of a natural flavonoid on gastric mucosal barrier. *Arzneimittel forschung*, 1989, **39**, 590-592.

55. Gracioso JS, Vilegas W, Hiruma-lima CA and Brito AR. Effects of tea from *Turneraulmi folia* L. on mouse gastric mucosa support the Turneraceae as a new source of antiulcerogenic drugs. *Biol. Pharm. Bull.*, 2002, **25**, 487-491.

56. Beil W, Birkholz C and Sewing KF. 1995. Effects off lavonoids on parietal cell acid secretion, gastric mucosal prostaglandin production and *Helicobacter pylori* growth. *Arzneimittel forschung*, 1995, **45**, 697-700.

57. Galvin G and Szabo S. Experimental gastric mucosal injury: Laboratory models reveal mechanism of pathogenesis and new therapeutic strategies. *FASEB J.*, 1992, **6**, 825–831.

58. Gryglewski RJ. Molecular mechanisms of inflammation. *Eur. J. Rheumatol. Inflamm.*, 1981, **4**, 153-159.

59. Freeman BD and Natanson C. Antiinflammatory therapies in sepsis and septic shock. *Expert Opin. Investigl. Drugs*, 2000, **9**, 1651-1663.

60. Gibaldi M. *Biopharmaceutics and Clinical Pharmacokineics*. Philadelphia, 1982, p 11-17.

61. Gibaldi M and Perrier D. *Farmacocinetica*, Barcelona, 1982, p 149-162.

62. Goodman Gilman A. The Pharmacological Basis of Therapeutics. McGraw-Hill, New York, 1966, p 144.

63. Rothwell NJ. Eicosanoids, Thermogenesis and Thermoregulation. *Prost. Lts. Ess. Fat. Ac.*, 1992, **46**, 1-7.

64. Di Rosa M, Giroud PJ and Willoughby DA. 1971. Studies of the mediators of acute inflammatory response induced in rats in different sites by carrageenan and turpine. *J. Pathol.*, 1971, **101**, 15-29.

65. Vinegar R, Schreiber W and Hugo R. Biphasic development of carrageenan edema in rats. *J. Pharmacol. Exp. Therapeutics*, 1969, **166**, 96-103.

66. Crunkhon, P. and Meaccock SER. Mediators of the inflammation induced in the rat paw by carrageenan. *Brit. J. Pharmacol.*, 1971, **42**, 392-402.

67. Vineagar R, Traux JF, Selph JH, Johnston PR, Vinable AH and Mckenzie KK. Pathway to carrageenan-induced inflammation in hind limb of the rat. *Fed. Proc.*, 1987, **46**, 118 -126.

68. Maria TZ and Ferreira H. Relationship between edema and plasma exudation in the rat paw carrageenan inflammation. *Infla. Res.*, 1978, **8(6)**, 606-609.

69. Di Rosa M, Giroud PJ and Willoughby DA. Studies of the mediators of acute inflammatory response induced in rats in different sites by carrageenan and turpine. *J. Pathol.*, 1971, **101**, 15-29.

70. Meade CJ, Turner GA and Bateman PE. The role of polyphosphoinositides and their break down products in A23187 induced release of arachidonic acid from rabbit polymorphonuclear leucocytes. *Biol. Che. J.*, 1986, **23**, 425-436.

71. Castro J, Sasame H, Sussman J and Bullette P. Diverse effects of SKF525A and antioxidants on [CCl.sub.4]-induced changes in liver microsomal p-450 content and ethylmorphine metabolism. *Life science*, 1968, 7, 129.

72. Della Loggia A, Tubaro A, Dri P, Zilli C and Del Negro P. The role of flavonoids in the antiinflammatory activity of *Chamomilla recutita*. *Biol. Res.*, 1986, **213**, 481-484

73. Mukherjee PK, Mukherjee K, Bhattacharya S, Pal M and Saha BP. Studies on the anti-inflammatory of *Drymaria cordata* Willd. *Nat. Prod. Sci.*, 1998, **4(2)**, 91-94.

74. Merskey H. Classification of chronic pain, description so chronic pain and definitions of pain terms. *Pain*, 1986, **3**, 1-226.

75. Garrett N and McShane F. The pathophysiology of pain. *AANA Journal*, 1999, **67(4)**, 349-57.

76. Deal CL and Moskowitz RW. Nutraceuticals as therapeutic agents in osteoarthritis. Rheum. *Dis. Clin. North Am.*, 1999, **25(2)**, 379-95.

77. Rothner AD. A practical approach to headaches in adolescents. *Pediatr. Ann.*, 1991, **20(4)**, 200-205.

78. Goodman and Gilman. *The pharmacological Basis of Therapeutics*. 19th edn., New York, Mc Graw-Hill, 1996, p 959-975.

79. Flower R.J and Vane JR. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). *Nature*, 1972, **240**, 410-411.

80. Joao JB, Scheidt C, Otuki M, Adair RS and Santos RS. Biological activity of plant extracts: novel analgesic drug. *Expert Opinion on Emerging Drugs*, 2001, **6(2)**, 261-279.

81. Rajnarayana K, Reddy MS, Chaluvadi MR and Krishna DR. Bioflavonoids classification, pharmacological, biochemicaleffects and therapeutic potential. *Indian J. Pharmacol.*, 2001, **33**, 2-16.

82. Trease GE and Evans WC. *Pharmacognosy*. 4th edn., ELBS Publication, Bailliere Tindall, London, 1985, p 344.

83. Samaranayake LP and Johnson NW. Guidelines for the use of antimicrobial agents to minimise development of resistance. *International Dental Journal*, 1999, **49**, 189-195.

84. Katzung BG. *Basic and clinical pharmacology*. 7th edn., Appleton & Lange, Stamford, Connecticut, U.S.A., 1998, p 62-67, 242-244.

85. Verpoorte R, Tjenatsoi A, Van-Doorne J and Svedsen AB. Medicinal plants of Suriname: Antimicrobial activity of some medicinal plants. *J. Ethnopharmacol.*, 1982, **5**, 221-226.

86. Akobundu IO and Agyakwa CN. *A handbook of West African Weeds*. International Institute of tropical Agriculture, 1987, p 240, 272, 348.

87. Rocio MC and Rion JL. (1989). A review of some antimicrobial substances isolated from medicinal plants reported in the literature 1978-1972. *Phytother. Rev.*, 1989, **3**, 117-125.

88. Almagboul AZ, Basho AK, Karim A, Salibm TA and Khalid SA. Antimicrobial activity of certain Sudanese plants used in folklore medicine. Screening for antifungal activity VI, *Fitoterapia*, 1988, **59**, 393-396.

89. Corthout B, Piefers L and Cleays M. Antiviral caffeoylester from *Spondias mornbin. Phytochemistry*, 1992, **31**, 1979-1981.

90. Misra TN, Sigh RS, Pandey NS, Prasal C and Singh BP (1992).Antifungal essential oil and a long chain alcohol from *Achyranthisaspera. Phytochemistry*, 1992, **31**, 1811-1812.

91. Hablemarian S, Gray AI and Waterman PG. A new antibacterial sesquiterpene from *Premma oligotrichia. J. Nat. Prod.*, 1993, **56**, 140-143.

92. Dixon RA, Dey PM and Lamb CJ. Phytoalexins: Enzymology and molecular biology. *Adv Enzymol.*, 1983, **55**, 1-69.

93. Taniguchi M and Kubo I. Ethnobotanical drug discovery based on medicine men's trials in the African savanna: screening of east African plants for antimicrobial activity II. *J. Nat. Prod.*, 1993, **56**, 1539–1546.

94. Lutterodt GD, Ismail A, Basheer RH and Baharudin HM. Antimicrobial effects of *Psidium guajava* extracts as one mechanism of its antidiarrhoeal action. *Malaysian J. Med. Sci.*, 1999, **6(2)**, 17-20.

95. Marjorie MC. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, 1999, **12(4)**, 564-582.

96. Miles RS and Amyes SGB. *Medical Microbiology*. Livingstone, Edinburgh, New York, 1996, p 151-177.

97. Lamanna C and Shapiro IM. Western blot analysis indicated migration of cytochrome. *J. Bacteriol.*, 1943, **45**, 385-394.

98. Garrod LP, Lambert HP, Grady F and Worth PM. *Antibiotics and chemotherapy*. Livingstone, Edinburgh, New York, 1981, p 405-408.

CHAPTER - 12

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 Studies on pharmacological screening of the bioactive molecules from some selected plants so as to explore the therapeutic values and development of some novel drugs from those indigenous plants

Chapter 1

Herbal medicine or phytotherapy is the science of using herbal remedies to treat the sick. It therefore covers everything from medicinal plants with powerful actions. A great variety of plants are used for medicinal treatments. Either the dried plant, or a specific part of it (root, leaves, fruit, flowers, seeds), is formulated into suitable preparations-compressed as tablets or made into pills, used to make infusions (teas), extracts, tinctures, etc., or mixed with excipients to make lotions, ointments, creams, etc. The active principle is extracted and purified from plant material for as long as that process remains economically viable compared with chemical synthesis. Herbal medicine has been used for centuries by people all over the world to treat disease and promote health. Both the west and the east have spent considerable time, research and energy developing the theories and applications within the field of herbal medicine. Herbs are generally easy to administer and cost-effective and when properly prescribed and used, have the advantage of being relatively free of side effects when compared to western pharmaceutical medicines. Herbal medicine is currently enjoying a revival in popularity in the west and in fact it is the primary form of medicine in many parts of the world. With the great reliance on this type of medicine, it becomes pertinent to search for potent, effective and relatively safe plant medicines as well as scientific validation of the success claims about plants already in use by traditional medicine practitioners in order to enhance their safety and efficacy. These are some of the problems making this alternative healthcare system less acceptable, especially by orthodox medicine practitioners. In Sikkim traditional healers and remedies made from plants play an important role in the health of people.

Medicinal plants are plants whose extracts can be used directly or indirectly for the treatment of different ailments. Therefore, the use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed. Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering humanity. Furthermore, in the world more than 30% of the pharmaceutical preparations are based on plants

Chapter 2

Various reports on phytochemical and pharmacological analysis of *K. rotunda* and *E. cannabinum* along with their allied species have been presented. The current updated status of natural origin used as antioxidant, wound healing, antiulcer, analgesic and antipyretics, anti-inflammatory and antimicrobials have been elaborated in the form of comprehensive review of literature. Though some reports on phytochemical and pharmacological analysis of *K. rotunda* and *E.cannabinum* exist in the literature, the present study revealed the presence of additional constituents with significant pharmacological activities.

Chapter 3

The modern methodologies for extraction and isolation of bioactive compounds from *K. rotunda and E. cannabinum* along with phytochemical group tests have been presented. The isolated bioactive compounds were analyzed by using physical spectroscopic methods like UV, IR, ^{1}HNMR, Mass and melting point determination etc. The evidence presented conclusively proved that the isolated bioactive compounds isolated were 2-hydroxy 4,4',6- trimethoxy chalcone from *K. rotunda* and 3′,4′,5,7–tetrahydroxy flavonol or quercetin from *E. cannabinum.*

Chapter 4

Toxicological investigation of methanol extract of *K. rotunda* and *E. cannabinum* were described. The MLD of methanol extract of *K. rotunda* rhizome was found to be 5 g/kg body weight and the MLD of the methanol extract of *E. cannabinum* leaves was to be 4.5 g/kg body weight in oral route whereas the MLD of isolated compound I and II were found to be 213.7mg/kg body weight and 196.3mg/kg body weight in 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

Antioxidant activities of methanol extract of *K. rotunda* and *E. cannabinum* presented. The TBARS and 4-HNE formation inhibitory effect of the methanolic extract of the plants of *K. rotunda* and *E. cannabinum* were significantly higher than that of control. The lipid peroxidation inhibition capacity of *K. rotunda* was studied by dose dependent manner and it was concluded that the antioxidant activity has inverse relationship with dose i.e. high at low dose and vice versa. The extract at 100µg/ml and 200µg/ml has significant lipid peroxidation inhibition activity in respect to 500 and 1000µg/ml. The lipid peroxidation inhibition activity of extract of *E. cannabinum* has observed in higher doses. The dose of 100µg/ml and 200µg/ml has insignificant activity whereas 500µg/ml and 1000µg/ml has moderate and significant lipid peroxidation activity respectively

Chapter 6

Wound healing activity of methanol extract of *K. rotunda* and *E. cannabinum* as well as compound I and II has been presented. Significant promotion of wound-healing activity was observed in both methanol extracts and isolated compound in all the three wound models such as excision, incision and dead space wound.

In excision wound model, the percentage closure of wound area was significantly increased by the methanol extracts of rhizome of *K. rotunda* and leaves of *E. cannabinum* and their isolated compounds in the respective animal groups. From the result it was revealed that, *K. rotunda* extract treated animals showed significant reduction in the wound area and faster rate of epithialisation as compared to *E. cannabinum* extract. The isolated compound treated animals showed faster epithelisation of wounds than the animals treated with methanolic extracts of both the plants.

In incision wound model the breaking strength of the 10^{th} day old restored incision wound was significantly increased in animals treated with isolated compounds and methanolic extracts of both the plants, when compared to control. The maximum breaking strength was observed in animals treated with isolated compound II in the dose of 20mg/kg bw. Significant breaking strengths were also observed in the animals treated

with isolated compound I followed by methanol extracts of rhizome of *K. rotunda* and leaves of *E. cannabinum*.

In dead space wound model, the extract of both the plants and their isolated compounds were assessed by the increase in the weight of granuloma, increase in breaking strength and hydroxylproline content of the granuloma tissue. This effect may be due to the enhancement of collagen maturation by increased cross linking of collagen fibers. The increased weight of the granuloma also revealed the presence of higher hydroxyl proline content. Among the methanolic extract and isolated compound treated groups the hydroxyl proline content was found to be more in isolated compound treated animals followed by methanolic extracts of *K. rotunda* and *E. cannabinum*.

In this study extracts as well as isolated compound significantly increased the granuloma tissue breaking strength and hydroxyproline content as compared to control. The result of the investigation provides pharmacological evidence on the folkloric use of both the plants for wound.

Chapter 7

Antiulcer activity of methanol extract of *K. rotunda* and *E. cannabinum* as well as compound I and II has been presented. Here the effect of methanolic extracts of *K. rotunda* and *E. cannabinum* and their isolated compound on pylorus ligated rat and aspirin induced ulcer model has investigated. The result of the present studies indicates that the isolated product of both the plant significantly reduces the total volume of gastric juice, free and total acidity of gastric secretion. The control animals had ulcers and hemorrhagic streaks whereas in animals administered with the methanolic extracts of *K. rotunda* and *E. cannabinum* and their isolated compound there was significant reduction in ulcer index. Thus in the present study concludes that both the above mention plant extracts and their isolated compounds possess significant antiulcer activity in different experimental models of ulcer in rats. The magnitude of activity obtained at the two dose levels used indicates high potency of anti-ulcer effect and together with the array of compounds already isolated from the plant provide impetus to continue the search for novel anti-ulcer constituents from this plant.

Chapter 8

This study was undertaken to evaluate the anti-inflammatory potential of the methanolic extract of *K. rotunda* and *E. cannabinum* on carrageenan-induced rat paw edema. The results of the present study suggest that the methanolic extract of *K. rotunda* and *E. cannabinum* at the dose levels of 200 and 400 mg/kg significantly suppressed carrageenan-induced paw edema in rats. On preliminary phytochemical screening the methanolic extract of, *K. rotunda* and *E. cannabinum* was found to contain flavonoid compounds. Flavonoids are known to target prostaglandins which are involved in the late phase of acute inflammation and pain perception. Hence, the presence of flavonoids may be contributory to the antiinflammatory and analgesic activities of *K. rotunda* and *E. cannabinum*. The study indicates the potential of these herbal drugs as anti-inflammatory drugs. Such drugs can be explored in various inflammatory diseases It can be concluded that methanolic extract of *K. rotunda* and *E. cannabinum* possess significant anti-inflammatory activity in rats. The extracts significantly reduce the increase in hind paw volume induced by carrageenan, compared to the vehicle treated control. The findings of the present work justified the use of this plant in the treatment of rheumatism and other inflammatory conditions for its anti-inflammatory action.

Chapter 9

This chapter carried out a preliminary screening for analgesic and antipyretic activities of the rhizome and leave extracts of *K. rotunda* and *E. cannabinum* plants in order to establish some of its pharmacological properties show the responses of mice to tail immersion and rats to yeast induced pyrexia method.

The present result showed that the methanol extract of *K. rotunda* and *E. cannabinum* possesses a significant antipyretic effect in yeast-provoked elevation of body temperature in rats and its effect is comparable to that of paracetamol. The present study has been undertaken to investigate the analgesic potential of methanol extract of *K. rotunda* and *E. cannabinum* by tail immersion method in comparison with pentazocine.

Of the two doses, the dose of 400mg/kg is found to be more potent and efficacious towards the analgesic and antipyretic, and the activity is in dose dependent manner. These data indicate the analgesic and antipyretic potential of *K. rotunda* and *E. cannabinum*. Generally, plants showing the antipyretic effect also possess analgesic

activity. The present analgesic and antipyretic activity of *K. rotunda* and *E. cannabinum* may be attributed to the presence of alkaloids, flavonoids and tannins and triterpenoid in their extract.

Chapter 10

Evaluation has done on the potential of plant extracts and phytochemicals on standard microorganism strains as well as multi-drug resistant bacteria, which were isolated from hospitals. Moreover, the study investigated the synergistic effects of extracts with antimicrobial activity in association with antibiotics against drugs resistant bacteria. Therefore, the results revealed the importance of plant extracts when associated with antibiotics, to control resistant bacteria, which are becoming a threat to human health. Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

All the experiments were performed both by using *in vitro* and *in vivo* model to establish 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 conclusion two medicinally important pytoconstituents 2-hydroxy 4, 4′, 6-trimethoxy chalcone (compound I) and 3′, 4′, 5, 7- tetrahydroxy flavonol (compound II) have been isolated from the rhizomes of *Kaempferia rotunda* and leaves of *Eupatorium cannabinum* respectively. The results of the studies performed in this thesis may contribute to the formulation of potential herbal medicine from the plants investigated.

LIST OF PUBLICATIONS

List of Publications in Journals

1. G. Mariappan, Nihar Bhuyan, **J.P. Mohanty**, Subarna Ganguli and D. Dhachinamoorthi. "An overview of the method of positional scanning synthetic combinatorial libraries". ***Indian Journal of Pharmaceitical Science***, 2006, **68(4)**, 420-424.

2. R. Chanda, **J.P. Mohanty**, N.R. Bhuyan, P.K. Kar, L.K. Nath. "Medicinal plants used against gastrointestinal tract disorders by the traditional healers of Sikkim Himalayas". ***Indian Journal of Traditional Knowledge***, 2007, **6(4)**, 606-610.

3. U.K.Nayak, G.Mariappan, **J.P.Mohanty**, N.R.Bhuyan and B.K.Patro. "Anti-lipid peroxidation activity of *Curcuma aromatic* Linn". ***Indian Drugs***, 2007, **44(6)**, 483-485.

4. **J.P. Mohanty**, L.K.Nath, N.R Bhuyan, S.K.Mohapatra. "Antibacterial spectrum of *Kaempferia rotunda* Linn. and *Eupatorium cannabinum* Linn." ***International Journal of Pharmacology and Biological Sciences***, 2008, **9(1)**, 45-50.

5. **J.P. Mohanty**, L.K.Nath, N.R.Bhuyan and U.K.Nayak. "A Preliminary Study on Gastric Antiulcer Activity of *Eupatorium Cannabinum* Linn. in rats". ***Advances in Pharmacology and Toxicology***, 2008, **2(1)**, 159-164.

6. **J.P. Mohanty**, L.K.Nath, N.R Bhuyan, G.Mariappan. "Studies on Antioxidant Potential of *Kaempferia rotunda* Linn." ***Indian Journal of Pharmaceutical Sciences***, 2008, **70(3)**, 362-364.

List of Presentations in conferences

1. R. Chanda, **J.P. Mohanty**, N.R. Bhuyan, A. Bakshi, S. Ganguli. "Market Survey of Sellings of Pharmaceutical Products in India". 58th Indian Pharmaceutical Congress (IPC), Mumbai, India, 1-3rd December, 2006.

2. **J.P. Mohanty**, Nihar Bhuyan, R. Chanda, Pinak Paul. "Antiulcer activity of *Kaempferia rotunda* Linn. of Sikkim Himalaya Region". 59th Indian Pharmaceutical Congress (IPC), Varanashi, India, 20-23rd December, 2007.

3. **J.P. Mohanty**, N.R. Bhuyan, L.K. Nath, K. Gautaman. "Studies on Analgesic and antipyretic of *Kaempferia rotunda* Linn". National Conference on Folk Medicine, 19-20th December, 2007, Gangtok, sponsored by Dept. of AYUSH, Govt. of India.

4. Hema Basnet, V.Ravi, S. VijayKumar, T.S. Mohamed Saleem, **JP. Mohanty**, S. Dutta, D.J. Sarkar, K.Gautaman. Cardio protective effect of benzene extract of *Hibiscusrosa sinensis* flower powder in-vitro model of myocardial ischemic-reperfusion injury in rats. National Conference on Folk Medicine, 19-20th December, 2007, Gangtok, sponsored by Dept. of AYUSH, Govt. of India.

5. Hema Basnet, **J.P. Mohanty**, S. Dutta, D. Sarkar, K.Gauthaman. Anti convulsant effect of *Drosera burmannii* Vahl. National Conference on Folk Medicine, 19-20th December, 2007, Gangtok, sponsored by Dept. of AYUSH, Govt. of India.

6. N.R. Bhuyan, T. Bera, B.P. Saha, **J.P. Mohanty**. "Plants and their constituents used to prevent Leishmaniasis". National Conference on Folk Medicine, 19-20th December, 2007, Gangtok, sponsored by Dept. of AYUSH, Govt. of India.