PHYTOCHEMICAL CHARACTERISATION AND *IN VITRO* ANTICANCER SCREEENING OF ETHANOL EXTRACT OF *CHROMOLAENA ODORATA* Linn.

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IN
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Submitted by
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MAY 2012

CERTIFICATES

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"PHYTOCHEMICAL CHARACTERISATION AND *IN VITRO* ANTICANCER SCREEENING OF ETHANOL EXTRACT OF *CHROMOLAENA ODORATA* Linn"** submitted by the student **CH ACHINTYA SINGH, [Reg. No: 26103033]** to "The Tamilnadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment for the award of degree of **Master of Pharmacy** in **Pharmaceutical Chemistry** was evaluated by us during the examination held on……………………….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"PHYTOCHEMICAL CHARACTERISATION AND *IN VITRO* ANTICANCER SCREEENING OF ETHANOL EXTRACT OF *CHROMOLAENA ODORATA* Linn"** is a bonafide research work carried out by **CH ACHINTYA SINGH, [Reg. No: 26103033]** in the department of Pharmaceutical Chemistry under my guidance and supervision for the partial fulfillment for the award of degree of **Master of Pharmacy** in **Pharmaceutical Chemistry** in J.K.K. Nattraja College of Pharmacy during the academic year 2011-2012.

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DECLARATION

I do hereby declare that the dissertation work entitled **"PHYTOCHEMICAL CHARACTERISATION AND *IN VITRO* ANTICANCER SCREEENING OF ETHANOL EXTRACT OF *CHROMOLAENA ODORATA* Linn"** submitted to "The Tamilnadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment to the requirement for the award of degree of **Master in Pharmacy** in **Pharmaceutical Chemistry**, is a bonafide work carried out by me during the academic year 2011-2012, under the guidance and supervision of **Dr. P. Perumal,** M.Pharm., Ph.D., Professor and Principal, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

I further declare that, this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associateship and fellowship.

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DEDICATED TO MY
BELOVED
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CHAPTER-1.

INTRODUCTION.

CHAPTER – 1

INTRODUCTION

1.1 History of Use of Traditional Herbal Medicines

" Traditional medicine is the sum total of the knowledge, skills and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether applicable or not, used in the maintenance of health as well as the prevention, diagnosis, improvement ore treatment of physical and mental illness" *(Anupama Singh, et al)*

In many countries, a large proportion of the population relies on traditional practitioners and their armamentarium of medicinal plants in order to meet health care needs. Herbal medicines have maintained their popularity for historical and cultural reasons. Such products have become more widely available commercially, especially in developed countries. *(International Agency for Research on Cancer, Volume 82)*

1.2 Herbal Medicinal System in India

Herbal medicines are "plant derived materials or products with therapeutic or other human health benefits which contain either raw or processed ingredients from one or more plants". The traditional Indian system of medicine has a very long term history of usage in a number of diseases and disorders, but lacks recorded safety and efficacy data. Development of standardized, safe and effective herbal formulations with proven scientific evidence can provide an economical alternative in several disease areas. This reveals the possibility to recast and develop the Indian traditional System of medicines in this manner with disease specific perspective. *(Kamlesh K Bhutani, et al).* Arunachal Pradesh in North East India is reported to have more than 545 species of orchids which is the highest number of orchid species known from any single state of India. This gives an initial advantage for observing and scrutinizing the rich flora and fauna for development of traditional knowledge. *(Chandra Prakash Kala, et al)*

Apart from the initiative to preserve the flora and fauna, various efforts have been undertaken by the Government of India in the form of a coordinated programme involving 19 CSIR laboratories, few academic departments and Research and Development (R&D) institutes in the field of traditional medicines. Their main aim is to discover new bioactive molecules from natural sources. Golden Triangle Partnership (GTP) has also been introduced for the validation of traditional Ayurvedic drugs. Its main objective is to bring safe, effective and standard Ayurvedic formulations along with Siddha, Unani and Homeopathy. *(K.K. Bhutani , et al)*

There are about 45,000 plant species in India mainly in the regions of Eastern Himalayas, Western Ghats and Andaman and Nicobar Island. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world. There are about 250,000 registered medical practitioners of the Ayurvedic system and in rural India 70 % of the population is dependent on the traditional system of medicine, the Ayurveda.

The major hindrance in the amalgamation of herbal medicines into modern medical practices is the lack of scientific and clinical data, and better understanding of efficacy and safety of the herbal products. To overcome these challenges and to ensure the quality and safety of its products and practices, standardization is of vital importance. *(S.D. Seth, et al)*

1.3 An overview on Current Status of Herbal Drugs in India

Herbal medicines are currently in demand and their popularity is increasing day by day. India is a vast repository of medicinal plants that are used in traditional medical treatments. The various indigenous systems such as Siddha, Ayurveda, Unani and Allopathy use several plant species to treat different ailments. The use of herbal medicine is becoming popular due to toxicity and side effects of Allopathic medicines. This led to the sudden increase in the number of herbal drug manufacturers. Herbal drug practices continue today because of its biomedical benefits as well as place in cultural beliefs in many parts of the world and have made

a great contribution towards maintaining human health. Currently majority of the world's population depends on plant derived medicine for the first line of primary health care for human alleviation because it has no side effects. *(Sheetal Verma, et al)*

Herbal drugs constitute a major share of all the officially recognized systems of health in India viz. Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy except Allopathy. More than 70% of India's population still use these non allopathic supplements, as per the Indian Drugs Act. However, there is vast experiential evidence based for many of the natural drugs. This offers immense opportunities for Observational Therapeutics and Reverse Pharmacology. Evidence based herbals are widely used in the diverse systems and manufactured, as per the pharmacopoeial guidelines, by a well organized industry. Significant basic and clinical research has been carried out on the medicinal plants and their formulations with the state-of-the-art methods in a number of Institutes/Universities. Indian medicinal plants also provide a rich source for antioxidants that are known to prevent/delay different diseased states. The medicinal plants also contain other beneficial compounds like ingredients for functional foods, spices, also used for home remedies. *(A.D.B. Vaidya, et al)*

1.4 Standardization of Traditional Medicine

Standardized herbal preparations are those which consist of complex mixtures of one or more plants which contain plant parts of plant material in the crude or processed state as active ingredients.

Plant constituents may be toxic but the adverse effect of such phytotherapeutic agents are less frequent compare to synthetic drugs. Plants contain several constituents some of which are present at very low concentrations. Very rarely phytochemical investigations succeed in isolating and characterizing all secondary metabolites present in the metabolites. Quality control and standardization of herbal medicines involved several steps out of which the source and quality of raw materials play a pivotal role in assuring the quality and stability of herbal preparations. Other factors such as use of fresh plants, temperature, light

exposure, water availability, nutrients, period and time of collection, method of collection, drying, packing, storage and transportation of raw materials, age and part of the plant collected etc can greatly affect the quality and therapeutic value of herbal medicines. Some plant constituents are heat labile so they need to be dried at low temperatures. Also, other active constituents are destroyed by enzymatic processes that continue for long time after plant collection. Thus proper standardization and quality control of raw materials and herbal preparations should be carried out. Marker substances are established for analytical purposes in case if the active principles of the plants materials are unknown. Also other factors such as method of extraction and contamination of microorganisms, heavy metals, pesticides etc can also interfere with the quality, safety and efficacy of herbal drugs. Thus, pharmaceutical companies prefer using cultivated plants instead of wild plants. Other process of standardization such as thin layer chromatography, gas chromatography, high performance liquid chromatography, mass spectrometry, infra red spectrometry, ultra violet visible spectrometry can used alone or in combination for standardization. *(J.B. Calixto, et al)*

1.5 Legal Status of Medicinal Plants in India

1.5.1 Background information

For centuries, Ayurveda, Siddha, and Unani systems of medicine have coexisted with yoga, naturopathy, and homeopathy.

Ayurveda means ‘science of life’. Ayurvedic philosophy is attached to sacred texts, the Vedas, and based on the theory of Panchabhutas - all objects and living bodies are composed of five basic elements: earth, water, fire, air and sky. Siddha means ‘perfection’. Siddha has close similarities to Ayurveda, the difference between these two systems being more linguistic. Unani is based on theory of the four bodily humours: blood, phlegm, yellow bile, and black bile. It is also called Arabic medicine. Yoga was propounded by Patanjali and is based upon observance of austerity, physical postures, breathing exercises, restraining of sense organs, contemplation, meditation and Samadhi. Naturopathy is a system of drugless treatment and a way of life. Homeopathy has few basic principles-law of similarity,

direction of cure, principle of single remedy, the theory of minimum diluted dose, and the theory of chronic disease.

1.5.2 Regulatory situation

Ayurveda, Unani, Siddha, Naturopathy, Homeopathy and Yoga are all recognized by the Government of India. The first step in granting this recognition was the creation of the Central Council of Indian Medicine Act of 1970. The main mandates of the Central Council are as follows-

- To standardize training by prescribing minimum standards of education in traditional medicine, although not all traditional medicine practitioners and homeopaths need be institutionally trained to practice;
- To advise the Central Government in matters relating to recognition/withdrawal of medical qualifications in traditional medicine in India:
- To maintain the central register of Indian medicine, revise the register from time to time, prescribe standards of professional conduct and etiquette, and develop a code of ethics to be observed by practitioners of traditional medicines in India. All traditional medicine practitioners and homeopaths must be registered to practice.

The Central Council of Homeopathy, constituted in 1973, has the same mandates. The Indian Government created the Department of Indian system of Medicines and Homeopathy in March 1995.The primary areas of work for the Department is education, standardization of medicines, enhancement of availability of raw materials, research and development, information dissemination, communication, and the involvement of traditional medicine and Homeopathy in National health care. More than 4000 personnel work in these areas.

The Indian Government seeks the active and positive use of traditional medicine and homeopathy in national health programmes, family welfare programmes, and primary health care.

1.5.3 Education and training

Through the Central Council of Indian Medicine and the Central Council of Homeopathy, the Indian Government is working to standardize the training of traditional medicine practitioners and Homeopaths. In support of this, seven national institutes are under the control of the Department of Indian Systems of Medicine and Homeopathy:

- National Institute of Ayurveda: established in 1976, located in Jaïpur, offers a PhD and MD in Ayurveda;
- National Institute of Homeopathy: established in 1975, located in Calcutta, offers Bachelors and MD degrees in Homeopathy;
- National Institute of Naturopathy: established in 1984, located in Pune, offers talks in Hindi and Marathi and programmes for teachers and doctors;
- National Institute of Unani Medicine: established in 1984, located in Bangalore, offers postgraduate research opportunities in Unani;
- National Institute of Postgraduate Teaching and Research in Ayurveda: located in New Delhi, offers PhD and MD degrees in Ayurveda;
- National Academy of Ayurveda: established in 1988, located in New Delhi, offers a Degree of Membership Certificate in Ayurveda;
- National Institute of Yoga: established in 1976, located in New Delhi, offers a one year diploma in Yoga. *(Dr. Xiaorui Zhang, et al)*

1.6 Legal Rights of Medicinal Plants in India

1.6.1 Intellectual Property Rights (IPR) for Herbal Medicine

The Intellectual Property Rights (IPR) for medicinal plants and herbal medicine emerges in two different backgrounds

1. Those that are found in nature as wild Medicinal Plants and collected for use, and

2. Those that are developed through plant breeding systems and used as cultivated Medicinal Plants. Plant breeding systems used so far are mostly conventional based on selection and control pollination.

Intellectual Property Rights was established in 1990 for uniting the indigenous people, scientific organisation and environmental groups to create and implement forceful strategies for the use of traditional knowledge and involvement of local people in conservation of medicinal plants. Traditional resource rights (TRP) also have many "Bundles of Rights" related to protection, conservation and compensation for the benefit of indigenous people.

1.6.2 Herbal Medicinal Plants and Intellectual property laws

It was thought that Agreement on Agriculture is the central focus of World Trade Organisation (WTO) negotiations, since agricultural is a lifeline of development of most countries in the world. It plays a pivotal role in ensuring food security, providing livelihoods, generating foreign exchange and determining the allocation of natural resources. Dominant interest within the Agreement on Agriculture circles around greater market access and increases volume of commodity flows. Agreement on Trade Related Intellectual Property Rights (TRIPS) is the outcome of WTO which is direct impact on farmer's livelihood, food security and economic development of the country. TRIPS provide common protection and enforcement of IPR such as copy rights, trademarks and patents and also make rules intended to limit international trade in counterfeit goods. *(Paramita Nandy, et al)*

1.7 Safety and efficacy of Herbal Medicine

1.7.1 Safety of Herbal Medicine

Many cases are reported on serious adverse effects after administration of herbal products. Toxicity has also been traced due to contamination and adulteration. Some herbal medicine can also be highly toxic. As a whole, herbal medicine can have various adverse effect and drug-food interactions. Therefore assessment of safety of herbal products is the first priority in herbal research.

The toxic effects of herbal preparation may be due to

A) Inherent toxicity of plant constituents and ingredients
B) Manufacturing malpractices and contamination
C) Evaluation of toxic effects of herbal formulation requires detailed phytochemical and pharmacological studies. However the use of toxic plants has been largely eliminated and recent reports of toxicity could be due to misidentification and overdosing of certain constituents. Potential contaminants include microorganisms, microbial toxins, pesticides, fumigation agents, radioactivity, and the presence of toxic compounds of toxic metals.

1.7.2 Assessment of toxicity

It can be done by the following technique

A) in vivo techniques
B) in vitro techniques
C) cell line techniques
D) micro array and other modern technique
E) standardization
F) techniques to adequately model toxicity.

1.7.3 Efficacy of Herbal Medicine

The efficacy of herbal medicine is the measure of its ability to improve health and well being. Traditional medicine usually takes a “holistic” approach where the physical, mental and social well being of an individual is treated. Thus medicinal value of herbal products is related to its nutritional and psychological aspects. The concept of World Health Organisation (WHO) is to emphasise the best art of physical and mental system through which it may be able to design appropriate indicators of efficacy and practical methodology to test them.

1.7.4 Assessment of efficacy

Presently, there is no way to assess efficacy of herbal medicines other than by conventional clinical trial methodologies, in which efficacy is assessed by clinical, laboratory, or diagnostic outcomes:

A) Clinical outcomes include parameters such as improved morbidity and mortality, reduced pain or discomfort, improved appetite and weight gain, reduction of blood pressure, reduction of tumour size and extent, and improved quality of life.

B) Laboratory/other diagnostic outcomes include parameters such as reduction of blood glucose, improvement of haemoglobin status, reduction of obesity as measured by radiological or imaging techniques, and improvement in electrocardiogram (ECG) findings. *(M. Mosihuzzaman, et al)*

1.8 Future status of Herbal Medicine

The uses of plants in medicine will continue in future, as a source of therapeutic agents, and as raw material based for the extraction of semi-synthetic chemical compounds such as cosmetics, perfumes and food industries. Recently even developed countries are using medicinal systems that involve the use of herbal drugs and remedies. Undoubtedly the demand for plant-derived products has increased worldwide. The demand is estimated to grow in the years to come fuelled by the growth of sales of herbal supplements and remedies. This means that scientists, doctors and pharmaceutical companies will be looking at countries like India, China etc. for their requirements, as they have the most number of medicinal plant species and are the top exporters of medicinal plants. *(Sheetal Verma, et al)*

1.9 Drug discovery from Plants

Drug discovery process from natural products has been undertaken by the Council of Scientific and Industrial Research along with various institutes and Golden Triangle Partnership. Apart from these, there are unfortunately, very few industries, when it comes to drug discovery from plants. Most of them restrict their

focus on herbal formulations. The enormous cost involved in isolation of pure compounds from plants is perhaps the prohibitive factor. Therefore, most of the natural products research for drug discovery perspective remains as an academic exercise rather than a full fledged program.

Traditional Indian Medicine		
Pro's	**Con's**	**Areas needing improvement**
Long term history of usage Efficacy Wider public acceptance Lower costs	Batch to batch variation Authentification of raw material Lack of clinical trials data Lack of reference standards Inherent photochemical variations Lack of proper pharmacopoeias	Quality control Regulatory controls Standardization of formulations Good agricultural practices Good manufacturing practices Development of monographs

Table No. 1.1 Traditional Indian Medicine

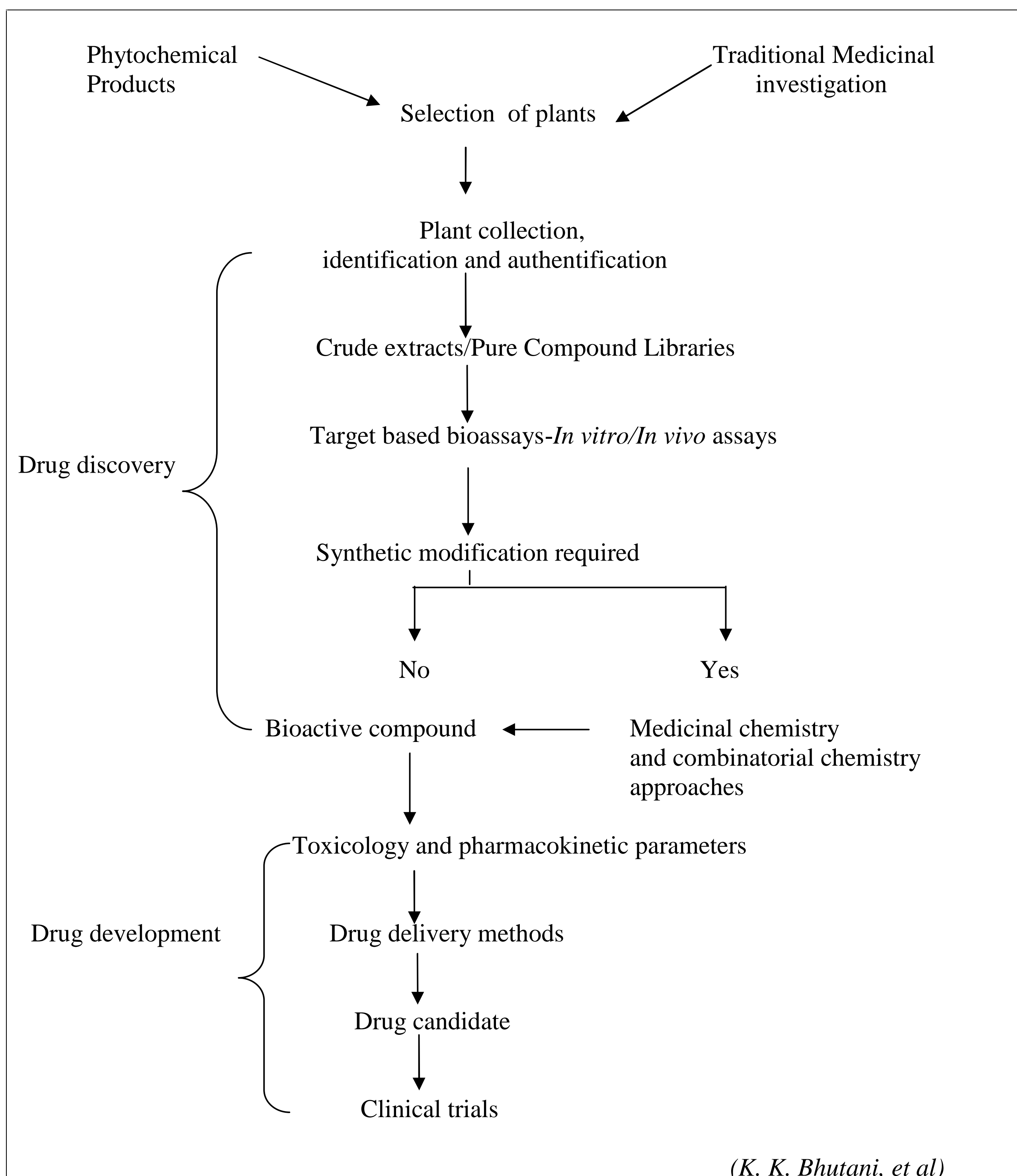


Table No. 1.2 Process of Drug Delivery from Plants

1.10 Cancer

Introduction

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancers cells can spread to other parts of the body through blood and lymph systems. *(Harold Varmus, et al)*

Cancer is a generic term used to encompass a group of diseases sharing common characteristics. It is mainly characterized by their unregulated growth and spread of cells to other part of the body. Its treatment is not only dependent upon which type of malignancy (cancer) they have, but also on the extent of its spread, together with its sensitivity to treatment. Assessment of cancer is done by meeting the physical, psychological and social needs of the patient. Any cell can undergo malignant changes and developed a carcinoma. Cancerous cell are not confined to localised overgrowth and infiltration of surrounding tissue, but can spread to other parts of the body via the lymphatic system and blood tissue, creating secondary deposits known as “metastases”. This occurs when normal cell control mechanism fails or disrupts. Microscopic examination of the surgical resection margins reveals the presence of malignant cells. Left untreated, these cells will result in localised recurrence of cancer and spread (metastasis). The earlier a cancer is detected, the less likely it is to metastasize, and so the more favourable will be the prognosis for the individual. *(Janice Gabriel, et al)*

Origins of Cancer

All cancers begin in cells, the body’s basic unit of life. To understand cancer, it’s helpful to know what happens when normal cells become cancer cells. The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells.

However, sometimes this orderly process goes wrong. The genetic material deoxyribonucleic acid (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form tissue called a tumor.

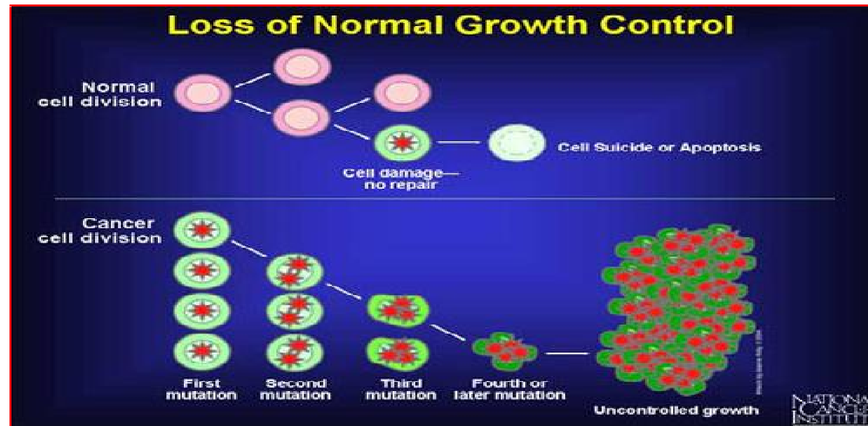


Fig. 1.1 Cancerous cell with loss of normal growth

Causes of Cancer

It was already known that cancer could be caused by chemicals, radiations, viruses and sometimes genetic. But with the growing knowledge of DNA and genes, it is known that damage to DNA by chemicals and radiation, or the introduction of new DNA sequences by virus is the most common cause of development of cancer.

Cancer causing agents are called carcinogens. These carcinogens causes genetic damage (mutations), these mutations lead to abnormal group of cells called clones. These mutant clones evolved to more malignant clones. Thus cancer progress by more genetic damage and mutations. Normal cells with damaged DNA die, cancer cells with damage DNA do not. *(American cancer society report)*

Cancer Control

Cancer control aims to reduce the incidence and mortality of cancer, and to enhance the quality of those affected by cancer, through an integrated and coordinated approach directed to primary prevention, early detection, treatment, rehabilitation and palliation.

If cancer control outcomes are to be improved, the population to be served must be the whole population comprising of: the healthy, the high risk, and those who harbour asymptomatic (pre-clinical) cancer; those with a cancer diagnosis who need treatment; those who are living with cancer as a chronic disease; those who are 'cured' of their cancer; and those who are dying of their cancer and require measures to bring dignity and comfort to the end of life. Cancer control is as much about

health, its promotion and maintenance, as it is about managing the disease, cancer. *(Mark Elwood, et.al)*

Symptoms

Since cancer can arise from a wide variety of sites and developed with many differing patterns of spread, there are no clear cut symptoms for cancer. Cancer is unlike many more specific disease or arthritic disease. The precise nature symptoms of cancer depends not only on primary site but specifically where the tumor is located in an organ, rate of development and also secondary spread is present or not. Many primary tumors cause swelling or lump if they arise at a visible or accessible part of the body, such as a skin, breast, testicle or oral cavity. A typical swelling due to a cancer is initially painless, through ulceration (skin breakdown) can occur, which may then become painful.

Staging of malignant disease

In order to ensure that a patient can be advised as to the most appropriate management of their particular disease, it is vital that the extent of their cancer is known. For example, if a patient presented with a breast lump, which proved to be malignant, it would be inappropriate to offer the patient a mastectomy if the cancer had already spread to the liver. Removal of the breast would not affect the patient's prognosis, because the cancer had already metastasized at the time of diagnosis. This is why it is so important to 'stage' a patient's cancer before detailed discussions can take place regarding the most appropriate treatment option.

Diagnosis of cancer

The following tests can confirm or eliminate a primary (diagnose), or determined the spread or the malignancy (stage). This investigation fall into three main groups-

1. Radiology
2. Pathology
3. Endoscopy

Radiology

It allows visualization of the internal structures as images. These images can be created in a number of ways such as X-rays, Computerised axial tomography (CAT or CT scan), Magnetic resonance imaging (MRI), Ultrasound, Nuclear medicine imaging, Positron emission tomography (PET).

Pathology

Pathology tests can confirm a clinical diagnosis and it can monitor a patient's disease and response to treatment. The various tests include biochemistry, monoclonal antibodies, tumor markers, biopsy, and cytology.

Endoscopy

It involves the passage of a long flexible bundle of fibre optic lights. It allows the operative to get a clear picture of the organs being examined thereby obtaining tissues sample for histological examinations. *(Janice Gabriel, et al)*

Treatment of Cancer

There are four standard methods of treatment of cancer: surgery, chemotherapy, radiation therapy and immunotherapy/biologic therapy. When initially diagnosed with cancer, a cancer specialist (called an oncologist) will provide the patient with cancer treatment options. The specialist will recommend the best treatment plan based on the type of cancer, how far it has spread, and other important factors like age and general health.

Ultimately, it is the patient who makes the treatment decisions based on doctor's recommendation, possible second opinions, and other information gathered from qualified professionals.

Cancer Prevention

Most people do not realise that cancer is preventable in many cases. The first step in cancer prevention is to learn what causes cancer and what its risk factors are. Many risk factors can be avoided, thus reducing the chances of cancer but cancer risks like genetics cannot be avoided. *(Lisa Fayed, et al)*

Benign and Malignant Tumors

Not all tumors are cancerous; tumors can be benign or malignant.

- **Benign tumors** aren't cancerous. They can often be removed, and, in most cases, they do not come back. Cells in benign tumors do not spread to other parts of the body.

- **Malignant tumors** are cancerous. Cells in these tumors can invade nearby tissues and spread to other parts of the body. The spread of cancer from one part of the body to another is called metastasis.

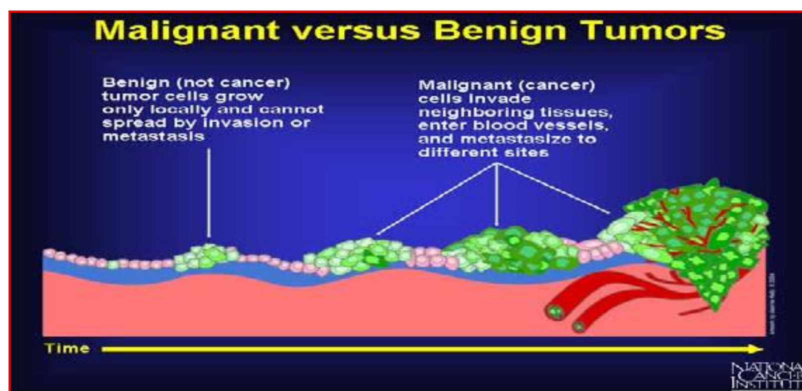


Fig. 1.2 Malignant versus Benign tumors

Types

Cancer types can be grouped into broader categories. The main categories of cancer include:

- **Carcinoma** - cancer that begins in the skin or in tissues that line or cover internal organs.

- **Sarcoma** - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.

- **Leukaemia** - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.

- **Lymphoma and myeloma** - cancers that begin in the cells of the immune system. *(Harold Varmus, et al)*

1.10.1 Cervical Cancer

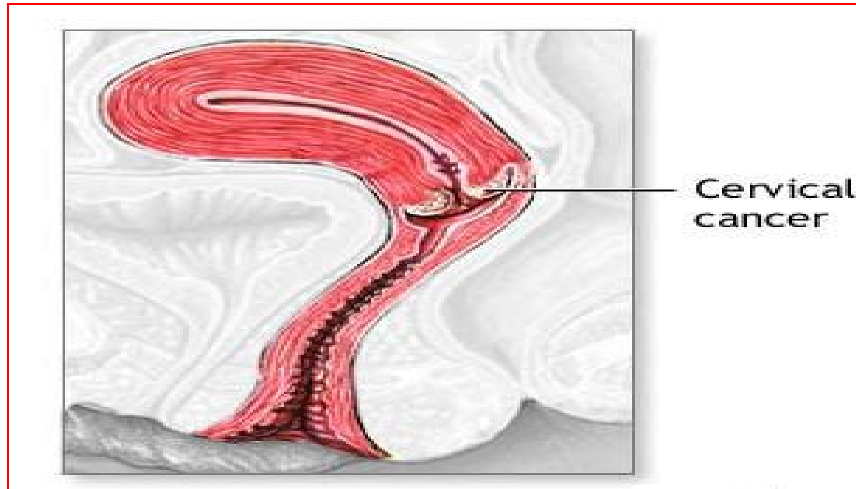


Fig. 1.3 Cervical Cancer

Definition

Cervical cancer is a cancer that starts in the cervix, the lower part of the uterus (womb) that opens at the top of the vagina. Cervical cancer is the third most common type of cancer in women.

Cervical cancers start in the cells on the surface of the cervix. There are two types of cells on the cervix's surface: squamous and columnar. The majority of cervical cancers are from squamous cells.

Development of cervical cancer

The development of cervical cancer is usually very slow. It starts as a pre-cancerous condition called dysplasia. This pre-cancerous condition can be detected by a Pap smear and is 100% treatable. That is why it is so important for women to get regular Pap smears. Most women that are diagnosed with cervical cancer today either have not had regular Pap smears or they have not followed up on abnormal results.

Undetected pre-cancerous changes can develop into cervical cancer and spread to the bladder, intestines, lungs, and liver. It can take years for pre-cancerous changes to turn into cervical cancer. Patients with cervical cancer do not usually have problems until the cancer is advanced and has spread.

Causes of cervical cancer

Almost all cervical cancers are caused by HPV (human papilloma virus). HPV is a common virus that is spread through sexual intercourse. There are many different types of HPV, and many do not cause problems. However, only certain strains of HPV actually lead to cervical cancer. Other strains may cause genital warts.

Risk factors of cervical cancer

Risk factors of cervical cancer include-

- Having sex at an early stage.
- Multiple sexual partners.
- Sexual partners who have multiple partners or who participate in high-risk sexual activities.
- Women whose mothers took the drug DES (diethylstilbestrol) during pregnancy in the early 1960's to prevent miscarriage.
- Weakened immune system.
- Poor economic status (may not be able to afford regular Pap smears).

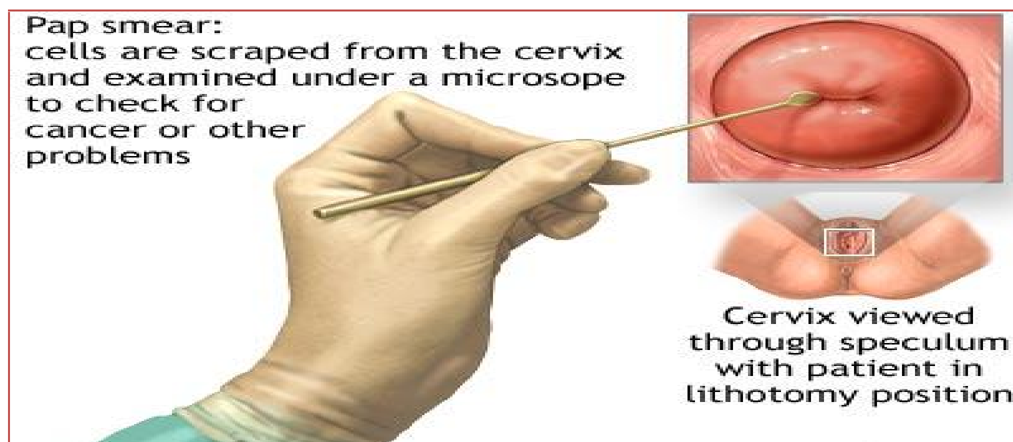


Fig. 1.4 Cervix view through speculum

1.10.2 Colon Cancer

Definition

Cancer that forms in the tissues of the colon (the longest part of the large intestine) is called colon cancer. Most colon cancers are adenocarcinomas (cancers that begin in cells that make and release mucus and other fluids).

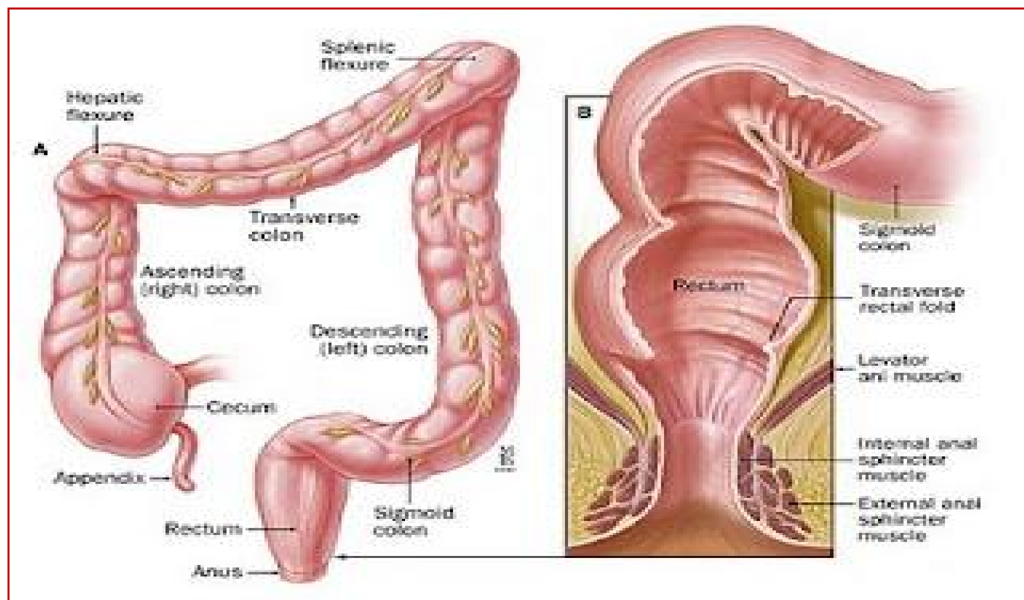


Fig. 1.5 Normal Colon and Rectum

Fig. 1.6 Colon tumour

Prevention

Colon cancer is often one of the most preventable cancers since it frequently develops from polyps that can be detected and removed before they become cancerous. To help prevent cancer of the colon-

- Get regular screening tests.
- Exercise regularly, and maintain a healthy weight.
- Eat a diet rich in fruits, vegetables and whole grains
- Do not smoke and do not drink alcohol excessively

Risk factors for colon cancer

A risk factor is something that may increase the chance of developing a disease.

- Men and women aged 50 and older: More than 90% of people with this cancer are diagnosed after the age of 50.
- People with a personal or family history of colon cancer benign (not cancerous) colorectal polyps.
- People with a personal family history of inflammatory bowel disease, ulcerative colitis or Crohn's disease.
- People who use tobacco.

Symptoms of colon cancer

The presence of a symptom does not mean that one has colon cancer but one should consult a physician if any of the following symptoms are noticed:

- Changes in bowel habits such as having diarrhoea or constipation.
- Stools that are narrower than usual (may be described as pencil-shaped).
- Frequent or general stomach discomfort (bloating, fullness or cramps).
- Diarrhoea, constipation or feeling that he bowel does not empty completely.
- Frequent gas pains.
- Weight loss for no apparent reason.
- Constant tiredness.
- Having nausea and/or vomiting. *(Abdallah Elsayd Mohamdeen, et al)*

1.11 Plant Source as Anticancer Agent

The use of natural substances to inhibit carcinogenesis is a rapidly evolving aspect of cancer research. Cancer is a major public health burden in both developed and developing countries. Plant derived agents are being used for the treatment of cancer. In recent years, medicinal plants have attracted a lot of attention globally. Since long time evidence has accumulated to demonstrate promising potential of medicinal plants used in various traditional, complementary and alternative systems especially for cancer treatment. Several medicinal plants have been studied for anti cancer pharmacological activity in recent years. To understand the mechanism of action, the researchers have worked at molecular level and several significant phytochemicals have been isolated based on the activity analysis of the medicinal plant extracts with different solvents.

Medicinal herbs are also significant source of synthetic and herbal drugs. So far, pharmaceutical companies have screened more than 25,000 plants for anti-cancer drugs. This should tell us that looking for single ingredients to attack cancer might be missing the point. Just as cancers are a product of disturbances in the body, so herbs can correct the disturbances as well as control many cancers. Herbal system of medicine has been practiced for thousands of years. Herbal medicines have a vital role in the prevention and treatment of cancer and medicinal herbs are commonly available and comparatively economical. A great deal of pharmaceutical research done in technologically advanced countries like USA, Germany, France, Japan and China has considerably improved quality of the herbal medicines used in the treatment of cancer. Some herbs protect the body from cancer by enhancing detoxification functions of the body. Certain biological response modifiers derived from herbs are known to inhibit growth of cancer by modulating the activity of specific hormones and enzymes. Some herbs reduce toxic side effects of chemotherapy and radiotherapy. Scientists all over the world are concentrating on the herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body.

Phytoconstituents derived from the herbs have been used in various formulations to enhance activity of immune cells of the body that promotes production of cytokines including interleukin, interferon, tumor necrosis factor and colony stimulating factor. These formulations help the body to fight cancer more effectively and reduce toxic side effects of chemotherapy and radiotherapy stages of cancer. *(Rajandeep Kaur, et al)*

1.11.1 Plant derived anticancer agents in clinical use

The isolation of the vinca alkaloids, vinblastine and vincristine from the Madagascar periwinkle, Catharanthus roseus G. Don. (Apocynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into clinical use for the treatment of cancer. Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukaemia, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma. The camptothecin derivatives, topotecan and irinotecan, and etoposide derived from epipodophyllotoxin are in clinical use all over the world. A number of promising agents such as flavopiridol, roscovitine, combretastatin, betulinic acid and silvestrol are in clinical or preclinical development. The discovery of paclitaxel from the bark of the Pacific Yew, Taxus brevifolia Nutt. (Taxaceae) is another evidence of the success in natural product drug discovery. Various parts of Taxus brevifolia and other Taxus species (e.g., Taxus Canadensis Marshall, Taxus baccata L.) have been used by several Native American Tribes for the treatment of some non-cancerous cases while Taxus baccata was reported to use in the Indian Ayurvedic medicine for the treatment of cancer.

1.11.2 Plant derived anticancer agents for future development

Numerous types of bioactive compounds have been isolated from plant sources. Several of them are currently in clinical trials or preclinical trials or undergoing further investigation. Flavopiridol is a synthetic flavone, derived from the plant alkaloid rohitukine, which was isolated from Dysoxylum binectariferum Hook. F. (Meliaceae). It is currently in phase I and phase II clinical trials against a broad range of tumors, including leukaemia, lymphomas and solid tumors. Synthetic

agent roscovitine which is derived from natural product olomucine, originally isolated from Raphanus sativus L. (Brassicaceae), is in Phase II clinical trials in Europe. Combretastatins were isolated from the bark of the South African tree Combretum caffrum Kuntze (Combretaceae) Combretastatin A-4 is active against colon, lung and leukemia cancers and it is expected that this molecule is the most cytotoxic phytomolecule isolated so far. *(Mohammad Shoeb, et al)*

1.11.3 Preventing cancer using folk remedies

Medicinal herbs of folk-origin are significant sources of synthetic and herbal drugs. In the commercial market, folk-medicinal herbs are used as raw drugs, extracts or tinctures. Isolated active constituents are used for applied research for finding their bioactivity. For the last few decades, phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of plausible anticancer compounds. To facilitate the readers to look at their areas of interest, we have tried to discuss potential anti cancer photochemical as drugs in this article. In this review anti-cancer pharmacological investigations of important phytochemicals for current status have been discussed. *In vitro* anti cancer studies have demonstrated that natural products of flavonoid type like luteolin and quercetin have the power to inhibit the proliferation of cells in human carcinoma of larynx and sarcoma-180 cell lines.

1.11.4 Natural products from folk medicines which are under trial or for approval as drugs

Some of the folk medicine described plant-derived secondary metabolites and their semi synthetic derivatives, are undergoing clinical trials as anticancer drugs (camptothecin, paclitaxel, epipodophyllotoxin, and vinblastine. In addition, the phytochemical combretastatin A4, was isolated from the South African medicinal tree, Combretum caffrum (Combretaceae), was derivatized to combretastatin A4 phosphate and AVE-8062. These analogs bind to tubulin leading to morphological changes and then disrupt tumor vasculature, and are in phase II trials.

1.11.5 Mechanism of some potential active ingredients

Pytochemicals isolated from folk medicines are found to act as potent antioxidants and free radical scavengers. These natural products are supposed to minimize DNA damage by reacting with free radicals and in this way they could prevent cancer. Some of the Pytochemical antioxidants of folk medicines are inhibitors of lipoxygenase and urokinase. Inhibition of these enzymes by folk medicines could prevent or reduce cancer growth and in this way their mechanism of action can be established. Natural products of folk-medicine, for example, genistein and curcumin, are found to decrease urokinase plasminogen activator thus helping to fight against cancer. *(G. Venkateshwar Rao, et al)*

1.11.6 Plants with anticancer activity

Sr. No.	Botanical name	Family	Common name	Active constituent
1	Allium sativum	Liliaceae	Garlic	Alliin ,allicin alliin, alliinase, S-allylcysteine (SAC), diallyldisulphide ,(DADS), diallyltrisulphide (DATS) and methylallyltrisuphide.
2	Actinidia chinensis	Actinidiaceae	China gooseberry, Kiwifruit	Polysaccharide known as "ACPS-R"
3	Aloe ferox, Aloe barbadenis	Liliaceae	Aloe vera	Aloe-emodin, emodin, aloin acemannan,
4	Ananas comosus	Bromeliaceae	Pine apple, Ananas	Bromelain
5	Angelica sinensis	Umbelliferae	Angelica	Polysaccharide fraction of known as "AR-4"

6	Annona species	Annonaceae	Monkey species	Acetogenins
7	Arctium lappa,	Compositae	Burdock	Potent anticancer factors.
8	Astragalus membranaceus	Papilionaceae	---	Swainsonine
9	Betula utilis	Betulaceae	Bhojpatra	Betulin
10	Camellia sinensis	Theaceae	Tea plant	Epigallocatechin gallate
11	Catharanthus roseus	Apocynaceae	Vinca	Vinblastine, Vincristine , Alstonine, Ajmalicine and Reserpine.
12	Chlorella pyrenoidosa	Oocystaceae	---	Lysine
13	Colchicum luteum	Liliaceae	Colchicum	Colchicines demecolcine
14	Combretum caffrum	Combritaceae	---	Combretastatin
15	Curcuma longa Linn.	Zinziberaceae	Turmeric	Tumerone, curcumine
16	Echinacea angustifolia	Asteraceae	Black Sampson	Arabinogalactan, Jucogalactoxyloglucans.
17	Fagopyrum esculentum	Polygonaceae	Vitamin p	Amygdalin, Rutin
18	Ginkgo biloba	Ginkoaceae	Kew tree	Ginkgolide-B, A, C and J
19	Glycyrrhiza glabra	Leguminosae	Liquorice	Glycyrrhizin.
20	Gossypium barbadense	Malvaceae	Raw cotton	Gossypol

21	Gyrophora esculenta	Umbilicariaceae	Mushroom	Polysaccharides ß-glucans, a-glucans, and galactomannans.
22	Linum usitatissimum	Linaceae	Flax seed, Linseed	Cynogenetic glycosides, Lignans
23	Mentha species	Labiateae	Pudina	Monoterpene ketones
24	Panax ginseng	Aralaceae	Ginseng	Ginsenosides, Panaxosides
25	Picrorrhizia kurroa	Scrophulariaceae	Picrorrhizia (kutki)	Picrosides I, II, III and kutkoside
26	Podophyllum hexandrum	Berberidaceae	Podophyllum	Podophyllin,astragalin

Table No. 1.3 Plants with anticancer activity *(D.M Sakarkar, et al)*

CHAPTER-2

LITERATURE REVIEW.

CHAPTER – 2

LITERATURE REVIEW

2.1 Plant Profile

Fig. 2.1 *Chromolaena odorata* leaves

2.1.1 Classification of the plant

Kingdom	Plantae
Subkingdom	Tracheobionta
Supervision	Spermatophyte
Division	Magniliprida
Class	Magnolipridа
Subclass	Astride
Order	Asterales
Family	Asteraceae
Genus	Chromolaena Dc
Species	*Chromolaena odorata* (L) King & Robins.
Synonyms	Eupatorium odoratum L,
	Eupatorium conyzoides Vahl,

		Eupatorium brachiatum Sw. ex Wikstr,
		Eupatorium atriplicifolium Vahl,
		Osmia odorata (L.) Schultz-Bip.
Vernacular Name	:	English- Siam weed, Christmass bush
		Tamil- Kambila
		Hindi- Bagh dhoka
		Malayalam - Communist pacha
Family	:	Asteraceae (Compositae)

2.1.2 Introduction

Chromolaena odorata is an aggressive species of perennial weed and belonging to the family Asteraceae, which widely occurs in the plantations of South India. *(G.O. Adesine, et al)*

It is fast growing oppressive, competitive occupying different kinds of arable land by forming dense strands that tends to prevent the growth of other flora. Therefore if affects plantation and other organisms in the ecosystem by its suppressive effects to young [plants as it is a growth inhibitor and has allelopathic capabilities. A lot of pharmacological and medicinal effects of extracts of *Chromolaena odorata* showed traditional uses of the plant especially in Asia to cure various disease ailments. Traditionally, boil fresh leaves of the plant has been used in many countries for treating various skin diseases. The leaf poultices of the plant were used in the treatment of fresh wounds and as an aid to stop bleeding. Its root can be used as analgesic and antipyretic. The extract of the leaves could be as gargle for treatment of sore throat and flu. One significant agronomic properties of the plant is that can be propagate either by seed or stem, but propagation by cutting is preferred mostly. *(W. Orapa, et al)*

2.1.3 General Description

Chromolaena Odorata is also known as Christmas bush, Bitter bush, Siam weed, Baby tea, Cariaquillo, and Santa Maria. It is a scrambling shrub. The plants have abundant, yellowish, fine lateral roots. Multiple sprouts arise from the root

crown and lower stems. Individual branches are long with relatively few branches. The opposite, three-nerved leaves are deltoid to ovate lanciolate, usually with a dentate margin and a long pointed tip. The leaves are aromatic when crushed. The inflorescences are corymbs of cylindrical heads located on the terminals of lateral branches. There are 15 to 20 tubular florets per head, white, lavender, pink or blue in colour. The seeds are a brownish gray to black that is 4 mm long with a pale brown pappus 5 or 6 mm long. *(C. Zacharaides, et al)*

Leaves

The leaves are soft, green, hairy and triangular in shape, with a distinctive three-vein (pitch fork) pattern. New growth exhibits a purple colouration. The stems are smooth, round and hairy brittle, becoming woody at the base when old.

Flowers

Flowers bloom from May to July again in September to October, producing masses of pale lilac flowers that appear white from a distance. They turn a darker lilac when matured.

Seeds

The plane produces huge numbers of windborne seeds within 8-10 weeks after flowering. Each seeds have a tuft of white hairs that allowed it to be transported by wind and water. Most seeds germinate immediately after rain, though some appear to remain dormant for several years. *(Warea Orapa, et al)*

Uses

- Fresh leaves or decoction have been used for the treatment of leech bite, soft tissue wounds, burn wound and skin infection.
- The leaf poultices of the plant were used in the treatment of fresh wounds and as an aid to stop bleeding.
- Root can be used as analgesic and antipyretic.
- The extract of the leaves could be as gargle for treatment of sore throat and flu.
- Macerated leaves are usually applied to swollen portion of the body to relieve inflammation.

2.2 Literature Review

Afolabi C. Akinmoladun et al., 2007 reported "Phytochemical constituent and antioxidant properties of *Chromolaena odorata* Linn"

"The author reported that aqueous and methanolic extracts of *Chromolaena odorata* were screened for phytochemical constituents. Tannins, steroids, terpenoids, flavonoids and cardiac glycosides were present in both the extract. Alkaloids were detected only in the methanolic extract. Antioxidant activity of *Chromolaena odorata* was performed and compared with standards (gallic acid and ascorbic acid). Results showed that percentage antioxidant activity of *Chromolaena odorata* is about one third of the value for gallic acid and ascorbic acid".

Alisi CS et al., 2011 reported "Antimicrobial action of methanol extract of *Chromolaena odorata* Linn"

"The author reported that the plant *Chromolaena odorata* contain flavonoids, tannins, saponins, glycosides, steroidal aglycones, alkaloids and 4-hydroxybenzoic acid. But methanol extract did not show positive reactions for saponins. Antimicrobial activity of *Chromolaena odorata* was performed and showed that methanol extract of *Chromolaena odorata* inhibited dehydrogenase activity in the organisms in a logistic dose dependant manner".

Alisi, C. S et al., 2008 reported "Nitric oxide scavenging ability of ethyl acetate fraction of methanolic leaf extracts of *Chromolaena odorata* Linn"

"The author reported the concentrating effect of ethyl acetate on plant phenolic compound. Nitric oxide scavenging ability of ethyl acetate fraction of methanolic extracts of *Chromolaena odorata* was performed. Results exhibited that ethyl acetate fraction demonstrated a marked ability to scavenge nitric oxide and/or inhibit nitrite formation. The ethyl acetate fraction of *Chromolaena odoratat* ability to scavenge nitric oxide generated *in vitro* by sodium nitroprusside was concentration dependent".

Anyasor, G.N et al.**,** 2011 reported "Phytochemical constituent, proximate analysis, antioxidant, antibacterial and wound healing properties of leaf extracts of *Chromolaena odorata* Linn"

"The author reported that, the aqueous and ethanolic leaf extracts of *Chromolaena odorata* indicated the presence of saponins, phenols and tannins. Phlobatannins and alkaloids were present in the aqueous extract while cardiac glycosides, anthraquinones and terpenoids were present in the ethanolic extract. Proximate analysis showed that *Chromolaena odorata* could serve as a good source of energy, flavour, minerals, protein and dietary fibre. Antioxidant assays and antibacterial studies of the plant extract were performed. Results exhibited that *Chromolaena odorata* shows good antioxidant and antibacterial action".

Bamidele Victor Owoyele et al., 2008 reported "Analgesics, anti-inflammatory and antipyretic activities from flavonoid fractions of *Chromolaena odorata* Linn"

The author reported that crude ethanolic extract of *Chromolaena odorata* was fractionated using water and four solvents- n-hexane, dichloromethane, ethyl acetate and n-butanol. Pytochemical screening, analgesics, anti-inflammatory and antipyretic activity of all the fractions were performed. Results exhibited that the active constituent responsible for analgesi, anti-inflammatory and antipyretic activities of *Chromolaena odorata* are mainly flavonoid compounds".

C. S. Alisi et al., 2011 reported "Normalisation of Lipoproteins Phenotypes by *Chromolaena odorata* Linn"

"The author reported that ethanolic extract of *Chromolaena odorata* has got the ability to prevent dyslipidemia resulted from carbon tetrachloride induced oxidative damage. Mechanism of action is not fully understood but the protection conferred on the liver by the extract would have preserved the liver cell to function in the maintenance of lipoprotein phenotype. Intake of *Chromolaena odorata* extract in diet may offer useful benefit in the preservation of lipoprotein phenotype, which will also be beneficial in the reduction of cardiovascular risk associated with dyslipidemia"**.**

Debashisha Panda et al., 2010 reported "Phytochemical analysis and investigation of anthelmentic and wound healing potentials of *Chromolaena odorata* Linn"

The author reported that petroleum ether, ethyl acetate and methanol extracts were analyzed for different phytoconstituents by the method of qualitative phytochemical analysis. Anthelmentic activity of different extracts of *Chromolaena odorata* was performed. Results exhibited that methanol extract showed potent anthelmentic activity when compared to petroleum ether and ethyl acetate extracts. In wound healing activity methanolic extract was endowed with potent wound healing property when compared to petroleum ether and ethyl acetate extracts".

G. Bedi et al., 2010 reported "Effect of essential oil of *Chromolaena odorata* Linn"

"The author reported that essential oil of *Chromolaena odorata* was obtained by using Clevenger apparatus. Cyclooxygenase function of prostaglandin-H synthase activity of essential oil of *Chromolaena odorata* was performed. Results showed that essential oil of *Chromolaena odorata* increased the activity of cyclooxygenase function of prostaglandin-H synthase. The concentration value of essential oil required to increase the activity of the cyclooxygenase function of prostaglandin-H synthase was found to be 87.5µg/ml".

Igboh M. Ngozi et al., 2009 reported "Chemical profile of *Chromolaena odorata* Linn"

"The author reported the proximate composition of *Chromolaena odorata*. This indicates that *Chromolaena odorata* may have a short shelf life due to its high moisture content. Phytochemical screening was carried out and results showed that *Chromolaena odorata* is highly rich in saponins, moderately rich in phytates and tannins, with little content of alkaloids, flavonoids and cyanogenic glycosides".

***K. K. Naidoo et al*.,** **2011** reported "Screening of *Chromolaena odorata* Linn for antibacterial and antifungal properties"

"The author reported that, various extracts of leaves of *Chromolaena odorata* inhibited several gram positive and gram negative bacteria. All gram positive

bacteria were inhibited by the ethyl acetate extracts obtained from the leaves, except for S. aureus and methanol extracts derived from the leaves inhibited all gram positive bacteria including one gram negative bacteria, *E.coli*. Antifungal and antimicrobial activity of ethanol and aqueous extract of the plant was performed. Results exhibited that ethanol extract was found to be more effective than aqueous extracts in the leaf and stem extracts".

Moses S. Owolabi et al., 2010 reported "Chemical composition and bioactivity of the essential oil of *Chromolaena odorata* Linn"

"The author reported that essential oil of *Chromolaena odorata* obtained by using Clevenger apparatus was light green oil. GC-MS analysis of the essential oil was carried out and led to the identification of 56 components, representing 99.3% of the oil. The oil was found to be rich in and -pinenes, germacrene D, -copaene and caryophyllene. Antimicrobial screening of the essential oil of *Chromolaena odorata* was performed and showed to exhibit marginal antibacterial activity against *Bacillus cereus* and antifungal activity against *Aspergillus niger"*.

Pierangeli G. Vital et al., 2009 reported "Antimicrobial activity and cytotoxicity of *Chromolaena odorata* Linn"

The author reported antimicrobial and cytotoxic properties of ethanol extracts of leaves of *Chromolaena odorata* and ethyl acetate extracts of stem bark of *Uncaria perrottetii.* The ethanolic extract of *Chromolaena odorata* leaves showed antimicrobial activity against *B.subtilis, S. aureus and S. typhimurium*. Phytochemical screening tests were also carried out and results exhibited that Chromolaena odorata showed the presence of flavonoids, saponins, tannins and steroids and *Uncaria perrottetii* showed the presence of alkaloids, tannins and leucoanthocyanin.

Rajmohon D et al., 2011 reported "Insecticidal properties of *Chromolaena odorata* Linn against the life cycle of the mosquito, *Aedes aegypti*"

"The author reported that, acetone extract of the leaves of *Chromolaena odorata* showed low percentage of egg hatchability and high percentage of larval and pupal mortality. Results indicated that biomass of *Chromolaena odorata* can be used as repellent to control mosquito population".

Tran Manh Hung et al., 2011 reported "Flavonoid Glycosides from *Chromolaena odorata* Linn leaves and *in vitro* Cytotoxic Activity"

"The author reported that, ethyl acetate soluble fractions of the 70% ethanolic extract of *Chromolaena odorata* resulted in the purification of thirteen compounds. The known compounds were identified as aromadendrin 4' methyl ether, eriodictyol 7, 4'-dimethy ether, 4' –methyl ether, isosakuranetin, quercetin 7, 4'-dimethy ether, kaempferide, acacetin, rhamnazin, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside and kaempferol 3-O-glucopside. Cytotoxic activity of ethanol extract of *Chromolaena odorata* was performed. Results showed significant cytotoxic activity in the inhibition of HL-60 cancer cell lines".

CHAPTER-3

AIM AND SCOPE OF WORK.

CHAPTER - 3
AIM AND SCOPE OF WORK

In India, drugs of herbal origin have been used in traditional system of medicine such as Ayurveda, Unani and Homeopathy since ancient times. Most common traditional system in India is Ayurveda and Homeopathic systems. Public seems to be fed up with modern medicine (synthetic drugs) mainly due to only symptomatic relief is experienced, completion of treatment is never a reality, more so in chronic ailments, frustrating side effects and high cost involved. The general opinion of the public is moving towards use of herbal drugs. This is due to the reason that Ayurveda is considered to be our own system and it relies only on natural remedies and reasons for homeopathy finding favours are its ease of administration of dose, high acceptability by children and no side effects (*Sunil Jawla, et al*).

Keeping above in mind, this study was conducted. The aim of the present study is to find out new anticancer substances from the indigenous plant, *Chromolaena odorata* Linn. (Asteraceae) a traditional medicinal plant consisting a wide range of pharmacological, biological activities and phytochemical constituents. Literature survey revealed that some amount of pharmacological work has been carried out on *Chromolaena odorata* such as anthelmentic, wound healing, antioxidants, antimicrobial, analgesics, antifungal and antibacterial activity. But no work has been found during literature survey in line of anticancer activity. Therefore it was thought worthwhile to carry out preliminary phytochemical screening, characterisation of the isolated ingredients by means of spectral studies and pharmacological investigation in line of anticancer activity. The selection of the plant was made on the basis of its availability, therapeutic value and the lesser degree of research work with reference to the said pharmacological aspects.

CHAPTER-4

PLAN OF WORK.

CHAPTER - 4

PLAN OF WORK

The research work entitiled "Phytochemical Characterization and *in vitro* anticancer screening of ethanol extract of *Chromolaena odorata* Linn" was carried with the following aspects.

Part 1: Phytochemical studies

- Collection and authentication of plant material.
- Solvent extraction of plant material with petroleum ether (60-80 °C).
- Solvent extraction of plant material with ethanol (95% v/v).
- Preliminary phytochemical screening of both extracts.
- Thin layer chromatography of the extracts.
- Isolation of plant constituent by column chromatography.
- Characterization of isolated compound by spectral studies.

Part 2: Pharmacological studies

In vitro studies

- Evaluation of anticancer activity of the ethanol extract by MTT assay using human colorectal adenocarcinoma cell line (HCT116) and human cervical cancer cell lines (HeLa).

CHAPTER-5

MATERIALS AND METHODS.

CHAPTER - 5

MATERIALS AND METHODS

5.1 Phytochemical Studies

Collection and authentication of plant

The leaves of plant *Chromolaena odorata* Linn (Asteraceae) was collected in the month of July 2011 from Perur, near Perur temple, Coimbatore, Tamilnadu and was authentified by Dr. M. Palanisamy, Scientist ‘C’ with reference number of No.BSI/SRC/5/23/2010-11/Tech.1089. Botanical Survey of India, Southern Regional Centre, Coimbatore – 641 003.

Preparation of plant sample

The collected leaves of *Chromolaena odorata* were dried under shade and using a mechanical grinder it was made into a coarse powder. The powder was passed through sieve No.40 and packed in thimble and stored for further use.

Extract procedure

The dried coarse powder material of *Chromolaena odorata* leaves (150gm) was deffatted with petroleum ether (60-80 °C) in a soxhlet apparatus and extraction was continued for 72 hours to remove waxy substances, which usually interfere in the isolation of phytoconstituents. Then the marc after defatted with petroleum ether was dried and extracted with ethanol (95% v/v) in a soxhlet apparatus. The solvent was then distilled off and the resulting semisolid mass was dried in a dessicator and the mass obtained was weighed.

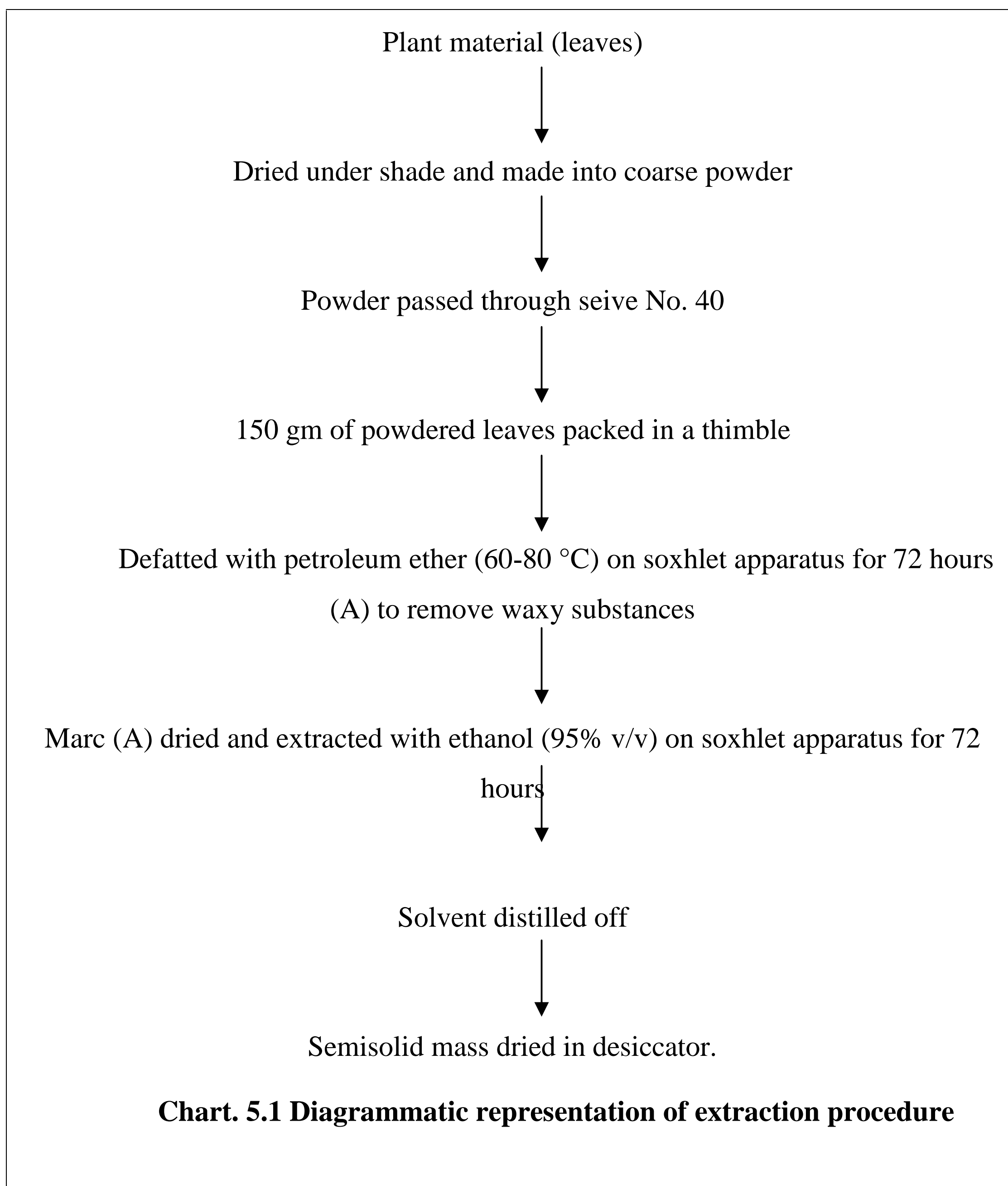


Chart. 5.1 Diagrammatic representation of extraction procedure

5.2 Preliminary phytochemical screening

The petroleum ether and ethanol extracts of *Chromolaena odorata* were subjected to qualitative test for the identification of various chemical constituents.

5.2.1 Test for alkaloids

Small amount of extract was treated with few drops of dilute hydrochloric acid and filtered. The filtrate was collected and subjected for following tests.

A. Mayer's Test: (Potassium Mercuric Iodide)

1 ml of the extract was added with 1 ml of Mayer's reagent (potassium mercuric iodide solution). Whitish yellow coloured precipitate indicated the presence of alkaloids.

B. Dragendroff's Test: (Potassium Bismuth Iodide)

To 1 ml of the sample solution 2 ml of Dragendroff's reagent was added. Formation of orange red precipitate indicated the presence of alkaloids.

C. Wagner's Test: (Iodide in Potassium Iodide)

Fraction of the extract was treated with 2 ml of Wagner's reagent and observed for the formation of reddish brown coloured precipitate.

D. Hager's Test: (Picric Acid)

Fraction of the extract was treated with Hager's reagent and observed for the formation of yellow coloured precipitate. *(Surekha, et al)*

5.2.2 Test for carbohydrates

A. Molisch's Test

1 ml of filtrate was mixed with 2 drops of Molish's reagent and 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Brown to violet ring indicates the presence of carbohydrates. (*C.K. Kokate, et al*)

B. Fehling's Test

Extract was treated with Fehling's solution A and B and heated on a water bath for few minutes and observed for the formation of red precipitate of cuprous oxide.

C. Barfoed's Test

Small portion of the extract was treated with Barfoed's reagent and observed for the formation of red precipitate.

D. Benedict's Test

Small portion of the extract was treated with Benedict's reagent and placed in boiling water bath for few minutes and observed for the formation of orange red precipitate. *(G A Ayoola, et al)*

5.2.3 Test for glycosides

A. Legal's Test

To the sample, 1ml pyridine and few drops of sodiumnitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour indicates the presence of glycoside.

B. Keller Killani Test

1 gm of powdered drug was extracted with 10 ml of 70 % alcohol for few minutes and filtered. To 5 ml of filtrate, 10 ml of hydrogen peroxide and 0.5ml of strong solution of lead acetate were added. The precipitate thus obtained was filtered. Filtrate was shaken with 5ml of chloroform and the layer was separated and to this 1ml of mixture, 1 volume of 5 % ferric sulphate and 99 volumes of glacial acetic acid were added.

To this mixture 1 or 2 drops of conc. Sulphuric acid was added. Appearance of blue colour confirmed the pressure of de oxy sugars.

C. Borntrager's Test

The extract was treated with chloroform and the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. Ammonia layer acquired rose pink colour indicates the presence of glycoside.

5.2.4 Test for fixed oil and fats

A. Stain test

Small quantity of the extract was separately passed between two filter papers. Appearance of stain on the paper indicates the presence of fixed oil.

B. Saponification Test

Few drops of 0.5 alcoholic potassium hydroxide was added to a small quantity of extract along with a drop of Phenolphthalein. The mixture was heated on water bath for 1-2 hours formation of soap neutralization of alkali indicated the presence of fixed oil and fats.

5.2.5 Test for tannins and phenolic compounds

A. Ferric Chloride Test

To the small quantity of extract few drops of 5% ferric chloride solution was added. Formation of brownish colour indicated the presence of phenolic compounds.

B. Lead acetate Test

To the extract, 10% of lead acetate solution was added. Formation of white precipitate indicated the presence of tannins.

C. Gelatin Solution Test

To the extract 1% solution of gelatin containing sodium chloride solution was added and observed for the formation of white precipitate.

D. Potassium dichromate test

5ml of filtrate was treated with 1ml of 10% aqueous potassium dichromate solution. If yellowish- brown precipitate was formed it suggested the presence of tannins. *(K.R. Khandelwal, et al)*

5.2.5 Test for triteprenoids

A. Salkowsky Test

A small quantity of extract was treated with 5ml of concentrated sulphuric acid, the solution turned yellow to red, indicated the presence of triterpenoids.

B. Liebermann – Burchard Test

A small quantity of extract was treated with 0.2ml concentrated sulphuric acid and 4ml acetic anhydride. The solution turned pink in colour and finally becomes purple showing the presence of triterpenoids.

5.2.6 Test for saponins

A. Foam Test

The extract was diluted with 20ml of distilled water and it was agitated on a graduated cylinder for 15 minutes. The formation of 1cm layer of foam showed the presence of saponins. *(C.K. Kokate, et al)*

5.2.7 Test for proteins

A. Millions Test

To the extract, little of water and Millon's reagent was added. Appearance of red colour showed the presence of proteins.

B. Ninhydrin Test

To the extract little of ninhydrin reagent was added. Appearance of purple colour showed the presence of proteins.

C. Biuret test

1ml of filtrate was taken in water and 1mnl of 4% copper sulphate was added to it, violet or pink colour is formed, indicates the presence of proteins. *(K.R. Khandelwal, et al)*

5.2.8 Test for flavones & flavonoids

A. Aqueous Sodium hydroxide test

To small amount of sample solution add aqueous sodium hydroxide solution. Formation of rellow colour indicates the presence of flavonoids.

B. Concentrated Sulphuric acid Test

To small amount of sample solution add 5 ml of dilute ammonia and 1 ml of concentrated sulphuric acid. Formation of yellow colour indicates the presence of flavonoids. *(G A Ayoola, et al)*

C. Schinodo's Test

To a small fraction of the extract add few magnesium turnings followed by concentrated hydrochloric acid and then slightly boiled. Red colour formation indicates the presence of flavonoids. *(K.R. Khandelwal, et al)*

5.3 Chromatographic studies

Introduction

Chromatography was introduced first by the Russian botanist Micharl Iswett. It is a method for separating the components of a mixture by differential distribution of the components of the mixture between a stationary phase and a mobile phase. Chromatography was initially used for the separation of coloured substances from the plants, but today it has become the most extensive technique of separation and purification of organic compounds.

The basic principle of separation is the difference in the affinity of the different compounds to stationary phase. They are then flushed through the system at different rates. These differential rates of migration as the mixture moves over adsorptive materials provide separation. Repeated sorption or desorption that take place during the movement of the sample over the stationary phase determines the rates. Thus, smaller the affinity a molecule has for the stationary phase, the shorter the time spent in a column. *(Geetu Gambhir, et al)*

5.3.1 Thin layer chromatography (TLC)

Thin layer chromatography is an important analytical tool in separation, identification and estimation of different components. It is a solid-liquid form of chromatography where the stationary phase is normally a polar adsorbent and the mobile phase can be a single solvent or combination of solvents. Thin layer chromatography is a quick, inexpensive micro scale technique that can be used to determine the number of components in a mixture. *(A.V Kasture, et al)*

General techniques in TLC

- Preparation of thin, uniform layer plate with adsorbent
- Activation of TLC plates
- Application of sample on TLC plates
- Selection of solvent system
- Separation chamber and development
- Detecting agents or visualizing agents
- Determination of R_f values *(Egon Stahl, et al)*

Procedure

- Required quantity of silica gel G was taken.
- Sufficient quantity of distilled water was added and mixed to produce slurry.

- The slurry was then poured into TLC plates by spreading technique to obtained uniform layer.
- The coated plates were allowed to dry in air and activated by heating in hot air oven at 100 °C for 1 hour.
- With the help of capillary tube the prepared extract previously dissolved in respective solvent was spotted about 1 cm from one end of the TLC plate.
- The plate was then placed in the development tank previously saturated by the mobile phase.
- This mobile phase rises by the capillary action through the plate.
- Development was continued till the solvent front reaches $3/4^{th}$ of the plate.
- The plate is removed from the chamber and the solvent front is marked.
- The plate is kept for air drying and detecting agent or visualizing agent is applied.
- R_f value of the spots are recorded and are used for qualitative identification of the compounds. *(Kasture A V, et al)*

Measurement of Retention factor (R_f)

The migration distances of substances on thin layer chromatograms are generally fixed as R_f values. It is called as retention factor and is calculated as follows.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent front}}$$

The R_f values depend on solvent system, adsorbent and amount of material spotted etc. *(JB Harborne, et al)*

5.3.2 Column chromatography

Column chromatography is also known as adsorption chromatography. The mixture to be separated is dissolved in a suitable solvent and allowed to pass through a tube containing the adsorbent. The component which has greater absorbing power is adsorbed in the upper part of the column. The next component is adsorbed in the lower portion of the column which has lesser adsorbing power than the first

component. This process is continued. As a result the materials are partially separated and adsorbed in the various parts of the column. The initial separation of the various can be improved by passing either the original or some other suitable solvent slowly through the columns. The various bands present in the column become more defined. The banded column of the adsorbent is termed as chromatogram, and the operation is known as development of chromatogram. The portion of a column which is occupied by a particular substance is called its zone. The narrower the zones, the longer the number of substances which can be separated in a column of a definite length, and the more concentrated are the eluates. *(Gurdeep R. Chatwal et al)*

Fig. 5.2 Column chromatography of ethanol extract of *Chromolaena odorata*

Materials and method

Adsorbent – Silica gel for column chromatography

Activation – 110 °C for 1 hour

Mobile phase – Petroleum ether, Benzene, Chloroform and Methanol

Quantity of extract – 4 grams

Extract used – Ethanol extract of *Chromolaena odorata* leaves

Length of column – 60 cm

Diameter of column – 2.5 cm

Rate of elution – 20 drops per minute

Volume of fraction – 20 ml each

Method of packing – Wet packing

Technique – Gradient elution

Procedure

Packing a silica gel column

- Column was taken, washed and rinsed water and petroleum ether and made to dry completely.
- After complete drying, a small piece of cotton wool was placed in the bottom of the column followed by whatmann filter paper.
- Then the column was filled with silica gel previously activated at 110 °C for 1 hour.
- Sufficient amount of solvent was made to run in order to make the silica gel completely settled.
- Once silica gel has settled, two whatmann filter papers were added carefully to the top of the column.

Loading a sample into the column

- 4 gm of extract was taken in a china dish and mixed with silica gel.
- The mixture was then stirred well until it forms a free flowing powder.
- The sample was then packed into the column with the help of a clean dry funnel.
- Two whatmann filters are then placed on to the top of the sample layer.

- Then a small piece of cotton wool is placed on to the top of whatmann filter paper.

Eluting the sample

- The column was eluted by gradient method in increasing polarity (petroleum ether, benzene, chloroform and methanol).
- The fractions were then collected in clean 100 ml labelled boiling tube up to 20 ml with the speed of 20 drops per minute.

Analysing the fractions

The collected fractions were analysed by thin layer chromatography to determine if the fractions contains more than one component and if the fractions can be combined. The Phytochemical tests were then performed as to confirm which phytoconstituent is present in the fraction. The fractions containing the similar R_f values were combined together. The collected fractions were made to dry and is used for spectral studies using UV, IR and NMR etc.

5.4 Pharmacological Screening

Materials

The human colorectal adenocarcinoma cell line (HCT116) and human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Sciences (NCCS), Pune. The HCT116 cells were grown in Dulbeccos modified eagles medium (DMEM) and HeLa cells were grown in Eagles minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37 °C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passages weekly, and the culture medium was changed twice a week. *(Monks A, et al* and *Mosmann T, et al)*

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a haemocytometer and diluted with medium with 5% fetal bovine serum (FBS) to give final density of 1×10^5 cells/ml. one hundred micro litres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37 °C, 5% CO_2, 95% air and 100% relative humidity. After 24 hours, the cell were treated with serial concentrations of the extracts and fractions. They were initially dissolved in neat dimethysulfoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred microlitres per well of each concentration was added to plates to abtain final concentrations of 500, 250, 125, 62.5 and 31.25 µg/ml respectively. The final volume in each well was 200 µl and the plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 48 hours. The minimum containing without samples were served as control. Triplicate was maintained for all concentrations.

MTT Assay

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population in response to external factors. The reduction of tetrazolium salt is now widely accepted as a reliable way to examine cell

proliferation. The yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilised and quantified by spectrophometric means.

The MTT cell proliferation assay measures the cell proliferation and conversely, when metabolic event leads to apoptosis or necrosis, the reduction in cell viability. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. *(American type culture collection, 2001)*

Principle

MTT is a yellow soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 hours of incubation, 15 µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 hours. The medium with MTT was then flicked off and the formed formazan crystals were solubilised in 100 µl of DMSO (dimethyl sulfoxide) and then measured the absorbance at 570 nm using micro plate reader. The percentage cell inhibition was determined using the following formula.

Percentage (%) cell inhibition = 100-Absorption (sample)/Absorption (control) × 100.

Nonlinear regression graph was plotted between percentahe cell inhibition and Log_{10} concentration and IC_{50} was determined using Graphpad Prism software.

CHAPTER-6

RESULTS AND DISCUSSIONS.

CHAPTER - 6

RESULTS AND DISCUSSION

6.1 Preliminary phytochemical screening of *Chromolaena odorata* extracts

Extraction of *Chromolaena odorata*

Dried crushed leaves of *Chromolaena odorata* was used for extraction by pet ether (60-80 °C) and ethanol (95% v/v) and the results are tabulated in Table No. 6.1.1.

Plant name	Part used	Extraction method	%w/w yield	
Chromolaena odorata	Leaves	Continuous hot percolation process	Petroleum ether (60-80 °C)	Methanol (95%v/v)
			0.75	5.80

Table No. 6.1.1 Data showing the extractive values of leaves of *Chromolaena odorata*

Preliminary phytochemical screening

The extracts of *Chromolaena odorat* were used for preliminary phytochemical screening such as alkaloids, flavonoids, phytosterols, glycosides, saponons, terpenoids and tannins etc as per the procedure discussed in topic 5.2 of chapter 5. The results are tabulated in Table No. 6.1.2. The extract shows the presence of alkaloids, flavonoids, glycosides, triterpenoids, tannins and phenolic acid, carbohydrates and proteins.

Sr. No.	Chemical Test	Petroleum ether Extract	Ethanolic Extract
1.	**ALKALOIDS**		
a.	Mayer's Test	+	+
b.	Dragendroff's Test	+	+
c.	Wagner's Test	+	+
d.	Hager's Test	+	+
2.	**CARBOHYDRATES**		
a.	Molisch's Test	-	+
b.	Fehlings Test	-	+
c.	Barfoed's Test	-	+
d.	Benedicts Test	-	+
3.	**GLYCOSIDES**		
a.	Legal Test	-	+
b.	Kiddes Test	-	+
c.	Born Tragers Test	-	+
4.	**FIXED OIL & FAT**		
a.	Stain Test	-	-
b.	Saponification Test	-	-
5.	**TANINS & PHENOLICS**		
a.	Ferric Chloride Test	-	+
b.	Lead Acetate Test	-	+
c.	Gelatin Solution Test	-	+
6.	**TRITERPENOIDS**		
a.	Salkowsky Test	+	+
b.	Libermann Burchard Test	+	+
7.	**SAPONINS**		
a.	Foam Test	-	-
8.	**PROTEINS**		
a.	Million's Test	-	+
b.	Ninhydrin Test	-	+

9.	**FLAVONS & FLAVONOIDS**		
a.	Aqueous NaOH test	-	+
b.	Conc.Sulphuric Acid Test	-	+
c.	Schinodo's Test	-	+

Table No. 6.1.2 Qualitative phtytochemical screening results of *Chromolaena odorata*

6.2 Thin Layer Chromatography (TLC)

The ethanolic extract of *Chromolaena odorata* was subjected for TLC by using various solvent systems. On the basis of trial and error method, the following solvent system showed 2 to 4 different spots with different R_f values on development. The results are tabulated in Table No. 6.2.

Solvent system	No. of spots	Colour of spots	Detecting agent	R_f value
Toluene : Ethyl acetate (5:5)	2	Green Green	Iodine chamber	0.72 0.88
Ethyl acetate : Methanol (3:7)	1	Brown	Iodine chamber	0.89
Ethyl acetate : Methanol (4:2)	4	Brown Brown Brown Brown	p-toluenesulphuric acid	0.42 0.57 0.71 0.96
Toluene : Chloroform Acetone (4:2.5:3.5)	1	Green	p-toluenesulphuric acid	0.73
Benzene : Ethyl acetate (7.5:2.5)	2	Brown Brown	Iodine chamber	0.59 0.72
Benzene : Ethyl acetate : Diethyl amine (7:2:1)	3	Brown Brown Brown	Iodine chamber	0.29 0.65 0.78

Table No. 6.2 TLC pattern of ethanol extract of *Chromolaena odorata*

The TLC study was carried out and reported as per the procedure described in topic 5.3.1 of chapter 5. From the TLC study of the ethanol extract of *Chromolaena odorata* showed the presence of four spots as the maximum number of spots with solvent system Ethyl acetate : Methanol (4:2) using p-toluenesulphuric acid as detecting agent. The R_f values of the spots were calculated and found to be 0.42, 0.57, 0.71 and 0.96 respectively.

6.3 Column Chromatography

Details of column chromatography of ethanol extract of *Chromolaena odorata*

Sr. No.	Frac-tions	Eluent	Ratio	Nature of residue	No of spot in TLC	R_f value	TLC eluting solvent system
1	1-4	Pet. Ether	100	Colourless	--	--	Ethyl acetate : Methanol
2	5-8	Pet. Ether : Benzene	9 : 1	Colourless	--	--	Ethyl acetate : Methanol
3	9-12	Pet. Ether : Benzene	8: 2	Colourless	--	--	Ethyl acetate : Methanol
4	13-16	Pet. Ether : Benzene	7 : 3	Colourless	--	--	Ethyl acetate : Methanol
5	17-20	Pet. Ether : Benzene	6 : 4	Colourless	--	--	Ethyl acetate : Methanol
6	21-24	Pet. Ether : Benzene	5 : 5	Colourless	--	--	Ethyl acetate : Methanol
7	25-28	Pet. Ether : Benzene	4 : 6	Colourless	--	--	Ethyl acetate : Methanol
8	29-32	Pet. Ether : Benzene	3 : 7	Colourless	--	--	Ethyl acetate : Methanol
9	33-36	Pet. Ether : Benzene	2 : 8	Colourless			Ethyl acetate : Methanol
10	37-40	Pet. Ether : Benzene	1 : 9	Colourless	--	--	Ethyl acetate : Methanol
11	41-44	Benzene	100	Colourless	--	--	Ethyl acetate : Methanol
12	45-48	Benzene : Chloroform	9 : 1	Light green colour	1	0.90	Ethyl acetate : Methanol

13	49-52	Benzene : Chloroform	8: 2	Green colour	1	0.89	Ethyl acetate : Methanol
14	53-56	Benzene : Chloroform	7 : 3	Green colour	--	--	Ethyl acetate : Methanol
15	57-60	Benzene : Chloroform	6 : 4	Light green colour	--	--	Ethyl acetate : Methanol
16	61-64	Benzene : Chloroform	5 : 5	Yellowish green colour	--	--	Ethyl acetate : Methanol
17	65-68	Benzene : Chloroform	4 : 6	Faint yellow colour	--	--	Ethyl acetate : Methanol
18	69-72	Benzene : Chloroform	3 : 7	Colourless			Ethyl acetate : Methanol
19	73-76	Benzene : Chloroform	2 : 8	Colourless	--	--	Ethyl acetate : Methanol
20	77-80	Benzene : Chloroform	1 : 9	Colourless	--	--	Ethyl acetate : Methanol
21	81-84	Chloroform	100	Colourless	--	--	Ethyl acetate : Methanol
22	85-88	Chloroform : Methanol	9 : 1	Light yellow colour	1	0.90	Ethyl acetate : Methanol
23	89-92	Chloroform : Methanol	8: 2	Yellow colour	2	0.89 0.76	Benzene : Ethyl acetate : Diethyl amine
24	93-96	Chloroform : Methanol	7 : 3	Yellow colour	1	0.78	Benzene : Ethyl acetate : Diethyl amine
25	97-100	Chloroform : Methanol	6 : 4	Light yellow colour	--	--	Benzene : Ethyl acetate : Diethyl amine

26	101-104	Chloroform : Methanol	5 : 5	Faint yellow colour	--	--	Ethyl acetate : Methanol
27	105-108	Chloroform : Methanol	4 : 6	Colourless	--	--	Ethyl acetate : Methanol
28	109-112	Chloroform : Methanol	3 : 7	Colourless	--	--	Ethyl acetate : Methanol
29	113-116	Chloroform : Methanol	2 : 8	Colourless	--	--	Ethyl acetate : Methanol
30	117-120	Chloroform : Methanol	1 : 9	Colourless	--	--	Ethyl acetate : Methanol
31	121-124	Methanol	100	Colourless	--	--	Ethyl acetate : Methanol

Table No. 6.3 Column Chromatography of ethanol extract on *Chromolaena odorata*

Analysis of the isolated compound CO 1

Thin layer Chromatography of the isolated compound CO 1

The isolated compound CO 1 obtained from column chromatography was analysed by thin layer chromatography using the solvent system Ethyl acetate : Methanol (4 : 2) and detected by using p-toluenesulphuric acid. It showed the presence of one brown colour spot with R_f value of 0.09.

Fig. 6.1 TLC of the isolated compound of CO 1

UV Spectroscopy

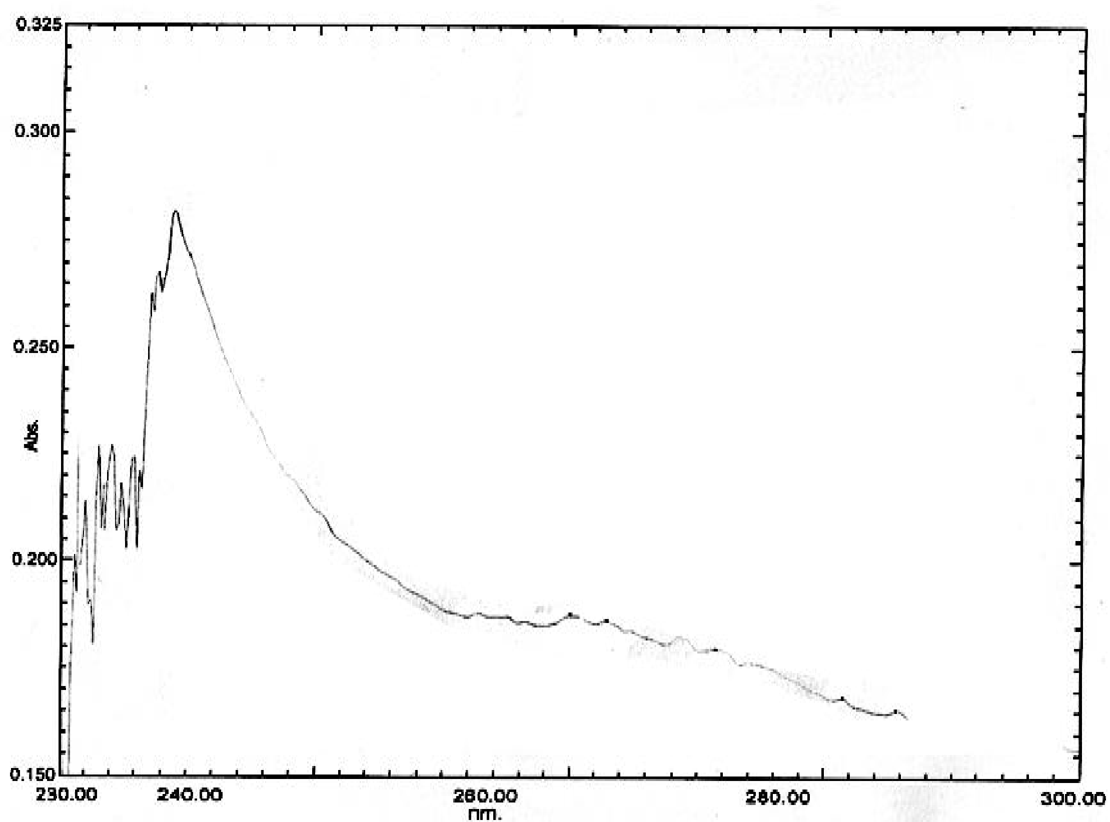


Fig. 6.2 UV spectroscopy of the isolated compound CO 1

Wavelength (max)   : 240 nm

Solvent **:** Chloroform.

IR Spectroscopy

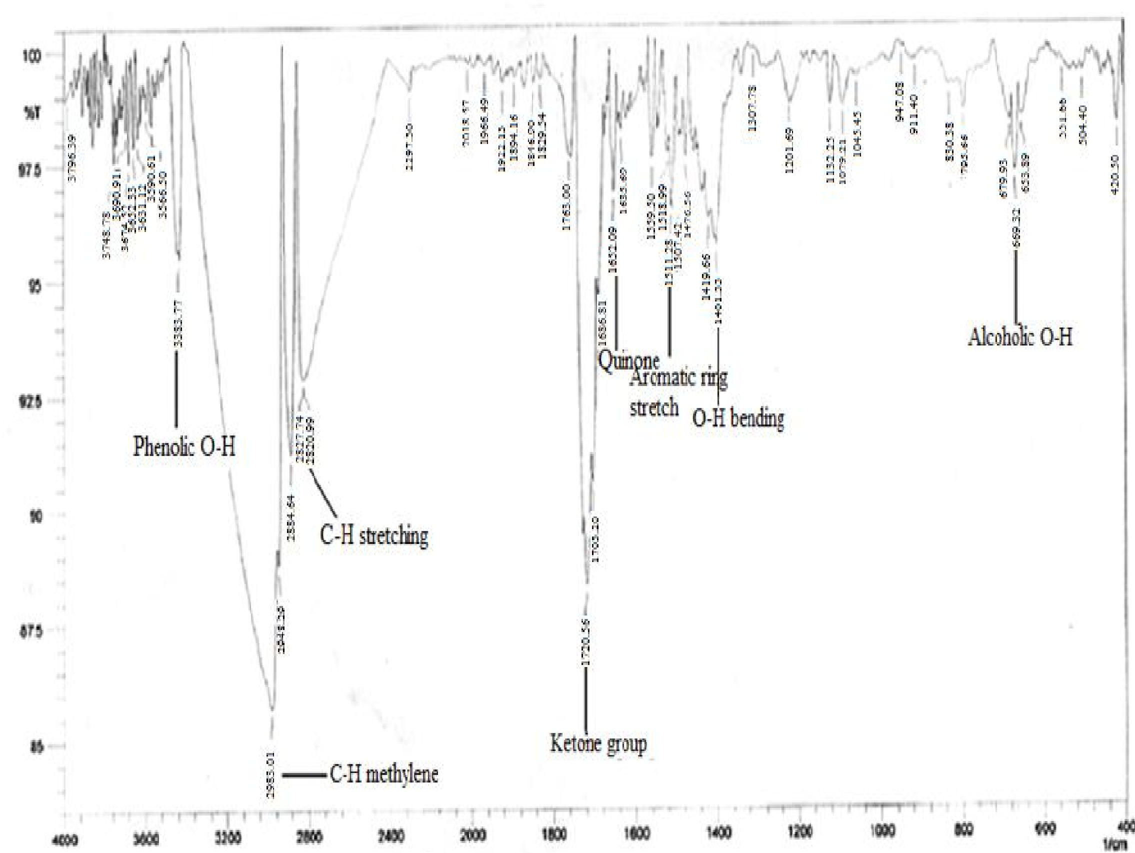


Fig. 6.3 IR Spectrum of isolated compound CO 1

NMR Spectroscopy

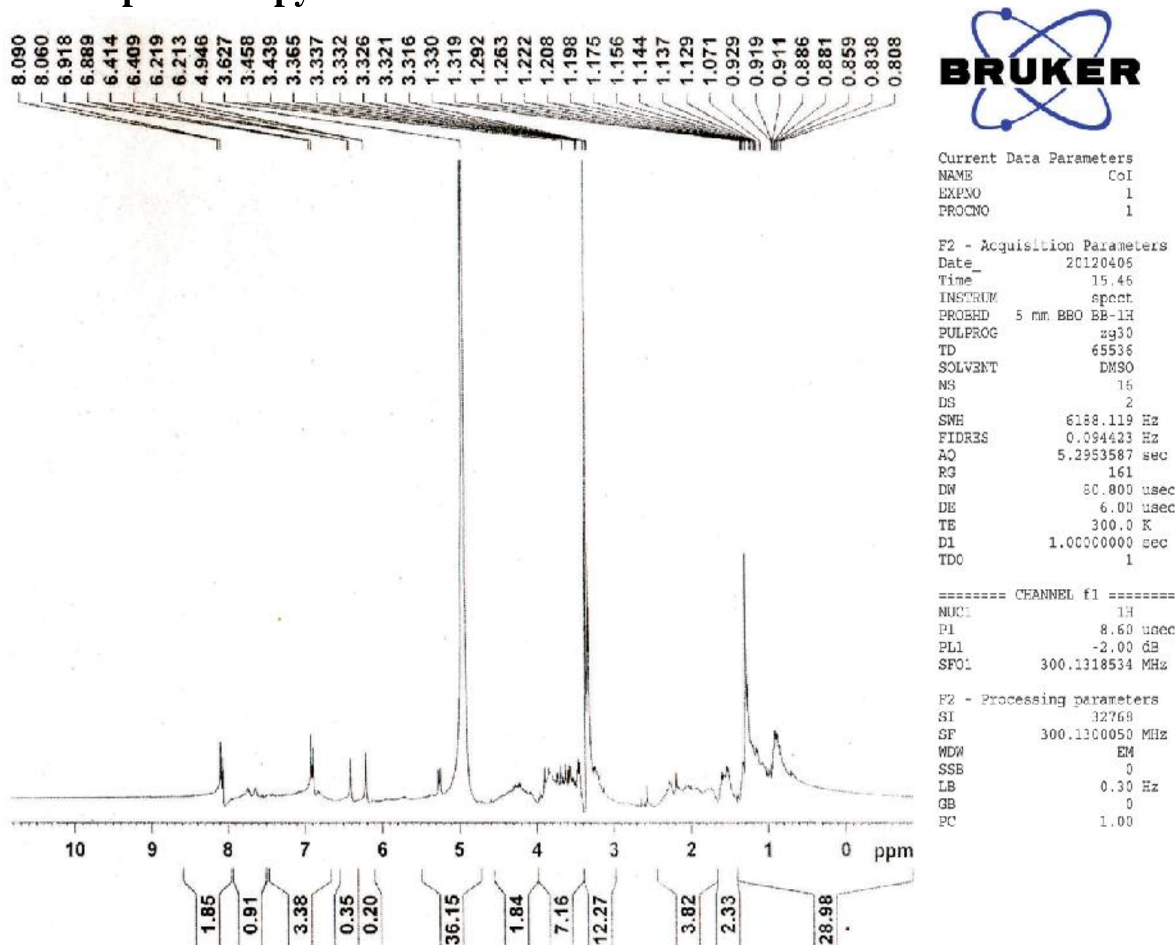


Fig. 6.4 NMR Spectrum of isolated compound CO 1

IR spectral data of the isolated compound CO 1

Sr. No	Functional groups	Frequency cm^{-1}
1	Phenolic O-H	3388.77 cm^{-1}
2	C-H methylene stretch	2983.01 cm^{-1}
3	C-H Stretching vibration	2827.74 cm^{-1}
4	Ketone group	1720.56 cm^{-1}
5	Quinone	1652.09 cm^{-1}
6	Aromatic ring stretch	1511.28 cm^{-1}
7	O-H bending	1401.33 cm^{-1}
8	Alcoholic O-H out of plane	669.32 cm^{-1}

NMR spectral data of the isolated compound CO 1

Sr. No	Types of proton	Chemical shift, ppm ( )
1	Ethers (CH-O-R)	3.3, 3.4
2	Phenolic (Ar-O-H)	4.9
3	Aromatic proton (AR-H)	6.2, 6.4

Results

The chemical characterization of the ethanol extract of *Chromolaena odorata* is summarized in Table No. 6.1.2. The results indicated the presence of alkaloids, carbohydrates, glycosides, tanins, triterpenoids, proteins and flavonoids. The TLC study was carried out and summarized in Table No. 6.2. From the TLC study of the ethanol extract of *Chromolaena odorata,* the presence of four spots were observed as maximum number of spots with Ethyl acetate : Methanol (4:2) using p-toluenesulphuric acid as detecting agent. The R_f values of the spots were calculated and found to be 0.42, 0.57, 0.71 and 0.96 respectively. Column chromatography of the ethanol extract of *Chromolaena odorata* is summarized in Table No.6.3. Phytochemical analysis of the isolated compound CO 1 gave positive result for flavonoids. Thin layer chromatography of the isolated compound CO 1 was carried out using Ethyl acetate : Methanol (4:2) and p-toluenesulphuric acid as detecting

agent and showed the presence of one brown colour spot. The R_f value was determined as 0.9.

UV spectra of the isolated compound CO 1 was carried out and data showed the maximum absorption at 240 nm. IR spectra of the isolated compound CO 1 was carried out and data showed the presence of phenolic O-H at 3388.77 cm^{-1}, C-H methylene stretch at 2983.01 cm^{-1}, C-H stretching vibration at 2827.74 cm^{-1}, functional group ketone at 1720.56 cm^{-1}, quinone functional group at 1652.09 cm^{-1}, aromatic ring stretch at 1511.28 cm^{-1}, O-H bending at 1401.33 cm^{-1}, alcoholic O-H out of plane bend at 669.32 cm^{-1}. NMR spectra of the isolated compound was carried out and data showed the presence of ether (CH-O-R) at 3.3, 3.4 ppm, phenolic (Ar-OH) at 4.9 and aromatic proton (Ar-H) at 6.2, 6.4 respectively.

The phytochemical screening and spectral data of the isolated compound suggested that the isolated compound resembles structural similarities with the flavonoids.

6.4 Pharmacological screening

In vitro* anticancer activity of *Chromolaena odorata

The purpose of this assay is to screen drugs for anticancer activity using cancer cell line of HCT-11 (human colorectal adrenocarcinoma cancer cell line) and HeLa (human cervical cell line) using MTT assay method.

Extract	**Cell line**	**Conc (µg/ml)**	**% Cell inhibition**	**IC_{50} (µg/ml)**
Ethanol extract of *Chromolaena odorata*	HCT116	31.25	26.96±2.008	60.18
		62.5	57.59±1.891	
		125	66.34±2.01	
		250	85.30±0.9313	
		500	92.90±0.5224	
	HeLa	31.25	21.92±1.652	71.74
		62.5	42.15±1.825	
		125	74.13±1.004	
		250	77.18±0.3528	
		500	89.13±0.5302	

Table No. 6.4.1 Percentage cell inhibition of different concentration of ethanol extract of *Chromolaena odorata*

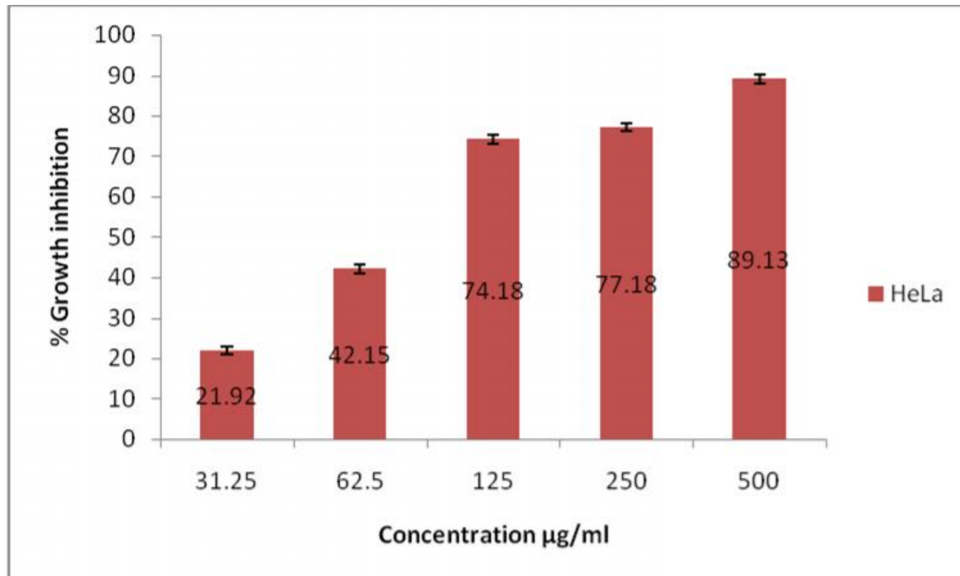


Chart No. 6.4.1 Percentage Cell inhibition of Anticancer activity of *Chromolaena Odorata* in human cervical cancer cell line (HeLa).

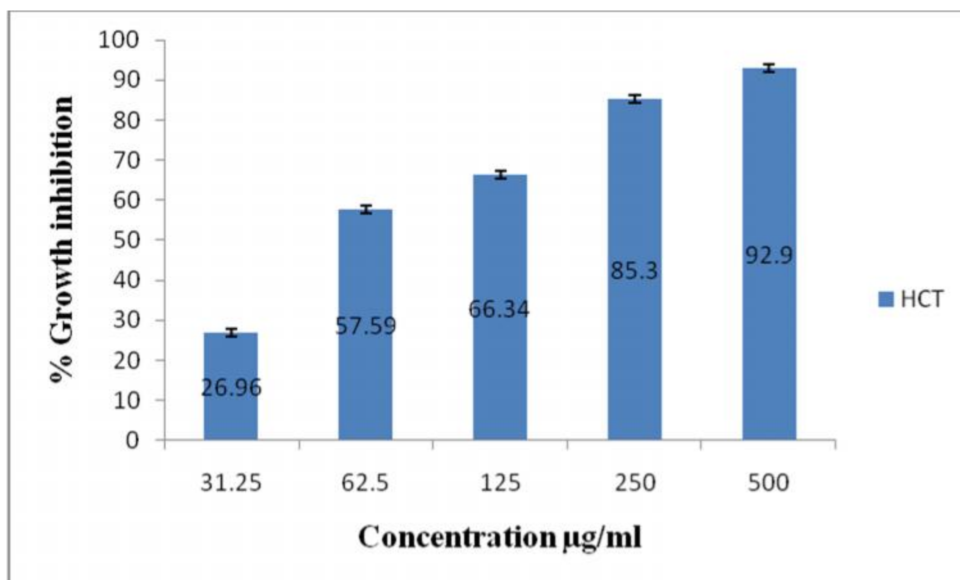


Chart No. 6.4.2 Percentage Cell inhibition of anticancer activity of *Chromolaena Odorata* in human colorectal adenocarcinoma cell line (HCT116).

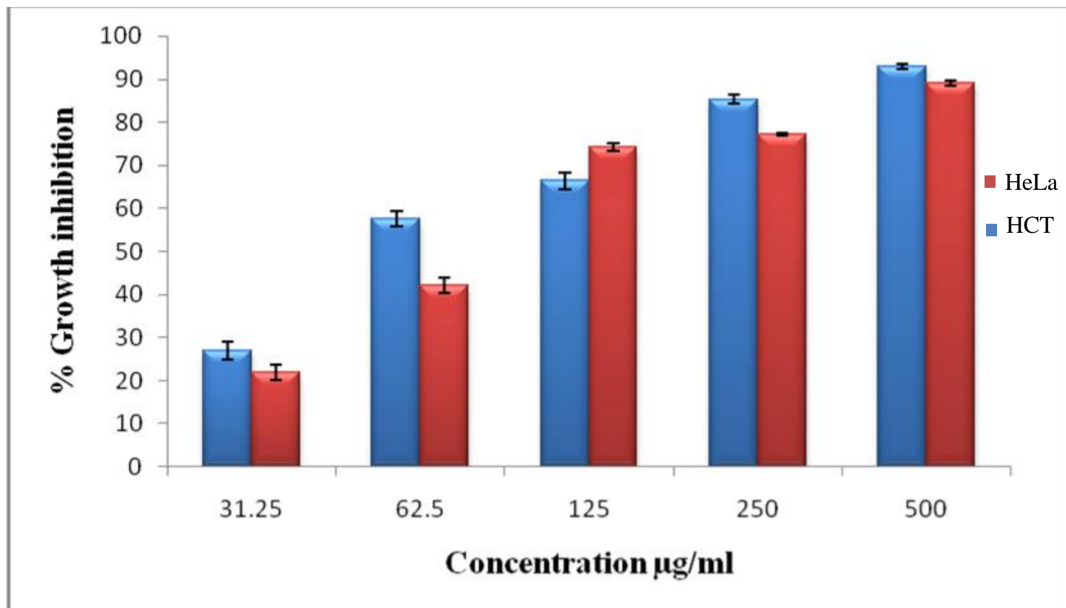
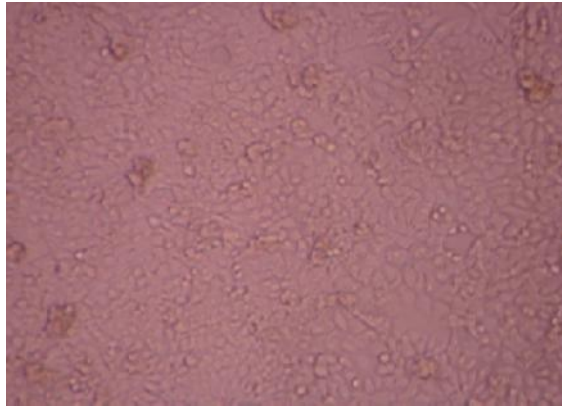
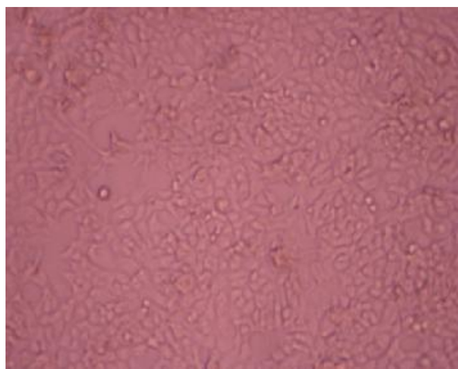


Chart No. 6.4.3 Percentage growth Inhibition of *Chromolaena odorata* in two cell lines (HCT-116 and HeLa)

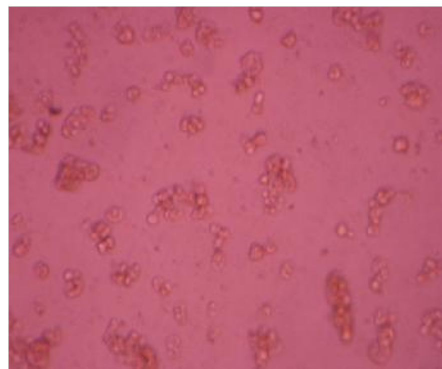
HCT- 116



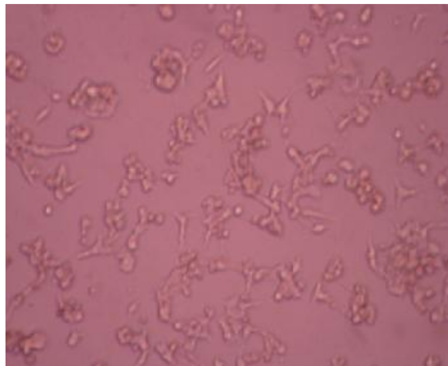
Control (without sample)



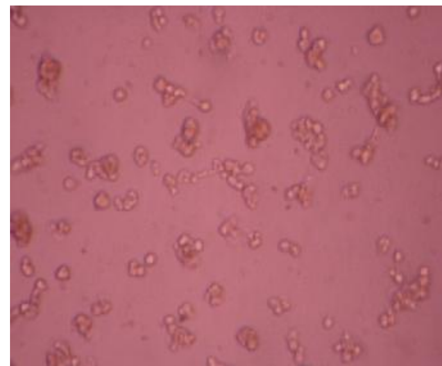
62.5 µg/ml



125 µg/ml



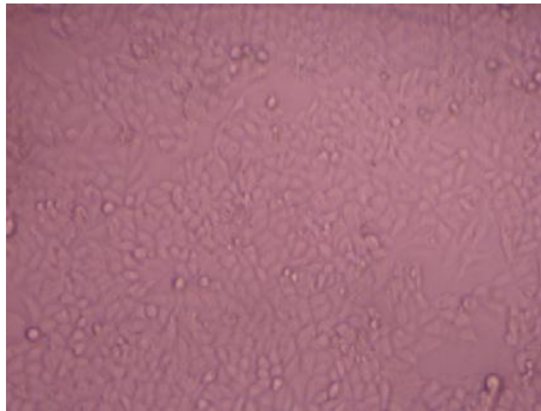
250 µg/ml



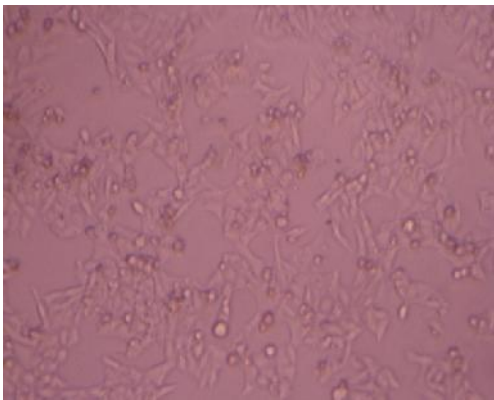
500 µg/ml

Fig. 6.4 Inhibition of cancer cell lines by different concentration of ethanol extract of *Chromolaena odorata*

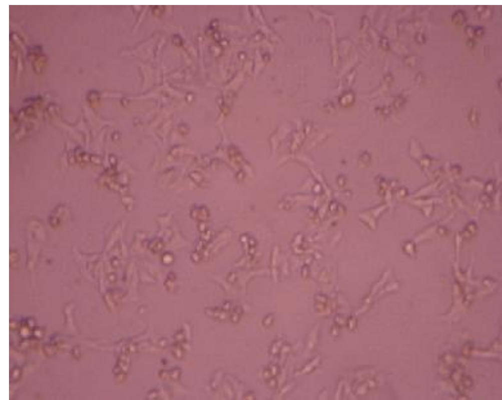
HeLa



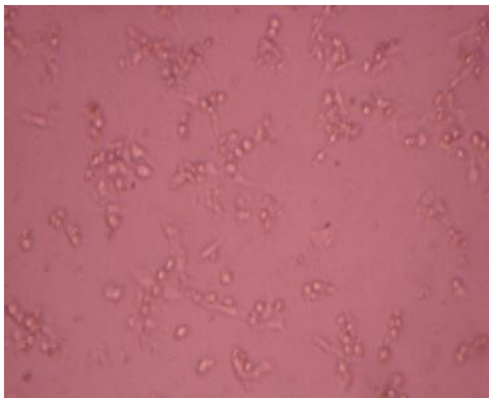
Control (without sample)



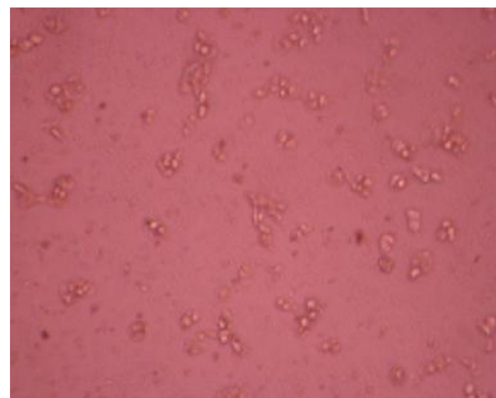
62.5 µg/ml



125 µg/ml



250 µg/ml



500 µg/ml

Fig. 6.5 Inhibition of cancer cell lines by different concentration of ethanol extract of *Chromolaena odorata*

Results

The pharmacological screening of the ethanol extract of *Chromolaena odorata* was performed as per reported in topic 5.4 of chapter 5. The data of percentage cell inhibition by ethanol extract of *Chromolaena odorata* at different concentrations were presented in Table No. 6.4.1 and Chart No. 6.4.1 and 6.4.2. The difference in inhibition of cell by ethanol extract of *Chromolaena odorata* was presented in Chart No. 6.4.3 Inhibition picture of cancer cell line HCT116 and HeLa was presented in figure 6.4 and 6.5. The results indicated that ethanol extract of *Chromolaena odorata* showed significant inhibition of cell on both cell lines with IC_{50} of 60.18 μg/ml in HCT116 and 71.74 μg/ml in HeLa respectively. The result also showed that percentage inhibition of cell by the ethanol extract of *Chromolaena odorata* increases with increase in concentration of extract.

6.5 Discussion

The leaves of *Chromolaena odorata* linn belonging to the family Asteraceae has been investigated in a systematic way covering the phytochemical screening and pharmacological studies.

From the literature survey, it revealed that very less amount of pharmacological work has been carried on *Chromolaena odorata.* But no work has been found during literature survey in the line of anti cancer activity. Therefore it was thought worthwhile to carry out preliminary phytochemical screening, isolation and characterization of the isolated compound and pharmacological investigation in the line of anti cancer activity.

Extraction

The leaves of *Chromolaena odorata* linn. were collected in the month of July 2011 from Perur, near Perur temple, Coimbatore, Tamilnadu and was authentified by Dr. M.Palanisamy, Scientist 'C' with reference number of No.BSI/SRC/5/23/2010-11/TECH.1089, Botanical Survey of India, Southern Regional Centre, Coimbatore-641 003. The collected leaves were dried and using a mechanical grinder it was made into a coarse powder. The powder was seive through seive No. 40. 150 gm of the powder was packed in a thimble and extracted with petroleum ether (60-80 ºC) by continous hot percolation process using Soxhlet apparatus for 72 hours. The resulting semisolid mass was vacuum dried in a dessicator to yield (0.75 gm) solid residue. After the extraction with petroleum ether the same plant material was dried and again extracted with ethanol (95% v/v) for 72 hours in Soxhlet apparatus. The solvent was removed by distillation under reduced pressure and resulting semisolid mass was vacuum dried to yield (5.80 gm) solid residue.

Phytochemical screening

Qualitative phytochemical screening were carried out to identify the phytoconstituents. The petroleum ether extract of *Chromolaena odorata* contained alkaloids and triterpenoids.While the ethanol extract showed the presence of alkaloids, carbohydrates, glycosides, tannins, triterpenoids, proteins and flavonoids.

Thin Layer Chromatography

The thin layer chromtography of the ethanol extract of *Chromolaena odorata* obtained showed the presence of four spots with Ethyl acetate : Methanol (4:2) using

p-toluene sulphuric acid as detecting agent. The R_f values were calculated and found to be 0.42, 0.57, 0.71 and 0.96 respectively.

Column chromatography

Compounds were isolated from Chromolaena odorata by column chromatography with gradient elution technique with the help of solvent system petroleum ether, benzene, chloroform and methanol. Phytochemical analysis of the isolated compound CO 1 showed positive result for flavonoid. TLC of the isolated compound CO 1 was also carried out using Ethyl acetate : Methanol (4:2) and p-toluenesulphuric acid as detecting agent and showed the presence of one spot. R_f value was determined as 0.90. The isolated constituents were purified and characterized by spectral studies using UV, IR and NMR spectroscopy etc.

UV spectral data showed the maximum absorption at 240nm.

IR spaectral data showed the presence of phenolic O-H at 3388.77 cm^{-1}, C-H methylene stretch at 2983.01 cm^{-1}, C-H stretching vibration at 2827.74 cm^{-1}, functional group ketone at 1720.56 cm^{-1}, quinone functional group at 1652.09 cm^{-1}, aromatic ring stretch at 1511.28 cm^{-1}, O-H bend at 1401.33 cm^{-1}, alcohol O-H out of plane bend at 669.32 cm^{-1} respectively.

NMR spectral data showed the presence of ether (CH-O-R) at 3.3, 3.4 ppm, phenolic (Ar-OH) at 4.9 and aromatic proton (Ar-H) at 6.2, 6.4 respectively.

From the study of phytochemical screening, thin layer chromatography and spectral studies suggested that the isolated compound resembles structural similarities with the flavonoids.

Pharmacological screening

Scientific strategies for the *in vitro* anticancer evaluation of natural products with biological activity have changed in the past few years. One recent development is the highly automated bioassay screening based on colorometric methods that quantify the proloferation of cells. Many biological assays require the measurement of surviving and/or proliferation of mammalian cells. This can be acheived by MTT assay, a non-radioactive, fast and economical assay widely used to quantify cell viability and proliferation.

MTT is a yellow water soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water insoluble dark blue farmazan by reductive cleavage

of the tetrazolium ring. MTT is cleaved by all living cells but not ny dead cells. The amount of farmazan generated is directly proportional to the cell number. *(DA Scudiero, et al)*

In the present study anticancer activity of ethanolic extact of Chromolaena odorata was evaluated with MTT assay using cancer cell line of HCT-11 (human colorectal adrenocarcinoma cancer cell line) and HeLa (human cervical cell line). The present study demonstrated that ethanol extract of *Chromolaena odorata* showed significant inhibition of both cell lines (HCT116 and HeLa). Inhibition of cell by the ethanol extract of *Chromolaena odorata* was found to concentration dependent. On comparison, it was found that percentage inhibition of cell in HCT116 cell line is more. Thus, in indicated that ethanol extract of *Chromolaena odorata* showed more effective in screening anticancer activity using HCT cell line when compared to HeLA cell line.

The result suggested that ethanol extract of *Chromolaena odorata* showed significant anticancer activity with IC_{50} values of 60.18 µg/ml for HCT116 cell line and 71.74 µg/ml for HeLA cell line respectively.

CHAPTER-7

SUMMARY AND CONCLUSION.

CHAPTER - 7

SUMMARY AND CONCLUSION

The work presented here dealt with the plant *Chromolaena odorata* Linn. towards the isolation of phytoconstituents, characterisation of the isolated compound by spectral studies using UV, IR and NMR spectroscopy and evaluation of anticancer activity using cell line HCT116 and HeLa by using MTT assay.

The plant was collected from Perur, near Perur temple, Coimbatore, Tamil Nadu during the month of July 2011 and was taxonomically identified and confirmed by the Botanical Survey of India, Southern Circle Tamil Nadu Agriculture University Campus, Coimbatore, India, with reference number of No.BSI/SRC/5/23/2010-11/Tech.1089.

The literature survey revealed that very less amount of pharmacological work has been carried on *Chromolaena odorata*. Therefore it was thought worthwhile to carry out preliminary phytochemical screening, isolation and characterization of the isolated compound and pharmacological investigation in the line of anticancer activity.

The project work was initiated with the collection of plant, dried under shade and then powdered with mechanical grinder and passed through sieve No. 40 and stored in an air tight container. The crushed plant material was extracted by using petroleum ether (60-80 °C) and ethanol (95% v/v) with the help of Soxhlet apparatus successively. The percentage yield of petroleum ether and ethanol extracts were 0.75 and 5.80 w/w respectively.

The crude extracts were subjected to preliminary phytochemical screening. The preliminary phytochemical screening revealed that the extract of *Chromolaena odorata* contains alkaloids, flavonoids, glycosides, triterpenoids, tannins and carbohydrates as major phytoconstituents.

Thin layer chromatography was carried out for ethanol extract by using various solvent systems. Ethyl acetate : Methanol (4:2) shows the maximum number

of spots with p-tolunesulphuric acid as detecting agent. The R_f values were calculated and found to be 0.42, 0.57, 0.71 and 0.96 respectively.

Further crude ethanol extract of *Chromolaena odorata* linn was subjected for isolation by using Column chromatography with gradient elution technique with the help of solvent system petroleum ether, benzene, chloroform and methanol. Phytochemical test of the isolated compound CO 1 was carried and showed positive test for flavonoids. Thin layer chromatography of the isolated compound CO 1 was also carried out by using solvent system Ethyl acetate : Methanol (4:2) and showed the presence of one spot with detecting agent p-toluenesulphuric acid and R_f value was determined as 0.90. The isolated constituents were purified and characterized by spectral studies using UV, IR and NMR spectroscopy etc.

UV spectral data showed the maximum absorption at 240 nm.

IR spectral data showed the presence of phenolic O-H at 3388.77 cm^{-1}, C-H methylene stretch at 2983.01 cm^{-1}, C-H stretching vibration at 2827.74 cm^{-1}, functional group ketone at 1720.56 cm^{-1}, quinone functional group at 1652.09 cm^{-1}, aromatic ring stretch at 1511.28 cm^{-1}, O-H bending at 1401.33 cm^{-1}, alcoholic O-H out of plane bend at 669.32 cm^{-1}.

NMR spectral data showed the presence of ether (CH-O-R) at 3.3, 3.4 ppm, phenolic (Ar-OH) at 4.9 and aromatic proton (Ar-H) at 6.2, 6.4 respectively.

From the study of phytochemical screening, thin layer chromatography and spectral studies suggested that the isolated compound resembles structural similarities with the flavonoids.

Pharmacological screening of the ethanol extract of *Chromolaena odorata* was carried out using using cancer cell line of HCT-11 (human colorectal adrenocarcinoma cancer cell line) and HeLa (human cervical cell line). The result showed significant inhibition of cell with IC_{50} values of 60.18 µg/ml and 71.74 µg/ml respectively. The result also showed that percentage inhibition of cell by the ethanol extract of *Chromolaena odorata* increases with increase in concentration of extract.

In conclusion, from literature survey, preliminary phytochemical screening, thin layer chromatography study, isolation of active compound by column chromatography and spectral studies of the isolated compound by UV, IR and NMR

spectroscopy, it reveals that flavonoids may be the secondary metabolite present in active crude extract of leaves of *Chromolaena odorata* linn which may be responsible for pharmacological activity in the line anticancer activity. Considering the lack and need of drugs, the specific effects of *Chromolaena odorata* reported in the present study is of enormous interest and deserves further investigation using more experimental paradigms for further confirmation to isolate and characterize the active compounds.

CHAPTER-8

BIBLIOGRAPHY.

CHAPTER 8

BIBLIOGRAPHY

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