

EVALUATION OF ANTICANCER ACTIVITIES OF ETHNOMEDICINAL PLANTS FROM WESTERN GHATS

Thesis Submitted to The
KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
(KAHER), BELAGAVI

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India

Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A' Grade by NAAC 2nd Cycle) [Placed in Category 'A' by MHRD (GOI)]



For the Award of The Degree of
Doctor of Philosophy
In the Faculty of
Pharmacy
By

Mr. Mahendra Kumar Chouhan M. Pharm.
(Full-Time Ph.D. Scholar, Registration No: KLEU/Ph.D./14-15/DO1214013)

Under the Guidance of

Dr. Pramod J. Hurkadale M. Pharm, Ph.D., PDCR
Professor and Head
Department of Pharmaceutical Biotechnology,
KLE College of Pharmacy, Belagavi-590010, Karnataka

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I hereby declare that the thesis entitled “**Evaluation of Anticancer Activities of Ethnomedicinal plants from Western Ghats**” is a bonafide and original research carried out by me under the guidance of **Dr. Pramod J. Hurkadale**, Professor and Head, Department of Pharmaceutical Biotechnology, KLE College of Pharmacy, Belagavi. The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.

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EVALUATED PARAMETERS

A. BODY WEIGHT

B. HAEMATOLOGICAL PARAMETERS

- a) RBC COUNT
- b) WBC COUNT
- c) % HAEMOGLOBIN

C. TUMOR PARAMETERS

- a) TUMOR WEIGHT
- b) TUMOR VOLUME
- c) KAPLAN MEIER SURVIVAL PLOT
- d) MEDIAN SURVIVAL TIME
- e) % INCREASED LIFE SPAN

**2. FIBROSARCOMA (SOLID TUMOR)
MODEL**

EVALUATED PARAMETERS

A. BODY WEIGHT

B. HAEMATOLOGICAL PARAMETERS

- a) RBC COUNT
- b) WBC COUNT
- c) % HAEMOGLOBIN

C. TUMOR PARAMETERS

- a) TUMOR BURDEN
- b) TUMOR PROGRESSION
- c) KAPLAN MEIER SURVIVAL PLOT
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List of Abbreviations

<i>A. coynei</i>	- <i>Achyranthes coynei</i> Sant.
A.C.	- <i>Achyranthes coynei</i> Sant.
A-549	- Lung Cancer Cells
BSL	- Brine Shrimp Lethality
<i>C. inerme</i>	- <i>Clerodendrum inerme</i> L. Gaertn.
C.I.	- <i>Clerodendrum inerme</i> L. Gaertn.
DAL	- Daltons Ascitic Lymphoma Model
DMSO	- Di methyl sulfoxide
<i>Daudi</i>	- Burkitt's Lymphoma Cells
High dose	- 400 mg per Kg
HT-1080	- Fibrosarcoma Cancer Cells
HT-29	- Colon Cancer Cells
HL-60	- Leukaemia (Blood Cancer Cells)
ILS	- Increased Life Span
IC ₅₀	- Inhibitory Concentration
Low dose	- 200 mg per Kg.
mg	- Milligram
ml.	- Milliliter
MTT	- 3-(4,5- dimethylthiazol-yl)-2, 5- diphenyltetrazolium bromide
MCF-7	- Breast Cancer Cells
MST	- Median Survival Time
Nm	- Nanometer

OECD	- The Organization for Economic Co-operation and Development
PBS	- Phosphate Buffer Saline
pH	- Hydrogen Potential
ppm	- Parts Per Million
RT	- Retention Time
Rpm	- Revolution Per Minute
SD	- Standard Deviation
Sec.	- Second
UV	- Ultraviolet Light
WHO	- World Health Organization
μg	- Microgram
μl	- Microliter
λ_{max}	- Wavelength of Maximum Absorption

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Abstract

Background

The present study envisages reviewing the available literature and focuses on the evaluation of the anticancer potential of the ethnomedicinal plant. It was indeed an effort to procure for the scientific validation of ethnomedicinal plants with well-accepted methods for its traditional claims. This study provides an insight into critical aspects of phytochemical content as well different cancer model with various study parameters and also initiates an area of research which is generally not attempted because of less spread of traditional information and knowledge.

Objectives

To perform the anticancer activity of *Achyranthes coynei* and *Clerodendrum inerme* ethnomedicinal plants by using *In-vitro* assay methods.

To evaluate the safety and efficacy of *Achyranthes coynei* and *Clerodendrum inerme* ethnomedicinal plants by *In-vivo* models.

Methodology

This experimental work was carried out using the maceration method for plant extraction followed by phytochemical investigation, HPLC profile for the presence of marker compounds in the plant extract. Dye exclusion assay, MTT assay using various cell lines viz, Lung A-549, breast cancer MCF-7, blood cancer HL-60, Colon cancer HT-29, Fibrosarcoma HT-1080, and Burkitt's lymphoma *Daudi* further, Brine shrimp lethality assay, Dalton's ascitic lymphoma (Liquid tumor) and fibrosarcoma (Solid tumor) were used for anticancer evaluation with various biochemical parameters.

Results

The findings suggested the promising activity by using the methods for anticancer evaluation of plant extracts.

Conclusion

Based on the results observed and inferred, there is indeed a lack of the consistency of scientific validation of ethnomedicinal plants and traditional practices. Thus, obviating the fact and requirement to evaluate and prove, the folklore claims and traditional practices with proper scientific reasons.

Keywords: *Achyranthes coynei*; Brine Shrimp; *Clerodendrum inerme*; Fibrosarcoma.

INTRODUCTION

1. INTRODUCTION:

1.1 Background

Role of Ethnomedicinal Plants in Cancer Treatment

The plant-based Indian medicine system & that has played a crucial role as most of the plant-derived compounds such as Camptothecin, Etoposide, Taxans, Vincristine, etc. have influential anti-cancer activity. [1] The numerous traditional medicines are widely used as an alternative therapy. Chemotherapy, radiotherapy & surgical excision are few conventional therapies frequently utilized but it seldom proves to be inefficient to solve the problem. [2] The traditional medicine systems in India are linked to the rich & diverse flora. One of the biodiversity hotspots happens to be in the Western Ghats of India.

The medical practices in India have a combination of traditional and modern medicine due to varied which reasons include an ecological, cultural and historical background that supports its efficacy as a therapeutic model. The rate at which these medicinal plants are being utilized is increasing with the advancement of days and thereby it becomes essential to document, analyze & evaluate the usage so as to realize the pattern of usage & also the commercial value. [3]

Achyranthes coynei belongs to the Amaranthaceae family and it has various uses in ethnic practice and folklore claims. It has the property to cure cough, fever, piles, urinary calculi, scorpion sting, anaemia - especially in obstetrics and gynecology, abortion, induction of labor, remedy for postpartum bleeding and wounds which is brought into the application by traditional practitioners. [4]

Clerodendrum inerme belongs to Verbenaceae family & plants included in this family have claims for their ethnomedicinal properties. [5] It is utilized in the treatment of chronic cough, varied skin diseases, venereal infection, worm infestations, beriberi, lymphadenopathy & buboes. [6] *C. inerme* contains essential phytoconstituents and

anti-cancer agents like Pentadecanoic acid β -D-glycoside, Betulinic acid and sterol compounds. [7, 8, 9]

The current study included animal models that were chemically (3-methacholanthrene) induced with progressive types of tumors i.e. Dalton's Ascitic Lymphoma (DAL) and fibrosarcoma. DAL multiplies in the lymphatic cells of the peritoneal cavity rapidly. The tumor that is induced by 3-methacholanthrene rapidly multiplies in female albino mice and that can be induced with a single subcutaneous administration of 200 mg dose at lower back or thigh region which is a better site for the development of fibrosarcoma tumor. [10] The fibrosarcoma tumor model, DAL model has not yet been demonstrated and evaluated in animals meticulously. Therefore, to evaluate the anti-cancer effect, a comparison for efficacy was made between the plant extract & ideal doxorubicin drug.

The present study aims at scientifically validating the ethnomedical claim of *Achyranthes coynei* and *Clerodendrum inerme* used as an anticancer medicine. Hence, there is a need for exploring the anticancer potential using various procedures.

The naturally originated medicines are the vital part of the pharmaceutical industries which have various biologically active molecules led towards the treatment of human ailments. The treatment for cancer, there are many useful drugs which are from naturally derived drugs that include viz. Podophyllotoxin, Camptothecin, Taxol, Vincristine, Vinblastine, etc. that are applied for the treatment of cancer. [11] India has a plethora of traditional medicine and conventional practices that are utilized by 70% of the world population for which the therapeutic efficacy of traditional medicines certain standards have to be established so as to obtain scientific validation. Hence, there is a need for reverse pharmacology approach for testing safety and efficacy and the concept has been followed globally for the revolution of traditional strategy. [12] Traditional medicine and folk practices (ethnomedicine) fall under the non-codified system of

medicine. Under which the crude drug or plant extracts are used for the treatment of various human ailments by most of the local traditional healers, practitioners & vaidyas in this system. The drug extracts and isolates or similarly structured compounds are also evaluated for various activity were some of the drugs which are used as anticancer agents for the treatment such as vincristine, vinblastine, taxol, podophyllotoxins, Camptothecin, gitoxigenin, digitoxigenin, digoxigenin, morphine, tubocurarine, codeine, atropine, aspirin, curcumin, allicin, pilocarpine, artemisinin & ephedra. [13] The systematic evaluation of herbal drugs as per the WHO guidelines with systematic criteria, that includes the pharmacognostic characters, identity, purity evaluation and biological evaluation [14] for which Medicinal plants & drug discovery play a principle role for which selection of the medicinal value of the plant is based on their ethnomedicinal use, references in the classical text and their preparations. The strategies involved in drug discovery from medicinal plant extracts is, to check for the presence of active moiety and lead molecules. The anticancer activity of any plant is correlated with the toxicity profiling of plant extract. If the plant material is toxic in nature, there may be a chance that the plant exhibits inhibition of cell growth and this could have a potential anticancer activity.

As per the World Health Organization, 80% of the population in the developing countries around the world are using the traditional system of medicine. The proper identification of the plant is important for authentic utilization [15] and further evaluation.

The codified and non-codified system is an ancient science which is effective for the treatment of human ailments. In a codified medicine system Ayush, Traditional medicines are covered and on the other hand, the non-codified system of medicine is practiced used by ethnic people, local practitioner and folklore healers.

Ethnomedicine has been defined and made popular because of the folklore, traditional healers and the day to day practices have shown its effectiveness towards varied ailments not only in humans but also in domestic animals.

The word cancer was coined by Hippocrates. The ancient Egyptians have recorded the description of cancer, but they did not use the term cancer. The Egyptian textbook dated around 3000 BC. has described trauma and surgery and the evidence of the same has been found in Egyptian mummies, while the head and neck region and fossilized bone tumors (Osteosarcoma) were studied. [16]

Cancer is defined as a genetic disease i.e. the abnormality in genes that regulate the growth and proliferation leading to a malignant condition. To understand the cause and the nature of malignancy, there is a need to see the root cause of the disease. The molecular biology techniques provide key information, to understand the root cause, clinical care, pathogenetic and prognostic analysis of tumors. In general, due to DNA damage, the normal cells become cancerous when the body is unable to repair the DNA damage. Exposure to the polluted environment, such as pollution, cigarette smoke, carcinogens, chemicals, dyes, etc., considered to be the chief cause, results in DNA mutation.

Tumors are generally of two types, benign and malignant. Benign tumors have the capacity to convert to malignant tumors. These tumors are capable of spreading through the bloodstream. High- quality health care, early detection and diagnosis with optimal treatment of cancer may help to overcome the disease [17, 18]

Due to remarkable achievements in cancer medicine, treatment with proper diagnosis, prevention and early detection is possible. Nowadays, scientists and clinicians are continuously working in the cancer research sector to provide better health care for

cancer patients. It is difficult to say on which site its effect is higher; due to its therapy on accurate target without less harming the other normal tissue, genetics in cancer, Immunotherapy, Nanotechnology-based therapy, surgery, gene expression studies and proteomics and the early detection and diagnosis of cancer forms an essential component in cancer treatment.

To understand the growth of uncontrolled cell division and related pathways by which it gets triggered. It also explores the epidemiology, anatomy and physiology that provides overall information for the cause of cancer and possible treatments.

Cancer has been the chief reason for deaths throughout the world and the WHO statistics accounted for 9.6 million deaths worldwide and 9% of deaths in India in recent 2019 of which the most common types of cancers include lung, liver, stomach, colorectal and breast cancer. [19]

Cancer [20-23]

Cancer is the 2nd prime reason for deaths and is accountable for 9.6 million deaths globally as recorded in 2018, which implies that one out of every six deaths occurs as a reason for cancer. Currently, in India, 2.5 million cases have been registered and around 0.4 million deaths in a year. Tobacco use is responsible for 22% of overall deaths & non-availability of pathological services, last stage presentation, delayed diagnosis and inaccessible treatment account for 26% of the deaths. Cancer encompasses a substantial set of diseases that may cause an affection in any part of the body. Neoplasms & malignant are the descriptions and are used interchangeably to describe cancer. Cancer is characterized by abnormal cells undergoing rapid & enormous cell division that is not bound by any boundary. It has the ability to spread to distinct organs through various routes & this process is termed as metastasis. Cancer of

breast, lung, prostate, colon & rectum, skin and stomach hold responsible as the principle forms of cancer. The normal cells get transformed into cancerous cells because of a multistage process. And most times it is a pre-cancerous lesion that forms a malignant type of tumor. The conditions change due to changes and interactions of external environment to genetic factors like U.V. radiation, ionizing radiation, physical carcinogens, chemical carcinogens including tobacco, asbestos, aflatoxin, heavy metals, arsenic and biological carcinogen such as parasite (Bacteria, Virus including H. pylori, HPV, Epstein-Barr virus, HIV etc.). Also, aging is a factor that develops the cancerous condition & the risk gets magnified because of physical inactivity, unhealthy diet, tobacco, alcohol consumption, and occupational hazards. The burden of cancer can be reduced by its proper management when it is detected in the early stage with proper diagnosis and timely treatment. By avoiding tobacco use, alcohol consumption, weight management, intake of vegetables and proper food, appropriate physical activity, vaccination against HPV and Hepatitis B, awareness, etc. will help improve and speed up the recovery process. Non-communicable diseases contribute as a major health problem in the public sector. The suggestions of cancer screening, identification, diagnosis with stages along with curative treatment helps. Screening for cancer detection with appropriate tests is helpful to arrive at a conclusion. A correct diagnosis is very essential and important for effective and appropriate cancer treatment. Surgical removal, radiotherapy and chemotherapy are the basic procedures which are effective in the early stage.

Cancer which forms solid tumor are masses of tissue overgrowth and blood whereas lymphatic tumors are not solid tumors. Malignant tumor spread and invade the nearby tissues and they can travel from one part of the body to another. The benign tumor can also invade nearby tissues. The striking distinction is that normal cells grow

under systematic and controlled pattern whereas tumor cells grow abruptly with an uncontrolled pattern. The immune system fails to fight the cancerous cells and thereby occurs the rapid spread of these cells to the distant areas resulting in metastatic cancer. The immune system does not kill the cancerous cells. When cancer spread through the body, the condition is called metastatic cancer. This causes functional changes and severe damage to the body. The major classification of cancer is focused on the nature and type of cell involved viz. carcinoma, myeloma, leukaemia, melanoma, sarcoma, lymphoma, brain & spinal cord tumor.

Carcinoma is the widespread form of cancer & is formed by epithelial cells. Adenocarcinoma is another type of epithelial cancer that produces fluid or mucous from glandular tissues. Commonly breast, colon, and prostate fill the category of adenocarcinoma. Basal cell carcinoma is the cancer of the outer layer of skin. Squamous cell or epidermal cell carcinoma refers to the cancer of the squamous cells. Organs mainly affected are stomach, intestine, lungs, bladder and kidneys. The transitional epithelium type tissue formed due to cancer is called transitional cell carcinoma. Sarcoma refers to the cancer of bone & soft tissues that include muscles, fat, lymph vessels & blood vessels & fibrous tissues that include tendons & ligaments. Osteosarcoma, is one of the major types of sarcoma & leiomyosarcoma, Kaposi sarcoma, malignant fibrous histiocytoma, fat, dermato-fibrosarcoma protuberances are common types of soft tissue sarcomas. Bone marrow cancer is called leukaemia which is a form of liquid cancer. In the blood & in bone marrow, many leukemic blast cells develop which results in crowding of abnormal cells.

Cancer in lymphocytes is called lymphoma. 'T' and 'B' lymphocytes are principle part of the immune system that is responsible for the defense mechanism of the body. In lymph nodes and lymph vessels, when abnormal lymphocyte cells build-

up, it leads to lymphoma. Lymphomas are mainly of 2 types, viz; Hodgkin (HL) and Non-Hodgkin lymphoma (NHL). In HL, abnormal lymphocytes are known as 'Reed-Sternberg' and in most cases, these are Beta cells. In a population with NHL, a large group of cancerous cells proliferates either quickly or slowly as B & T cells.

Multiple myelomas are cancers that get initiated in plasma cells, which is an immune cell. The abnormal plasma cells which are now called myeloma cells are formed in the bone marrow leading to the tumor in bones throughout the body. This type of myeloma is popularly known as Kahler's disease/ plasma cell myeloma.

Melanoma is the cancer of melanocytes – the specialized cells that produce melanin. Normally melanomas are formed in the skin but can often be found in regions where pigmentations are observed significantly as in the eyes.

There are various types of brain & spinal cord tumor and their nomenclature depends on the type of cell where they are generated or on the source of the first tumor found in the CNS. A brain tumor can either be benign or malignant. The tumor which initiates in the cells that give emergence to sperms or eggs are called as germ cell tumors. A neuroendocrine tumor is a tumor that is formed from cells that release hormones into the bloodstream as a response to the signal obtained from CNS. Tumors of such origin may result in higher than normal quantity of hormones leading to many different symptoms. Another type of neuroendocrine tumor is known as a carcinoid tumor. These tumors usually progress slowly and are mostly found in the gastrointestinal system, commonly in the rectum or small intestine. They may metastasize to the liver or other sites in the body resulting in the secretion of substances like serotonin and prostaglandin which are responsible for causing carcinoid syndrome.

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REVIEW OF LITERATURE

1.2 Literature Review

This study has been intended to perform phytochemical evaluation and anti-cancer potential by various scientific and well-accepted methods by using *Achyranthes coynei* Sant. Family Amaranthaceae and *Clerodendrum inerme* Gaertn. Family - Verbenaceae. These ethnomedicinal plants were used to understand their effect on cancer by using well-established methods and strategies for scientific validation of the traditional claims.

As of now the treatment available for cancer is chemotherapy, radiotherapy and excision of the tumor. The cytotoxic drugs are very harmful to the body unless they are very specific to the cancer cells. The long-term treatment with anti-cancer drugs is strongly associated with serious & severe side effects. The use of traditional medicines and conventional practices has attracted attention to improving healthcare outcomes, by curing various diseases. [24]

The study of traditional knowledge is the preservation of a rich repository of empirical knowledge about plants and their medicinal uses. On the perspective of traditional medicine is its potential contribution to integrative medicine. Recently the uses of traditional medicines have come into codifying and non-codifying systems practice & have attracted the attention in relation to improving the healthcare outcomes. [25] Biodiversity refers to the variability among all the living sources including both – the terrestrial and the aquatic ecosystems. It also includes diversity that exists within species, between species and also that which exists between ecosystems. The biodiversity hotspot concept includes rich biodiversity of unique species, the endemic plant species (~1500 species). The global diversity hotspots comprise 2.3% of the earth's land area. There is a total of 34 biodiversity hotspots in the world, out of that India has 2 biodiversity hotspots, one is the Himalayan region and second in the Western

Ghats region. [26] These regions have a plethora of plants with medicinal use that have been used for varied purposes in the traditional medicine system.

As per WHO traditional medicines include heterogenous health practices, varied approaches, and abundant knowledge based on plants, animal, minerals, spiritual therapy, manual therapy techniques & exercises; either applied solely or in combination [27, 28] Traditional medicine and practices are classified into (1) Codified system of medicine, which has pharmacopeias and ancient script (2) Non-Codified system of medicine that includes folk medicine that is administered by oral means & is acquired by trial & error approach (3) Spiritual and shamanistic medicine that has religious/spiritual faith (4) Allied form of health knowledge which includes Yoga, Tai-Chi, different forms of meditation and breathing techniques [29] .

The traditional knowledge and ethnic practices are diminishing with time because of inadequate support and practice. Therefore, there is a serious need for proper documentation to preserve this ancient, non-codified traditional knowledge. Despite of the available treatment, approximately 80% of the population still prefers the traditional medicine system over modern medicine in primary healthcare. [30, 31, 32] The traditional system of medicine is linked to the rich biodiversity of the Western Ghats. Various attempts have been made to document the medicinal property and related information on tribal case studies and cure for selected ailments. There are very few references available for studying and understanding various aspects of cancer treatment using medicinal plants. The current study, emphasizes to understand the anti-cancer potential of the selected ethnomedicinal plants for scientific validation of folklore claims. The present work is also an attempt to analyze and evaluate the traditional knowledge, practice and application of ethnomedicinal plants in the treatment of cancer.

The plants selected for the present study were *Achyranthes coynei* Sant. Family Amaranthaceae and *Clerodendrum inerme* Gaertn. Family Verbenaceae. These plants were used as ethnomedicine to cure various ailments. *Achyranthes coynei*, is a wild shrub species that belongs to Verbenaceae family. [31, 32] The mention of this plant is present in the ancient Indian literature like Ayurveda and has been used in various traditional medicinal preparations.

1.2.1 Description of Plants

1. *Achyranthes coynei* Sant.



Figure 1: Aerial parts of *Achyranthes coynei* Sant.

Names of *Achyranthes coynei* Sant. in Different languages [33,34]

Sanskrit : Apamarg

Hindi : Apamarg

Kannada : Uttarani, Keuvemp uttarani

English : Chaff-flower, Prickly chaff flower, Devil's horsewhip

Synonyms: - *Achyranthes aspera*, *Achyranthes porphyristachya* Wall

Taxonomical Classification [34]

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Caryophyllales

Family : Amaranthaceae

Genus : *Achyranthes*

Species : *Achyranthes coynei* Sant.

Achyranthes coynei Sant. belongs to the Amaranthaceae family that has 850 species that belong to 71 different genera, of which India is enriched with 60 species that belong under 20 genera. In the world, around 15 species are distributed in the tropical and subtropical regions. [35-37] In India, *Achyranthes aspera* and *Achyranthes bidentata* is found and reported from Karnataka, India. *Achyranthes coynei* Santapau, a perennial shrub which grows up to a height of 2-4.5 meters with profuse branching. In 1949, it was first reported from Khandala, Maharashtra by Santapau. This species has been recorded from Raigad, Sindhurg, Thane and Amravati, making it endemically distributed. It is considered as red-listed endemic plant species and it has been mentioned in the Red Data Book of Indian Plants, and in ENVIS Centre on floral diversity. Normally this plant species is found along the roadside, canal bunds and under the shade. [38-41]

Achyranthes coynei is a small beautiful shrub that blooms to give a reddish coloured flower that is very attractive. This reddish flower gives this plant a distinguished character and is useful for medicinal purpose. [42]

A. coynei is used for the treatment of various ailments viz. cough, fever, piles, urinary calculi, scorpion sting, anaemia and wounds by traditional practitioners; leaves and stem are used in the form of decoction, paste/poultice, or ash. The literature available on *A. coynei* is very limited. Studies have mostly been concentrated on pharmacognostic, phytochemical and antioxidant properties. Due to similar morphological characteristics as *Achyranthes aspera*, this plant has been exploited for cancer treatment by traditional practitioners. In Ayurveda, *Achyranthes aspera* is a medicinal plant that is used in cancer therapy.

The methanolic leaf extract exhibits anti-proliferative potential on human epithelial cancer cells. This plant has an antiviral potential against Herpes Simplex Virus - type 1 i.e. HSV-1 & type 2 i.e. HSV-2 due to the presence of Oleanolic acid (OA). This is a major component in the plant, and it has potent anticancer properties. [43] Other studies based on real-time PCR suggest the expression of metastatic & angiogenic genes & transcription of metallo-proteases, inhibitors of MMPs & angiogenic factors VEGF-A & VEGF-B. This study also suggests the need for *in vivo* activity. [44] The plant exhibited cytotoxic activity against colon cancer i.e. COLO-205 cells by clonogenicity, migration and apoptosis. The mechanisms were based on caspase - 9, caspase - 3, p16, p21, p27, Bax & Bcl-2 gene expression, chromatin condensation and DNA fragmentation. The flow cytometric analysis detected the cell cycle to be arrested at S phase. [45] The methanol extract of the plant also exhibited wound healing activity. [46]

A. coynei Santapau is a species endemic to Maharashtra state. The plant is a profusely branched shrubby habit approximately 3.0 - 3.5 m tall, initially erect, later patent & finally it is deflexed on the inflorescence, the opened flowers have a diameter of 2 cm & its colour is rosy-purplish. These features distinguish it from all other species

that are enclosed under *Achyranthes*. It has been reported to be present in 3 new localities of Belagavi district, highlighting the extent of distribution of *A. coynei* in Karnataka State. [47] The plant is also recently found in the Kutch desert. It is endemic to India and is a threatened species. [48] As per reports from Karnataka and Maharashtra states of India, *A. coynei* is a rare & endemic perennial shrub that is used to treat various disorders, with antimicrobial and antioxidant properties. Also, it was reported for its distinguishing morphological characters like Perennial, shrubby nature and woody stem. In the preliminary phytochemical screening, ash & extractive values, macro as well as micro elements & nutritive factors along with secondary metabolites that include alkaloids, saponins & triterpenoids were found. The presence of oleanolic acid was analyzed by HPTLC. It was suggested that for identification and authentication the stem part was useful. [49] The plant, importantly leaves, are utilized for the treatment of varied ailments by ancient folk healers. Pharmacognosy includes morphological, physicochemical parameters & microscopic characters of the leaf of *A. coynei*. HPTLC analysis was performed, for identification and authentication of the plant. [50, 51] *In-vitro* rapid multiplication protocol for ex-situ conservation was carried out to protect the plant from extinction. [52]

The plant *Achyranthes aspera* and *Achyranthes coynei* have similar chemical content but they are different from each other and both are used in traditional medicine. Further, *Achyranthes coynei* and *Achyranthes aspera* leaf and stem materials were evaluated for HPTLC analysis to evaluate and elucidate differences within and among the species. The Densitometric analysis showed chemical variation in the species. [53] The anti-oxidant activity & total phenolic content of *A. coynei* were tested since it is used on the same lines as *A. aspera* by the traditional practitioners of Belgaum. Various extraction procedures viz. continuous shaking, ultra-sonic extraction technique &

microwave-assisted technique were carried out at different time periods. It was found that the phenolic content, tannic and caffeic acid were equivalent. [54] *A. coynei* was tested for the accumulation & trends in the distribution of triterpenoids: Betulinic, Ursolic acids (BA, OA, and UA) & Oleanolic acid in leaves, stem, root & inflorescence by using Reversed Phase-Ultra Flow Liquid Chromatographic (i.e. RP-UFLC) technique. [55] The plant is traditionally known and acknowledged for curing and treating the various health ailments. The root of the plant is used for medicinal purposes. [13, 56]

2. *Clerodendrum inerme* (L.) Gaertn.

The word *Clerodendrum* was originated from the Greek words - 'kleros', which means chance/ fate & 'dendron' which implies to be a tree.



Figure 2: Aerial parts of *Clerodendrum inerme* (L.) Gaertn.

The Common Names: [57-59]

Sanskrit : Kundali

Hindi : Sankuppi

English : Wild jasmine, Indian privet, Garden queen

Synonyms: *Volkameria inermis* L

Taxonomical Classification [59, 60]

Kingdom : Plantae,

Subkingdom : Tracheobionta,

Superdivision : Spermatophyta,

Division : Magnoliophyta,

Class : Magnoliopsida,

Subclass : Asteridae,

Order : Lamiales,

Family : Verbenaceae,

Genus : *Clerodendrum* L.,

Species : *Clerodendrum inerme* (L.) Gaertn.

It is a tropical plant and widely distributed across India, Sri Lanka, Southeast Asia, Nepal, Bangladesh, and the Mediterranean. *C. inerme* is 1-1.8 m tall evergreen shrub with woody stems. The leaves are simple, (5-10 cm) long smooth, slightly shiny, ovate to elliptical, tip acute to acuminate, venation pinnate, margins of entire leaves are opposite. It comprises of three flowers connected at a common point at the base, usually cyme or umbel, 5 lobes, fused white corolla, 4 stamens, upwardly curved and reddish to purple. Fruit 1 – 1.5 cm long, obovoid, green turning black. [61,62]

Clerodendrum inerme has various traditional uses as a febrifuge, antiseptic, bleeding control, uterine stimulant, pest control agent, asthma, ringworm, hepatitis &

stomach pains [63], for scrofulous and venereal infections, antidote for fish, crabs, toadstools poisoning [64], leaf juice for skin diseases. The oil boiled roots are used in rheumatism. [65, 66]

HPTLC work is reported for 2 sterols, triterpene, stigmasterol glucoside, betulinic acid, pentadecanoic acid- β -D-glucoside, acacetin, and apigenin was isolated. [67] The *Clerodendrum* species - *C. indicum*, *C. trichotomum*, *C. chinense*, *C. phlomidis*, *C. serratum*, and *C. petasites* are utilised for rheumatism, coughs, asthma, skin conditions, malaria, inflammatory diseases, febrifuge & vermifuge as a traditional & alternative medicine [68, 69, 70] treatment of inflammation-related diseases. [71] The plant extract exhibited cytotoxic activity *in-vitro* when a normal fibroblasts (mouse), human cancer-cells - HT-29, AGS & MDA-MB-435S. [72] This plant extract also exhibits genomic stability, chromosomal aberration and tissue protection [73], chemo preventive and anti-lipid peroxidative effects [74], skin cancer and RBC membrane integrity potential [75] effective against oral carcinogenesis animal model [76, 77] using the plant for pharmacognostic studies, the Ursolic acid identification was performed [78] apart from this Pentadecanoic acid- β -D-glucoside, acacetin and apigenin are also found. [79]

Clerodendrum inerme is used in the treatment of piles, urinary calculi, scorpion sting, anemia, wound, cough, fever, and antitumor agent in traditional practices. It also exhibits antimalarial, antioxidant hepatoprotective, anti-inflammatory, antimicrobial, antiviral, anti-haemolytic, anti-hypotensive, an antifungal activity. Some of the reported research work suggests it has anti-cancer activity. Standardized drugs have not been used for testing the anticancer activity using animal models in most of the studies. Also, there is a need for proper scientific validation for its traditional claim. Therefore, the present study is design based on well-accepted *in-vitro* and *in-vivo*

models. Phytoconstituents which possess anti-cancer potential viz. Oleanolic Acid, Ursolic Acid, Betulinic Acid Caffeic Acid, Tannic Acid, etc. are present in the plant.

The potency of Ursolic acid against breast cancer along with anti-inflammatory effects was studied. The results showed that it affects the proliferation through G1/G2 arrest and regulates the expression of key proteins in signal transduction pathways. [80] The structural modifications of Ursolic acid also exhibited inhibition of growth on diverse cancer cells with the least harmful effects on normal cells. The mechanism of action is by the depolarization of membrane potential of mitochondria, cell arrest in the G0/G1 phase & apoptosis/necrosis found in HepG2 cells and H22. [81] A study was performed on the efficacy of Ursolic acid against tumor growth and angiogenesis and its molecular mechanisms.[82] Oleanolic acid has consequential pharmacological activities that include regulating blood sugar level, anti-tumor properties & liver protection. It also has anti-proliferation and apoptosis potential on human bladder cancer through mTOR, Akt, ERK1/2 & S6K protein phosphorylation. [83] Studies on Betulinic acid revealed its anticancer potential through mitochondrial Bax/Bak-independent release of cytochrome-c, which is the death signal for cancer cells [84] Tannic acid also exhibits antitumor activity on human breast cancer cells and on animals with DMBA-induced mammary tumor. The effect of tannic acid was identified using the western blotting technique to check for expression of Bcl-2, PARP-1, Bax, & p53. The results showed a reduction in Bax, activation of PARP-1 & an increase in Bcl-2 expression as seen in the H9c2 cells. In a mammary tumor, there was increased expression of p53 and a reduction in tumor volume. [85] A brief review was attempted for studying the anti-tumor activities of Ursolic, Oleanolic & glycyrrhetic acid. These 3 acids & their derivatives show consequential anti-tumor promoting & cytotoxic activities, inducing apoptosis, inhibiting proliferation & preventing invasion. This

suggests that they could be developed as the anti-cancer and cancer chemo-preventive agents. [86]

1.3 Justification

The ethnomedicinal plants are used for various disease treatment because of their availability in their region. The geographical area, its usage and practice vary accordingly at plants and from the literature, it is evident that there are various ethnomedicinal being used in various parts of India. From the Western Ghats, there is no evidence reported for scientific validation of anticancer property used by the traditional healers and local practitioners.

The long-term cancer treatment with cancer treatment is associated with severe side effects because they are not specific to only cancer cells. Hence, there is a need for the pharmacological approach to scientifically validate their safety and efficacy of the ethnomedicinal plants. For the present study, *Achyranthes coynei* and *Clerodendrum inerme* are selected. These ethnomedicinal plants are used in the treatment of cough, fever, piles, urinary calculi, scorpion sting, anaemia, wound and antitumor agent. Which also exhibits antimalarial, antioxidant hepatoprotective, anti-inflammatory, antimicrobial, antiviral, anti-haemolytic, anti-hypotensive and antifungal activity evaluated by various studies. The phytoconstituents (Oleanolic Acid, Ursolic Acid, Betulinic Acid Caffeic Acid, Tannic Acid) are present in both the plant and they possess anticancer potential. Plant-based drugs are not toxic in comparison with modern drugs which are the first line of treatment.

Ethnomedicinal plants were used by the non-codified practitioners since ages to cure various diseases. Hence, there is a need for the pharmacological approach to scientifically validate their safety and efficacy of the ethnomedicinal plants. Further, this study aims to provide scientific validation to its folklore claim with reference to the non-codified practice of traditional medicine. The medicinal plants are highly

recognized and well known for therapeutic purpose. Due to the increasing population, there is a need for a substantial number of medicinal plants as they are quite popular amongst traditional practitioners. Therefore, the chance of adulteration and substitution is increased. The ethnomedicinal plants used for the treatment of cancer do not have a proper scientific validation in comparison with standard drugs used in modern medicine system. The various approaches to evaluate the anticancer potential by using different *in-vitro* methods using various cancer cells, different *in-vivo* methods with different approaches and network pharmacology aspects are helpful and these are the well-known and acceptable methods are available. Various approaches are needed to evaluate the anticancer potential of ethnomedicinal plants for its traditional claim. In view of the above investigations, there is a need to conduct a scientific study on its anticancer potential.

AIM & OBJECTIVES

1.4 Objectives of the study

Aim: Evaluation of the selected ethnomedicinal plants for the anticancer property by performing the anticancer activity of *Achyranthes coynei* Sant. Family Amaranthaceae and *Clerodendrum inerme* (L.) Gaertn. family Verbenaceae, ethnomedicinal plants by using *in-vitro* assay methods and *in-vivo* models.

Objectives

Primary Objectives

To evaluate the anticancer activity of *Achyranthes coynei* Sant. and *Clerodendrum inerme* (L.) Gaertn. ethnomedicinal plants by *in-vitro* assay methods

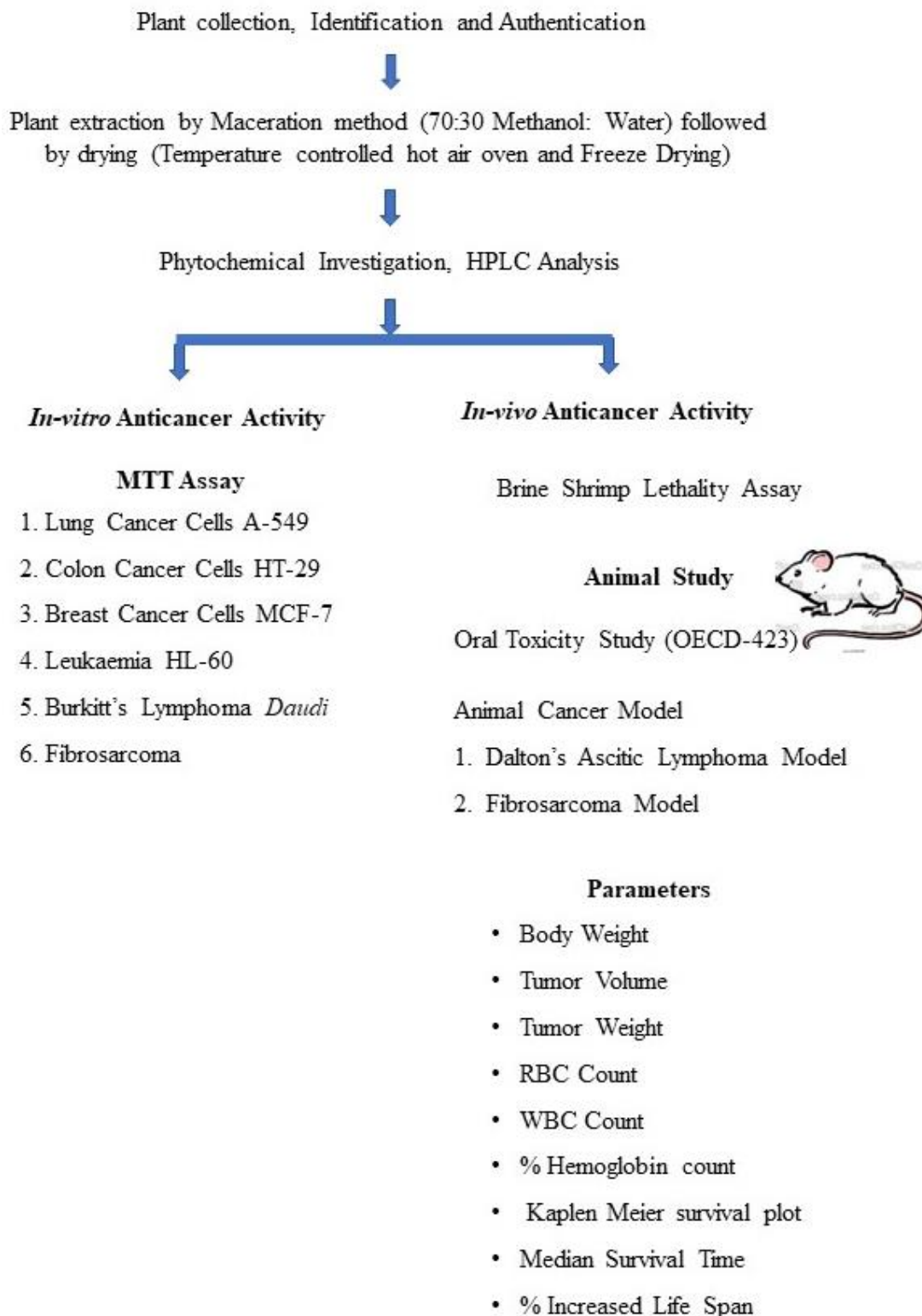
Secondary Objectives

To evaluate the safety and efficacy of *Achyranthes coynei* Sant. and *Clerodendrum inerme* (L.) Gaertn. ethnomedicinal plants by *in-vivo* models.

MATERIALS & METHOD

2. Materials and Methods

Schematic representation of the proposed research work



2.1 Selected Medicinal Plants

1. *Achyranthes coynei* Sant. (Family: Amaranthaceae)
2. *Clerodendrum inerme* (L.) Gaertn. (Family Verbenaceae)

1. *Achyranthes coynei* Sant.



Figure 3: Aerial parts of *Achyranthes coynei* Sant.

2. *Clerodendrum inerme* (L.) Gaertn

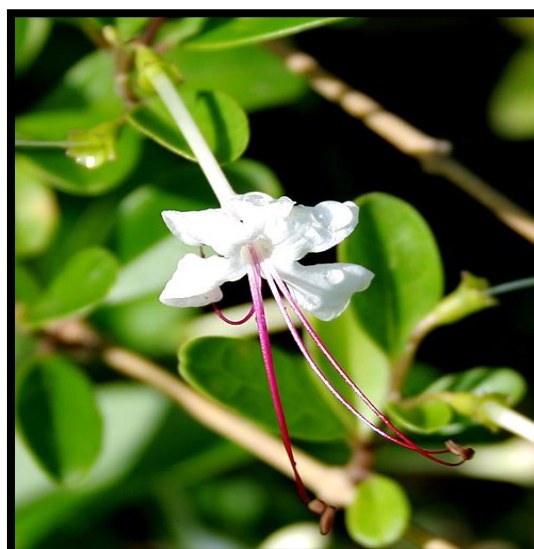


Figure 4: Aerial part of *Clerodendrum inerme* (L.) Gaertn.

2.2 Ethnomedicinal Plant - Collection & Authentication

Ethnomedicinal plant *Achyranthes coynei* was collected from Dandeli-Anshi tiger reserve area and *Clerodendrum inerme* were collected from Sulebhavi region of Belagavi, Karnataka, INDIA. Further plant material was authenticated by taxonomist and herbarium submitted at the National Institute of Traditional Medicine- Indian Council of Medical Research (ICMR-NITM), Belagavi, Karnataka, India.

S. No.	Place	Latitude (°N)	Longitude (°E)	Altitude (m)	Remarks
01.	Dandeli-Anshi Tiger reserve	15.01148° N	74.39502° E	529	Forest
02.	Sulebhavi	15.8946° N	74.6561° E	764	Village Side

Table 1: Geographical coordinates of plant collection sites

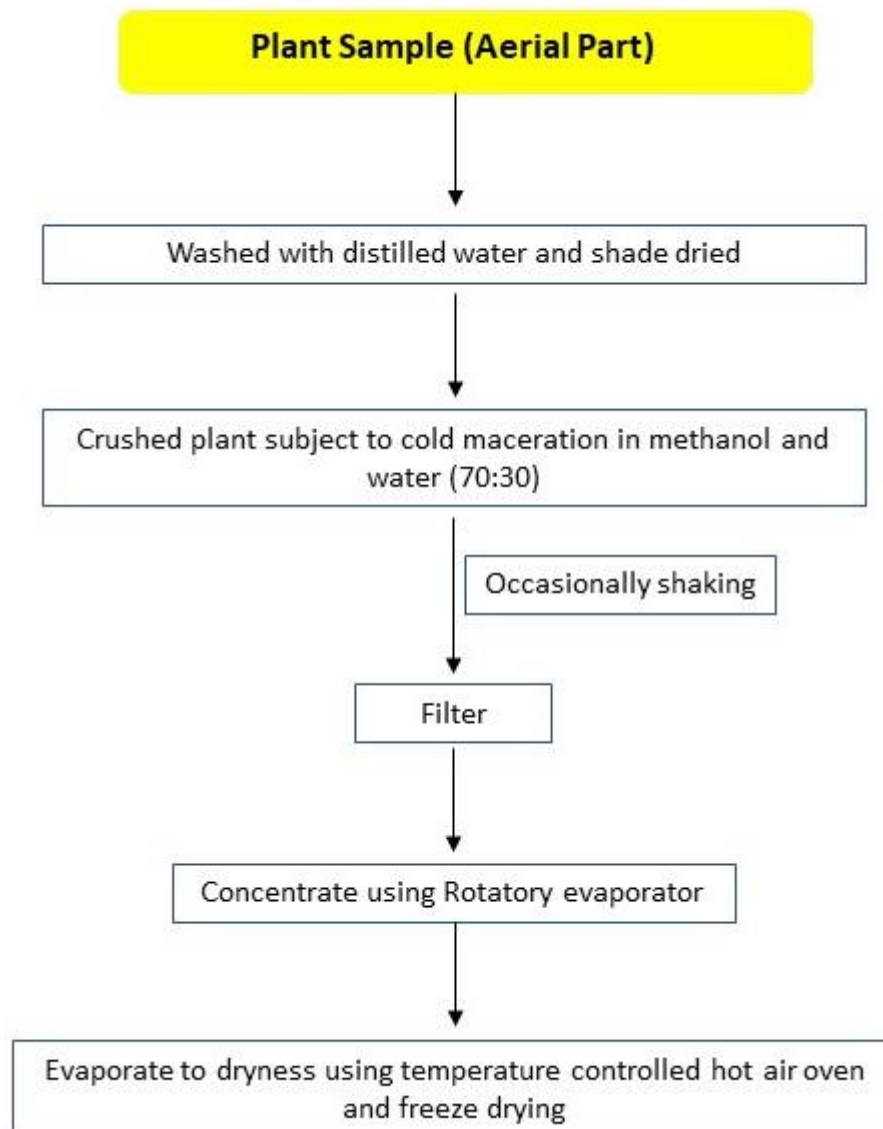


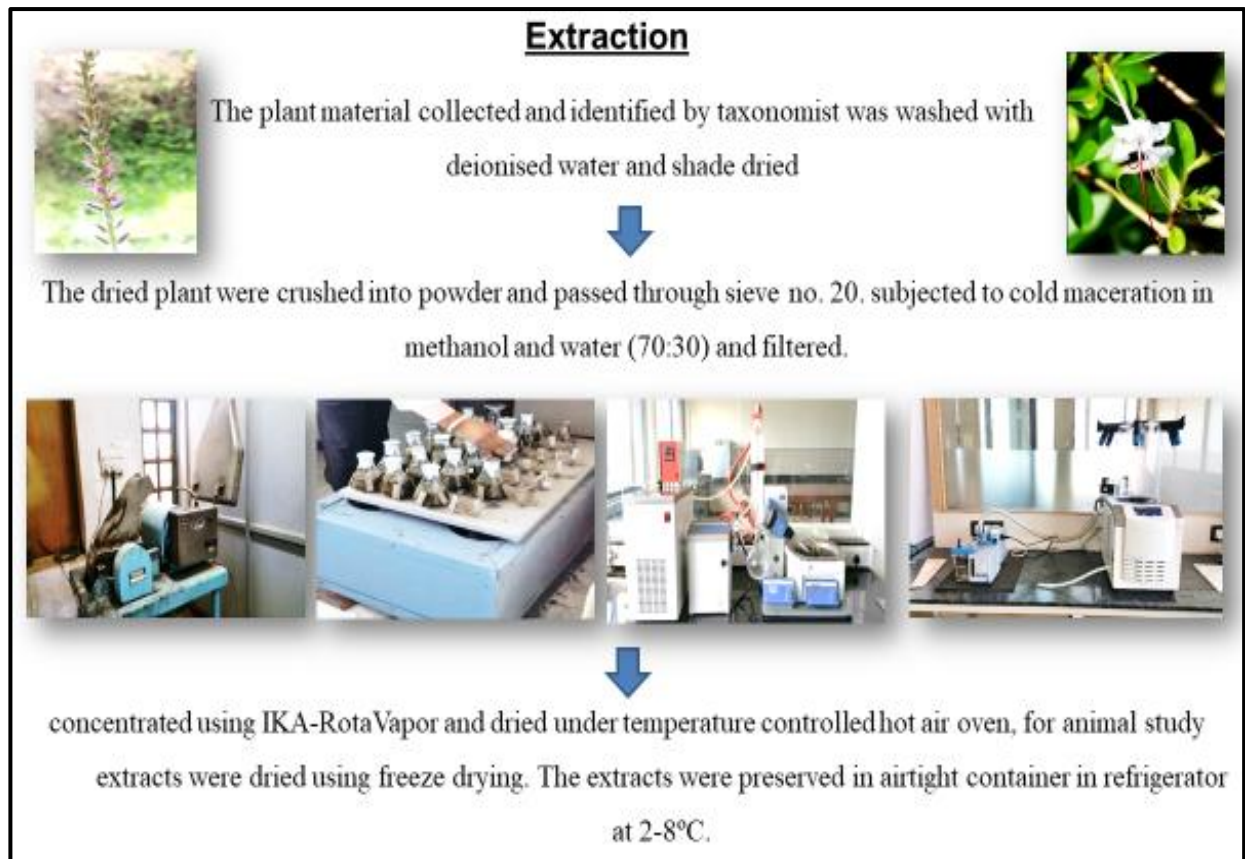
Figure 5: Map of Western Ghats and Plant collection sites.

2.3 Extraction of Medicinal Plant Material

Aerial part of plant material was washed using distilled water and shade dried. This shade-dried plant was crushed and sieved (No. 20). 100 g dried coarse plant material was macerated in a mixture of methanol and water (in the ratio of 70:30) for 3 days with occasional stirring.

After maceration, Whatman filter paper no.1 was used for filtration and concentrated using IKA-Rotatory evaporator and, dried under temperature-controlled hot air oven, freeze-dried extracts were used for animal study. Storage was done at 2-8°C. [87]





A. Coynei

Shade Dried Plant



C. inerme



Crushing



Maceration



Shaking



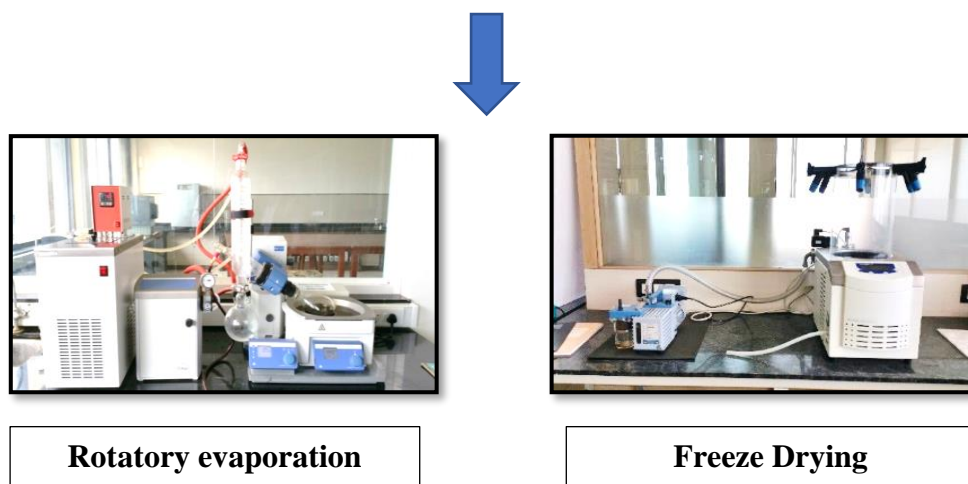


Figure 6: Different process of extraction.

2.4 The Yield of Plant Extract

The yield of plant extract was calculated at per 100 gm by the following formula.

$$\% \text{ yield} = \frac{\text{Total weight of extract in gms}}{\text{Total weight of plant material in gms}} \times 100$$

2.5 Preliminary Phytochemical Investigation of Plant Extract [49, 88]

The preliminary phytochemical & qualitative investigation was performed for the presence of secondary metabolites like carbohydrates, amino acids, protein, steroids, alkaloids, tri-terpenoids, flavonoids, glycosides, tannin, phenolic compounds, fats & oils by using the standard procedures.

Preliminary Phytochemical Investigations

Test for Carbohydrate-

Molisch Test-

The extract solution added a few drops of alcoholic alpha-naphthol, then added few drops of concentrated sulphuric acid through sides of the test tube. Purple to violet color ring appears at the junction which indicates the presence of carbohydrates.

Fehling's Test-

One ml of Fehling's A and Fehling's B solutions was mixed and boiled for one minute. An equal volume of test solution was added, heated in boiling water bath for 5-10 min. Observed for yellow, then brick-red precipitate which indicates the presence of carbohydrate.

Barfoed's Test-

Barfoed reagent and test solution heated for 1-2 min, in boiling water bath and cooled. Observed for red precipitate which indicates the presence of carbohydrate.

Cobalt-Chloride Test-

Test solution three ml mixed with two ml of cobalt chloride, boiled and cooled. FeCl_3 drops were added on NaOH solution. Observed for greenish-blue which indicates the presence of glucose, purplish which indicates the presence of fructose and upper layer greenish-blue and lower layer purplish which indicates the presence of a mixture of glucose and fructose.

Test for Starch:

Tannic Acid Test- With 20% tannic acid, was added to test the solution. The formation of a precipitate indicates the presence of starch.

Test for Proteins-

Tri Chloro Acetic Acid Test- To the extract solution tri chloro acetic acid was added. The formation of precipitation indicates the presence of protein.

Biuret Test- To the 2 ml. extract solution 2 ml Biurate reagent produces violet color which indicates the presence of protein.

Test for Amino Acids-

Ninhydrin Test-

To the test solution, the ninhydrin solution was added and boiled. Violet color Indicates the presence of amino acids.

Test for Steroids and Triterpenoids-

Salkowski Test-

To 2 ml of extract solution, 2 ml of chloroform and H_2SO_4 were added, and shake well, whether two layers as chloroform layer appeared red in color which indicates the presence of steroids and acid layer appeared greenish-yellow fluorescence which indicates the presence of triterpenoids.

Libermann-Burchard Test-

Extracts were treated with a few drops of acetic anhydride, boiled and cooled, then concentrated H_2SO_4 was added from the side of the test tube wall. Brown ring formed between junction two layers and the upper layer turns in to green color which shows the presence of steroids and red color which indicates the presence of triterpenoids.

Test for Glycosides

Test A-

Extract (200 mg) was treated with 5 ml of sulphuric acid by warming on a water bath. Filtered and neutralized with 5% sodium hydroxide solution and 0.1 ml of Fehling's

solution A and B were added until it became alkaline and heated on a water bath for 2 min. The quantity of red precipitate formed was compared with that of form in Test B. If the precipitate in test A is greater than in Test B which indicates glycosides present.

Test B-

Extract (200 mg) with 5 ml. of water instead of sulphuric acid, boiled and equal amount of water was added. 0.1 ml Fehling's solution A and B were added until alkaline and heated on a water bath for 2 min. Test B represents the amount of free reducing sugar already present in the crude drug, whereas Test A. The quantity of red precipitate formed, was compared with the quantity of precipitate formed in Test B with that of form in Test A. Represents free reducing sugar plus those related to acid hydrolysis of any glycosides in the crude drugs

Saponin Glycosides-

Froth Formation Test-

Plant extracts 2 ml was Placed in the test tube and shake well formation of froth indicates the presence of saponins.

Test for Flavonoids

Shinoda Test-

To the test solution, few magnesium turnings and con. Hcl drop wise was added. Pink scarlet, crimson red or occasionally green to blue color appears after a few minutes which indicates the presence of flavonoids.

Alkaline Reagent Test:

To the test solution, a few drops of sodium hydroxide solution was added. The intense yellow color is formed which turns to colorless on the addition of a few drops of dilute acid. Indicate the presence of flavonoids.

Zinc Hydrochloride Test:

To the test solution a mixture of zinc dust and conc. Hydrochloric acid was added. Gave a red color indicates the presence of flavonoids.

Test for Alkaloids

Dragendorff's Reagent Test-

To Plant extract, Dragendorff's reagent (Potassium bismuth iodide solution) was added. Reddish-brown precipitate indicates the presence of alkaloids.

Mayer's Reagent Test-

To plant extract Mayer's reagent (Potassium mercuric iodide solution) was added. Cream color precipitate forms which indicate the presence of alkaloids.

Wagner's reagent Test-

To plant extract Wagner's reagent (Iodine – Potassium iodide solution) was added. Reddish-brown precipitate indicates the presence of alkaloids.

Hager's Reagent Test-

To plant extract Hager's reagent (Saturated solution of picric acid) was added. Yellow precipitate indicates the presence of alkaloids.

Tannins and Phenolic Compound-

Ferric Chloride Test-

Plant extract treated with ferric chloride Blue color present if hydrolyzable tannins and green color appear if condensed tannins are present.

Gelatin Test-

To the plant extract 1% gelatine solution containing 10% sodium chloride was added. Gives precipitate Presence of tannin.

Test for Catechin-

Dipped match stick into the plant extract solution, dried and moisten with concentrated HCl. Then warmed near the flame. The color of wood changes to pink due to phloroglucinol indicates the presence of tannin.

Test for Chlorogenic Acid-

Plant extract solution treated with aqueous ammonia and exposed to the air gradually Green color is developed indicates the presence of tannin.

Test for Fat and Oil-

Saponification Test- Added few drops of 0.5 N alcoholic potassium hydroxide to a small number of extracts along with phenolphthalein separately and heated on a water bath for 1-2 hours. The formation of soap or partially neutralization of alkali The presence of fixed oils and fats in extracts.

High-Performance Liquid Chromatography (HPLC)

Chromatographic system (Shimadzu, Japan) was utilized to perform HPLC investigation (LC-20 AD) with degasser (DGU-20A5), Rheodyne injector, quaternary pump, diode-array detector (SPD-M20A). LC solution software version 1.25 was used for data analysis. Detection wavelength at 254 with a PDA detector was executed for 20 minutes.

Standard and samples were prepared in methanol at concentration of 10 mcg per ml. filtered through 0.2-micron syringe filter. [55]

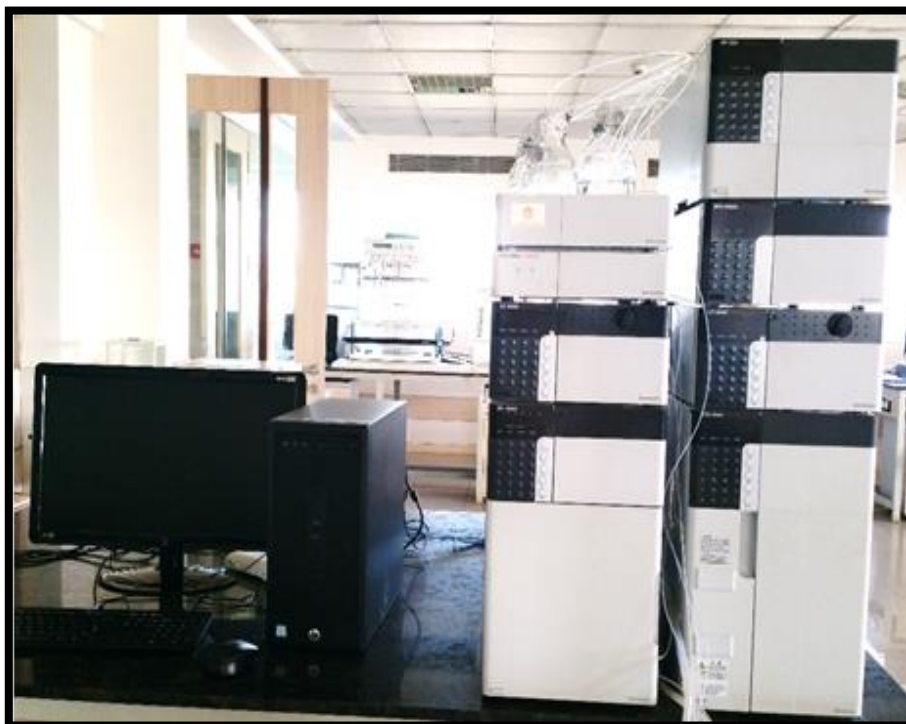


Figure 7: High-Performance Liquid Chromatography (HPLC)

Instrument Chromatographic Conditions:

1.	Instrument	HPLC
2.	Make	Shimadzu
3.	Model	LC-20 AD
4.	Column	THERMO Hypersil-Keystone C-18 250×4.6 mm Particle size- 5 μ
5.	λ_{max}	210 nm
6.	Mode	Isocratic
7.	Mobile phase	Methanol: Water (90:10) pH-5 (Glacial acetic acid)
8.	Injection volume	20 μ l.

2.6 *In-vitro* Studies

2.6.1 Dye Exclusion Assay [89]

This assay provides the number of viable cells that are available in a cell suspension for live cell count.

Cells were trypsinized and trypan blue dye was used for counting in Neubauer chamber



Counted cells were further calculated for live cells present in suspension by using the following formula

$$\text{No. of live cells present per ml} = \frac{\text{Live cell count}}{4} \times \text{Dilution factor} \times 10^4 (\text{Neubaur correction factor})$$

2.6.2 MTT Assay [90]

The MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first assay which is widely adopted. The viable cells containing active metabolism (mitochondrial enzymes) convert MTT to formazan crystals (violet in color) which can be detected at specific wavelengths.

MTT assay has been performed on following cancer cell lines

1. Burkitt's lymphoma cell lines –Daudi
2. Fibro sarcoma cell lines - HT-1080
3. Lung cancer cell lines - A-549
4. Breast cancer cell lines – MCF-7
5. Colon cancer cell lines – HT-29
6. Leukemia cancer cell lines – HL-60

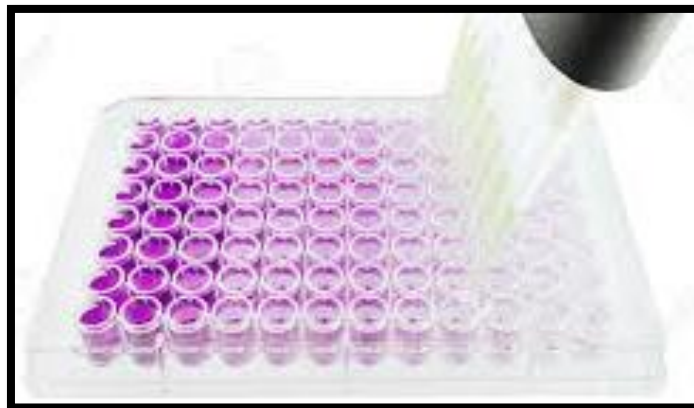
Cells count were performed by using trypan blue dye assay followed by cells were seeded into 96 well plate for MTT assay.

MTT Assay Procedure

10^4 cells/well were counted by the help of trypan blue dye exclusion assay and seeded in 96-well microplate.



24 hrs. of incubation then cells treated with various concentrations 1000,500, 250,125, 61.5, 31.25, 15.6 mcg/ml (two-fold dilution) of plant extracts for 24 hours.



MTT was added to each well and incubated for another 4 hours. The formazan products were solubilized by adding DMSO.



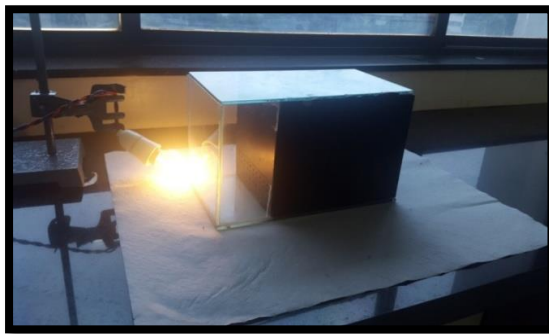
The optical density was measured at 570 nm using a microplate reader. The percent cell cytotoxicity calculated by the following formula of inhibition of growth:

$$\% \text{ Cell cytotoxicity} = 100 - \frac{(A_{control} - A_{Test})}{A_{control}}$$

2.7 *In-vivo* Studies

2.7.1 Brine Shrimp Lethality Assay [91]

Artemia salina Eggs (brine shrimp) were hatched by placing the eggs in 1 L of sea water with constant aeration in a fish tank and illuminated by a lamp to keep them warm.



Brine shrimp growing chamber



Eggs of brine shrimp



Brine shrimp at 20X



The brine shrimps were exposed 24 hours to control and plant extracts in various concentrations (Triplicates).



The viability of the brine shrimp is assessed based on movement.

2.7.2 Animal Study

Housing and Feeding Conditions:

Animals of the same gender were housed in small groups. The temperature in the experimental room was at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ with relative-humidity at 48% & the sequence lighting i.e. 12 hours dark & 12 hours light. A laboratory-based diet with easily available drinking water was followed. The Acclimation time was 7 days for laboratory conditions and each animal assigned a unique marked with a marker for identification.

2.7.3 Oral Toxicity Study [92]

OECD guideline 423 was followed with the initial extract concentration of 2000 mg/kg which was given to three female mice orally. The animal was critically observed after the oral dose and up to 14 days observation was performed to find any adverse or toxicity effect of the extract. The test procedure is mentioned in Figure:8.

OECD 423

As per the principle of the test, the stepwise procedure was followed. A defined amount of extract as per body weight was orally given to the female albino mice. The test compound-related mortality was recorded to determine the steps like-

1. No further testing of test compound is required
2. Additional dosing of three animals
3. Another three animals for dosing at the next higher or lower dose level

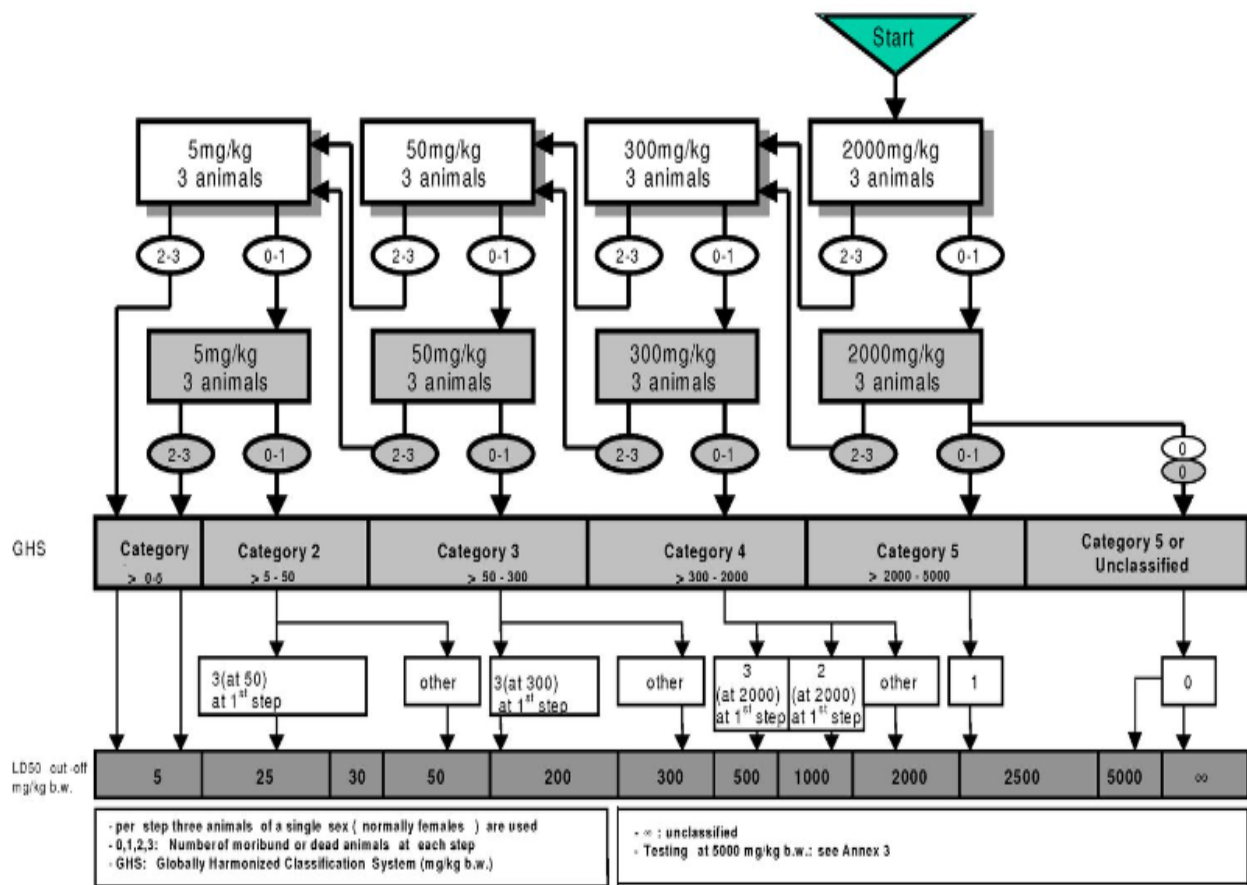


Figure 8: OECD 423 guideline flowchart

Animal selection- nulliparous female albino mice (8-12 weeks old & weight interval b/w \pm 20% of the mean weight) were selected for the procedure. The housing of the animal experiment room was maintained at 22°C & light-dark cycle of 12 hrs with laboratory animal diet and water was followed.

The preparation of dose as per 2 ml/100 gm (Should not exceed) body weight was considered. the dose was given by gavage (stomach tube). The single dose of 2000 mg per kg (highest starting dose) was followed as per chart to three animals followed by a limit test conducted. The time duration of the test was based on the onset, duration, and toxic sign severity. Observation of individual animals was required for the first thirty minutes/kg and the first four hours to twenty-four hours, special attention was needed. If no toxicity viz. skin-fur changes, eye, mucous membrane, circulatory, respiratory,

autonomous, central, somatomotor activity, behavior changes, diarrhea, convulsions, salivation, lethargy, drowsiness and coma are observed then the dose will be selected.

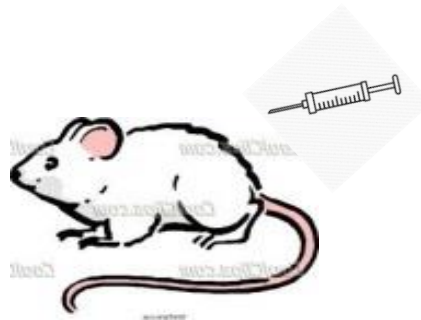
2.7.4 Preparation of Doses:

According to the OECD guidelines, doses of plant extracts were prepared & then administered orally in a continual volume over the dose range (1ml to 2 ml/100g body weight). The 22-gauge sized, bent and beaded oral needle was used for dosing.

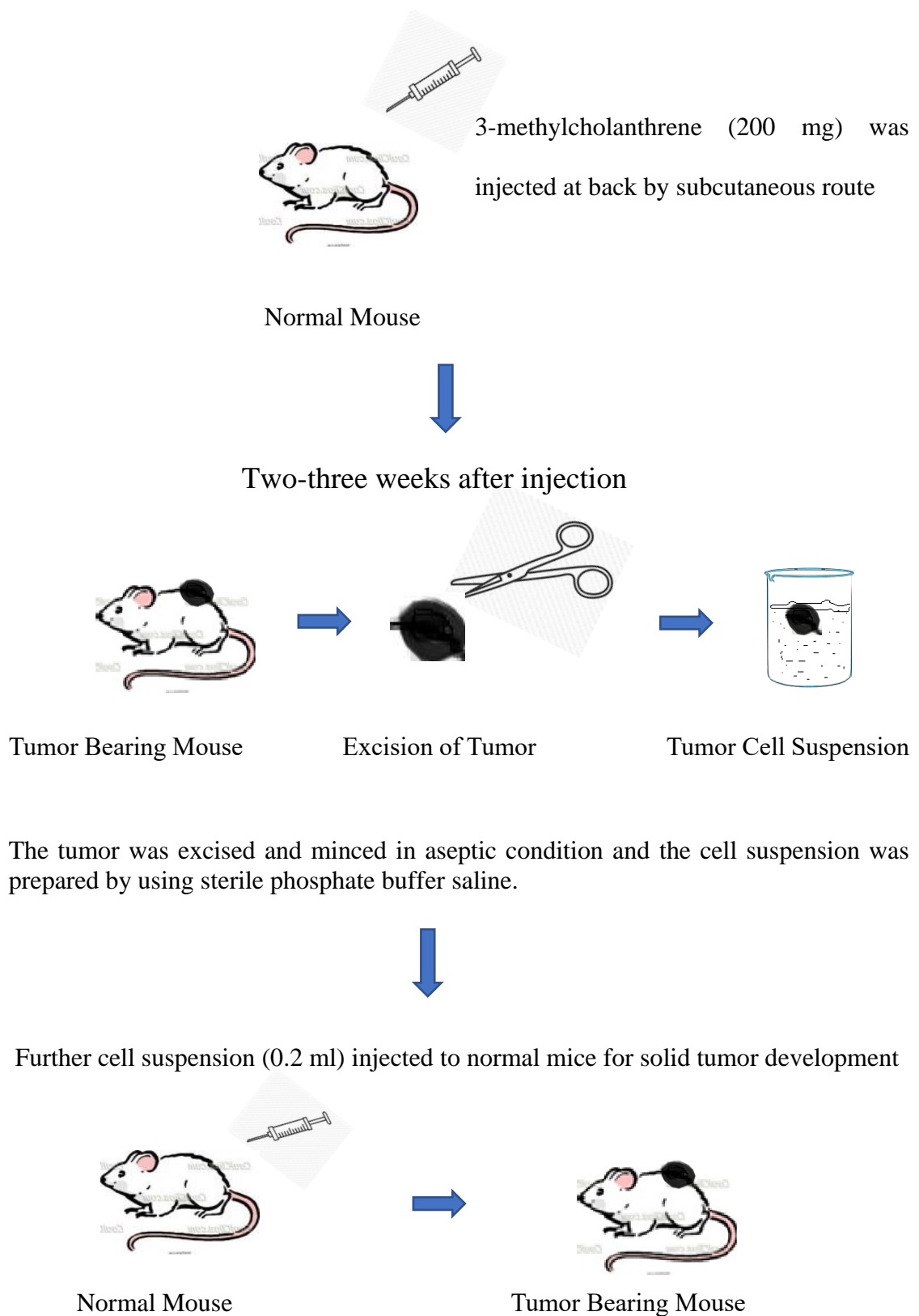
2.7.5 Tumor Induction

1. Dalton's Ascitic Lymphoma: [93,94]

For tumor induction cell suspension (0.2 ml of 2×10^5 cells/mouse) from tumor-bearing mouse was injected intraperitoneally to the animals. The injected cancerous cells multiply and induce the cancerous condition and the growth of the tumor is very high.



2. Fibrosarcoma (Solid Tumor): [95, 96]



2.7.6 Group distribution for cancer model in mice

Groups	Treatment	No. of Animals
Group- I	Normal saline (2ml/kg), p.o	6
Group-II	Control group	6
Group-III	Standard Drug Doxorubicin (2.5 mg/kg), i.v.	6
Group-IV	<i>Achyranthes coynei</i> extract (200 mg/Kg) p.o	6
Group-V	<i>Achyranthes coynei</i> extract (400 mg/Kg) p.o	6
Group-VI	<i>Clerodendrum inerme</i> extract (200 mg/Kg) p.o	6
Group-VII	<i>Clerodendrum inerme</i> extract (400 mg/Kg) p.o	6

Group-III Standard drug	Daltons Ascitic Lymphoma Model	Fibrosarcoma Model
Doxorubicin (2.5 mg/kg) [97]	I.P Route First dost- 24 hrs. later on every 4 th day 15 days of study	I.V. Route (Tail Vein) First dost- 24 hrs. later on every 4 th day 15 days of study

2.7.7 Evaluated Parameters

(A) Body weight

(B) Haematological parameters

- a) RBC Count
- b) WBC Count
- c) % Haemoglobin

(C) Tumor parameters

- a) Tumor weight
- b) Tumor volume
- c) Kaplan Meier survival plot
- d) Median survival time
- e) Percent increased life span

(A) Body Weight:[98]

The female albino mice selected for study and same weight ($\pm 20\%$ of weight variation acceptable) of the animal selected for the study. Weight has been recorded using weighing balance before the experiment and after the experiment.

(B) Haematological parameters

a) Red Blood Cell Count (RBC Count):[99]

RBC count was evaluated using a hemocytometer counting chamber which is made up of thick glass slid & a central platform that is surrounded by a gutter which is divided into two segments. The platform is 0.1 mm deep from below formed when covered with a coverslip. The engraved area measures 3 mm \times 3 mm (i.e. 9 mm²). By triple lines, the central area gets divided into 25 equal squares which further have 16 very small squares with an area of 0.0025 mm². RBC counting standard procedure is as given below.

Procedure:

Materials: Blood in Eppendorf tube, reservoir and capillary pipette, hemocytometer and its cover, a box of Kimwipes, microscope, hand counter, plasticine in a small plastic tray, Tallquist test paper, Tallquist reference scale, Tallquist test paper, Tallquist reference scale.

Red blood cell production occurs in the bone marrow and is dependent on a number of factors including iron (a component of haemoglobin), vitamin B₁₂ and folic acid (which are necessary for normal mitosis). Erythropoietin, a hormone produced by the kidney, regulates the rate of red cell production.

The hemocytometer counting chamber is a special thick glass slide with a central platform, divided into two and surrounded by gutters. The platform is exactly 0.1 mm below the surface of the slide. When the special thick (and expensive!) coverslip is placed on the slide, a chamber 0.1 mm deep is formed. In the center of each half of the platform is an engraved area 3 mm x 3 mm (9 mm²) (Figure 9). It is divided into 9 equal areas, each 1 mm². The central area is further divided (by triple lines) into 25 equal squares; each of these is again divided into 16 very small squares, each with an area of 0.0025 mm.

1. Examine the Unspotted Reservoir for red blood cell determinations and the capillary pipette assembly. Identify the reservoir chamber, diluent fluid, protective shield on the capillary pipette and the pipette itself. Note the color of the reservoir's bottom surface.
2. Hold the reservoir on a flat surface in one hand, and hold the pipette assembly on the other hand. Push the tip of the pipette shield firmly through the diaphragm in the reservoir neck. Pull out the assembly unit from the reservoir and remove the protective shield from the pipette assembly with a twist.

3. Inverted gently the Eppendorf tube containing the blood 2-3 times (to avoid blood cells settling at the bottom of the tube).
4. Open the Eppendorf tube containing ovine blood. Holding the pipette almost horizontally, touch the tip of it to the surface of the blood. The pipette was filled by capillary action. When the blood reaches the end of the capillary bore in the neck of the pipette, the filling action stopped. Carefully wipe any excess blood from the pipette's surface with a Kimwipe.
5. Squeeze the reservoir slightly, to force out a small amount of air. While still maintaining pressure on the reservoir, cover the opening of the overflow chamber of the pipette with your index finger and push the pipette securely into the reservoir neck.
6. Release the pressure on the reservoir, and remove your finger from the pipette opening. Negative pressures draw the blood into the diluent fluids.
7. Mix the contents of the reservoir chamber by squeezing the reservoir gently two or three times. Squeeze gently so that the diluent is not forced out of the chamber. Also, inverted the reservoir gently a few times.
8. Remove the pipette from the chamber, reverse its position, and replace it in the reservoir. This converts the system into a dropper assembly.
9. Squeeze a few drops out of the system into a container, or wipe with a tissue to clean the capillary bore.
10. Hold the end of the pipette and squeeze the sides of the reservoir so that you can deposit a small drop of the diluted specimen onto the polished surface of the counting chamber, next to the edge of the cover.
11. Carefully, place the hemocytometer on the microscope stage, and focus with the low-power objective to bring into clear view.

12. Then move the high-power objective (X10) into place, and count the number of cells in each of the five specified areas marked R in. On the edges of the squares, counted only the cells that touch the lines on the left and topsides. Omit the cells touching the lines at the bottom and right side.

Calculations: Calculate the number of red blood cells per mm^3 in sheep and human blood. Each tiny square has an area of 0.0025 sq. mm. and depth of 0.1 mm.

The volume is therefore 0.00025 mm^3 .

Thus, the number of cells per mm^3 in the diluted blood is: $N \times (1/0.02) = N \times 50$ (N being the number of cells counted in the 5 RBC squares of the hemocytometer)

$N \times 50 \times 200$, or $N \times 10,000 =$ number of RBC per mm^3 of whole blood.

Reminder: $1 \mu\text{l} = 1 \text{ mm}^3$

b) White Blood Cells Count (WBC Count): [99, 100]

The method used for the WBC count is almost similar as it is for RBC count. The standard procedure for WBC counting was followed.

The method used is similar to that used for the red blood cell count. There are a few differences. The white and red blood cell diluents differ. The white blood cell diluent haemolysis the red blood cells and thus they will not interfere with the white blood cell counting process. The number of cells will be counted in each of the four specified areas of the hemocytometer – 4 corners of the hemocytometer grid marked as a ‘W’.

1. Place the hemocytometer on the microscope stage. With the low power objective, focus on the chamber area to bring the four large (w) corner regions into view.
2. Determine the number of cells in each of the four specified areas. On the edges of

the squares, count only the cells that touch the lines on the left and topsides. Omit the cells touching the lines at the bottom and right side. It may be necessary to wait a few minutes before counting to permit the cells to settle.

3. Rinse the hemocytometer and wipe it dry.

Calculations: Each of the 4 W squares has an area of 1mm^2 and a depth of 0.1 mm .

The total volume is therefore $4 \times 1 \times 0.1 = 0.4\text{ mm}^3$.

The number of cells per mm^3 in the diluted blood is:

$N \times (1 / 0.4) = N \times 2.5$ (N being the number of cells counted)

Dilution in the reservoir was 1:20; therefore, there are $N \times 2.5 \times 20 = N \times 50$ WBC per mm^3 of blood.

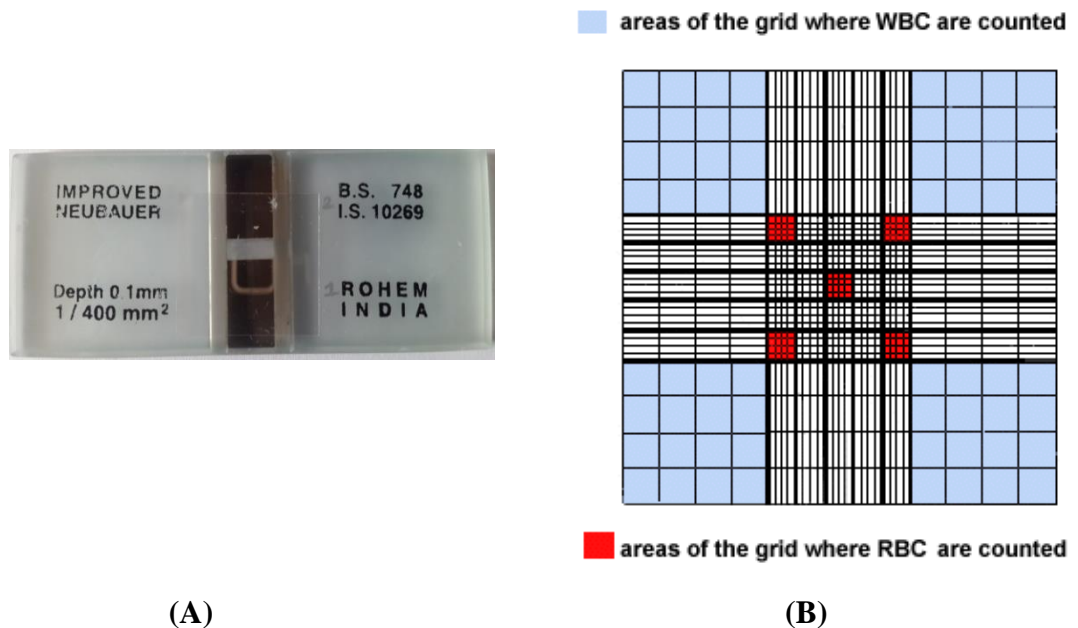


Figure 9: (A) Hemocytometer (Neubauer Chamber) (B) Areas of RBC and WBC count. [101]

c) Percent Haemoglobin: [99, 102]

Haemoglobin is the blood protein and important component of blood which is responsible for the transport of oxygen. The oxygen-carrying capacity of blood is determined by haemoglobin percent present in RBC. The standard procedure is given below.

Determination of Haemoglobin Concentration:

Since haemoglobin is the RBC protein responsible for oxygen transport, the most accurate way of measuring the oxygen-carrying capacity of the blood is to determine its haemoglobin content.

Tallquist Method:

1. Removed a piece of test paper from a Tallquist booklet and placed it on a flat surface.
2. Gently inverted the Eppendorf tube containing the blood 2-3 times (to avoid cells settling at the bottom of the tube).
3. Spot a drop of blood on the Tallquist blotting paper.
4. Compared the colour with the Tallquist colour reference standard and record the % haemoglobin.

(C) Tumor Parameters

a) Tumor Weight:

For liquid tumor, falcon tube was filled with ascitic fluid & then subjected to centrifuge, further, the supernatant was discarded and the weight of the tumor cells was recorded.

b) Tumor Volume and Tumor Size: [103, 104]

Tumor volume was measured for DAL model on the 15th day of the experiment by exploring the peritoneal cavity and filling the peritoneal ascitic fluid in a graduated falcon tube to measure the volume.

For solid tumor, (Fibrosarcoma) model, the tumor was measured at an interval of 72 hours over the study duration. Vernier scale was used for measurement of tumor size & following formula was applied for the calculation.

$$\text{Measurement of Tumor Volume} = \frac{\text{Length} \times (\text{Width})^2}{2}$$

c) Kaplan Meier Survival Plot: [105, 106, 107]

The Kaplan-Meier estimate is applied to calculate the fraction of subjects that survived over a period of time after treatment. In animal experiments, the effect of treatment is measured by calculating the number of subjects that survive post-treatment. Survival time is defined as time starting from a specified stage until the incident occurs & is termed as survival analysis. The observation was continued till the day of the death of the last animal of each group. The animal that has been omitted from the study is termed as a censored observation. The Kaplan-Meier estimate, because of its simplicity, is used for computing the survival of animals irrespective of the difficulties associated with it. For various situations, such as death events, the survival proportion curve can be generated. This analysis is applicable for comparison of the effect of two drugs and analyses of the survival of subjects.

Tumor growth and host survival, median survival time and % increased life span parameters were evaluated. From day zero to day of death of animals were taken and

compared between treatment and control group. By the help of formula, % Increased Life Span was calculated.

$$\% \text{ Increase Life Span} = \frac{\text{Median Survival of Treatment Group}}{\text{Median Survival of Control Group}} \times 100$$

3. Statistical Analysis:

- MTT assay results has been interpreted by transforming data and non-linear regression analysis.
- The values of animal parameters were calculated using one-way ANOVA & Dunnett's test, the data were described in terms of mean & standard deviation. Where $*=P<0.05$, $**=P<0.01$, $***=P<0.001$.
- Graph Pad Prism® version 5.0 software was used.

RESULTS

4. Results

In this chapter, the research results were disclosed, such as collection, phytochemical investigation, HPLC qualitative analysis was done for the presence of marker compounds, *in-vitro* anticancer activity of various cancerous cells & *in-vivo* anticancer activity like brine shrimp lethality assay, animal study, oral toxicity study, DAL model, fibrosarcoma model in mice, blood-parameters that include RBC count, WBC count, % haemoglobin count & tumor parameters.

4.1 Plant Collection and Authentication of Plants

Plant collected from the Dandeli-Anshi tiger reserve and Sulebhavi (Belagavi) Western Ghats region. Plant herbarium prepared and submitted at ICMR-NITM (Formerly RMRC) Belagavi, authenticated by Taxonomist, (herbarium voucher No.-RMRC-1271 and RMRC- 1272) Annexure- 1

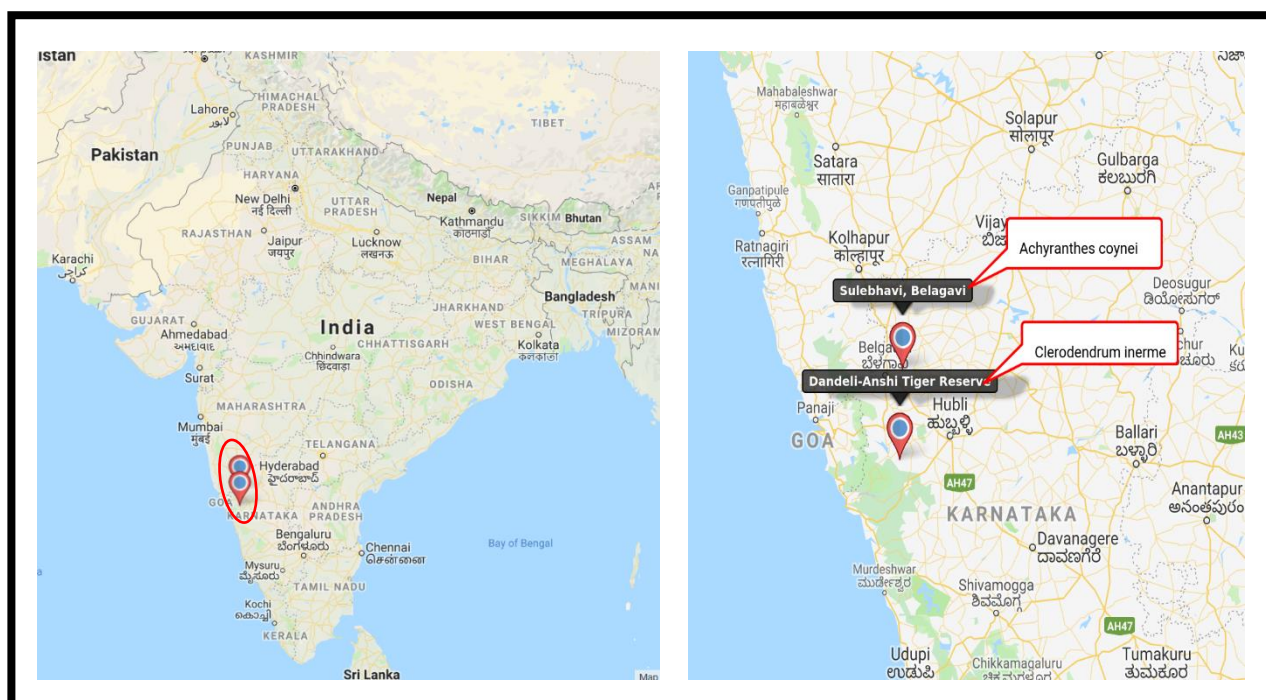


Figure 10: Geographical region and collection site of plants Dandeli-Anshi Tiger reserve 15.01148(°N), 74.39502 (°E) and Sulebhavi 15.8946° N, 74.6561° E.

4.2 The Yield of Plant Extract

Extracts of both plants were calculated for per 100 grams yield and the nature of the plant extract mentioned in Table no. 2 and Figure 11.

$$\% \text{ Yield} = \frac{\text{Obtained extract quantity in gms}}{\text{Plant material taken (100 gm)}}$$

S. No.	Plant	% Yield of Plant Extract	Nature of extract
1.	<i>Achyranthus coynei</i>	14.9	Dark brown viscous, sticky
2.	<i>Clerodendrum inerme</i>	1.84	Brown Hygroscopic

Table 2: Percent yield and physical nature of plant extracts.

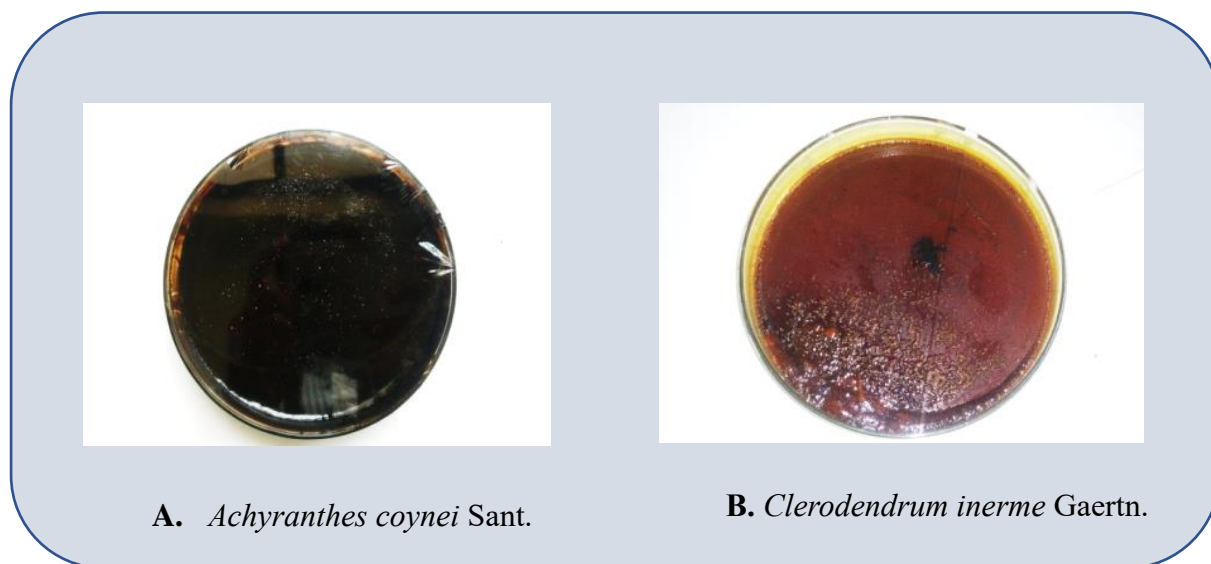


Figure 11: Dried Plant Extracts **A.** *Achyranthes coynei* Sant. and **B.** *Clerodendrum inerme* Gaertn.

4.3 Preliminary Phytochemical Investigation - Plant Extract

The qualitative phytochemical investigation was performed to induce the presence of different secondary metabolites like carbohydrate, proteins, steroids, triterpenoids, amino acids, glycosides, alkaloids, flavonoids, tannins and phenolic compounds, oil & fats. Table 3. defines the presence of secondary metabolites.

S. No.	Test	<i>Achyranthus coynei</i>	<i>Clerodendrum inerme</i>
1.	Carbohydrates	+	+
2.	Proteins	+	+
3.	Amino acids	+	+
4.	Steroids	+	+
5.	Triterpenoids	+	+
6.	Glycosides	+	+
7.	Flavonoids	+	+
8.	Alkaloids	+	+
9.	Tannins & Phenolic compounds	+	+
10.	Fat and Oils	–	–
11.	Saponins	+	+

Table 3: Preliminary phytochemical investigation findings for presence of secondary metabolite in plants.

4.4 Chromatographic (HPLC) Analysis –

Analysis of the HPLC for marker compound was carried out which has the anti-cancer potential was present in both of the plant extracts. Which are confirmed by HPLC fingerprinting with the following (Table 4) chromatographic conditions and their retention time compared with standards. Figure 12 denoting the retention time for plant extracts of *Achyranthus coynei*, *Clerodendrum inerme*, standards Betulinic acid, Oleanolic acid, Ursolic acid and mixture of standards, along with mobile phase for comparison and qualitative analysis.

S.No.	Instrument	HPLC
1.	Make	Shimadzu
2.	Model	LC-20 AD
3.	Column	THERMO Hypersil-Keystone C-18 250×4.6 mm Particle size- 5 μ
4.	λ_{\max}	210 nm
5.	Mode	Isocratic
6.	Mobile phase	Methanol: Water (90:10) pH-5 (Glacial acetic acid)
7.	Injection volume	20 μ l.
8.	Betulinic acid standard R_t	9.735 min.
9.	Oleanolic acid standard R_t	11.076 min.
10.	Ursolic acid standard R_t	11.805 min.
11.	A mixture of standard R_t	9.723, 11.103, 11.783 min.
12.	<i>Achyranthes coynei</i> R_t	9.707, 10.321, 11.155 min.
13.	<i>Clerodendrum inerme</i> R_t	9.703, 10.346, 11.034 min.

Table 4: Chromatographic conditions and retention time of standard compound and plant extracts.

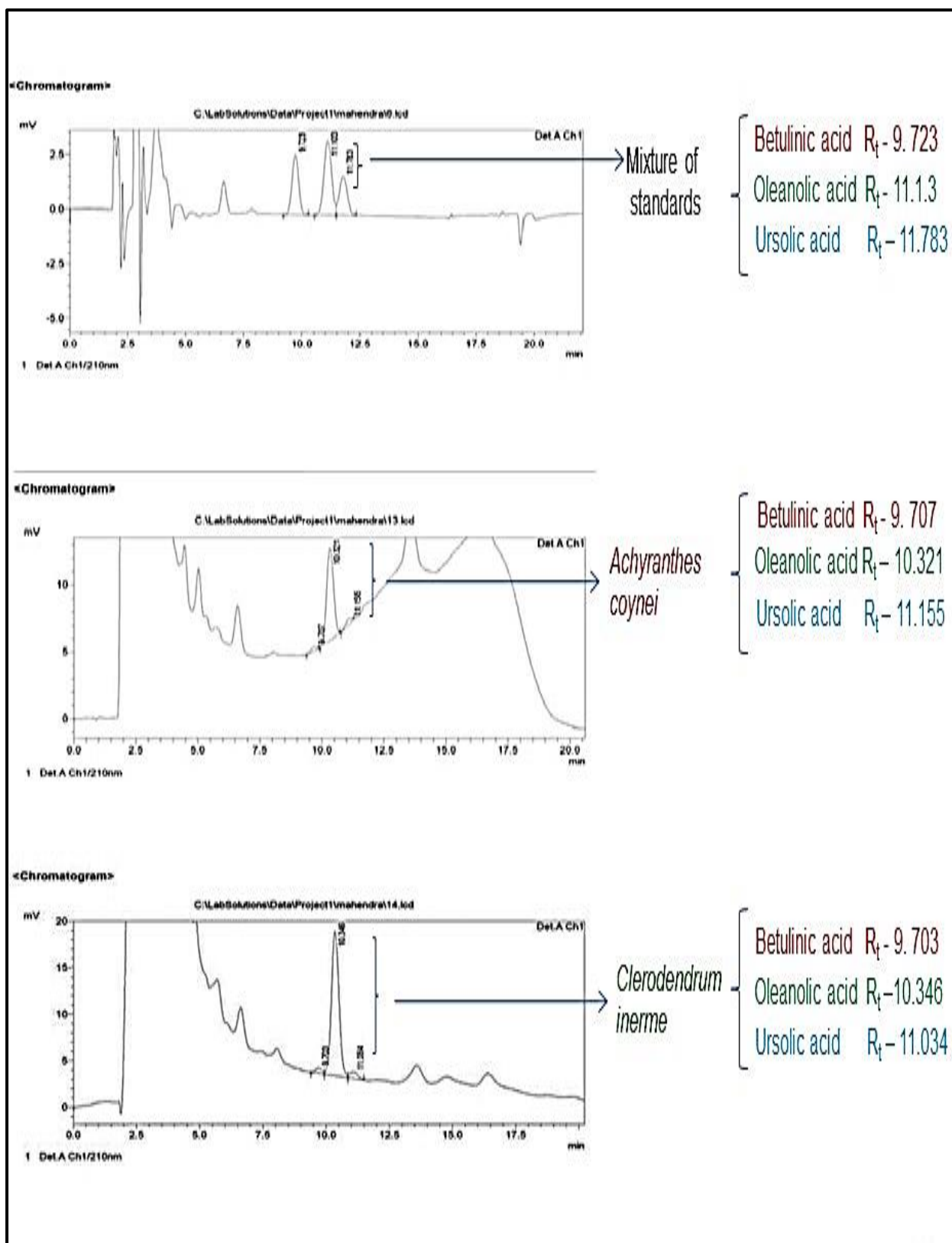


Figure 12 (B): Chromatograms plant extracts.

4.5 *In-vitro* Anticancer Activity

4.5.1 Trypan Blue Dye Exclusion assay

All cancerous cells were counted for MTT assay cell count, trypan blue dye helps to differentiate between live and dead cells. Live cells can be distinguished by blue-colored dye at the outer wall and dead cells were blue from inside. For MTT assay 10,000 cells were counted and cells were seeded.

4.5.2 Cytotoxicity Assay (MTT assay)

MTT assay has been performed on following cancer cell lines

1. Burkitt's lymphoma cell lines –Daudi
2. Fibro sarcoma cell lines - HT-1080
3. Lung cancer cell lines - A-549
4. Breast cancer cell lines – MCF-7
5. Colon cancer cell lines – HT-29
6. Leukemia cancer cell lines – HL-60

The obtained crude extracts were subjected to MTT assay using cancer cell lines which were procured from NCCS, Pune. The cell lines used for *In-vitro* anticancer activity were treated with plant extracts at a different concentration of 1000, 500, 250, 125, 61.5, 31.25, 15.6 µg/ml and standard drug Doxorubicin at 250, 125, 61.5, 31.25, 15.6 µg/ml concentration as well (two-fold dilution). The results in percent inhibition were calculated in Mean \pm S.D. (Table 5) followed by transforming data in log form and nonlinear regression analysis for IC₅₀ values. The Inhibitory concentration value (IC₅₀ Value) for *Achyranthus coynei*, *Clerodendrum inerme* extract and standard drug doxorubicin were obtained for Burkitt's lymphoma cell lines –Daudi 198.2, 213.2 and 62.36 µg per ml respectively, Fibrosarcoma cell lines - HT-1080 values were 192.4,

204.8 and 78.96 μg per ml respectively, the Lung-cancer cell lines - A-549 values were 222.2, 259.5 and 88.05 μg per ml respectively, Breast-cancer cell lines – MCF-7 values were 183.2, 219.6 and 73.10 μg per ml respectively, Colon-cancer cell lines – HT-29 values were 190.4, 225.7 and 78.82 μg per ml, Leukemia cancer cell lines – HL-60 values were 254.6, 281.2 and 60.6 $\mu\text{g}/\text{ml}$ obtained, which shown in Table 6 to 11 along with graphs in Figure 13 to 18.

		Treated Concentration in $\mu\text{g/ml}$																		
		<i>Achyranthus coynei</i> Extract							<i>Clerodendrum inerme</i> Extract							Standard Drug Doxorubicin				
Percent Inhibition	Cells	1000	500	250	125	62.5	31.25	15.6	1000	500	250	125	62.5	31.25	15.6	250	125	62.5	31.25	15.6
	<i>Daudi</i>	31.00	44.00	54.00	70.00	94.00	99.00	99.00	26.00	40.00	51.00	69.00	76.00	92.00	93.00	19.00	30.00	44.00	69.00	74.00
	HT-1080	18.98	39.00	47.76	67.80	73.60	91.954	98.084	19.15	26.54	40.34	66.45	71.45	80.50	90.43	19.5	31.30	51.40	64.45	76.5
	A-549	18.90	35.72	49.82	61.78	81.73	90.632	93.876	14.70	37.80	51.87	63.78	72.87	88.97	96.87	12.65	26.45	47.876	66.70	75.50
	MCF-7	25.876	40.965	54.00	60.54	78.46	88.54	93.00	18.123	39	48.90	66.80	78.90	91.95	98.084	14.80	26.80	49.70	64.00	78.00
	HT-29	13.995	31.6	46.32	59.78	71.7	89.75	93.876	26.00	47.00	51.00	69.00	77.76	92.00	93.00	19.00	30.00	47.876	69.00	74.00
	HL-60	12.65	39.65	51.65	60.54	71.87	89.87	95.76	15.98	40.43	57.23	61.78	78.98	93.20	98.084	17.65	26.76	49.76	60.01	76.6

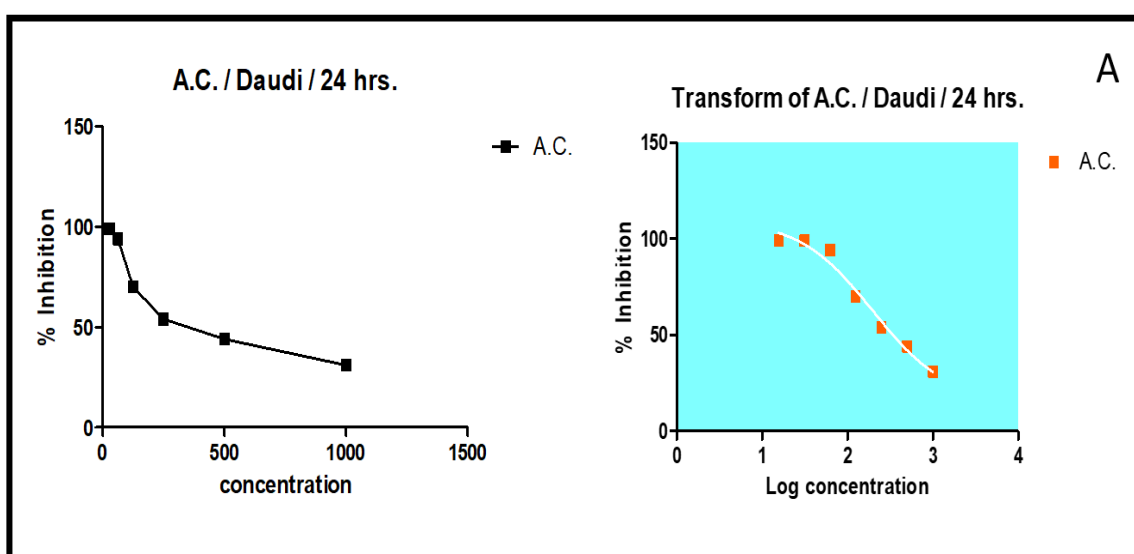
Table 5: The percent inhibition of different cell lines at different concentration of *Achyranthus coynei*, *Clerodendrum inerme* extract and standard drug Doxorubicin respectively.

1. Burkitt's Lymphoma Cells –Daudi

The Burkitt's Lymphoma cells were treated with plant extracts at 1000, 500, 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$ and standard drug doxorubicin at 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$. The IC_{50} values were obtained for *Achyranthes coynei* Sant., *Clerodendrum inerme* (L.) Gaertn. and Standard (Doxorubicin) as 198.2, 213.2 and 62.36 respectively. Results were calculated in mean \pm S.D. of absorbance values followed by transform the data and non-linear regression analysis by using graph pad prism 5.0 software.

S. No.	Test	IC_{50} value ($\mu\text{g/ml}$)
1.	<i>Achyranthes coynei</i> Sant.	198.2
2.	<i>Clerodendrum inerme</i> (L.) Gaertn.	213.2
3.	Standard (Doxorubicin)	62.36

Table 6: The IC_{50} value ($\mu\text{g/ml}$) of *Achyranthus coynei*, *Clerordendrum inerme* Doxorubicin respectively on Daudi cells.



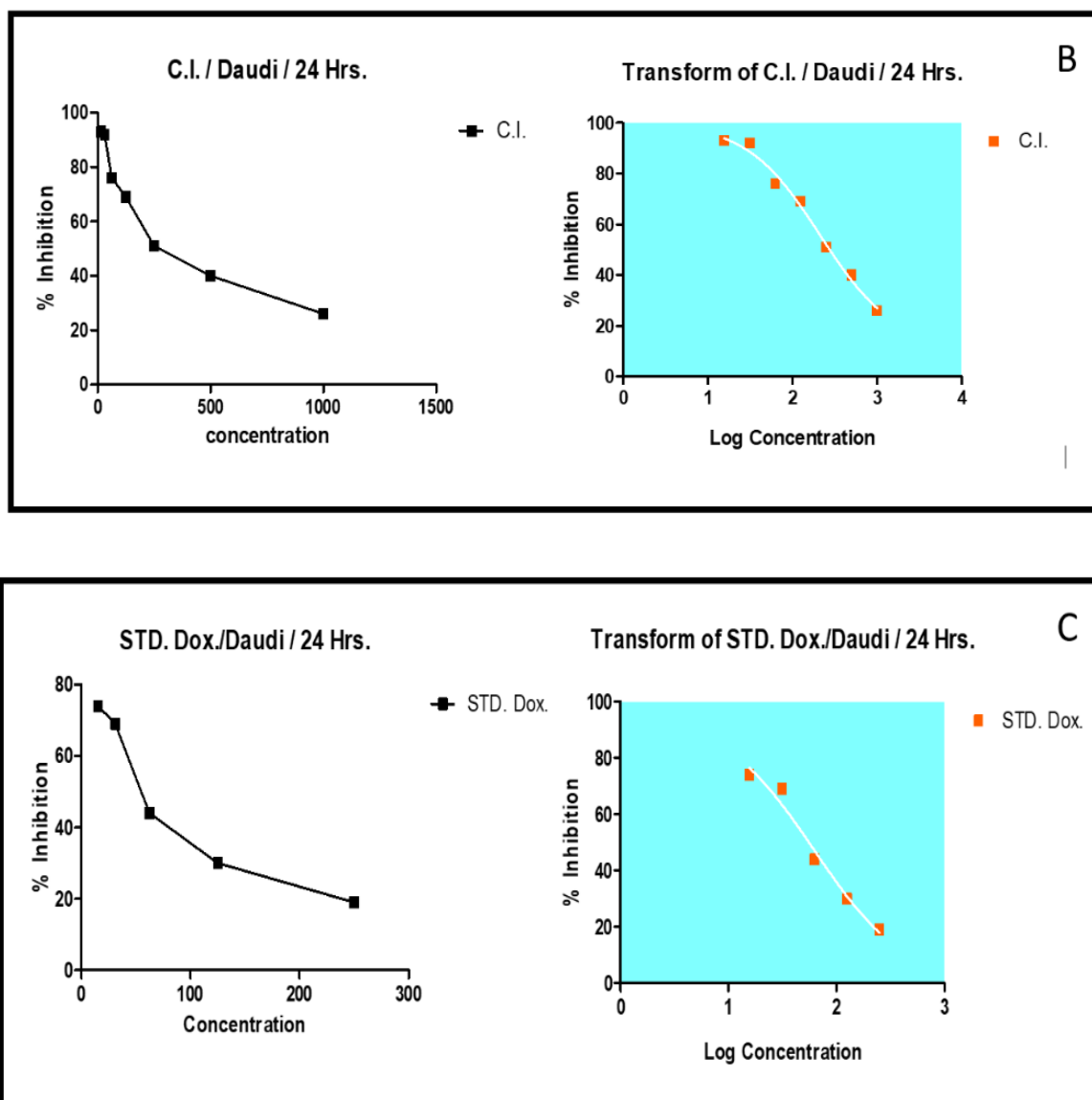


Figure 13: The percent inhibition of *Daudi* cells at different concentrations of (A) *Achyranthus coynei*, (B) *Clerodendrum inerme* extract & (C) standard drug Doxorubicin respectively.

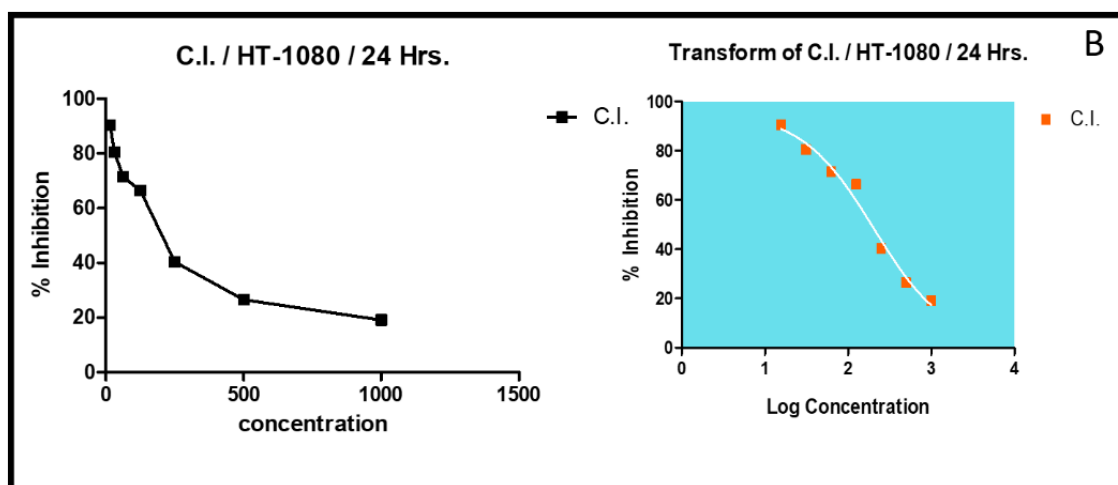
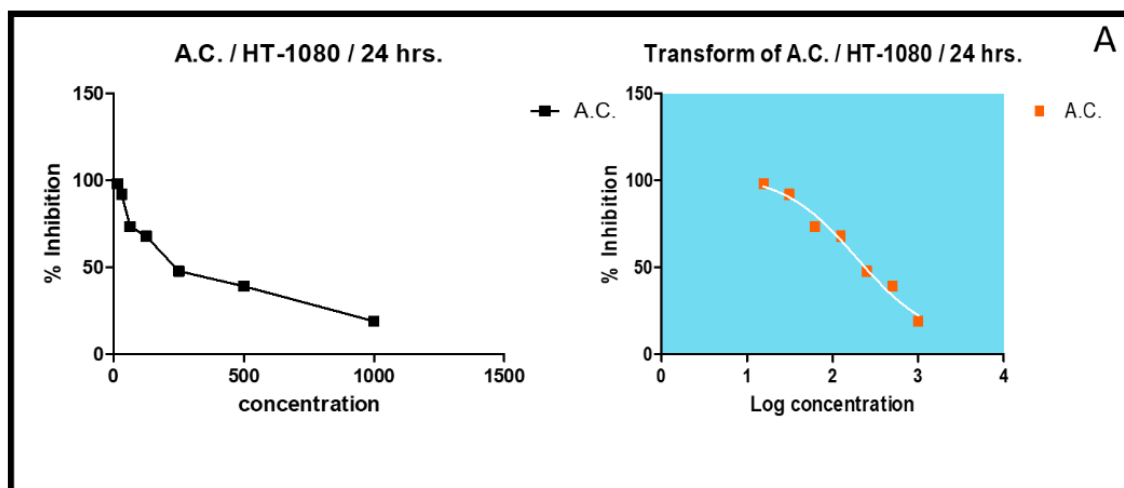
2. Fibrosarcoma Cells - HT-1080

Fibrosarcoma Cells - HT-1080 cells were treated with plant extracts at 1000, 500, 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$ and standard drug doxorubicin at 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$. The IC_{50} values were obtained for *Achyranthes coynei* Sant., *Clerodendrum inerme* (L.) Gaertn. and Standard (Doxorubicin) as 192.4, 204.8 and

78.96 respectively. Results were calculated in mean \pm S.D. of absorbance values followed by transform the data and non-linear regression analysis by using graph pad prism 5.0 software.

S. No.	Test compound	IC ₅₀ value ($\mu\text{g/ml}$)
1.	<i>Achyranthes coynei</i> Sant.	192.4
2.	<i>Clerodendrum inerme</i> (L.) Gaertn.	204.8
3.	Standard (Doxorubicin)	78.96

Table 7: The IC₅₀ value ($\mu\text{g/ml}$) of *Achyranthus coynei*, *Clerordendrum inerme* plant extract and standard drug Doxorubicin respectively on Fibrosarcoma cells - HT-1080 cells.



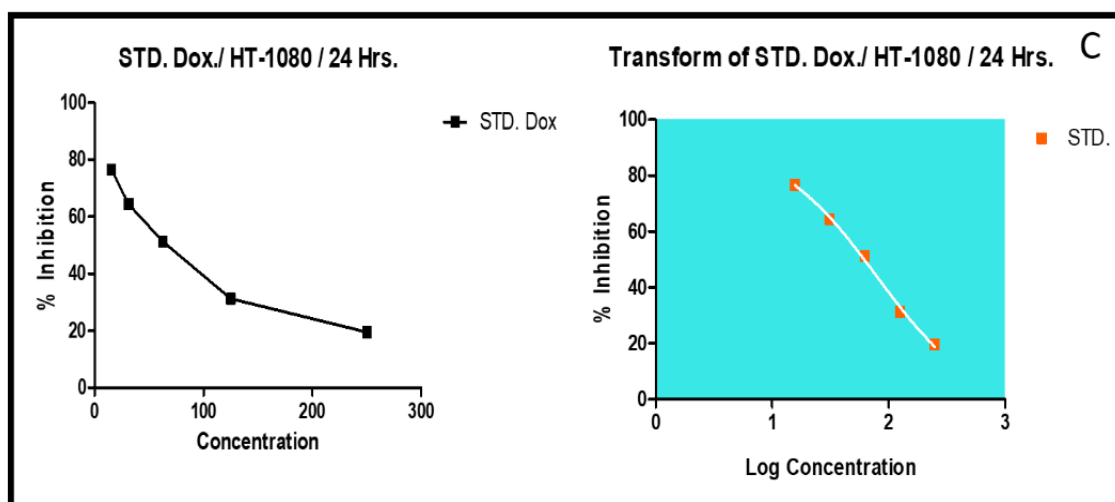


Figure 14: Percent inhibition of Fibrosarcoma cells - HT-1080 at different concentrations of (A) *Achyranthus coynei*, (B) *Clerodendrum inerme* extract & (C) Doxorubicin respectively.

3. Lung Cancer Cells - A-549

Fibrosarcoma Cells - HT-1080 were treated with plant extracts at 1000, 500, 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$ and standard drug doxorubicin at 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$. The IC_{50} values were obtained for *Achyranthes coynei* Sant., *Clerodendrum inerme* (L.) Gaertn. and Standard (Doxorubicin) as 222.2, 259.5 and 88.05 respectively. Results were calculated in mean \pm S.D. of absorbance values followed by transform the data and non-linear regression analysis by using graph pad prism 5.0 software.

S. No.	Test compound	IC_{50} value ($\mu\text{g/ml}$)
1.	<i>Achyranthes coynei</i> Sant.	222.2
2.	<i>Clerodendrum inerme</i> (L.) Gaertn.	259.5
3.	Standard (Doxorubicin)	88.05

Table 8: The IC_{50} value ($\mu\text{g/ml}$) of *Achyranthus coynei*, *Clerodendrum inemre* plant extract & Doxorubicin respectively on A 549 cells.

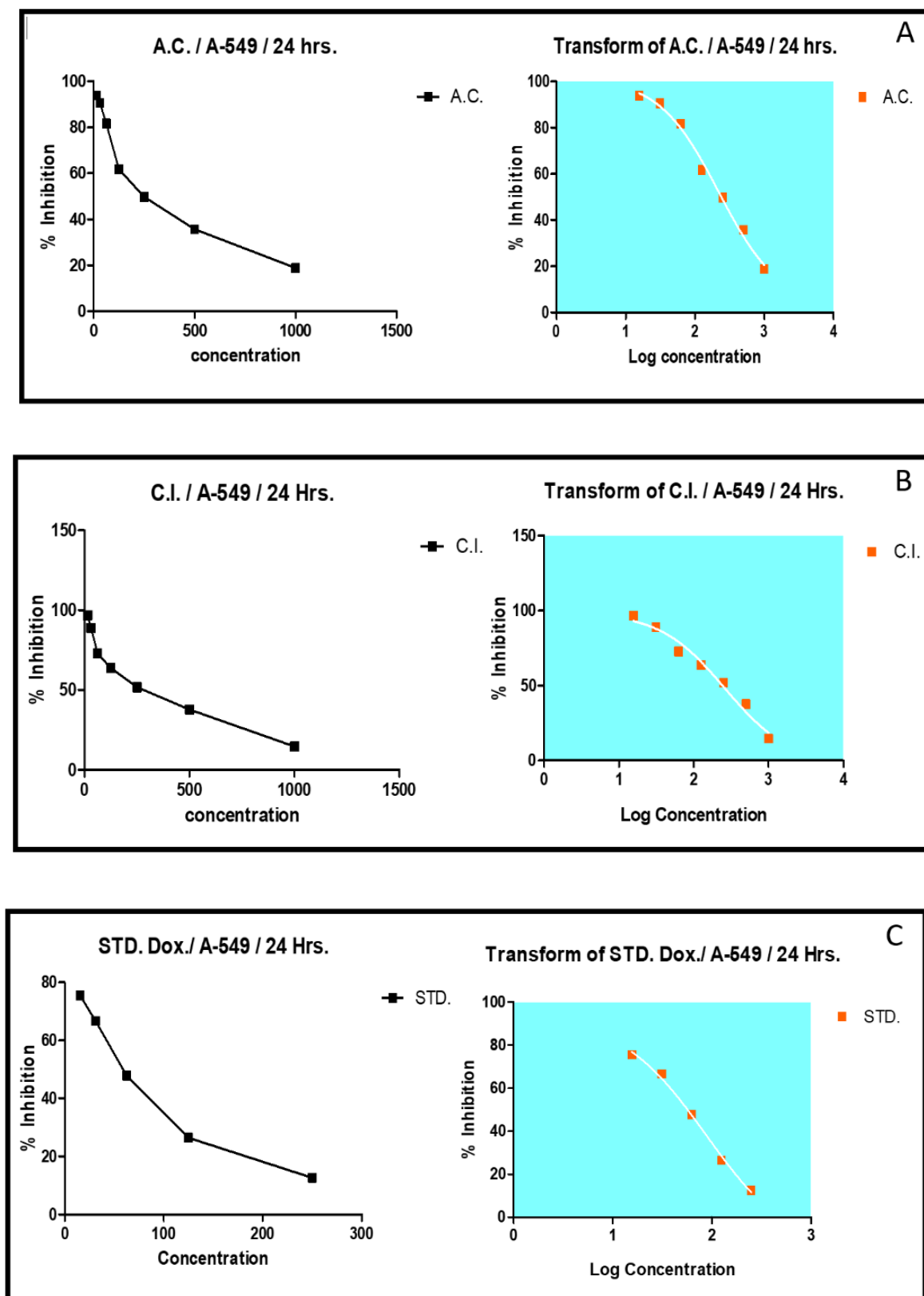


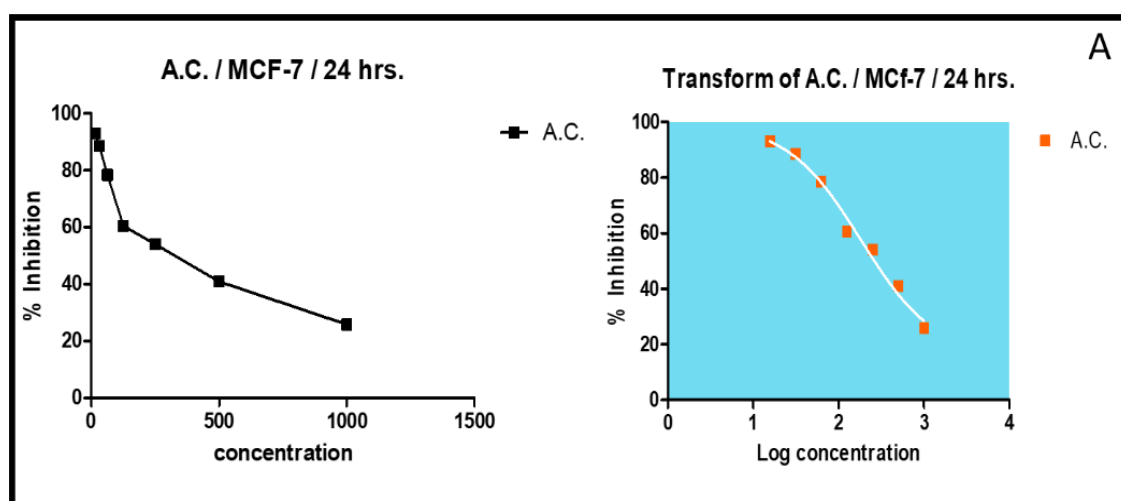
Figure 15: Percent inhibition of A-549 cells at different concentrations of (A) *Achyranthus coynei*, (B) *Clerodendrum inerme* extract & (C) Doxorubicin respectively.

4. Breast Cancer Cells– MCF-7

Breast Cancer Cells– MCF-7 were treated with plant extracts at 1000, 500, 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$ and standard drug doxorubicin at 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$. The IC_{50} values were obtained for *Achyranthes coynei* Sant., *Clerodendrum inerme* (L.) Gaertn. and Standard (Doxorubicin) as 183.2, 219.6 and 73.10 respectively. Results were calculated in mean \pm S.D. of absorbance values followed by transform the data and non-linear regression analysis by using graph pad prism 5.0 software.

S. No.	Test compound	IC_{50} value ($\mu\text{g/ml}$)
1.	<i>Achyranthes coynei</i> Sant.	183.2
2.	<i>Clerodendrum inerme</i> (L.) Gaertn.	219.6
3.	Standard (Doxorubicin)	73.10

Table 9: IC_{50} value ($\mu\text{g/ml}$) of *Achyranthus coynei*, *Clerordendrum inerme* plant extract & Doxorubicin respectively on MCF-7.



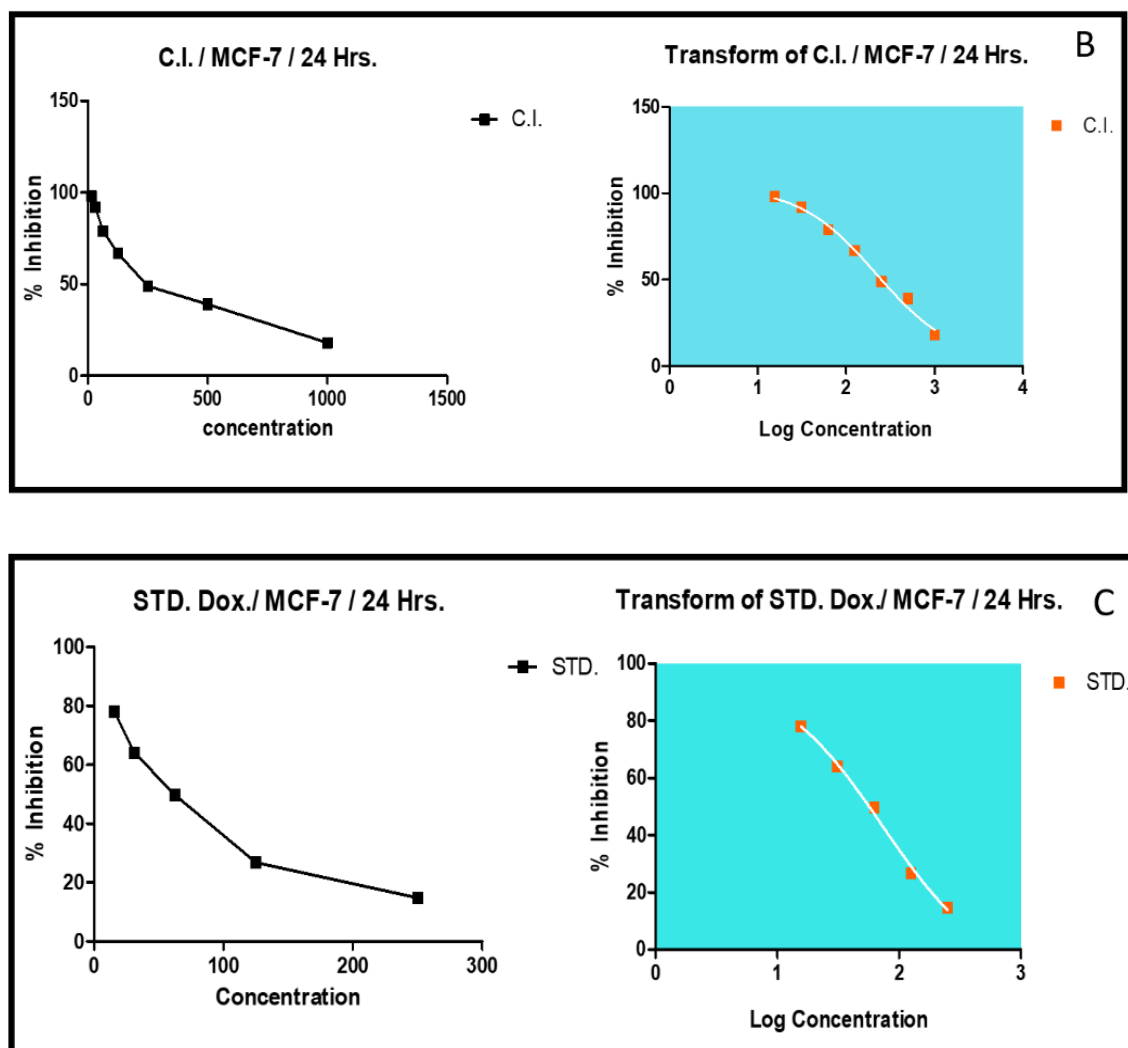


Figure 16: Percent inhibition of MCF-7 cells at different concentrations of (A) *Achyranthus coynei*, (B) *Clerodendrum inerme* extract & (C) Doxorubicin respectively.

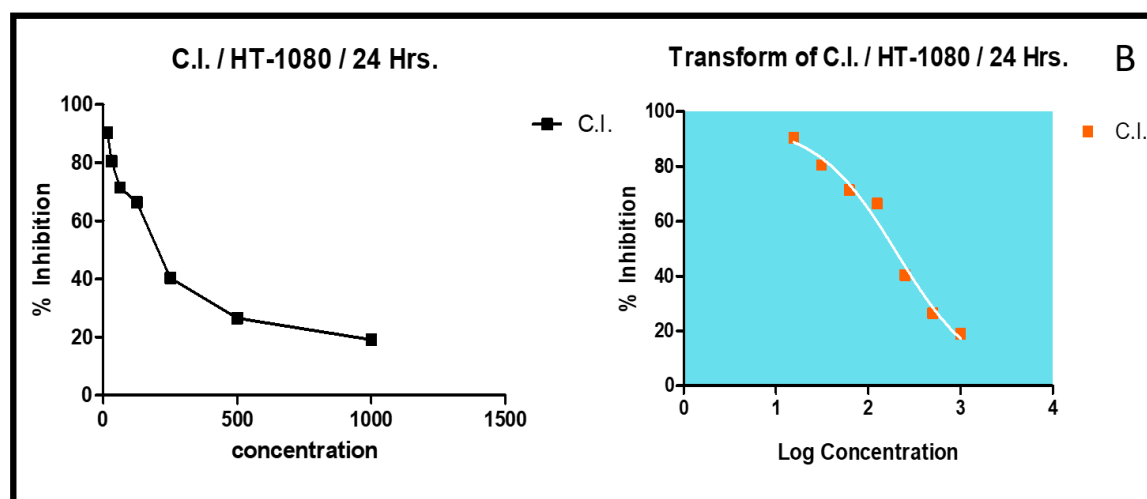
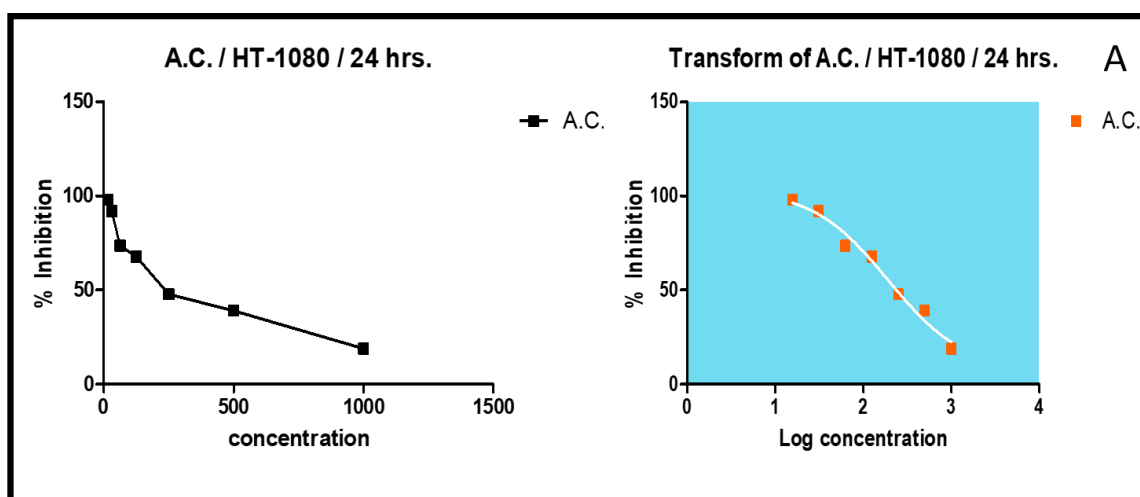
5. Colon Cancer Cells – HT-29

Colon Cancer Cells – HT-29 were treated with plant extracts at 1000, 500, 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$ and standard drug doxorubicin at 250, 125, 61.5, 31.25, 15.6 $\mu\text{g/ml}$. The IC_{50} values were obtained for *Achyranthes coynei* Sant., *Clerodendrum inerme* (L.) Gaertn. and Standard (Doxorubicin) as 190.4, 225.7 and 78.82 respectively. Results were calculated in mean \pm S.D. of absorbance values

followed by transform the data and non-linear regression analysis by using graph pad prism 5.0 software.

S. No	Test compound	IC ₅₀ value (µg/ml)
1.	<i>Achyranthes coynei</i> Sant.	190.4
2.	<i>Clerodendrum inerme</i> (L.) Gaertn.	225.7
3.	Standard (Doxorubicin)	78.82

Table 10: IC₅₀ value (µg/ml) of *Achyranthus coynei*, *Clerordendrum inerme* plant extract & Doxorubicin respectively on HT-29 cells.



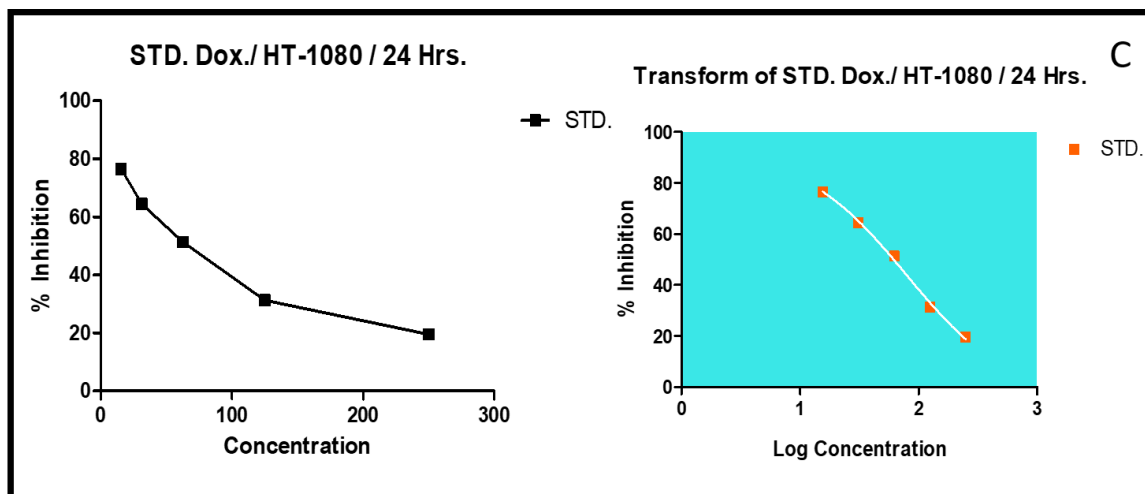


Figure 17: Percent inhibition of HT-29 cells at different concentrations of *Achyranthus coynei*, *Clerodendrum inerme* extract & Doxorubicin respectively.

6. Leukemia Cells – HL-60

Colon Cancer Cells – HT-29 were treated with plant extracts at 1000, 500, 250, 125, 61.5, 31.25, 15.6 µg/ml and standard drug doxorubicin at 250, 125, 61.5, 31.25, 15.6 µg/ml. The IC₅₀ values were obtained for *Achyranthes coynei* Sant., *Clerodendrum inerme* (L.) Gaertn. and Standard (Doxorubicin) as 190.4, 225.7 and 78.82 respectively. Results were calculated in mean ± S.D. of absorbance values followed by transform the data and non-linear regression analysis by using graph pad prism 5.0 software.

S. No.	Test compound	IC ₅₀ value (µg/ml)
1.	<i>Achyranthes coynei</i> Sant.	254.6
2.	<i>Clerodendrum inerme</i> (L.) Gaertn.	281.2
3.	Standard (Doxorubicin)	60.6

Table 11: IC₅₀ value (µg/ml) of *Achyranthus coynei*, *Clerodendrum inerme* plant extract & Doxorubicin respectively on HL-60 cells.

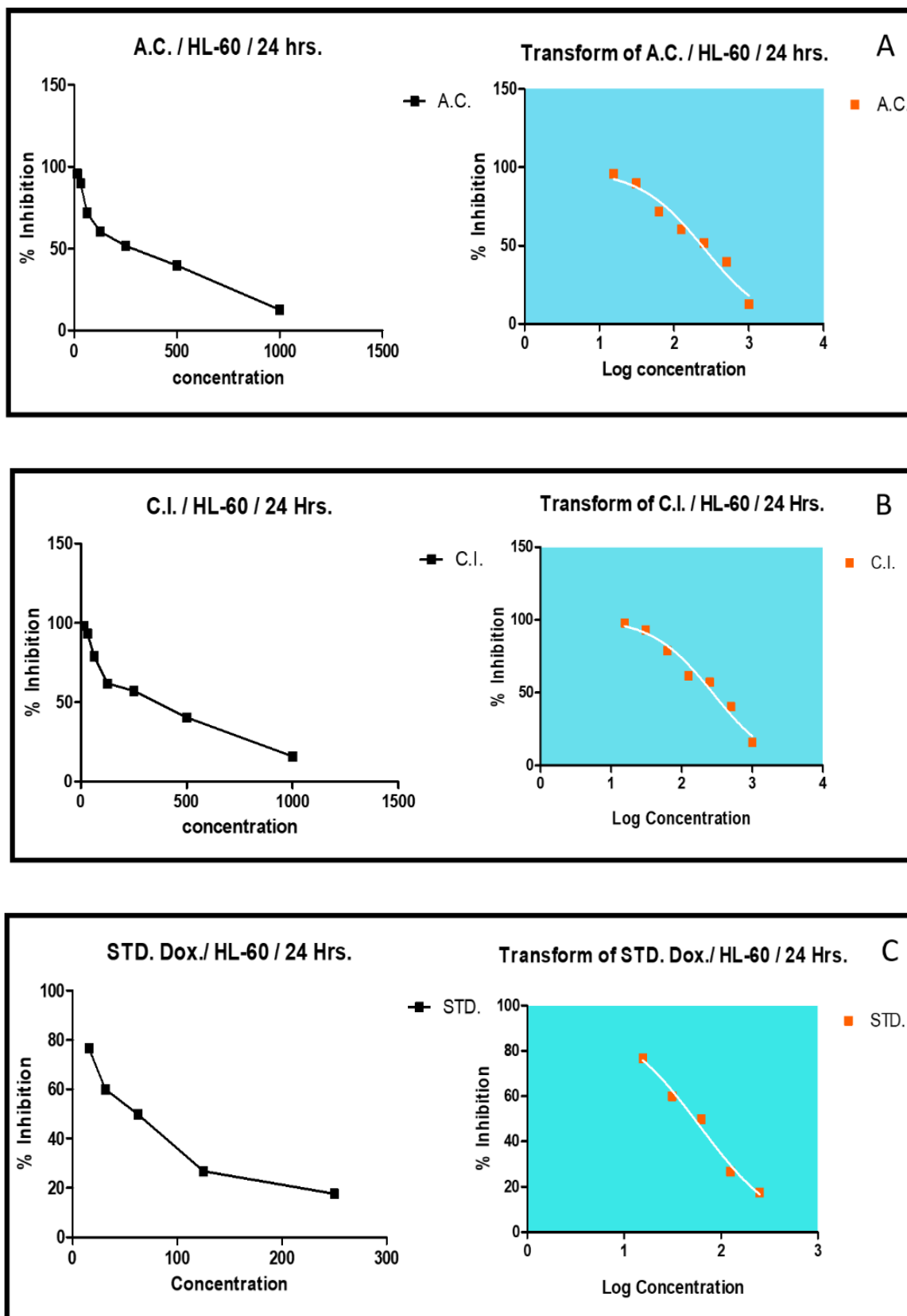


Figure 18: Percent inhibition of HL-60 cells at different concentrations of *Achyranthus coynei*, *Clerodendrum inerme* extract & Doxorubicin respectively.

4.6 *In-vivo* Studies of anticancer activity

4.6.1 Brine Shrimp Lethality Assay

Toxicity assessment of plant extract was evaluated using brine shrimp and the LD₅₀ (Lethal dose) value at 50% population get killed was measured which is below 100 ppm found in both of the extracts. The number of live and death brine shrimps were calculated based on no movement was considered as dead. Percent cytotoxicity values are calculated in triplicate, Mean \pm S.D which is given in Table 12.

<i>Achyranthes Coynei</i> - Percent Cytotoxicity							
Conc. (PPM)	No. of Deaths			Mean	S.D.	% Mean	% S.D
10	3	3	2	2.666667	0.57735	26.66667	5.773503
20	4	5	4	4.333333	0.57735	43.33333	5.773503
30	6	4	4	4.666667	1.154701	46.66667	11.54701
40	6	6	5	5.666667	0.57735	56.66667	5.773503
50	7	7	5	6.333333	1.154701	63.33333	11.54701
60	6	7	7	6.666667	0.57735	66.66667	5.773503
70	6	8	7	7	1	70	10
80	9	7	8	8	1	80	10
90	9	8	9	8.666667	0.57735	86.66667	5.773503
100	9	10	10	9.666667	0.57735	96.66667	5.773503

Table 12: Lethality and percent cytotoxicity of *Achyranthus coynei* extract at different concentration.

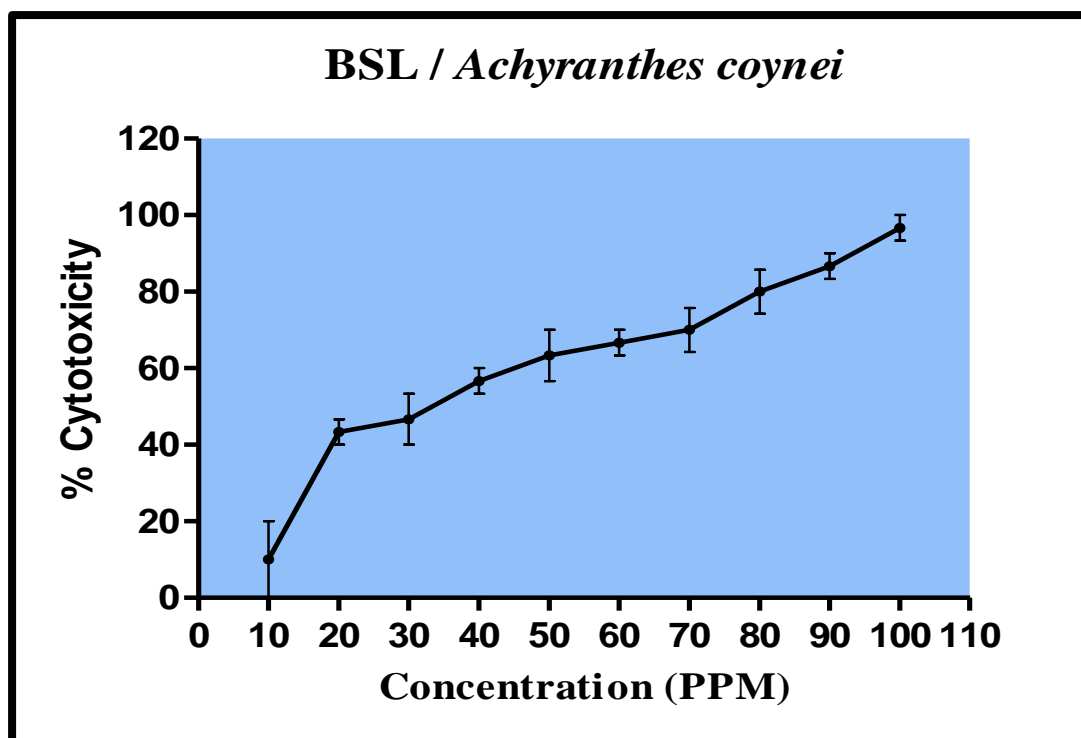


Figure 19: percent cytotoxicity graph pattern on Brine Shrimp at different concentrations of *Achyranthus coynei* extract.

<i>Clerodendrum inerme</i> Percent Cytotoxicity							
Conc. (PPM)	No. of Death			Mean	S.D.	% Mean	% S.D
10	2	2	3	2.333333	0.57735	23.33333	5.773503
20	2	4	3	3	1	30	10
30	5	5	6	5.333333	0.57735	53.33333	5.773503
40	4	7	7	6	1.732051	60	17.32051
50	6	6	8	6.666667	1.154701	66.66667	11.54701
60	5	10	8	7.666667	2.516611	76.66667	25.16611
70	6	9	9	8	1.732051	80	17.32051
80	6	9	10	8.333333	2.081666	83.33333	20.81666
90	10	10	7	9	1.732051	90	17.32051
100	10	10	10	10	0	100	0

Table 13: Lethality and percent cytotoxicity of *Clerodendrum inerme* extract at different concentration.

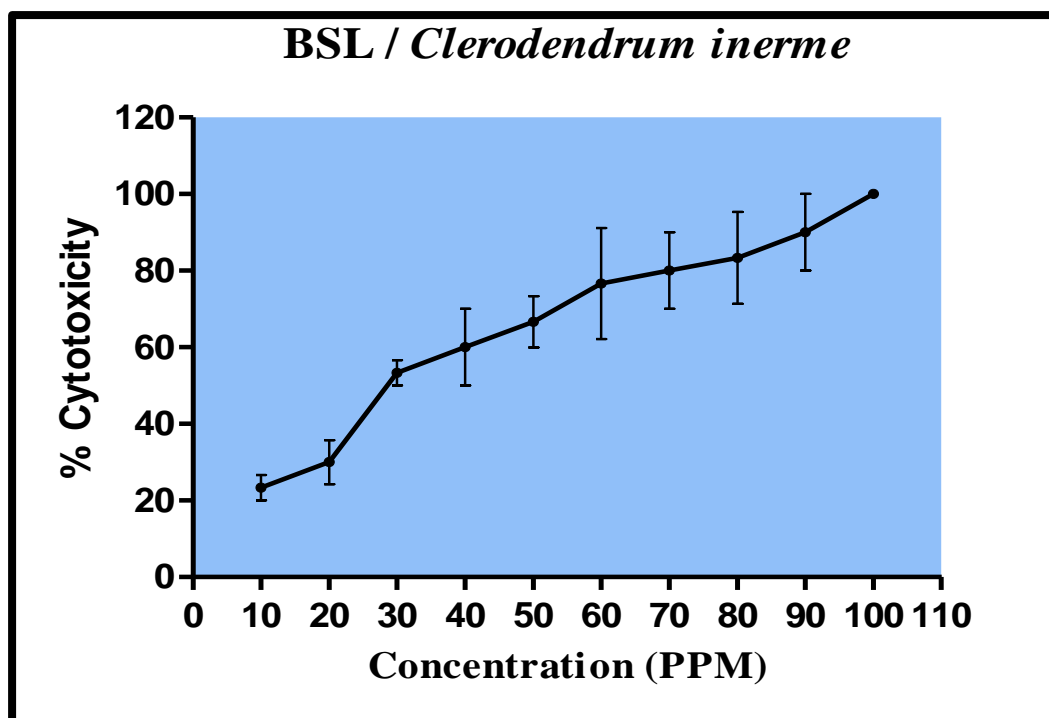


Figure 20: Percent cytotoxicity graph pattern on Brine Shrimp at different concentration of *Clerodendrum inerme* extract.

4.6.2 Animal Studies

The present study was endorsed by the Institutional Animal Ethical Committee with reference no. (KLECOPIAEC/Res.22-10/10/2015) (Annexure-II) for animal experimentation and animals were procured and acclimatize for 7 days at lab condition. Female albino mice were housed and provided conventional diet ad-libitum and free water was provided with sequence lighting i.e. light for 12 hours & dark for 12 hours, $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ & relative-humidity at 48%.

4.6.3 Oral Toxicity Studies:

Oral toxicity (OECD 423) was conducted and it was discovered that both extracts were safe up to a dose of 2000 mg/kg body weight. The female albino mice used were

nulliparous, 8 to 12 weeks old & with the weight of animals between $\pm 20\%$. This finding suggested the dose levels for the further conduct of the experiment.

S. No.	Plant Extract at 2000 mg/kg Body weight	Observation for 14 Days	Dose
1.	<i>Achyranthes coynei</i>	No mortality found	1/10 th of 2000 mg/kg= 200 mg/kg (Low Dose) 2/10 th of 2000 mg/kg= 400 mg/kg (High Dose)
2.	<i>Clerodendrum inerme</i>	No mortality found	1/10 th of 2000 mg/kg= 200 mg/kg (Low Dose) 2/10 th of 2000 mg/kg= 400 mg/kg (High Dose)

Table 14: Selected dose of plant extracts.

4.6.4 Experimental Animal Model for Cancer studies

Animals were selected as per the criteria of body weight & at 8-12 weeks of age after which induction of tumor was done by the procedure as mentioned in the methodology. These animals were grouped when the tumor reached 0.5 cm in size (in case of the fibrosarcoma tumor model) and the weight variation also took not more than $\pm 20\%$ variation of body weight.

For tumor models were selected for the anticancer activity was take for the study as-

1. Dalton's Ascitic Lymphoma (Liquid Tumor) Model
2. Fibrosarcoma (Solid Tumor) Model

1. DAL Animal Model

The female mice have injected the liquid tumor as mentioned in the methodology and divided it into groups accordingly. The duration of the study was 15 days. Various parameters were evaluated viz. body weight, haematological parameters, tumor weight, tumor volume, Kaplan Meier survival plot, percent increased life span, median survival time with statistically analyzed. These parameters provided the effect of the plant extract is in a dose-dependent manner.

(A) Body Weight:

Body weight parameter was executed and before the experiment and after the experiment the weight was recorded and compared using statistical analysis of significant changes between groups. When the obtained data were calculated the change in body weight was significantly increased in the normal group v/s control animals group ($p < 0.001$) and the body weight was reduced compared to the control group for doxorubicin-treated animals group ($p < 0.001$); *C. inerme* (Low Dose) body weight decreased but not statistically significant ; *C. inerme* high dose $p < 0.01$; *A. coynei* low dose and High Dose $p < 0.05$ and $p < 0.01$ respectively.

S. No.	Groups (n)	Weight of Animals in grams Before Experiment	Weight of Animals in grams After Experiment
1	Normal	20.50 ± 0.4472	26.35 ± 1.362
2	Control	20.58 ± 0.4167	39.70 ± 1.145 a***
3	DOX	20.58 ± 0.4215	28.42 ± 0.9314 b***
4	A.C. (200 mg/kg)	20.70 ± 0.6356	38.57 ± 1.965 b ^{ns}
5	A.C. (400 mg/kg)	20.35 ± 0.3391	33.60 ± 0.9265 b**
6	C.I. (200 mg/kg)	20.95 ± 0.6442	38.47 ± 0.8227 b*
7	C.I. (400 mg/kg)	20.33 ± 1.138	35.40 ± 1.698 b**

Table 15: Body weight of animals. Where each group n=6 animals; values expressed in mean ± SD. *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$. a – Compare with Normal animals group, b- Compared with Control animals group, ns- Non significant.

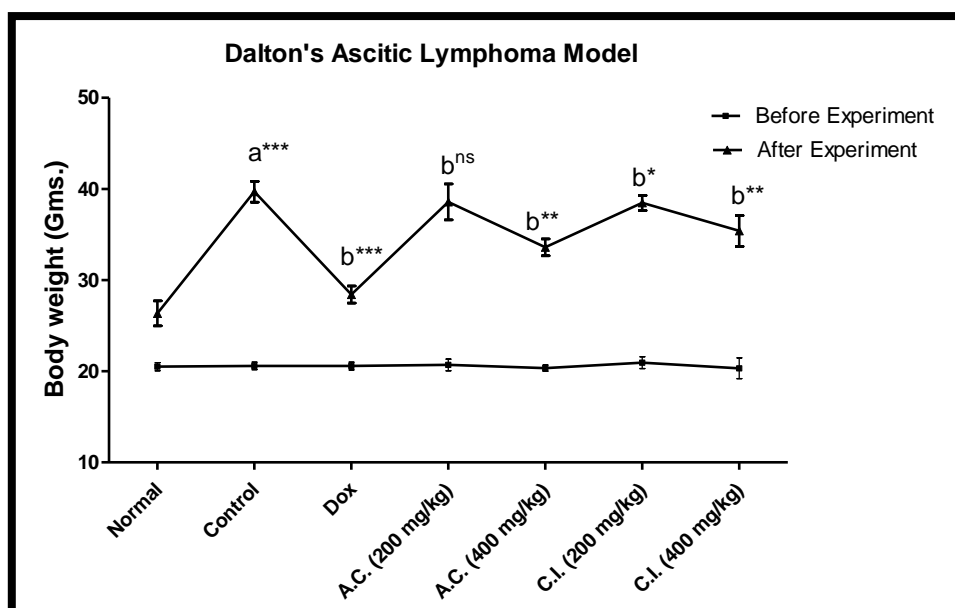


Figure 21: Body weight pattern graph of animals before & after the experiment; Where each group n=6 animals; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compare with Normal group, b- Compared with Control group, *ns*- Non significant.

(B) Haematological Parameters

Haematological parameters viz. RBC count, WBC count & percent haemoglobin were calculated using standard methods that provide the changes occurred between groups. The difference between groups was analyzed using one-way ANOVA & Dunnett's test. When the control animals group was compared to normal animals group, there were significant reduction for RBC ($P < 0.01$), % Hb ($P < 0.01$) and significant increase for WBC ($P < 0.001$) was observed in the haematological parameters. It was observed that doxorubicin group when compared with the disease control animals showed significant ($P < 0.001$) reduction in haematological parameters & the plant *A. coynei* and *C. inerme* extracts treated groups exhibited no significant changes when compared with the control group. The values of RBC, WBC and percent haemoglobin for each group mentioned in Table 16 and comparative graphs were mentioned in Figures 22, 23 and 24 respectively.

S. No.	Groups (n)	RBC Count	WBC Count	% HB Count
1.	Normal	10.72±0.3971	4.7± 0.2449	13.97 ± 0.2805
2.	Control	9.867 ± 0.3559 a**	5.333 ± 0.4179 a***	10.7 ± 0.405 a***
3.	DOX	5.517 ± 0.4355 a*** b***	3.117 ± 0.2639 a*** b***	5.567 ± 0.6593 a*** b***
4.	A.C. (200 mg/kg)	9.488 ± 0.6512 b ^{ns}	5.117 ± 0.3971 b ^{ns}	10.02 ± 0.8841 b ^{ns}
5.	A.C. (400 mg/kg)	9.183 ± 1.182 b ^{ns}	4.85 ± 0.4037 b ^{ns}	9.333 ± 0.6593 b ^{ns}
6.	C.I. (200 mg/kg)	9.367 ± 0.689 b ^{ns}	5.133 ± 0.4274 b ^{ns}	10.07 ± 1.166 b ^{ns}
7.	C.I. (400 mg/kg)	9.067 ± 0.4719 b ^{ns}	4.897 ± 0.6067 b ^{ns}	9.45 ± 0.946 b ^{ns}

Table 16: RBC, WBC and percent Haemoglobin Count of normal, control & treatment groups of animals post-treatment. Where each group n=6 animals; values expressed in mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

a) Red Blood Cell Count:

The change in red blood cell count was observed as when the control group compared with normal there was a significant ($p < 0.01$) decrease in the count was observed. When the doxorubicin treated group was compared with normal as well control group there is more significantly ($p < 0.001$) decreased in the count was observed and when plant extract treated groups were compared with control group count were decreased but not significantly.

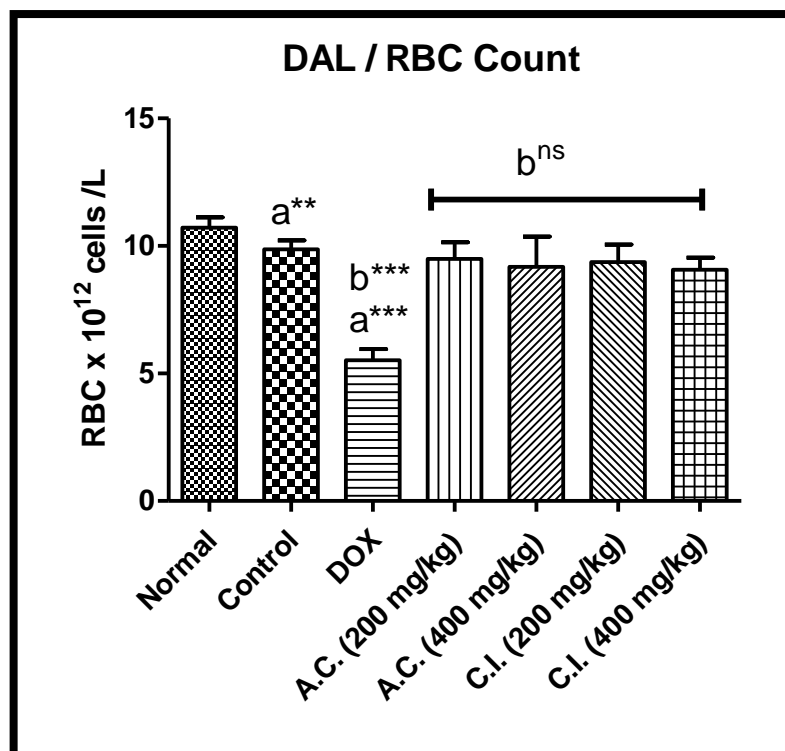


Figure 22: Red Blood Cell count of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

b) White Blood Cell Count:

The change in percent haemoglobin count was observed as when the control group compared with normal there is significant (p <0.001) increased in the count was observed. When the doxorubicin treated group was compared with normal as well control group there is more significantly (p <0.001) decreased in the count was observed and when plant extract treated groups were compared with control group count were decreased but not significantly.

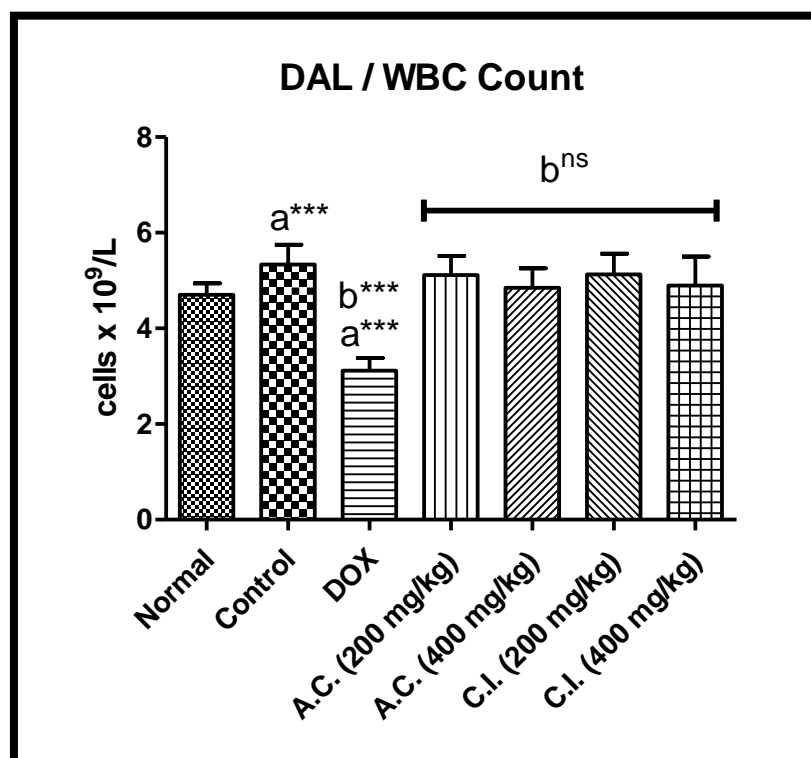


Figure 23: White Blood Cell count of different groups. The values are calculated by using GraphPad Prism 5.0 using one-way ANOVA followed by Dunnett's test, where each group $n=6$ animals; values expressed in mean \pm SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

c) Percent Haemoglobin Count:

The change in percent haemoglobin count was observed as when the control group compared with normal there is significant ($p<0.001$) decrease in the count was observed. When the doxorubicin treated group was compared with normal as well control group there is more significantly ($p<0.001$) decreased in the count was observed and when plant extract treated groups were compared with control group count were decreased but not significantly.

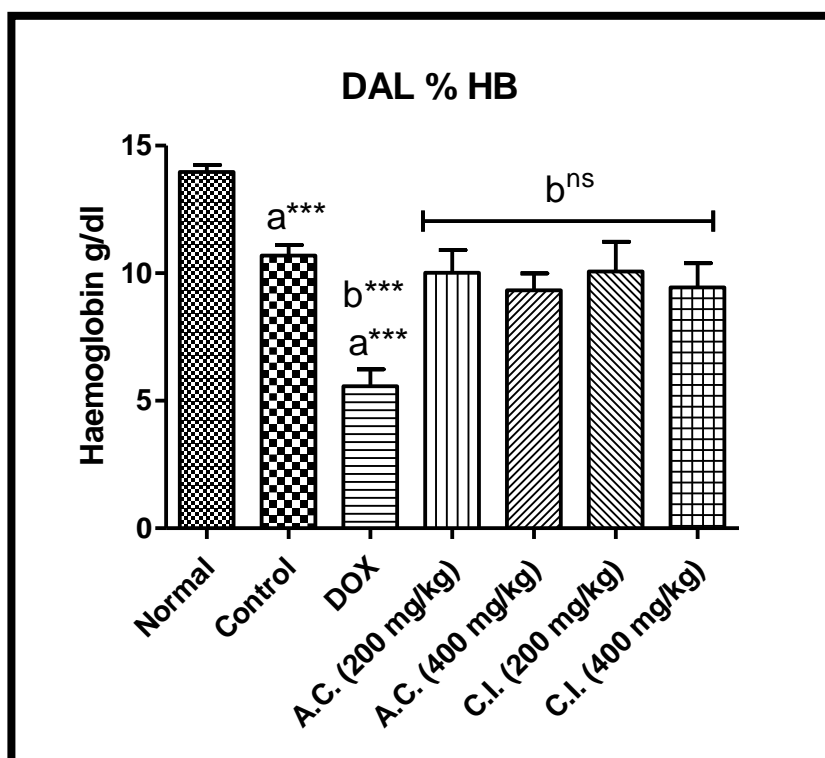


Figure 24: Percent haemoglobin count of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

(C) Tumor Parameters:

After the last dose of the experiment (15th day of the experiment), on the next day, CO₂ asphyxia was given to the animals followed by collection of ascitic fluid through a cut in the abdomen at the lower side. The liquid was further subjected to centrifuge & sedimentation, the solid cells & supernatant was removed & weighed. The tumor parameters - weight & volume of tumor values were given in Table 17.

S. No.	Groups (n)	Tumor Weight (gm.)	Tumor Volume (ml.)
1.	Normal	Non-Tumor Group	Non-Tumor Group
2.	Control	7.37 ± 0.2613	11.95 ± 0.8264
3.	DOX (2.5 mg/kg)	2.94 ± 0.2763 b***	4.9 ± 0.4604 b***
4.	A.C. (200 mg/kg)	6.57 ± 0.3939 b ^{ns}	11.17 ± 0.948 b ^{ns}
5.	A.C. (400 mg/kg)	5.147 ± 0.583 b**	8.283 ± 0.5845 b**
6.	C.I. (200 mg/kg)	6.7 ± 0.5688 b*	10.95 ± 0.6565 b*
7.	C.I. (400 mg/kg)	5.42 ± 0.8676 b**	9.417 ± 0.6432 b**

Table 17: Tumor weight and Tumor volume of tumor-bearing mouse groups after the experiment. Where each group n=6 animals; values expressed in mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compared with Normal group, b- Compare with Control group, ns- Non significant.

a) Tumor Volume

The animals were given the appropriate dose of extracts & standard drug showing the significant changes in tumor volume. The DOX (2.5 mg/kg) group exhibited significant ($p < 0.001$) decrease in tumor volume; *A. coynei* low dose treated group revealing not significant (ns) difference and *A. coynei* high dose expressing significant ($p < 0.01$) decrease; *C. inerme* low dose treated group was at significant ($p < 0.05$) decrease and *C. inerme* high dose treated group was showing significant ($p < 0.01$) decrease in tumor volume respectively when compared with control animal group. The significant difference denoted in Table 17.

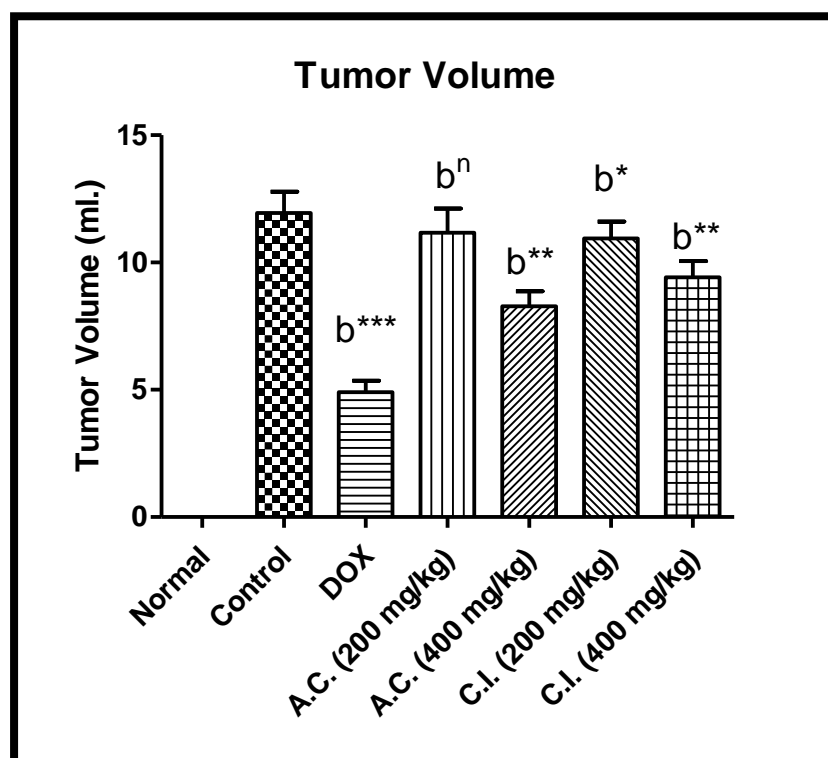


Figure 25: Tumor volume of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compare with Normal group, b- Compared with Control group, ns- Non significant.

b) Tumor Weight

The plant extracts and standard drug-treated groups showing significant changes in tumor weight and results are showing the effect in a dose-dependent manner. When the DOX (2.5 mg/kg) exhibiting significant (p <0.001) decrease in weight of tumor; *A. coynei* low dose treated group revealing not significant (ns) difference and *A. coynei* high dose revealing significant (p <0.01) decrease also, *C. inerme* low dose treated group was showing significant (p <0.05) decrease and *C. inerme* high dose treated group was showing significant (p <0.01) decrease in tumor weight respectively when groups compared with control animals group. The significant difference between each treatment group shown in Table 17.

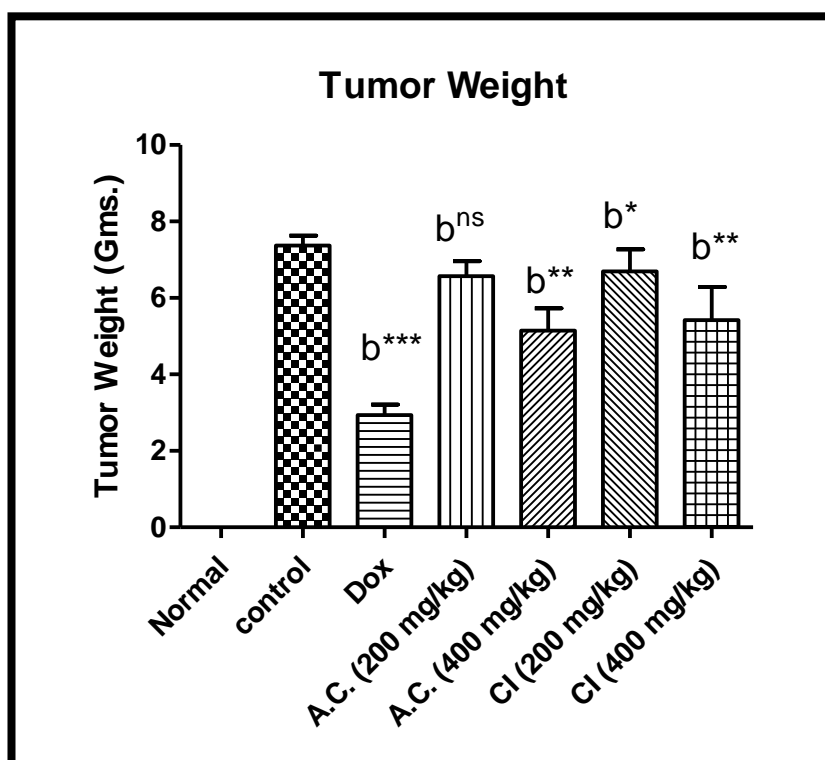


Figure 26: Tumor weight of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compare with Normal group, b- Compared with Control group, *ns*- Non significant.

c) Kaplan- Meier Survival Analysis

This analysis estimates and calculates the survival of subjects after treatment for over a period. The number of subjects survived post-treatment was calculated and the pattern was plotted as survival proportions from first death to last death of animals from each group. The simplicity of this estimate to calculate and compute the animal survival irrespective of the difficulties associated with it. The median survival time data provides the median time of survival for the whole group, which estimated by the first death and last death of animals from a particular group. When we compared with the control group, we get the percent life span.

Therapeutic efficacy of extracts and doxorubicin the effects were evaluated by the Kaplan Meier survival plot which representative for percent survival proportion of animals belong to normal, control and treatment group. Tumor burden parameter for 22

days of maximum animals survival. The Kaplan Meier survival plot which explained that pattern of different groups of animals survived. The control group of animals survived up to the 17th day the median survival time (MST) for the control group was 16 days, this median survival data was considered 100% for calculation of percent increased life span for treated groups. The animals lasts survived for Dox (2.5 mg/kg) treated group was 22 days and MST was 21 days, for *Achyranthes coynei* (Low Dose) treated group was 19 days and MST was 18 days, for *Achyranthes coynei* (High Dose) treated group was 21 days and MST was 20 days and for *Clerodendrum inerme* (Low Dose) treated group was 19 days and MST was 18.5 days, for *Clerodendrum inerme* (High Dose) treated group was 20 days and MST was 19 days respectively. The percent increased life span for Dox (2.5 mg/kg) treated group was 31.25%, for *Achyranthes coynei* (Low Dose) treated group was 12.2%, for *Achyranthes coynei* (High Dose) treated group was 25%, for *Clerodendrum inerme* (Low Dose) treated group was 15.62% and for *Clerodendrum inerme* (Low Dose) treated group was 18.75% obtained.

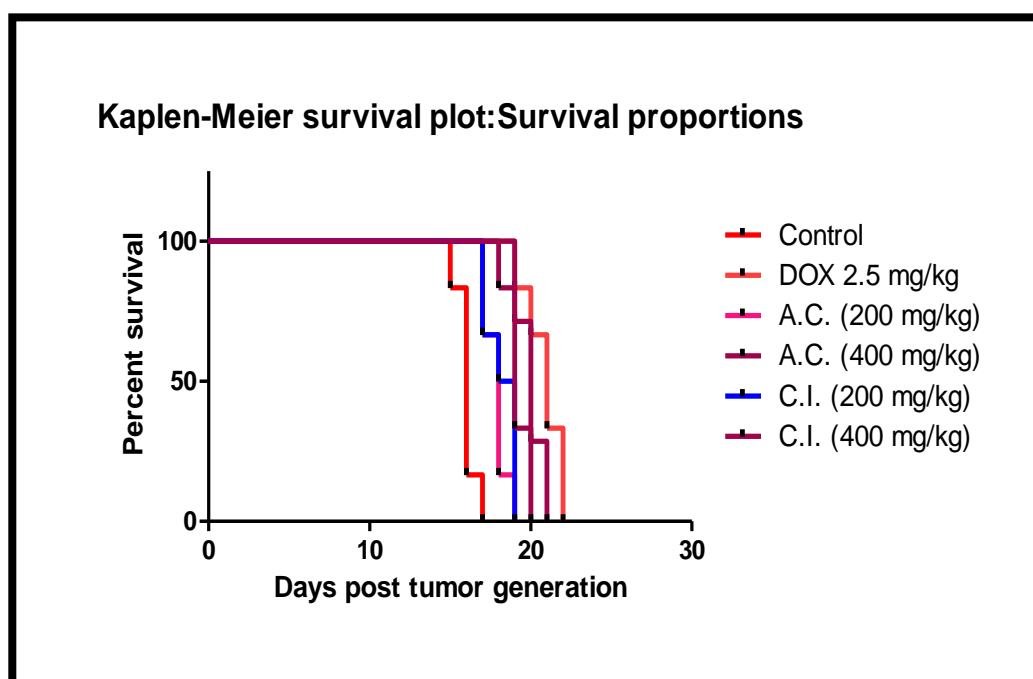


Figure 27: Kaplan-Meier survival estimate for different survival proportions at different groups.

$$\% \text{ Increase Life Span} = \frac{\text{Median Survival of Treatment Group}}{\text{Median Survival of Control Group}} \times 100$$

Survival Proportions						
Days Post Tumor Generation	Control	DOX (2.5 mg/kg)	A.C. (200 mg/kg)	A.C. (400 mg/kg)	C.I. (200 mg/kg)	C.I. (400 mg/kg)
0.000	100	100	100	100	100	100
15.000	83.33					
16.000	16.66					
17.000	0.00		66.66		66.66	
18.000			16.66		50	83.33
19.000		83.33334	0.00	71.42857	0.000	33.33
20.000		66.66		28.57143		0.00
21.000		33.33		0.00		
22.000		0.00				

Table 18: Percent survival proportions of different groups at different days.

d) Median Survival Time

Median survival time data provides the survival time of a group which obtained by first and last animal death. The obtained data is demonstrated in Table 19.

Median Survival Time						
Survival Data Summary	Control	DOX 2.5 mg/kg	A.C. (200 mg/kg)	A.C. (400 mg/kg)	C.I. (200 mg/kg)	C.I. (400 mg/kg)
Number of rows	55	55	55	55	55	55
# of blank lines	49	49	49	48	49	49
# rows with impossible data	0	0	0	0	0	0
# censored subjects	0	0	0	0	0	0
# deaths/events	6	6	6	7	6	6
Median survival	16	21	18	20	18.5	19

Table 19: Median survival time of different groups

e) Percent increased life span

The percent increased life span parameter provides the estimate of changes in the life span of individual treatment groups when the median survival time of a group compared with the control group. This indicates the effect of the intervention on animal groups by using the following formula.

Percent Increased Life Span			
S.No.	Treatment Group (n)	% Life Span	% Increased Life Span
1.	Control	100	---
2.	DOX 2.5 mg/kg	131.25	31.25
3.	<i>Achyranthes coynei</i> (200 mg/kg)	112.2	12.2
4.	<i>Achyranthes coynei</i> (400 mg/kg)	125	25
5.	<i>Clerodendrum inerme</i> (200 mg/kg)	115.62	15.62
6.	<i>Clerodendrum inerme</i> (400 mg/kg)	118.75	18.75

(Note: Calculations are based such that the life span of the control group is 100%)

Table 20: Percent increased life span of different groups.

2. Fibrosarcoma (Solid Tumor) Animal Model in Swiss Mice

The female mice (6-8-week-old) have injected the tumor suspension (0.2 ml) as described in methodology and divided into groups when tumor size reached 0.5 cm accordingly. The duration of the study was 15 days. Various parameters were evaluated viz. body weight, haematological parameters viz. RBC, WBC and percent haemoglobin Count, tumor parameters like tumor size at different time intervals (Tumor progression), percent tumor burden, Kaplan Meier survival plot, percent increased life span, median survival time with statistically analyzed. These parameters provided the effect of plant extract is in a dose-dependent manner.

(A) Body Weight:

Body weight parameter was executed and before the experiment and after the experiment the weight was recorded and compared using statistical analysis for significant changes between groups. When the obtained data were calculated it found that the change in body weight was significantly increased in control group ($p < 0.001$) in comparison of normal animals and significantly decrease in doxorubicin-treated animals group ($p < 0.001$); *A. coynei* low dose ($p < 0.05$), high dose ($p < 0.01$); *C. inermis* low dose ($p < 0.01$); high dose ($p < 0.01$) respectively in comparison with control group. The values of body weight for each group in mean \pm standard deviation with a significant marking are mentioned in Table 21 and the Figure represents the graphical representation of different bar graphs of treatment pattern before and after the experiment.

Body Weight (gm.)			
S. No.	Groups (n)	Weight of Animals in grams Before Experiment	Weight of Animals in grams After Experiment
1.	Normal	21.23 ± 0.5086	28.87 ± 1.692
2.	Control	21.18 ± 0.7548	31.75 ± 0.9354 a***
3.	DOX	21.38 ± 0.9908	27.88 ± 0.8329 b***
4.	A.C. (200 mg/kg)	21.25 ± 0.7791	30.95 ± 1.299 b*
5.	A.C. (400 mg/kg)	22.15 ± 1.300	29.98 ± 0.5879 b**
6.	C. I (200 mg/kg)	21.58 ± 0.9683	31.65 ± 1.276 b*
7.	C. I (400 mg/kg)	22.18 ± 0.5707	30.92 ± 1.113 b**

Table 21: Demonstration Body weight values in gram of different groups of animals at before and after the experiment. Where each group n=6 animals; values expressed in mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compare with Normal group, b- Compared with Control group, ns- Non significant.

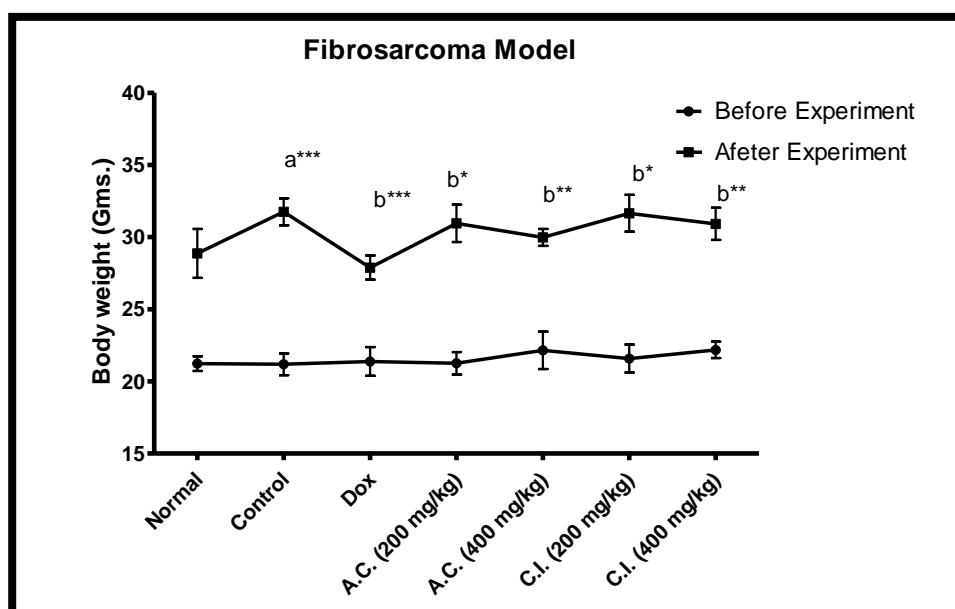


Figure 28: Body weight pattern graph of different groups of animals at before and after the experiment; Where each group n=6 animals; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compare with Normal group, b- Compared with Control group, ns- Non significant.

Haematological Parameters:

The experiment finding suggesting the effect of treatment on various treatment groups on haematological parameters viz. RBC count WBC count and percent haemoglobin data calculated and compared by using one-way ANOVA & Dunnett's test showing the significant changes. When the disease control group compared with normal animal group significant reduction found in view of RBC ($p < 0.05$), percent haemoglobin content ($p < 0.001$) & significant increase found in WBC count ($p < 0.001$). The DOX (2.5 mg/kg) treatment group decreased RBC, WBC and percent haemoglobin content significantly ($p < 0.001$) because of the side effects of the drug, subsequently the plant extracts treated groups *Achyranthes coynei* (Low and High Dose), *Clerodendrum inerme* (Low and High Dose) were showing decrease in values but values are not significant (*ns*).

The study finding suggested the plants *Achyranthes coynei* (Low and High Dose), *Clerodendrum inerme* (Low and High Dose) extracts were expressing the change in RBC, WBC count and, percent haemoglobin content from normal values but not significantly like DOX (2.5 mg/kg) treated group. This finding suggested that the diseased condition changes the normal physiology and biochemical changes which might be due to the different pathways and mechanisms. It was observed that when the doxorubicin group exhibits significant ($P < 0.001$) reduction in RBC, WBC count and percent haemoglobin count but not in the plant treated groups when compared to the control group. The values of RBC, WBC and percent haemoglobin for each group mentioned in Table 22 and comparative graphs were mentioned in Figures 29, 30 and 31 respectively.

S. No.	Groups (n)	RBC Count	WBC Count	% HB Count
1.	Normal	11.12 ± 0.4167	5.383 ± 0.09309	15.1 ± 0.5292
2.	Control	9.617 ± 0.4622 a*	6.383 ± 0.2483 a***	12.18 ± 0.4355 a***
3.	DOX	5.317 ± 0.4355 a*** b***	2.767 ± 0.4227 a*** b***	6.067 ± 0.6743 a*** b***
4.	A.C. (200 mg/kg)	8.883 ± 0.6113 b ^{ns}	6.067 ± 0.4082 b ^{ns}	10.72 ± 1.207 b ^{ns}
5.	A.C. (400 mg/kg)	8.65 ± 0.4087 b ^{ns}	5.85 ± 0.3886 b ^{ns}	10.73 ± 0.9245 b ^{ns}
6.	C.I. (200 mg/kg)	9.383 ± 0.2858 b ^{ns}	6.033 ± 0.5164 b ^{ns}	11.22 ± 0.9766 b ^{ns}
7.	C.I. (400 mg/kg)	9.317 ± 0.4956 b ^{ns}	5.933 ± 0.3445 b ^{ns}	11.23 ± 1.521 b ^{ns}

Table 22: RBC, WBC and percent Haemoglobin Count of different groups of animals after the treatment. Where each group n=6 animals; values expressed in mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

a) Red Blood Cell Count:

The change in red blood cell count was observed when the control group compared with normal there is a significant ($p < 0.05$) decrease in the count that was observed.

When the doxorubicin treated group was compared with normal as well control group there is more significantly ($p < 0.001$) decreased in the count was observed and when plant extract treated groups were compared with control group count were decreased but not significantly.

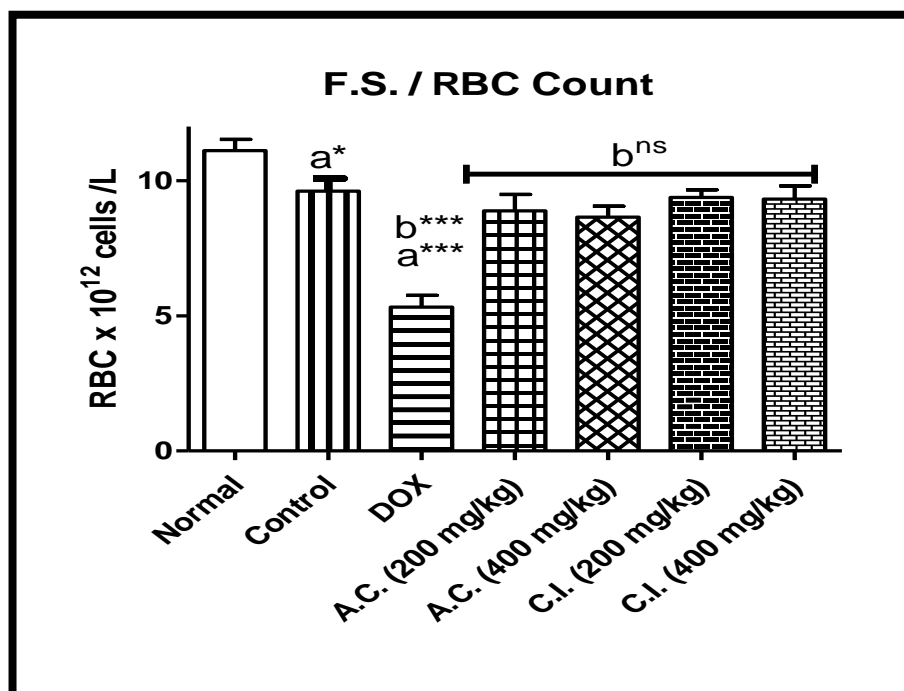


Figure 29: Red Blood Cell count of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

b) White Blood Cell Count:

The change in white blood cell count was observed when the control group compared with normal there is significant (p <0.001) increase in the count was observed. When the doxorubicin treated group was compared with normal as well control group there is more significantly (p <0.001) decreased in the count was observed and when plant extract treated groups were compared with control group count were decreased but not significantly.

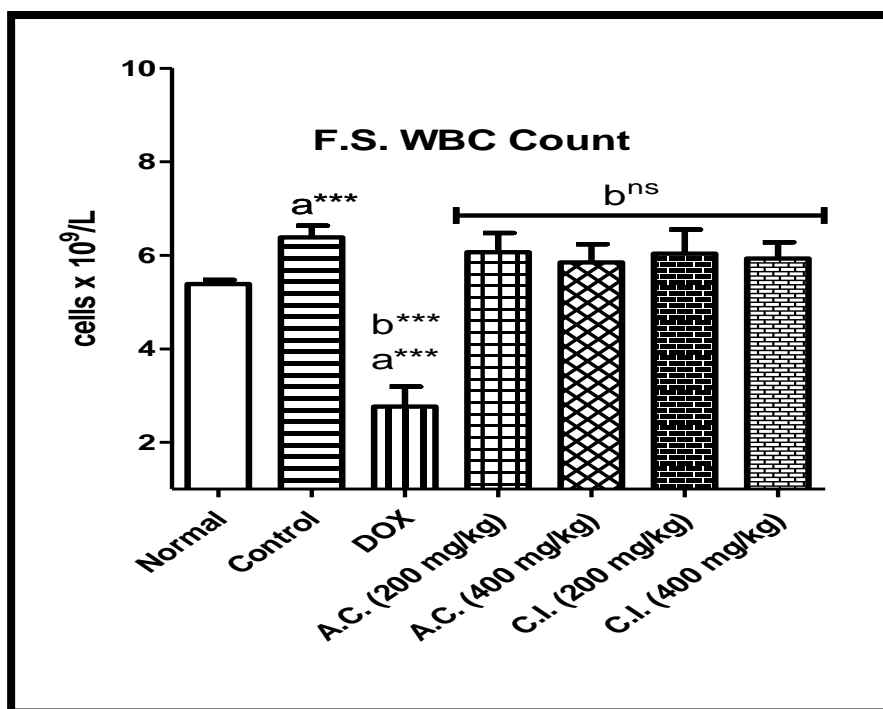


Figure 30: White Blood Cell count of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

c) Percent Haemoglobin count:

The change in percent haemoglobin count was observed as when the control group compared with normal there is significant (p <0.001) decrease in the count was observed.

When the doxorubicin treated group was compared with normal as well control group there is more significantly (p <0.001) decreased in the count was observed and when plant extract treated groups were compared with control group count were decreased but not significantly.

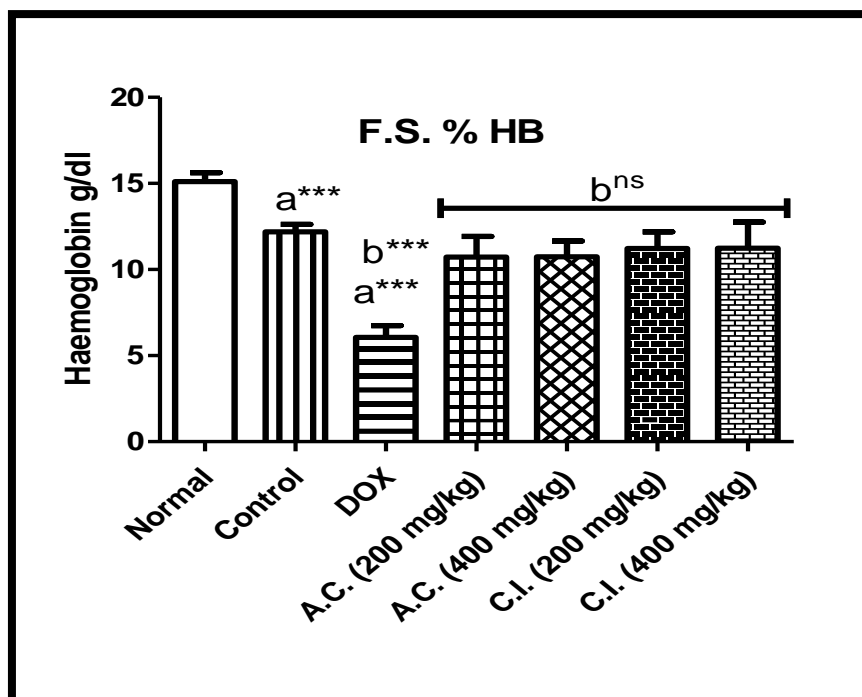


Figure 31: Percent haemoglobin content of different groups. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

(B) Tumor Parameters:

The tumor parameters revealed the effect of various treatment groups. The tumor progression pattern is analyzed by recording the size of the tumor at different time intervals in days as mentioned in Figure number 32. The percentage tumor burden parameter was calculated using tumor progression data, which revealed or expressed the proportional changes as per the treatment administered. The DOX (2.5 mg/kg) treated group showed significantly (p <0.001) decreased, subsequently the plant extracts treated groups *A. coynei* at low dose also reduced significantly (p <0.05), and the same observation was made for high dose of *A. coynei* with a significance (p <0.01) decrease, *C. inerme* low dose decreased significantly (p <0.05), high dose reduced significantly (p <0.05) when compared with the control group. Excised tumors photographs were given in Figure 33.

a) Tumor Burden Parameters

The tumor burden parameter was calculated by the percent change in tumor size at the day 1st and 15th day of the experiment. The pattern of percent change in tumor size is expressed as tumor burden which data is given in Table 23 and Figure 34.

Percent Tumor Burden		
S. No.	Treatment Group (n)	Percent Tumor Burden
1.	Control	496.0 ± 35.3
2.	DOX 2.5 mg/kg	250.0 ± 21.3 b****
3.	<i>Achyranthes coynei</i> (200 mg/kg)	458.0 ± 7.3 b*
4.	<i>Achyranthes coynei</i> (400 mg/kg)	420.0 ± 7.2 b**
5.	<i>Clerodendrum inerme</i> (200 mg/kg)	452.0 ± 9.6 b*
6.	<i>Clerodendrum inerme</i> (400 mg/kg)	436.0 ± 16.9 b*

Table 23: The Percent Tumor Burden of each experimental group. Where each group n=6 animals; values expressed in mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. a – Compare with Normal group, b- Compared with Control group, ns- Non significant.

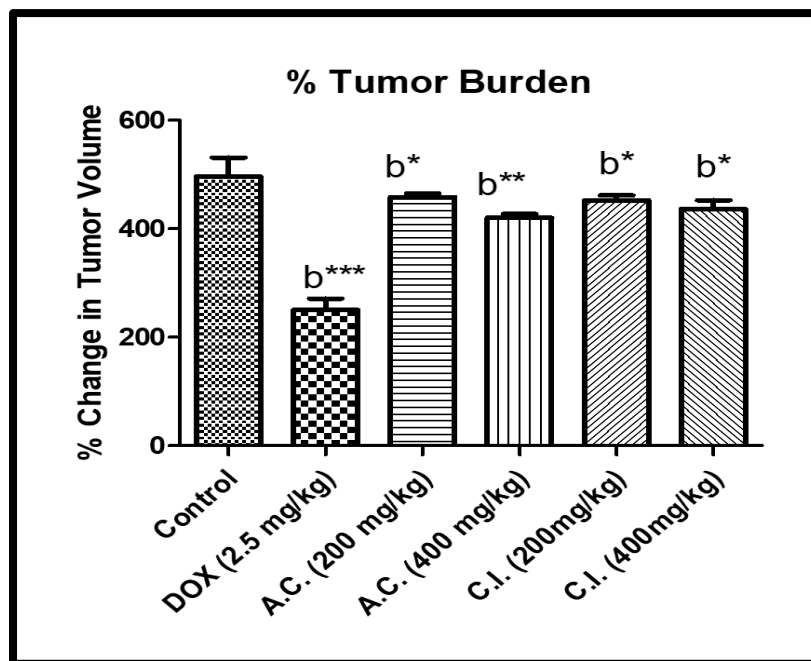


Figure 32: The percent change in tumor burden at the end of the study. Where each group n=6 animals; values expressed in mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001. a – Compared with Normal group, b- Compare with Control group, *ns*- Non significant.

b) Tumor Progression

Tumor progression from day one when the tumor has been grown up to 0.5 cm or 5 mm. after that gap of 2 days the size was measured till the 15th day of the experiment. The data of tumor progression is given in Table 24 and the percent tumor progression data is given in Table 25 and Figure 35 respectively.

Tumor Progression (mm)																		
Days	Control			DOX (2.5 mg/kg)			A.C. (200 mg/kg)			A.C. (400 mg/kg)			C.I. (200mg/kg)			C.I. (400mg/kg)		
	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	N	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
1	5	0.105	6	5	0.06	6	5	0.095	6	5	0.1	6	5	0.05	6	5	0.055	6
3	8.2	1.27	6	6.1	1.17	6	7.9	0.985	6	7.2	1.425	6	7.5	1.17	6	7.7	0.7	6
6	12.7	1.025	6	7.9	1.07	6	10.1	0.61	6	9.8	1.215	6	10.9	0.38	6	10.2	0.62	6
9	15.9	0.76	6	8.5	1.015	6	14.3	0.47	6	13.9	0.43	6	14.9	1.56	6	14.1	0.395	6
12	19.8	1.49	6	9.8	0.73	6	18.8	0.425	6	17.8	0.455	6	19.1	0.73	6	18	1.275	6
15	24.8	1.765	6	12.5	1.065	6	22.9	0.365	6	21	0.36	6	22.6	0.48	6	21.8	0.845	6

Table 24: Tumor size in mm; Tumor progression of experimental animals at different intervals of days. where each group n=6 animals; values expressed in mean \pm SD.

Percent Tumor Progression																		
Days	Control			DOX (2.5 mg/kg)			A.C. (200 mg/kg)			A.C. (400 mg/kg)			C.I. (200mg/kg)			C.I. (400mg/kg)		
	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	N	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
1.0	100	2.1	6	100	1.2	6	100	1.9	6	100	2.0	6	100	1.0	6	100	1.1	6
3.0	164	25.4	6	122	23.4	6	158	19.7	6	144	28.5	6	150	23.4	6	154	14.1	6
6.0	254	20.5	6	158	21.4	6	202	12.2	6	196	24.3	6	218	7.6	6	204	12.4	6
9.0	318	15.2	6	170	20.3	6	286	9.4	6	278	8.6	6	298	31.2	6	282	7.9	6
12.0	396	29.8	6	196	14.6	6	376	8.5	6	356	9.1	6	382	14.6	6	360	25.5	6
15.0	496	35.3	6	250	21.3	6	458	7.3	6	420	7.2	6	452	9.6	6	436	16.9	6

Table 25: Percent tumor progression of experimental animals at different days. where each group n=6 animals; values expressed in mean \pm SD.

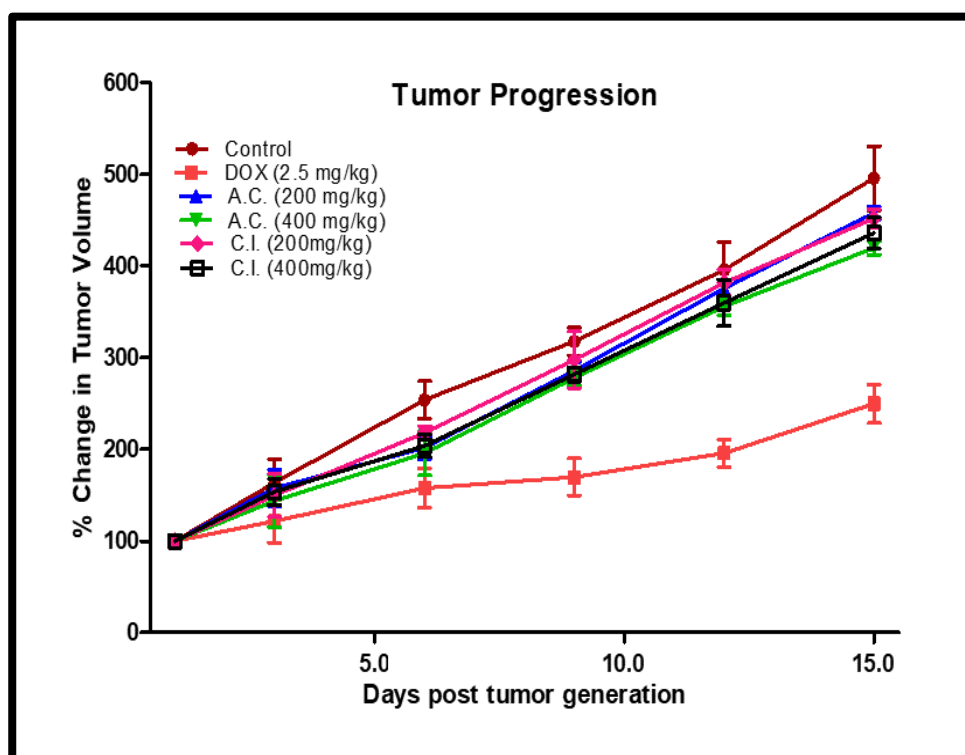


Figure 33: The percentage tumor progression pattern of experimental animals at different intervals of days. where each group n=6 animals; values expressed in mean \pm SD.

c) Kaplan- Meier Survival Plot

This analysis estimates and calculates the survival of subjects after treatment for over a period. The number of subjects survived post-treatment was calculated and the pattern was plotted as survival proportions from first death to last death of animals from each group. The simplicity of this estimate to calculate and compute the animal survival irrespective of the difficulties associated with it. The median survival time data provides the median time of survival for the whole group, which estimated by the first death and the last death of animals from a particular group. When we compared with the control group, we get the percent life span.

The Kaplan Meier survival plot which explained that pattern of different groups of animals survived. The therapeutic efficacy of plant extracts *Achyranthes coynei* (Low and High Dose), *Clerodendrum inerme* (Low and High Dose) and standard drug Doxorubicin [Dox (2.5 mg/kg)] treatment effects were evaluated by a Kaplan Meier survival plot which representative for percent survival proportion of animals belong to normal, control and treatment group. Tumor burden parameter for 45 days of maximum animal survival. The control group of animals survived up to the 21st day the median survival time (MST) for the control group was 19 days, this median survival data was considered 100% for calculation of percent increased life span for treated groups. The animals last surviving for Dox (2.5 mg/kg) treated group was 45 days and MST was 40 days, for *A. coynei* (Low Dose) treated group was 26 days and MST was 25 days, for *A. coynei* (High Dose) treated group was 40 days and MST was 26.5 days and for *C. inerme* (Low Dose) treated group was 24 days and MST was 23.5 days, for *C. inerme* (High Dose) treated group was 26 days and MST was 26.5 days respectively. The percent increased life span for Dox (2.5 mg/kg) treated group was 105.128%, for *A. coynei* (Low Dose) treated group was 28.205%, for *A. coynei* (High Dose) treated group was 35.897%, for *C. inerme* (Low Dose) treated group was 20.512% and for *C. inerme* (Low Dose) treated group was 30.769% obtained.

$$\% \text{ Increase Life Span} = \frac{\text{Median Survival of Treatment group}}{\text{Median Survival of Control Group}} \times 100$$

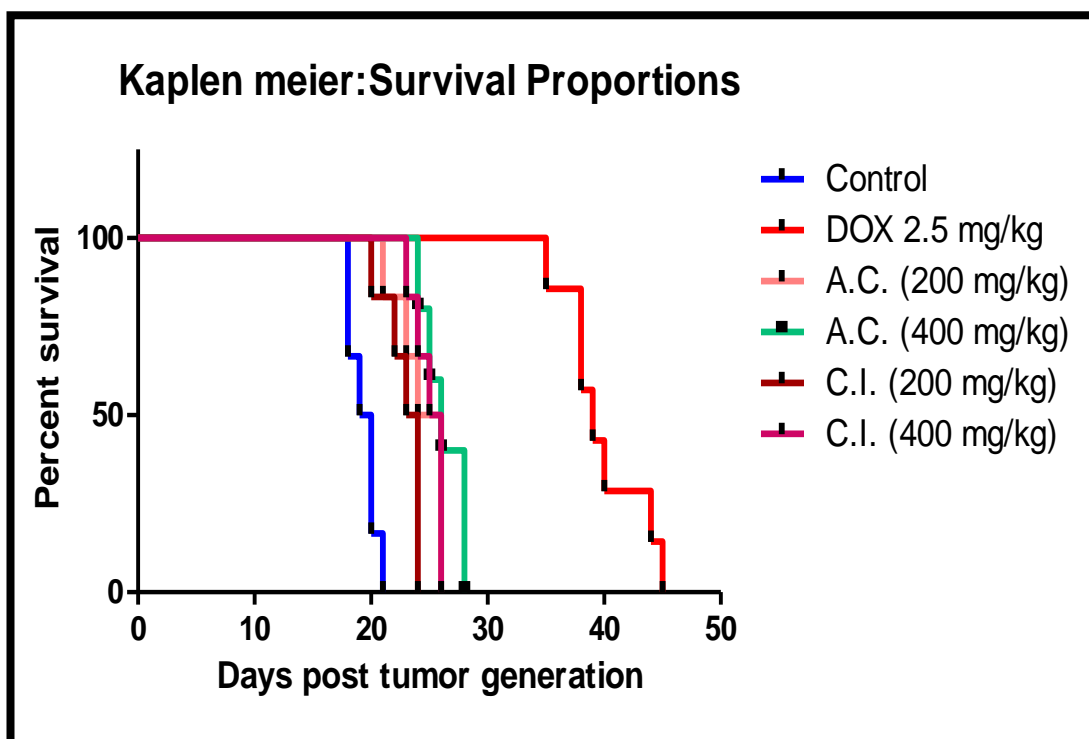


Figure 34: Kaplan-Meier survival estimate for different survival proportions at different groups

Survival Proportions (%)						
Days Post Tumor Generation	Control	DOX (2.5 mg/kg)	A.C. (200 mg/kg)	A.C. (400 mg/kg)	C.I. (200 mg/kg)	C.I. (400 mg/kg)
0	100.00	100.00	100.00	100.00	100.00	100.00
18	66.66					
19	50					
20	16.66				83.33	
21	0		83.33			
22					66.66	
23			66.66		50	83.33
24			50	80	0	66.66
25				60		50
26			0	40		0
28				0		
35		85.71				
38		57.14				
39		42.85				
40		28.57				
44		14.28				
45		0.000				

Table 26: Percent survival proportions of different groups at different days.

d) Median Survival Time

Median survival time data provides the survival time of a group which obtained by first and last animal death.

Median Survival Time						
Survival Data Summary	Control	DOX 2.5 mg/kg	A.C. (200 mg/kg)	A.C. (400 mg/kg)	C.I. (200 mg/kg)	C.I. (400 mg/kg)
Number of rows	195	195	195	195	195	195
# of blank lines	189	189	189	189	189	189
# rows with impossible data	0	0	0	0	0	0
# censored subjects	0	0	0	0	0	0
# deaths/events	6	6	6	7	6	6
Median survival	19.5	40	25	26.5	23.5	25.5

Table 27: Median survival time of different groups.

e) Percent Increased Life Span

The percent increased life span parameter provides the estimate of changes in the life span of individual treatment groups when the median survival time of a group compared with the control group. This indicates the effect of the intervention on animal groups by using the following formula.

Percent increased life span			
S. No.	Treatment Group (n)	% life span	% increased life span
1.	Control	100	---
2.	DOX (2.5 mg/kg)	205.128	105.128
3.	<i>Achyranthes coynei</i> (200 mg/kg)	128.205	28.205
4.	<i>Achyranthes coynei</i> (400 mg/kg)	135.897	35.897
5.	<i>Clerodendrum inerme</i> (200 mg/kg)	120.512	20.512
6.	<i>Clerodendrum inerme</i> (400 mg/kg)	130.769	30.769

Table 28: Percent increased life span of different groups.

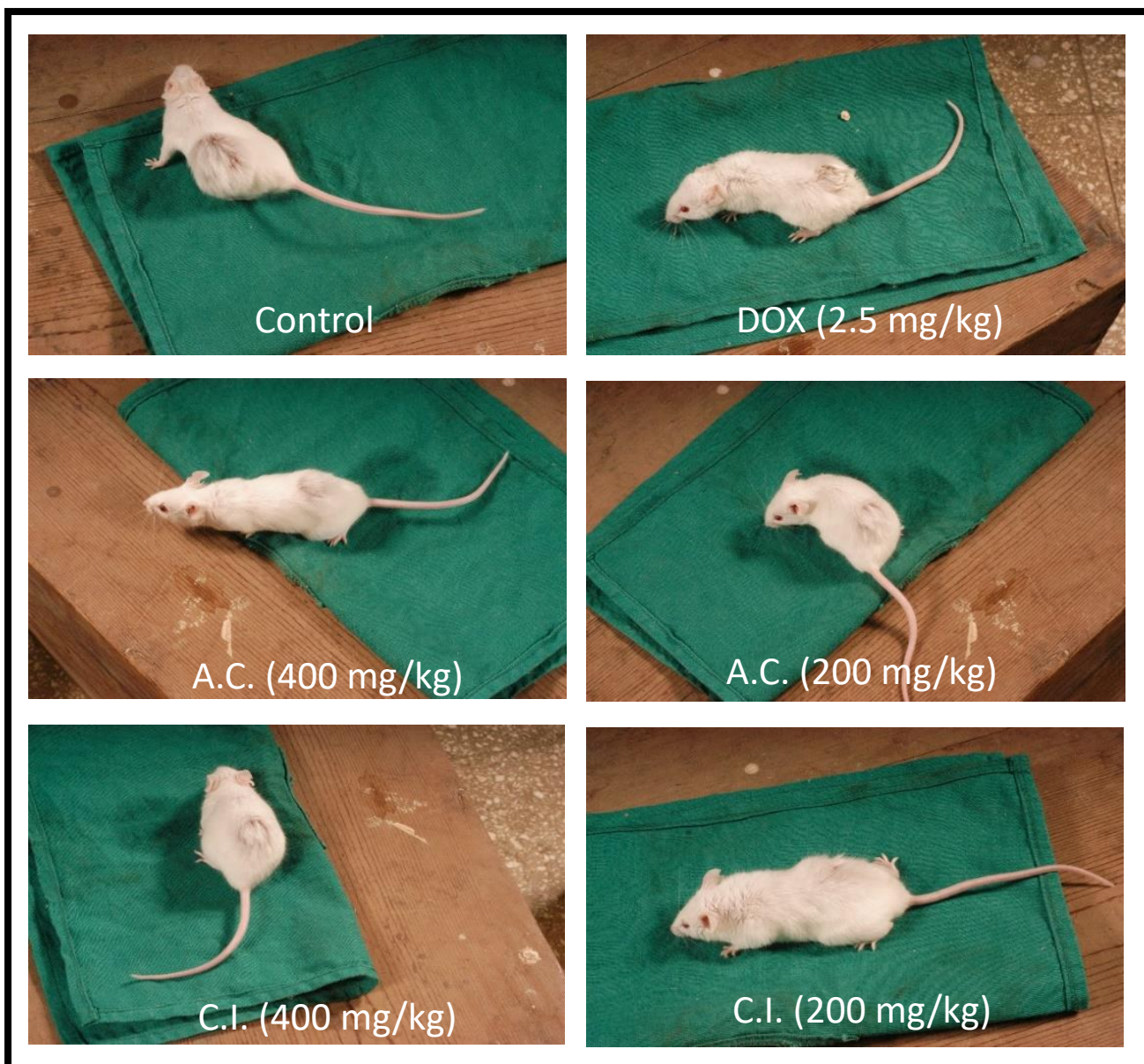


Figure 35: Tumor-bearing (induced) mice of different groups.

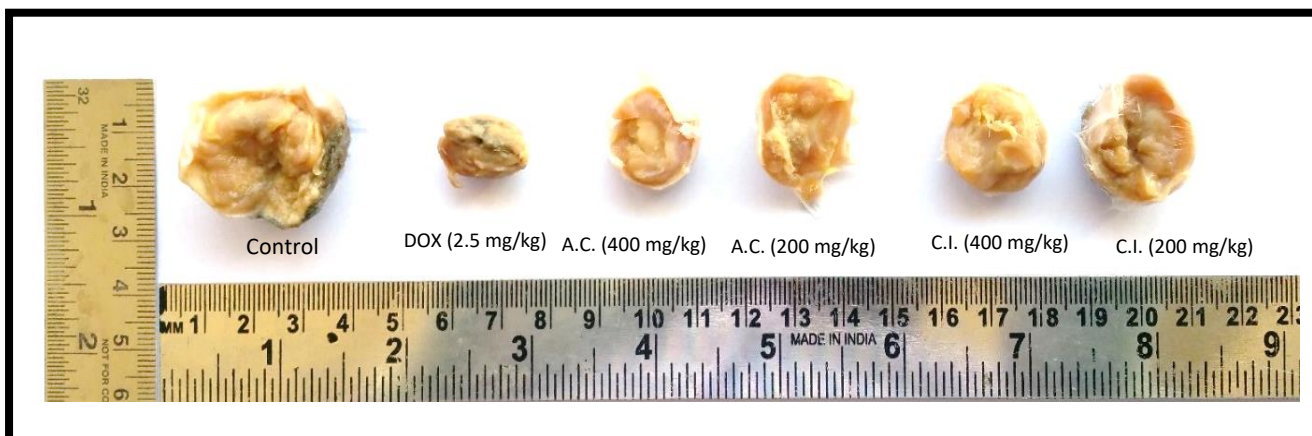


Figure 36: Excised tumors of different group of animals.



DISCUSSION

5. Discussion

Achyranthes coynei Sant. and *Clerodendrum inerme* Gaertn. have ethnomedicinal properties for various ailments and are being used by traditional healers for cancer treatment. Also, these plants were used in various ayurvedic as well as traditional medicinal practices. The exploitation of *A. coynei* Sant. plant has resulted in to be a threatened species (red-listed plant) while *Clerodendrum inerme* (L.) Gaertn. is found abundantly and profusely. In this study, evaluation of anti-cancer activity of *Achyranthes coynei* Sant. and *Clerodendrum inerme* (L.) Gaertn. has been done to provide scientific validation to the folklore claims by various well-accepted *in-vitro* and *in-vivo* models, outcome of which suggests that these plants have anticancer potential.

5.1 Identification of Plants:

The identification of plant material for its authentication was one of the important parameters. *Achyranthes coynei* Sant. Family Amaranthaceae is very similar to *Achyranthes aspera* which belongs to the same family and has similar morphological characters which creates difficulty for the identification and collection. Hence, due to some specific characters, features of *Achyranthes coynei* Sant. which have been described by various reported studies are proven to be similar to *Achyranthes aspera*. [38] The collection and authentication of the plant was performed and the herbarium was submitted at the National Institute of Traditional Medicine – Indian Council for Medical Research, Belagavi, Karnataka with herbarium voucher No.-RMRC-127 and RMRC- 1272.

5.2 Extraction

The dried and coarse powder was extracted by maceration method using methanol and water (70:30), selected for maximum extractive value. The maceration was performed

for 72 hrs. [87] and followed by filtration using Whatman filter paper no.1, the extracts were dried using rotatory evaporator & freeze-drying. The final extract obtained was hygroscopic and sticky in nature.

5.3 Phyto-Chemical Tests

The plant extracts were qualitatively analyzed to evaluate the presence of carbohydrates, proteins, steroids, amino acids, flavonoids, alkaloids, triterpenoids, glycosides, tannins, phenolic compounds, fats & oil. The secondary metabolites like flavonoids, triterpenoids, and alkaloids have anti-cancer potential; in addition, these secondary metabolites also play an essential role in cancer & treatment of various ailments. [42] Further, the extracts of both ethnomedicinal plants & were qualitatively analyzed using HPLC for the presence of phytochemicals and oleanolic acid, betulinic acid & ursolic acid in both extracts which comply with the previously reported outcomes. [54, 55]

5.4 *In-vitro* Studies

5.4.1 Anticancer/ Cytotoxicity Assay (MTT Assay)

The dye exclusion assay was performed for cell count that helps in the differentiation of live and dead cells. The 1×10^3 cells were counted and used for MTT assay, to evaluate the inhibitory concentration of plant extract at various concentrations viz. 1000, 500, 250, 125, 61.5, 31.25 and 15.6 $\mu\text{g/ml}$ and standard drug doxorubicin on various cancer cell viz. *Daudi*, HT-1080, A-549, MCF-7, HT-29, and HL-60. These results demonstrated that both plant extracts sensitized cell growth & multiplication in a dose-dependent way. *Achyranthes coynei* showed the anti-proliferative activity on *Daudi*, HT-1080, HT-29, A-549, HL-60, MCF-7 at their respective IC_{50} values which represents that the plant extract in its crude form shows anticancer potential by inhibiting cell growth and inhibition was shown in the following order: MCF-7 > HT-

29 > HT-1080 > *Daudi* > A-549 > HL-60. These results comply with a previous study performed on other species of *Achyranthes coynei* that is *A. aspera* that showed anticancer potential on pancreatic cell line MiaPaCa-2, Panc, Ht-29, breast cancer SKBR, prostate cancer cell line PC-3 and A-549 lung cancer cell lines. *A. aspera* is used in many ayurvedic formulations and is given routinely to the cancer patients by ayurvedic physicians [44]; *Achyranthes coynei* is also used due to the same morphological characteristics. [38] Our observation in this study serves as an additional independent report on anticancer potential of these two plants. *Achyranthes coynei*, a threatened species, was for the first time reported from the Western Ghats region [38] and this study was performed for first time for *Achyranthes coynei* plant extract to evaluate for its anticancer potential as is claimed according to traditional folklore.

Clerodendrum inerme Gaertn. extract also showed the anti-proliferative activity and the effect observed was in the following order: HT-1080 > *Daudi* > MCF-7 > HT-29 > A-549 > HL-60. The findings of MTT assay suggest that, *Clerodendrum inerme* Gaertn. has anticancer potential and it sensitizes the cancer cells in a dose-dependent manner which confirms its toxic character. The previous study reports that *Clerodendrum inerme* is a conventional herbal medicine and its effect against lung adenocarcinoma epithelial cell line has also been highlighted. [116, 117, 118] Different studies have suggested the anticancer potential of other species of same genus of the plant for its pharmacological efficacies and benefits. The MTT assay is one of the highly sensitive measures with different treatment. This assay calculates the end point measure / estimate which is not an immediate change in metabolic and structural integrity of cells. [44, 107] In the present study, doxorubicin showed to be most effective against HL-60 followed by *Daudi*, MCF-7, HT-29, HT-1080 and A-549 with the effect being decreased

with the order. Doxorubicin is a well-established drug for the treatment of cancer; its effect on cancer cells is higher than the plant extract because plant extracts are in crude form and contain Betulinic acid, Ursolic acid, Oleanolic acid and other phytoconstituents. Therefore, they need to be characterized for a single compound for further determination of their anticancer potential. [9, 80, 86] The outcome of the *in-vitro* anticancer profiling suggested that plant extract sensitizes the proliferation and has anticancer potential. Our study results suggest that, both plants may hold favorable as a new treatment / therapeutic option.

5.5 *In-vivo* Studies

5.5.1 Brine Shrimp Lethality Assay

The bioassay provides the toxicity assessment of any plant extract of chemical compounds. The assay provides the toxicity of extract at a different concentration which was performed in triplicate. The test gives the criteria of the category or character of any compound or plant extract to be analyzed. The brine shrimps were exposed to plant extracts (*Achyranthes coynei* and *Clerodendrum inerme*) & the lethal dose (50% of the treated population died) for extracts was found to be below 100 ppm. As per the literature *A. aspera*, extract was exposed to the brine shrimp at different time intervals and the results showed that, the mortality rate of brine shrimp was found to be elevated with growth in concentration of the extract. Hence, these findings seem to be in accordance with the results of our study. [119] The performed studies for various *Clerodendrum inerme* extract effects also suggest the same effect which supports the performed bioassay results. [110, 111] The previous study evaluated the same bioassay on *Clerodendrum infortunatum* Linn., a traditional medicine, observations for its cytotoxicity on brine shrimp nauplii was observed and similar effects were noted. [109]

This study for cytotoxicity of extracts at their lethal dose value follows good correlation with the previous studies and also suggests the potential chemical compound present in the crude extract responsible for that effect. The lethality assay suggests that both plant extracts have cytotoxic potential.

5.5.2 Animal Study

5.5.3 Oral Toxicity Studies

As per OECD guideline 423, animals were kept on standard protocol for food, water and light-dark cycle with the proper temperature and humidity. The animals were given an initial dose of 2000 mg/kg of animal body weight. The observation suggested absence of toxicity.

It was found that extracts of *Achyranthes coynei* Sant. and *Clerodendrum inerme* Gaertn. were found safe at 2000 mg/kg dose. There was no toxicity found. This dose was taken as reference dose and one-tenth of it i.e. 200 mg/kg as low dose & two-tenth i.e. 400 mg/kg as high dose was considered for further study.

5.5.4 Animal Cancer Model

- 1. DAL Model (Dalton's Ascitic Lymphoma Model)**
- 2. Fibrosarcoma Model**

1. DAL Model

The female mice were injected with the cell suspension of ascitic fluid from the tumor-bearing mouse. The study duration was 15 days and the animals divided into different treatment groups as per the materials and methods.

In DAL tumor, the spreading of cancer to the abdominal lining (peritoneum) can result in irritation which is soothed by production of a fluid within the abdomen. The spread of cancer continues to the portal vein resulting in damaged circulation due to increased blood force in the liver that results in pooling of fluid in the abdomen. Damaged liver produces fewer blood proteins leading to a disrupted fluid balance in the body & this further causes fluids to accumulate in the abdomen and body tissues. The blockage of lymphatic system due to tumor growth hinders the drainage of excess fluid resulting in fluid accumulation in the abdomen. Hence, a rapid increase in the volume of ascites tumor was observed in DAL tumor developing mice. Tumor growth is directly proportional to the amount of ascites fluid because ascites fluid serves as a direct dietary supply for cancer cells. [120, 121] The various parameters were examined like body weight before and after the experiment, a comparison between the groups which explained the change in body weight pattern and the effect of various treatments on it. There were significant changes that occurred in treatment groups. In bodyweight parameters, when control & normal groups were compared, a significant increase ($p < 0.001$) was observed. This change may have been due to the aggressiveness of lymphoma, which speeds very fast & changed the physiology of control group animals. Doxorubicin (2.5 mg/kg) was given intraperitoneal, treated groups animal weight decreased significantly ($p < 0.001$) when a comparison was made with the control group. The changes in body weight suggests of its strong cytotoxic effect on cancer cells as well normal cells, which resulted in many complications like myelosuppression,

reduction of RBC and WBC and other complications. *Achyranthes coynei* (Low Dose) showed insignificant decrease in the body weight while *Achyranthes coynei* (High Dose) treated group exhibited a significant decrease in body weight. The *Clerodendrum inerme* treated low & high doses showed significant decrease in body weight parameter. Similarly, *Mimosa pudica* extract was evaluated for the liquid tumor model and the findings also showed the same effect on blood parameters [122]. The favorable changes in body weight in plant extract-treated groups may be due to the anticancer principle compound present in plant extracts.

In the haematological parameters, it was observed that the RBC count and percent haemoglobin was decreased significantly in the control group when compared to the normal group. Doxorubicin (2.5 mg/kg) treated group exhibited significant decrease in RBC count compared to control group. The *Achyranthes coynei* low & high doses as well as *Clerodendrum inerme* low & high doses showed an insignificant decrease in RBC count and haemoglobin content when compared with the control group. The changes in RBC count and haemoglobin content were not significant in the case of plant extract treated groups which implies that plant extracts do not induce RBC and haemoglobin destruction.

For WBC count, the control group exhibited significant increase as compared to the normal group. When the Doxorubicin (2.5 mg/kg) treated group was compared with the control group, significant reduction in WBC count was observed. The WBC count for *Achyranthes coynei* low & high doses and *Clerodendrum inerme* low & high doses indicated insignificant decrease in comparison to the control group.

In the control group, an increase in WBC count was noted as a result of natural bodily immune response. The doxorubicin treated group demonstrated a drastic decrease in all haematological parameters due to its toxicity. The plant extract treated groups as well

expressed a decrease in haematological parameters, but it was insignificant which may have been due to the presence of natural antioxidants, nutraceuticals and other contents. Myelosuppression and anemia are the major problems encountered in cancer chemotherapy wherein doxorubicin is administered widely. The anaemia faced in tumor-bearing mice is either due to a reduction in RBC or haemoglobin which may be a result of iron deficiency or due to haemolytic or myelopathic conditions. [123] Upon treatment with *Ammannia baccifera* L., the haemoglobin content was restored; similarly, even RBC and WBC counts were restored to normal levels. [124] In another study, *Bauhinia variegata* was found to have protective action on the hemopoietic system. [125] The effect of *Achyranthes coynei* extract on blood parameters was the first kind of study and both extracts have shown identical results as previous studies. [112] The possible reason for similar effect may be due to the presence of anticancer properties.

Tumor parameters i.e. tumor volume & weight findings were found to be reduced significantly in the doxorubicin treated group in comparison to the control group. When mice were treated with *Achyranthes coynei* low dose, the volume and weight reduced insignificantly while for *Achyranthes coynei* high dose significant reduction ($p<0.01$) was observed; *Clerodendrum inerme* low dose treated group reduced significantly ($p<0.05$), *Clerodendrum inerme* high dose reduced significantly ($p<0.01$) when compared with the control group respectively. The findings suggest that treatment with the standard drug is improving the diseased condition. The plant extract-treated group as well showed a similar effect which suggests that the plant extract possesses the same effect as was observed in the previous study on DAL model, where *Streblus asper* inhibited the tumor volume and the DAL tumor-bearing animals exhibited a regular rapid increase in ascitic tumor volume. [128] In a similar study, DAL model was used

where *Premna herbacea* Roxb. inhibited the tumor volume and weight, indicating that the compound prevented the cancer cells from proliferating in the peritoneal region of the mice. [127] Ascitic fluid forms the direct nutritional source for tumor cells and a rapid increase in an ascitic fluid with tumor growth would suggest its means to meet the nutritional requirement. [120, 121] These similar findings suggest that the change in the tumor volume and weight are a result of extracts being administered in a dose dependent manner.

Kaplan-Meier survival data provides the survival proportion of different groups which is given in Figure 27. The survival proportions of different groups as per the treatment were given. At 15th, 16th, 17th day, the survival proportion was 83.33, 16.66 and 0% respectively; for Doxorubicin (2.5 mg/kg) treated group at 19th, 20th, 21st and 22nd day it was 83.33, 66.66, 33.3 and 0% respectively; for *Achyranthes coynei* (Low Dose) treated group at 17th, 18th and 19th day it was 66.66, 16.66 and 0% respectively; for *Achyranthes coynei* (High Dose) treated group at 19th, 20th and 21st day it was 71.428, 28.571 and 0% respectively; for *Clerodendrum inerme* (Low Dose) treated group at 17th, 18th and 19th day it was 66.66, 50 and 0% respectively and for *Clerodendrum inerme* (High Dose) treated group at 18th, 19th and 20th day it was 83.33, 33.33 and 0% respectively. The median survival time for control group was 16 days, 21 days for doxorubicin (2.5 mg/kg) treated group, 18 days for *Achyranthes coynei* (Low Dose) treated group, 20 days for *Achyranthes coynei* (High Dose) treated group, 18.5 days for *Clerodendrum inerme* (Low Dose) treated group and 19 days for *Clerodendrum inerme* (High Dose) treated group; which explains the median survival time of each group. The percent increased life span due to the different treatment was observed. The control group's life span was considered 100% and on the basis of it calculated the values for other groups. The percentage increase in life span was 31.25% for doxorubicin (2.5

mg/kg) treated group; 12.2% for *Achyranthes coynei* (Low Dose) treated group, 25% for *Achyranthes coynei* (High Dose) treated group, 15.62% for *Clerodendrum inerme* (Low Dose) treated group and 18.75% for *Clerodendrum inerme* (High Dose) treated group. The Kaplan-Meier survival data summary explained the effect of treatment and the subject at risk. The findings of the Kaplan-Meier survival data summary suggested that doxorubicin (2.5 mg/kg) treated group survived more than *Achyranthes coynei* and *Clerodendrum inerme* treated groups. Prolongation of life span of the tumor bearing animal forms a reliable criterion to estimate the worth of any anticancer drug. [126] An increase in life span of the tumor bearing animal more than 25% indicates considerable antitumor activity. [128] The findings of our study show the anti-tumor potential in terms of median survival time and percent increased life span which is increased; the *Achyranthes coynei* 400 mg/kg dose is significantly effective which shows that the effect is dose dependent.

The reduced tumor volume and extended survival time of both extracts treated tumor bearing mice is suggestive of the delaying impact on tumor proliferation. The DAL model findings suggested that both plant extracts sensitize the tumor growth pattern as well other parameters which supports to anticancer potential.

2. Fibrosarcoma (Solid Tumor) Model

The female mice were injected with the cell suspension of fibrosarcoma tumor. When the tumor developed & reached the size of 0.5 cm as was calculated with the help of Vernier calipers, these animals were divided into different groups. The extent of the study stayed 15 days.

The animal body weight of different groups was taken before and after the experiment to observe the change in body weight pattern. When the comparison was made between the groups, a significant increase ($p < 0.001$) was recorded; doxorubicin (2.5 mg/kg) treated group exhibited significant ($p < 0.001$) decrease compared to control animals' group. Reduced body weight due to doxorubicin has a strong cytotoxic effect on cancer cells as well as normal cells, which resulted in many complications like myelosuppression, reduction of RBC, WBC and other complications. [113] In terms of body weight, *Achyranthes coynei* at low dose exhibited significant decrease ($p < 0.05$), *Achyranthes coynei* high dose exhibited significant decrease ($p < 0.01$); *Clerodendrum inerme* low & high doses showed significant increase $p < 0.05$ & $p < 0.01$ in comparison to control group. Some previous studies results support our findings. Complex metabolic disturbances are observed, in humans as well as experimental animals, due to malignancies. It results in rapid decline in body weight and causes tissue wasting. The body weight progressively decreased in tumor hosts and comparatively it increased routinely in controls. In another study, similar pattern was reported, body weights steadily increased after the treatment of *Indigofera aspalathoides* extracts rather than the fibrosarcoma bearing animals. The study recorded marked loss of body weight. [129, 130] The change in body weight in treatment group exhibits a pattern that is supported by the previous studies. Body weight reduction in tumor bearing mice may be attributed to the generation of ATP by the liver through the Krebs's cycle. Another probability

suggests that there may be reduced muscle protein synthesis and muscle breakdown of proteins due to increased tumor burden. [131] These amino acids that are a result of protein breakdown are subsequently utilized by the liver, further increasing the host's metabolic burden. [132] Anticancer principle compounds present in plant extracts may be responsible for the observed changes in body weight.

The hematological parameters when observed showed the following results: RBC count of the control group declined significantly ($p < 0.05$) when compared with normal group. When the group treated with doxorubicin (2.5 mg/kg) was compared with normal & control groups, RBC count declined significantly ($p < 0.001$). *Achyranthes coynei* at low & high doses and *Clerodendrum inerme* low & high doses showed a decrease in RBC count but it was not statistically significant. The changes in RBC count were not significant in the case of plant extract treated groups which showed that the plant extracts did not induce any significant destruction of RBC. The WBC count recorded a significant ($p < 0.05$) increase in the control group as compared to the normal group. The doxorubicin treated group (2.5 mg/kg) exhibited significant ($p < 0.001$) decrease when compared to the normal & control group. The group treated with *Achyranthes coynei* low & high doses and *Clerodendrum inerme* low & high doses showed decrease but it was not statistically significant. The hemoglobin content declined in the control group significantly ($p < 0.05$) when compared to the normal group. The doxorubicin treated group exhibited significant ($p < 0.001$) decline when compared with normal & control animal groups. *Achyranthes coynei* low & high doses and *Clerodendrum inerme* low & high doses showed decreases but it was not statistically significant.

The increase in WBC count in the control group was due to immune response, the doxorubicin treated group showing the drastic decrease in RBC, WBC count and hemoglobin content due to its toxicity. The plant extract treated groups showed

decreases in RBC, WBC count and haemoglobin content when compared to control group. The findings of previous study suggests that similar pattern was recorded and can be interpolated with the study where the RBC count and percent hemoglobin were decreased in control group and the treated groups overcome the condition and maintain normal level, in case of WBC the level was increased due to immunological response in control group, distinguished difference was recorded in treated group. [99, 100] These findings strongly support our study findings and help to conclude that the hematological parameters were improved due to the plant extract containing anticancer phytoconstituents. The reduction in RBC levels and hemoglobin percentage constitutes to be the major problem in cancer bearing animals. [100, 133] This can be attributed to iron deficiency as observed in hemolytic and myelopathic conditions. [134] Normalization of RBC and percent hemoglobin occurs with treatment with plant extracts. WBC levels increase with the growth of tumor; the rise in WBC count of the solid tumor bearing treated mice with extracts follows a much slower rate when compared to the control group. The presence of natural antioxidants & nutraceuticals and other content may be the reason behind it. These result findings support the previous results. [114, 115] The effect of *Achyranthes coynei* extract on fibrosarcoma (Solid tumor model) model's various parameters are evaluated for the first time in this study.

The tumor progression pattern revealed the pattern on different days, intervals and increase in the size of the tumor. This gives the data with day wise increase in tumor progression pattern. At the end of this study, tumor burden in doxorubicin (2.5 mg/kg) treated group exhibited significantly ($p < 0.010$) decrease; *Achyranthes coynei* low dose treated group exhibited significant decrease ($p < 0.05$), high dose as well revealed significant ($p < 0.01$) decrease; *Clerodendrum inerme* group treated with low & high dose exhibited significant ($p < 0.05$) decrease when compared with control group. These

findings suggest that treatment with the standard drug as well plant extract-treated group improved the disease condition. The study wherein *Ginkgo biloba* extract's effect on solid tumor was observed, it exhibited similar findings related to the pattern of tumor progression i.e. increased time for tumor growth suggesting reduction in metastatic condition due to tumor growth. [135] The reduced pattern of tumor progression or tumor burden was similarly observed in our study which forms the conclusion for *Achyranthes coynei* and *Clerodendrum inerme* extracts.

Kaplan-Meier survival data provides the survival proportion of different groups which given in Figure 36. The survival proportions of different groups as per the treatment were given. In the control group at 18th, 19th, 20th and 21st day the survival proportion was 66.66, 50, 16.66 and 0%; doxorubicin (2.5 mg/kg) treated group at 35th, 38th, 39th, 40th, 44th, and 45th day it was 85.71, 57.17, 42.85, 28.57, 14.28 and 0%; *Achyranthes coynei* (Low Dose) treated group at 21st, 23rd, 50th, and 26th day it was 83.33, 66.66, 50 and 0%; *Achyranthes coynei* (High Dose) treated group at 24th, 25th, 26th and 28th day it was 80, 60, 40 and 0%; for *Clerodendrum inerme* (Low Dose) treated group at 20th, 22nd, 23rd and 24th day it was 83.33, 66.66, 50 and 0%; *Clerodendrum inerme* (High Dose) treated group at 23rd, 24th, 25th and 26th day it was 83.33, 66.66, 50 and 0% respectively. The MST as noted for the control group was 19.5; doxorubicin (2.5 mg/kg) treated group 40 days; *Achyranthes coynei* (Low Dose) treated group 25 days; *Achyranthes coynei* (High Dose) treated group 26.5 days; *Clerodendrum inerme* (Low Dose) treated group 23.5 days; *Clerodendrum inerme* (High Dose) treated group 25.5 days respectively. This explains the MST of each group. The percent increased life span due to different treatment was observed. The control group's life span was considered 100% and on the basis of it calculated the values for other groups. The percent increased life span for group treated with doxorubicin (2.5 mg/kg) it was

105.125%; 28.205% for *Achyranthes coynei* (Low Dose) treated group, 35.897% for *Achyranthes coynei* (High Dose) treated group, 20.512% for *Clerodendrum inerme* (Low Dose) treated group and 30.769% for *Clerodendrum inerme* (High Dose) treated group. The Kaplan-Meier survival data summary explained the effect of treatment and the subject at risk. The findings of the Kaplan-Meier survival data summary suggest that doxorubicin (2.5 mg/kg) treated animals survived higher than *Achyranthes coynei* and *Clerodendrum inerme treated* groups which confirmed that the plant extracts increased the life span when compared to control group. The outcome of our study complies with another study where two cancer models were performed and it was found that the extract of medicinal mushroom *Cordyceps taii* inhibits tumor growth in tumor-bearing mice profoundly. It was also observed that the survival time and the survival rate was increased in both the models. [136] Similarly, a plant-based study where *Withania somnifera* was experimented on, the related outcome where the same tumor model was used and the tumor growth pattern was inhibited and the percent survival exhibited the same death pattern outcome and the median survival was increased in extract treated animals. The percentage increase in life span was remarkably similar to our study. [137] Previous study suggests tumor suppression due to the anticancer potential of plant extract and this outcome resonates with our study in terms that the pattern of tumor suppression can be observed with dose dependent manner.

The selection of Dalton's ascitic lymphoma model and fibrosarcoma model are well accepted and established method with most mimicking models which have similarity with human cancer, this is one of the selection criteria for studying the preclinical anticancer investigation and evaluate the genetics for anticancer therapies. The various criteria help to understand the changes, because of various levels of treatment. The possible mechanism of action of the effect of plant extracts on cancer cells may be

because of cellular interactions of active compounds by endocytosis followed by generation of reactive oxygen species (ROS). ROS has different interactions like mitochondrial dysfunction and activation of caspase cascade which induce DNA fragmentation and chromosomal abrasion. ROS directly also causes fragmentation of DNA. The intracellular interactions of ROS with molecules like protein, lipids form apoptotic body to lead to cell death. Lipid peroxidation of cell membrane lipids causes the change in membrane integrity and cellular leakage which further leads to cell death. These are the possible pathways of apoptosis induced by the active principle present in the plant extract. The possible mechanism of action is mentioned in the graphical representation given below in Figure 37. The findings of fibrosarcoma model suggest that both the plant extracts improve the life span as well the tumor growth and other parameters which evidenced that plants have anticancer potential.

The future scope of the study can be in the direction of evaluation of change in cellular integrity parameters and checkpoints like mitochondrial dysfunction which cause of caspase cascade activation and proteins which are responsible for apoptosis can be studied further. The cellular macromolecule interactions with ROS (Reactive oxygen species) and cell membrane damage because of lipid peroxidation also can be scope for future study which can be given a clear picture of conclusion for a possible mechanism of the anticancer potential of these ethnomedicinal plants.

Graphical Representation

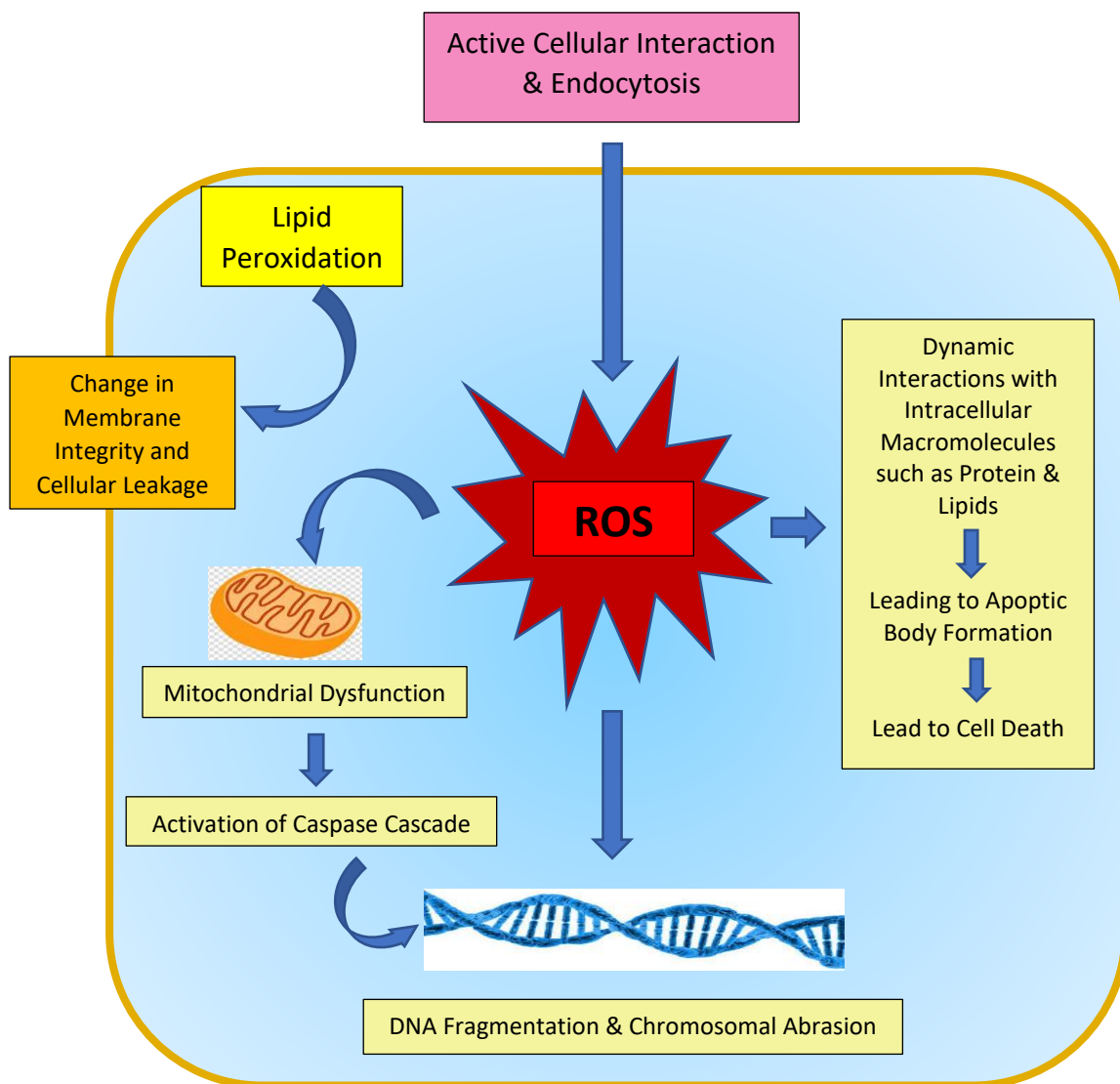


Figure 37: Possible Mechanism for Anticancer Activity

The word "SUMMARY" is centered within a red double-line border. The border consists of two parallel red lines, one slightly offset from the other, creating a rectangular frame around the text.

SUMMARY

7. Summary

The ethnomedicinal plants selected for this study were known for various ailments and to cure diseases. The study focus was on the evaluation of ethnomedicinal plants for their folklore claims and the selected ethnomedicinal plants *Achyranthes coynei* Sant. family Amaranthaceae and *Clerodendrum inerme* Gaertn. family Verbenaceae. The *A. coynei* Sant. chosen for the study has morphologically similar characteristics to that of *Achyranthes aspera*. plant. These plants are well documented in the Indian system of medicine as a potential anticancer remedy, this has also been documented in other traditional transcripts as well. The present study focused on the scientific validation of the ethnomedicinal plants taking into account the folklore claims and ethnic practice, so as to preserve and assimilate the knowledge. For this scientific work, the available information was collected along with published information for the studies of the various activities of plants. The previous study suggested that the plant extracts have some potential anti-cancer compounds namely betulinic, oleanolic and ursolic acid. These phytochemicals were evaluated using HPLC (standard quantitative method) in this study. The analysis suggested that all three compounds are present in both the plant extracts. The plant extracts were evaluated for their antiproliferative effect using various cancer cells, namely Burkitt's lymphoma- *Daudi*, Fibrosarcoma HT-1080, Colon cancer HT-29, Lung cancer A-549, Breast cancer MCF-7 and Leukaemia HL-60. These cancer cells were exposed to extracts and doxorubicin. The results revealed that plant extracts sensitize the cancer cells in a dose-relation manner. As a future scope, *the in-vitro* study can be taken up to isolate the single compound that sensitizes the cancer cells in a manner similar to that of anti-cancer drugs. Furthermore, the *in-vivo* anticancer activity was performed using Brine shrimp lethality assay, oral toxicity study in female albino mice, followed by two cancer models viz, Dalton's ascitic lymphoma and Fibrosarcoma

(Soft tissue tumor) animal models which are well accepted globally. The Brine shrimp lethality assay finding suggested that both plant extracts exhibited the LD₅₀ value below 1000 ppm concentration, which makes both the plant extracts possess cytotoxicity. From Dalton's ascitic lymphoma models and Fibrosarcoma (Soft tissue tumor) animal models, it was found that the plant extract-treated groups exhibited an increased median life span of animals according to the administered dose. Also, Animal life span was improved by comparing the distinct groups with the control group. The Blood parameters proposed that no important or much harmful change was made in the haematological profile by the plant extracts. The findings of the study lead to the conclusion that these plant extracts possess anticancer potential which complies with their traditional and folklore claims.

CONCLUSION

8. Conclusion

The present study revealed that the selected ethnomedicinal plants extract from *Achyranthes coynei* Sant. and *Clerodendrum inerme* Gaertn. exhibit anti-cancer potential which is supported with the study findings, which includes identification and authentication of these ethnomedicinal plants and the extraction of the aerial parts of the plant was performed using methanol and water as the solvent system which was selected on the basis of maximum extractive value. The qualitative phytochemical screening confirms the presence of carbohydrates, proteins, amino acids, steroids, triterpenoids, glycosides, saponins, flavonoids, alkaloids, tannins and phenolic compounds as secondary metabolites. The bioactive extracts were further analyzed by HPLC for the presence of various tri-terpenoidal compounds such as betulinic acid, oleanolic acid, and ursolic acid and which showed the anticancer potential. The *in-vitro* MTT assay was performed using various cancer cell lines Burkitt's lymphoma cell lines –Daudi, Fibrosarcoma cell lines - HT-1080, Lung cancer cell lines - A-549, Breast cancer cell lines – MCF-7, Colon cancer cell lines – HT-29, Leukemia cancer cell lines – HL-60 for the antiproliferative effect which revealed that these extracts showed sensitization and inhibition of proliferative cells. The *in-vivo* studies were performed by brine shrimp assay method which showed extracts have inhibitory concentration below 100 ppm which inhibited 50% of the population that confirms the cytotoxic nature of the ethnomedicinal plant extracts.

The pharmacological screening using female Swiss mice for liquid tumor (Dalton's ascitic lymphoma) and Solid tumor (Fibrosarcoma cancer) model. In the Dalton's ascitic lymphoma method there was a significant decrease in body weight, tumor volume, tumor weight and increased life span, median survival time when compared with control group animals which reveals that there is the inhibition of tumor

growth. In the fibrosarcoma model the findings of various tumor parameters revealed that the tumor size, tumor burden was decreased significantly and increased life span, median survival time when compared with the control group animals. The haematological parameters- RBC count, WBC count and percent haemoglobin count of both animal models were significantly decreased in doxorubicin treated groups but not in extract treated group. Which revealed that plant extracts does not significantly alter the haematological parameters when compared with the control group. In a nutshell the result findings suggested that both the ethnomedicinal plants *Achyranthes coynei* Sant. and *Clerodendrum inerme* extracts are effective as anticancer potential agents which provides a scientific framework and validation to the traditional folklore claims.



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ANNEXURES

Annexure- I

Certificate of Authentication



क्षेत्रीय आयुर्विज्ञान अनुसंधान केंद्र
REGIONAL MEDICAL RESEARCH CENTRE

भारतीय आयुर्विज्ञान अनुसंधान परिषद
Indian Council of Medical Research

नेहरु नगर, बेळगाव - ५९० ०१०.
Nehru Nagar, BELGAUM - 590 010.

Tel : 0831-2475477-78
Fax : 0831-2475479
E-mail : icmrblm@yahoo.co.in

Date: 22-01-2015

AUTHENTICATION

This is to authenticate that the plants brought by Mr. Mahendra Kumar Chouhan, Ph. D. Scholar, KLE University, Belgaum, are identified as *Achyranthes coynei* Sant. (Amaranthaceae) and *Clerodendrum inerme* (L.) Gaertn. (Verbenaceae). The voucher specimens of the same have been deposited in our herbaria with accession numbers RMRC-1271 and RMRC-1272 respectively.

Harsha Hegde
Scientist 'C'

Annexure-II

Institutional Animal Ethical Committee Approval



KLE University's
College of Pharmacy
(Recognized by PCI, AICTE & Accredited by NBA & 'A' grade By NAAC)
A constituent Unit of KLE Academy of Higher Education and Research
[Under section 3UGC Act, 1956 vide Govt. of India Notification No. F.9-19/2000-U.3 (A)]
Nehru Nagar, Belagavi - 590 010, Karnataka, India



Phone: 0831-2471399 Fax: 0831-2472387 Web: <http://www.klepharm.edu> E-mail: principal@klepharm.edu

Ref: No. KLEUCOP/_____ Date: 10/10/15

CERTIFICATE

This is to certify that the research project, "Evaluation of anti-cancer activities of ethanomedical plants from western ghats.", Submitted by Mr. Mahendra Kumar Chouhan has been approved in the Institutional Animal Ethics Committee meeting held on 10th October 2015, resolution No. KLEUCOP/IAEC/Res.22-10/10/2015 and was permitted to use 114 female mice
----- Rats/ Mice/ Rabbits/Guinea pig.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.



MEMBER SECRETARY
Institutional Animal Ethical Committee,
KLES's College of Pharmacy,
BELGAUM - 590010



CPCSEA Nominee
Institutional Animal Ethics Committee
KLES's College of Pharmacy,
BELGAUM.

Annexure-III

Training. Workshop & Conferences

1. Training on HPLC, HPTLC & U.V. spectrophotometer at Chika Overseas PVT. LTD. Ahmedabad.
2. Training on zebra fish, mouse tumor model, comet assay and micronucleous assay techniques at Bhabha Atomic Research Centre (BARC) & Tata Institute of Fundamental Research (TIFR), Mumbai.
3. Training on CAMAG HPTLC by ANCHROME, Mumbai.

**Chika
Overseas Pvt. Ltd.**



Marketing Off. : C-1/401,G.I.D.C. Phase II, Vatva, Ahmedabad-382 445, INDIA
Corp Off. : Plot No 68, Sector No.1, Dronagiri Node, Navi Mumbai-400 707 INDIA

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Certificate of Training

This to Certify that Mr. Mahendra Kumar Chouhan a, Ph.D. Scholar Student of 2014-2015 batch in College of pharmacy, KLE University Belgaum has successfully undergone the project training programme in, 'ANTI-CANCER ACTIVITIES OF ETHNOMEDICINAL PLANTS FROM WESTERN GHAT'.

On HPTLC method Development and Validation of 'HPTLC FINGER PRINT PROFILING OF PLANT EXTRACTS' at our Company's Quality Control Laboratory, The Training has been given on

1. PERKIN ELMER HPLC,
2. CAMAG HPTLC
3. PERKIN ELMER UV-Visible Spectrophotometer

Instruments for his project subject Analysis, under the guidance of Mr.M.G.Nandanwadkar – General Manager –Q.A. during the period of 7 days in the month of September-2015.

For Chika Overseas Pvt Ltd.

M.G.Nandanwadkar

M.G.Nandhanwadkar
General Manager -Q.A
Regulatory Compliances.





Government of India
Bhabha Atomic Research Centre
Trombay, Mumbai - 400 085, India
Radiation Biology & Health Sciences Division



Ref.: RB&HSD/16/ 1143

Nov 15, 2016

CERTIFICATE

This is to certify that Shri Mahendra Kumar Chouhan, Ph.D. Scholar, K.L.E. University, Belagavi, Karnataka, has carried out part of his Ph.D. Research work on "Evaluation of Anticancer Activities of Ethnomedicinal Plants from Western Ghats" at Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Trombay, Mumbai, under my guidance in the Radiation Biology and Health Science Division, Bhabha Atomic Research Centre, during October 27th to November 15th, 2016.

During this period of his research work, Shri Chouhan has gained experience of working on Zebra fish and mouse tumor models, and learnt techniques of Comet Assay and Micronucleus Assay.

Dr. Haladhar Dev Sarma, MVSc, PhD, FNAVS
Scientific Officer (G) and
Head, Animal House Facility & Radioisotope
Laboratory
Radiation Biology and Health Sciences Division
Bhabha Atomic Research Centre
Mumbai – 400 085, India
Tel : (+91-22) 2559 2376 / 25592978 / 25592949
Email : hdsarma@barc.gov.in

ANCHROM

TLC/HPTLC Training Certificate

June 15, 2019

Cert. No. 5275

ON-SITE HPTLC TRAINING CERTIFICATE

This is to certify that

Mr. Mahendra Kumar Chouhan

from Camag **HPTLC** customer **KLE Academy of Higher Education and Research, Belagavi**

has received a three-days free training from 12/06/2019 to 14/06/2019

at the hands of our **Application Specialist Mr. Pratik Sinalkar**

as part of our after sales lab support.



Akshay Charegaonkar
Director



Anchrom Enterprises (I) P. Ltd. Mumbai - 400 081. India.

Technologists, not Traders!

Annexure-IV

Publications

1. Chouhan Mahendra Kumar, Hurkadale Pramod Jayadevappa, Hegde Harsha Vasudev. Evaluation of *Clerodendrum inerme* (L.) Gaertn. on Burkitt's Lymphoma Cancer. Indian Journal of Pharmaceutical Education and Research. 2018 Apr 1;52(2):241-7.
2. Mahendra Kumar Chouhan, Pramod Jayadevappa Hurkadale, Harsha Vasudev Hegde. *Clerodendrum inerme* (L.) Gaertn. Extract Exerts Anticancer Activity on Lung Cancer Cells. Dhaka University Journal of Pharmaceutical Sciences. 2018 Dec 4;17(2):191-6..

Evaluation of *Clerodendrum inerme* (L.) Gaertn. on Burkitt's Lymphoma Cancer

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ABSTRACT

According to current census every year six million deaths occur in the world due to cancer. Hence, there is need of discovery and development of novel, safe and efficacious drugs for the treatment of various cancer. In the present study, an attempt has been made to explore the medicinal aspects of *Clerodendrum inerme* (L.) Gaertn.. Verbenaceae plant involving cytotoxic and antiproliferative potential as hydroalcoholic extract using methanol and water (70:30 V/V). Preliminary phytochemical investigation confirms the presence of the moiety in hydro-alcoholic extract and cytotoxic analysis using brine shrimp lethality assay, *Daudi* cell line culture, dye exclusion assay, MTT assay and animal model. The extract showed cytotoxicity below 100 ppm and LD₅₀ at 30 ppm level on brine shrimp lethality assay and on being subjected to MTT assay. A decrease in cell viability was observed at 213 µg/ml concentration and showing antitumor efficacy against Burkitt's lymphoma cells as well tumor model in female mice which showing the increased life span, as compared to control group, when treated with *C. inerme* (200 and 400 mg/kg body weight) and doxorubicin (2.5 mg/kg body weight) exhibited significant effect on tumor parameters. Hence it can be stated that *C. inerme* has potent antitumor properties.

Keywords: *Clerodendrum inerme*, Cytotoxicity, Antiproliferative, Lethality, MTT, *Daudi*

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INTRODUCTION

Cancer is the most common cause of death of millions across the world. WHO statistics demonstrates the same as well since control and recurrence of cancer goes parallel, other contributions factors such as age, family history and also genetic abnormalities predominates.¹ Around 80% of the world's population depends upon traditional medicine system and primary health centers in a typical Indian scenario. Medicinal plants have the potent activity towards to maintain the healthcare.² In Ayurveda, the Indian medicine system is based on plant-based medicines. It played a pivot like role because most drugs are plant based. Camptothecin, Etoposide, Taxanes, Vincristine are plant derived compounds which have potent anti-

cancer activity.³ Conventional and traditional medicines also widely used as complementary or alternative therapy. Conventional therapies were like surgical removal, chemotherapy, and radiotherapy but sometimes these therapies are inadequate to cure the disease, so new drug discovery and development are in need to provide another alternative to provide better help to the community.⁴ Various plants are used in the various system of medicine like Ayurveda, Siddha, Unani and Homeopathy to treat cancer. The traditional system of medicine specially herbal medicine in India is directly linked to it's rich floral diversity, the Western Ghats of India is one such high biocultural diversity region, which is one of



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the global biodiversity hotspots. Medicine in contemporary India is a fascinating blend of a traditional system with conventional one and often been used for various historical, cultural and ecological and socioeconomically reasons. Even through the rate of medicinal plant utility is even increasing very little, is known about its, use patterns. It is very important to document, analyze and evaluate their knowledge not only for their cultural reasons but also for their commercial value, as ethnomedicinal uses of plants are one of the most essential critical used by the pharmaceutical industry in finding the new therapeutic agents.⁵ *Clerodendrum inerme* plant belongs to family Verbenaceae, and plants belong to this family plants have various useful ethnomedicinal properties.⁶ This plant is used as an ethnomedicine to treat cough, scrofulous infection, buboes problem, venereal infection, skin diseases, vermifuge and in beriberi.⁷ *Clerodendrum inerme* plant containing some important phytoconstituents viz. Pentadecanoic acid β -D- glycoside, Stigmasterol, 4 α -methyl-24 β -ethyl-5 α -cholesta-14, 25-dien-3 β -ol, 24 β -ethylcholesta-5, 9(11), 22-trien-3 β -ol, Betulinic acid.^{8,9} These constituents have potential effects towards cancer cure.¹⁰ Dalton's ascitic lymphoma (DAL) is a very progressive cancer, which increases the lymphatic cells in the peritoneal cavity. However no proper systematic study was been evaluated for the lymphatic cancer activity using Burkitt's lymphoma (DAL) cells and in animal experimentation, comparison was made between effect of plant extract and standard drug doxorubicin on tumor parameters using Swiss mice. The present study is based on the ethnomedicinal claim of various species of *Clerodendrum* used in traditional system of medicine by various tribes. Hence the scientific validation of these claims with various evaluation parameters for anticancer potential has to be explored.

MATERIALS AND METHODS

Collection of Plant Material and Extract Preparation

Plant material *Clerodendrum inerme* aerial parts were identified and collected from Dandeli-Anshi forest, North Karnataka region in the month of January, and authenticated by comparing with earlier collection by taxonomist Dr. Harsha V. Hegde, Scientist -D Regional Medical Research Centre, (ICMR-RMRC), Belagavi, Karnataka with herbarium specimen no RMRC-1272. Shed dried plant material was crushed and subjected to extraction using maceration process, in methanol and water (70:30 V/V). The solvent was recovered using rotary evaporator (IKA) and the concentrated extract

was dried using hot air oven at 40 °C. The extract was kept at -20° C until its uses.

Phytochemical Investigation

Phytochemical screening was carried out on crude extract using appropriate procedure to evaluate phytochemical constituents such as carbohydrates, proteins, amino acids, steroids terpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic compounds, fat, oil, and saponins.^{11,12,13}

Brine Shrimp Lethality Bioassay

The cytotoxic study was carried out by brine shrimp lethality bioassay, using the *Artemia salina*. Cysts were incubated in 1L of sea water for 24 hours, with aeration and light source to provide temperature and to attract nauplii. After hatching, matured nauplii were swimming and gathered near to light source. The plant extract was prepared concentration in triplicates ranging from 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μ g/ml or ppm using sea water in 5ml test tubes. 10 nauplii were added into each of the test tubes. Without test, substance served as the control. A drop of dry yeast suspension (3 mg in 5 ml sea water) was added to each test tube as a food for shrimps. This setup was left for 24 hrs incubation under continuous illumination of light source near test tube to provide temperature. After 24 hrs the live and dead nauplii were counted. Extract have shown significant cytotoxic activity (LC₅₀ < 100 ppm). The extract was almost 100% lethal to the brine shrimp at the concentration of 1000 μ g/ml. and LC₅₀ < 100 ppm was considered significant. Results are expressed as the mean \pm standard error of the mean (S.E.M.) of three independent experiments. Cytotoxicity (mean % death after 24 h with LC₅₀ values) of the extract was compared with those of the control and is shown.¹⁴

In-Vitro Cytotoxicity Activity- MTT Assay

Cell line and cell culture

The Burkitt's lymphoma (*Daudi*) cells were purchased from NCCS Pune, Maharashtra, INDIA. Cells were cultured in RPMI-1640 (SIGMA) medium, supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) 100 units penicillin-streptomycin (GIBCO, INVITROGEN, USA); gentamycin and amphoterecin (Himedia, India) at pH 7.4, 37°C and humidified atmosphere of 95% air and 5% CO₂ in incubator (NEW BRUNSWICK SCIENTIFIC, GERMANY), Doxorubicin hydrochloride obtained as sample from RPG Life Sciences Limited, Mumbai, India, and cells were grown in 75 semi-square tissue culture flask and used for experiments when in exponential growth phase.

Trypan Blue Dye Exclusion Assay

Burkitt's lymphoma cells *Daudi* were made in the density of 6×10^3 cells/ml cells in PBS (SIGMA) medium and 10 μ l of cell suspension was mixed with 10 μ l of 0.4% trypan blue solution in PBS (SIGMA) for 1min. The cells were counted using Neubauer chamber (Roheam, Germany) examined under an inverted light microscope (LABOMED INC., USA), the dead cells were visible blue in color whereas the live cells did not absorb the dye because of trypan blue dye differentiated the live cells and dead cells. Live cell does not absorb dye inside and in dead cells dye penetrate inside, so this difference easily differentiates the live and dead cells. Distinct cell type and cell viability were asses using Neubauer counting chamber

MTT Assay

Hydro-alcoholic extract of *Clerodendrum inerme* on antiproliferative activity was determined by MTT assay, *Daudi* cells were seeded into flat bottom 96 well plate (Corning, Cell bind, U.S.A.) with lid were seeded with 6×10^3 cells per well with 150 μ l of DMEM growth medium with 10% FBS (Gibco, Invitrogen, USA). The plate was incubated for 24 hrs at 37°C under 5% CO₂ in a humidified atmosphere. After 24 hrs test and standards in 1000, 500, 250, 125, 62.5, 31.25, 15.625 μ g/ml concentration were added for 24 hrs in triplicates, after incubation at 37°C under 5% CO₂, 20 μ l of 5 mg/ml MTT at pH 7.4 was added in per well, and kept in dark for another 4 hrs towards color reaction development. An equal volume 100 μ l of DMSO (SIGMA-ALDRICH) was added to stop the reaction and to solubilize the purple-blue crystals. The absorbance was measured at 630 nm using Elisa plate reader (Lisa 300, Germany).¹⁵

$$\% \text{ cell viability} = \frac{\text{Absorbance of test}}{\text{Absorbance of negative control}} \times 100$$

Change in Cellular Morphology

Daudi cells 6×10^3 were seeded in 24 well plate and kept for incubation for 24 hrs at 37 °C, 5% CO₂. Cells were treated with IC₅₀ of extract for 24 hrs. Cells were observed for cellular morphology changes using an inverted microscope (Labomad, USA) at 40 \times .

In-Vivo Antitumor Activity

For the animal experiment animals were procured from the central animal facility from the institute, female Swiss albino mice 30 \pm 2 g were selected for the study to enhance sensitivity than male used, and housed under standard laboratory condition 25°C \pm 2°C temperature

RH 50%-60% and 12 hr natural light and dark condition, animals were free to access to standard pellet diet and water *ad libitum*. All animals were acclimatized for two weeks before the experiment. For acute oral toxicity and dose determination OECD guideline 423 was followed. Animals were divided into five groups and each group contains six animals.^{16, 17} Group I was a normal group, group II was a positive control group, group III was received doxorubicin (2.5 mg/kg body weight) and IV & V were received low and a high dose of *Clerodendrum inerme* (200 and 400 mg/kg body weight) respectively. Animal experiment protocol was performed by Institutional Animal Ethical Committee (IAEC) K.L.E. University's College of Pharmacy Belagavi, India. The Burkitt's lymphoma cell suspension (0.2 ml of 2×10^5 cells/ml) was injected intraperitoneally into respected groups of animals. Treatment was given after 24 hrs of tumor inoculation to animal groups. Group III received (Doxorubicin 2.5 mg/kg body weight) IV & V were received *Clerodendrum inerme* plant extract in low dose and high dose (200 and 400 mg/kg body weight). With prior calculations of dose as per body weight extract treatment were daily orally administered and doxorubicin once in 4 days for 4 dose treatment by intraperitoneal route. After experiment animals were sacrificed by CO₂ asphyxia and tumor parameters body weight, tumor volume, tumor weight, and hematological parameters were observed after 15 days. Body weights of all groups of mice were measured before the experiment and after the 15 days of treatment. Sacrificed the animal by CO₂ asphyxia ascitic fluid was collected into falcon tubes (Corning, USA) to measure the tumor volume and weight. Blood parameters like total blood count RBC, WBC, Haemoglobin count by using bright-field microscopy method to calculate, the number of RBC and WBC count in blood per liter Neubauer chamber (Haemocytometer) was used.

STATISTICAL ANALYSIS

Data were expressed as mean \pm Standard Deviation, transform data and non-linear regression analysis, one-way ANOVA followed by Tukey-Kramer multiple comparison tests using GraphPad Prism® version 5.01, GraphPad Software, San Diego, California, USA. $P < 0.05$ was considered as statistically significant

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening of hydroalcoholic extract of *C. inerme* aerial part showed the presence of carbo-

Table 1 : The presence of phytoconstituent was representing using the symbol "+" and the absence was shown using the symbol "-".

S.No.	Test	Observation
1.	Carbohydrate	+
2.	Proteins	+
3.	Amino acids	+
4.	Steroids	+
5.	Triterpenoids	+
6.	Glycosides	+
7.	Flavonoids	+
8.	Alkaloids	+
9.	Tannins and Phenolic compounds	+
10.	Fat and Oil	-
11.	Saponins	+

hydrate, protein, amino acid, steroids, triterpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic compounds, and saponins. (Table 1)

Brine Shrimp Lethality Bioassay

Extract have shown significant cytotoxic activity ($LC_{50} < 100$ ppm). The extract was almost 100% lethal to the brine shrimp at the concentration of 1000 $\mu\text{g}/\text{ml}$. and $LC_{50} < 100$ ppm was considered significant. Results are expressed as the mean \pm standard deviation (S.D) of three independent experiments. Cytotoxicity (mean % death after 24 h with LC_{50} values) of the extract was compared with those of the control and is shown LC_{50} 30 ± 0 ppm was found.

MTT Assay

The percent cell viability of the hydroalcoholic extract of *Clerodendrum inerme* and doxorubicin were observed 213.2 $\mu\text{g}/\text{ml}$ and 62.36 $\mu\text{g}/\text{ml}$ which was the concentration that causes the significant decrease in proliferation by 50% i.e. IC_{50} of each one showed in Figure 1. The optimal cell number were seeded for the study and was found to be promising results, in order to evaluate the dose-dependent effect against Burkitt's lymphoma (*Daudi*) cells showed.

Change in Cellular Morphology

Cellular morphology changes of treated and untreated Burkitt's lymphoma cells, Figure 2 were observed under inverted microscope at 40 \times after treatment with IC_{50} if plant extract for 24 hrs Figure 2 (a) represents normal morphology of control untreated cells and treated cells whether lost their normal morphology Figure 2 (b) & (c), due to cell death most of them were floating and rounded in the medium.

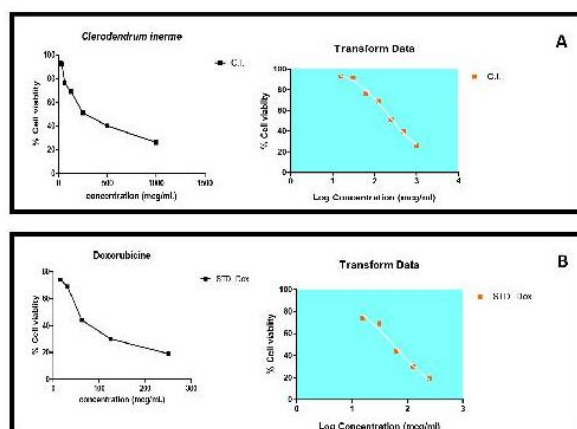


Figure 1: Representative graphs of the percentage of cell viability at different concentration (A) treatment with *C. inerme*, (B) treatment with doxorubicin, data were expressed as mean \pm Standard Deviation, transform of data and non-linear regression analysis

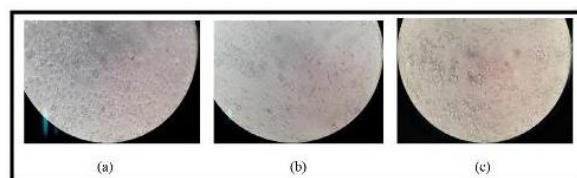


Figure 2: Representative images of cellular morphology of Burkitt's lymphoma cells (*Daudi*) (a) normal control cells, (b) treated with doxorubicin for 24 hrs (c) treated with *C. inerme* IC_{50} concentration for 24 hrs.,

In-Vivo Antitumor Activity

Both of the concentrations of *Clerodendrum inerme* extract revealed significant ($P < 0.05$) inhibition of ascitic tumor when the effect on body weight, tumor volume, tumor weight parameters compared with diseased control group. *Clerodendrum inerme* at low dose 200 mg/kg body weight showed lesser antitumor activity when compared with higher dose 400 mg/kg body weight. Hematological parameters also showing the effect on hemoglobin count of group III, IV and V compared with control group, showing decrease in hemoglobin count. MTT assay is the most important parameter to be evaluated for *in-vitro* anticancer effects. The present investigations provide the importance for biomedical research for safe and efficacious towards cancer. Antitumor activity of *C. inerme* extract at 200, 400 mg/kg body weight treated to burkitt's lymphoma induced female mice and effects revealed significant ($P < 0.05$) tumor inhibition (tumor volume, tumor weight) when compared with disease control group. Figure 3, the percent survival of all groups repre-

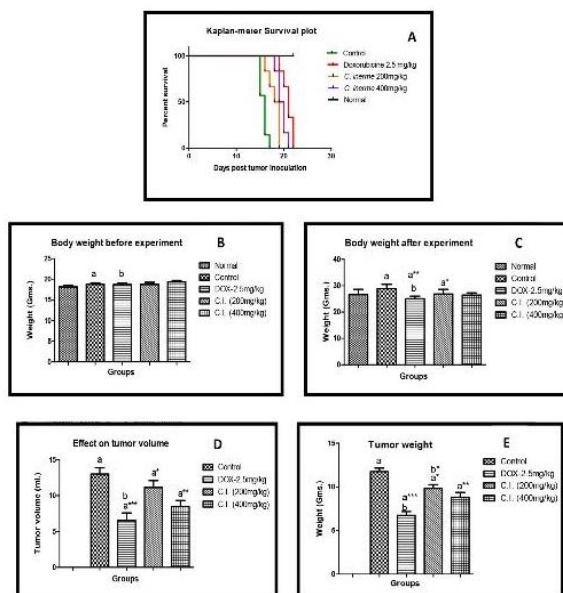


Figure 3: Therapeutic anticancer effect of *C. inerme* and doxorubicin; (A) Kaplan-meier survival plot of treatment with *C. inerme* extract and doxorubicin on different groups represents percent survival of animals after post treatment, (B) and (C) Difference in body weight before and after the treatment, (D) and (E) Tumor volume and tumor weight [I] Normal group [II] Control group [III] mice treated with Doxorubicin 2.5 mg/kg body weight [IV] and [V] mice treated with *C. inerme* 200 mg/kg and 400mg/kg body weight. * $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; a: disease control group, b: tumor induced group treated with doxorubicin, each data points represents mean \pm standard deviation, (n=6)**

sented by Kaplan-Meier survival plot of treatment with plant extracts and standard drug doxorubicin group. This antitumor effect could be due to presence of antitumor compounds present in the plant extract. Figure 3(A) shows the Kaplan-Meier survival plot of experimental group for the period of 30 days, which revealed that, there was 100% survival of normal group animals and treatment group III, doxorubicin 2.5 mg/kg body weight treated and group IV and group V at *C. inerme* 200, 400 mg/kg body weight treated also revealed increased the percentage survival life span, when compared with disease control group. However poor survival of disease control due to higher mortality of burkitt's lymphoma cancer. The suppression of blood component characterized by decrease in the production of WBC, RBC and Haemoglobin content which responsible for providing immunity and carrying oxygen. This type of condition showed, when infection in the body occurs or anticancer treatment done. In case of anticancer drug treatment like doxorubicin the reduction in the blood components due to toxicity of drug.¹⁸ Doxorubicin is the choice of drug in many cancer conditions, but it has severe side effects also. In the present investigation

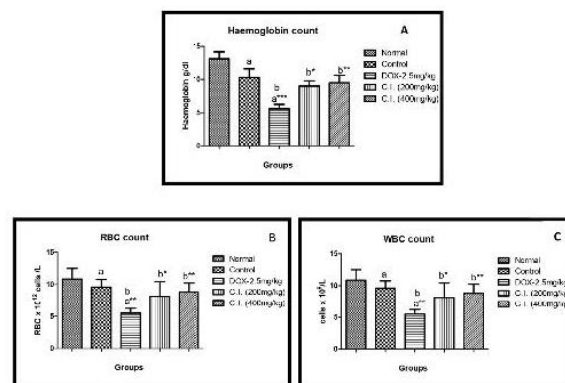


Figure 4: Representation of haemoglobin content and blood cell count, A: Haemoglobin content, B: red blood cells, C: white blood cells, mice treated with *C. inerme* extract at 200, 400 mg/Kg body weight and doxorubicin (2.5 mg/Kg body weight) * $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; a: disease control group, b: tumor induced group treated with doxorubicin, each data points represents mean \pm standard deviation, (n=6).**

the extract was showing antitumor potential with less toxicity or side effects. Figure 4 represents haemoglobin content, WBC and RBC count, it was found that the significant ($P < 0.05$) decrease in percent haemoglobin count, WBC and RBC count, when doxorubicin group compared with disease control group, and the *C. inerme* extract 200 and 400 mg/kg body weight treated groups showing significant ($P < 0.05$) difference when compared with doxorubicin treated group. However it was observed that, the plant extract treated group showing not significant ($P < 0.05$) decrease blood components when compared with disease control group. In disease control group the higher level of WBC were observed, which could be due to different pathway and different mechanism in animals due to cancer.¹⁹ This study was found that the hydroalcoholic extract of *Clerodendrum inerme* extract has cytotoxic effect which demonstrated by Brine shrimp lethality bioassay and anti-proliferative effect has shown by MTT assay and morphological changes on Burkitt's lymphoma cell line. In the study, doxorubicin was used as standard drug, analyzed results are statistically significant. In conclusion, this study has been supporting the ethnomedicinal value of *Clerodendrum inerme* for the anti-cancer potential, and it can be used for the benefit for the sustainable use of traditional medicinal plants. The mechanism of action is still not cleared that how the active principle of the plant is showing effect. Further studies can be done to evaluate and demonstrate the mechanism pathway of biological anticancer activity of *Clerodendrum inerme* plant. The potent anticancer properties of *Clerodendrum inerme* hydroalcoholic extract showed the presence of antioxidant and anticancer principles. The

results of the study were consistent and support the earlier studies, which further support the anticancer potential of *Clerodendrum inerme* plant. Nowadays phytochemicals derived from plant source are being intensively investigated for cancer. Cell death and apoptosis is the important physiological and pathological condition, which involved in various importance protein-expression, condensation fragmentation of DNA and shrinkage of cells. As reported in literature the single compound could have more potential anticancer activity, the evaluation of anticancer compound is necessary for the proof of concept. The further scope of this study could be helpful to assess the exact mechanism of action.

CONCLUSION

The result of anti-proliferative or cytotoxic effect of *Clerodendrum inerme* inhibit the growth of cells and indicated the anticancer potential of plant extract, also showing a toxic effect on brine shrimp lethality assay, MTT assay morphological changes, and Daltons ascitic lymphoma model in Swiss mice. These all parameter showed the anticancer effect of plant extract. This is an indigenous plant and used as folk medicines for years together, this study provides the scientific validation to its traditional claims. Other anticancer activity are also done on this plant using other extracts but without using any standard anticancer drug treatment and this is the loop in research, so there was a need to fill up this gap and this study provides the anticancer effect of extract with the standard anticancer drug.

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Kumar *et.al.*:Evaluation of *Clerodendrum inerme* (L.) Gaertn. on Burkitt's Lymphoma Cancer



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***Clerodendrum inerme* (L.) Gaertn. Extract Exerts Anticancer Activity on Lung Cancer Cells**

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ABSTRACT: Cancer is the leading cause of death world wide. Recently there are no new drugs for safe and efficient treatment. *Clerodendrum inerme* (L.) Gaertn. (Verbenaceae) plant is being used by the ethnic people for cancer treatment. In this study, cytotoxic and antiproliferative potential of hydroalcoholic (methanol and water; 70:30 v/v) extract of *C. inerme* were evaluated. Various anticancer investigations performed like, lung cancer cell A-549 culture, dye exclusion assay, MTT assay, morphological changes and compatibility with RBC, confirmed the presence of the moiety that have the cytotoxic and antiproliferative potential. Compatibility with RBC was observed, when treated with standard drug doxorubicin, and hydroalcoholic extract of *C. inerme* at 259.5 µg/ml concentration (IC₅₀). In addition, the same treatment revealed, decrease in cytotoxic efficacy and cell viability against lung cancer cells. Furthermore, change in the cell morphology also suggesting potent antitumor properties of *C. inerme*.

Key words: *Clerodendrum inerme*, RBC aggregation, MTT, Cytotoxicity.

INTRODUCTION

As per WHO statistics cancer is the leading cause of death. Every year, millions death occur due to its recurrence and other contributing factors like age, genetic abnormalities and family history.¹ Plant derived drugs like camptothecin, taxanes, etoposide, vincristine are important medicines that have potent anticancer activity.² Conventional and traditional medicines are also used widely as complementary or alternative medicines³, also to cure this deadly disease, surgical removal, chemotherapy and radiotherapy are most of the time not adequate or effective to overcome.⁴ Traditional medicine system like Ayurveda, Siddha, Unani, Homeopathy and other

systems (Folk practices) of medicine were based on most of plant derived medicines. In the large section of the population, plant based traditional systems of medicine playing a vital role to provide health care which increasing the over harvesting of natural products. Traditional systems of medicines are playing an important role to meet the global health care burden.⁵ The richest source of medicinal value and their enormous potential medicinal plants are used for phenolic compounds, flavonoids, alkaloid, terpenoids, vitamins, tannins etc.. Secondary metabolites are interest of herbal-based medicines over the globe.⁶ In case of new medicines, plant derivatives and metabolites have a significant place for preparation of drugs.⁷ Naturally available sources are the hot topic and to identify the normal secondary metabolites is the intensive research topic now days.⁸ *C. inerme* reacts on DNA and cause the

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fragmentation by some signaling pathways, in this context this study has been designed. The present study is focused on the effect of *C. inerne* extract on Lung cancer A-549 cell line to evaluate the DNA damage and their effect as anticancer agents. *C. inerne* plant belongs to family Verbenaceae, and plants belong to this family, have various useful ethnomedicinal properties.⁹ This plant is used as an ethnomedicine to treat cough, scrofulous infection, buboes problem, venereal infection, skin diseases, vermifuge and in beriberi.¹⁰ *C. inerne* plant contains some important phytoconstituents viz. pentadecanoic acid- β -D-glicoside, stigmasterol, 4 α -methyl-24 β -ethyl-5 α -cholesta-14, 25-dien-3 β -ol, 24 β -ethyl-cholesta-5,9(11),22-trien-3 β -ol, betulinic acid.^{11,12} These constituents have potential effects towards cancer cure.¹³ The plant extract have shown significant cytotoxic activity (LC₅₀ < 100 ppm). The extract was lethal to the brine shrimp at the concentration of LC₅₀ < 100 ppm.

MATERIALS AND METHODS

Collection of plant material and extract preparation. The *C. inerne* plant aerial parts were collected from Dandeli-Anshi forest, the North Karnataka region in the month of January, and authenticated by taxonomist Dr. Harsha V. Hegde, Scientist D Regional Medical Research Centre, (ICMR-RMRC), Belagavi, Karnataka with herbarium specimen no RMRC-1272. Plant material was shed dried and crushed, subjected to maceration process, (70:30 v/v in methanol and water). The extract concentrated using rotary evaporator and dried using hot air oven at 40°C.

Phytochemical investigation. Phytochemical investigation for carbohydrates, proteins, amino acids, steroids terpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic compounds, fat, oil, and saponins showed the presence of these moieties.²

In vitro cytotoxicity activity

Cell line and cell culture. The A-549 Lung cancer cells were procured from NCCS Pune, Maharashtra, INDIA. The cells were cultured in T-75 cell culture flask using DMEM (Gibco, Invitrogen,

USA) medium, with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) penicillin-streptomycin 100 units (Gibco, Invitrogen, USA); gentamycin and amphoterecin (Himedia, India) at pH 7.4, 37°C and air: CO₂ (95: 5) condition in incubator (New Brunswick Scientific, Germany).¹⁴

Trypan blue dye exclusion assay. The A-549 Lung cancer cells were cultured at the density of 6 \times 10³ cells /ml.. The A-549 lung cancer cells were used when reached in exponential phase. The trypan blue dye exclusion assay was implemented to evaluate the number of viable cells present in the cultured cell suspension. Trypsinized cells were counted using Neubauer chamber under an inverted light microscope (Labomed Inc., USA) cells were dyed using trypan blue, which is vital dye, live cells cell wall does not allow to enter the dye inside and the dead cells are differentiated by blue in color.¹⁵

MTT assay. The antiproliferative potential was evaluated using MTT (3-(4,5 dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The yellow MTT, a dark purple colored formazone, which is produced by reaction with mitochondrial succinate dehydrogenase was noted by colorimetric analysis. Hydro-alcoholic extract of *C. inerne* on antiproliferative activity was determined by MTT assay, A-549 cells were seeded into 96 well plate (Corning, Cell bind, U.S.A.). The density of cells was 6 \times 10³ cells per well with 150 μ l of DMEM growth medium, 10% FBS (Gibco, Invitrogen, USA) and penicillin-streptomycin (Invitrogen, USA). The plate was kept for incubation at 37°C, 5% CO₂ in a humidified incubator for 24 hrs. After the incubation, cells were treated in triplicates with test *C. inerne* extract in various concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625 μ g/ml concentration for 24 hrs at pH 7.4, temperature 37°C, 5% CO₂, in the incubator. Doxorubicin was used as the positive control. After 24 hrs incubation, 20 μ l MTT reagent at concentration 5 mg/ml, pH 7.4 was added in each well, and kept in the dark for another 4 hrs for formazone crystal formation (color reaction). After 4 hrs. to stop the reaction and solubilize the formazone crystals, 100 μ l of DMSO (SIGMA-ALDRICH) was

added to each well. The absorbance was measured at 630 nm on Elisa plate reader (Lisa 300, Germany) and the % cell viability were calculated using the formula. The dose response curve was plotted to determine 50% inhibition of cancer cells at inhibitory concentration IC₅₀ values for plant extract and doxorubicin.^{16,17}

$$\% \text{ Cell viability} = \frac{\text{Absorbance of test}}{\text{Absorbance of negative control}} \times 100$$

Change in cellular morphology. The A-549 lung cancer cells were analyzed for the change in morphology. Cells were treated for 24 hrs at IC₅₀ values of *C. inerme* extract and standard drug doxorubicin which shows the changes in the cell morphology, attached and detached cells due to the effect of inhibitory concentration by using inverted microscope at 40x.¹⁸

Erythrocyte aggregation assay. An equal volume of fresh blood, PBS and 10 mM EDTA were added and centrifuged at 3000 rpm for 10 min at 37°C. The obtained erythrocytes pellet was washed in PBS twice. The fresh erythrocytes and plant extract were mixed at 1:1 ratios which were incubated at 37°C for 1hr. After incubation of samples, slides were prepared and imaging were taken, by using contrast imaging inverted microscope (Labomed, USA).¹⁹

Statistical analysis. Data were expressed as mean ± standard deviation, transform data and non-linear regression analysis, using GraphPad Prism® version 5.01, GraphPad software, San Diego, California, USA.

RESULTS AND DISCUSSION

Extract preparation and phytochemical screening. Aerial part of *C. inerme* were identified and shed dried plant material was subjected to crush and macerate in methanol and water at 70: 30 ratio for three days with occasional stirring, further extract was filtered through whatman filter paper no 1. Further solvent was evaporated using rota vapor instrument and dried in a temperature controlled hot

air oven to dry extract. The yield from 100 g plant material was 1.84 g extract. The extract was brown in color and hygroscopic in nature. The extract was stored in airtight container at 2-8 °C in refrigerator.

Phytochemical screening showed the presence of carbohydrate, protein, amino acid, steroids, triterpenoids, glycosides, flavonoids, alkaloids, tannins, phenolic compounds, and saponins in the aerial part hydroalcoholic extract of *C. inerme*.²

Trypan blue dye exclusion assay. The A-549 Lung cancer cells were cultured in a culture T flask. Cells were counted using Neubauer chamber under an inverted light microscope. Cells were dyed using trypan blue, which is vital dye, live cells cell wall does not allow to enter the dye inside and the dead cells are differentiated by blue in color, the average number of differentiated live cells in per ml were counted and 6×10³ cells per ml accordingly were prepared for seed in 96 well plate.

MTT assay. The cell toxicity assay is one of the vital determinant for toxicity analysis, which explain the proliferation response to any anticancer agent and provide the information for survival, metabolic activity and cell death.²⁰ Recently, it has been reported that *C. inerme* have potent antiproliferative activity on liquid tumors also.² Earlier studies have demonstrated that *C. inerme* have chemical moiety, have anticancer effect in dose dependent manner which was evaluated in the present study using different extract and cell line.²¹ *In-vitro* cytotoxic activity against A-549 lung cancer cell line was evaluated against different concentration of hydro alcoholic extract of *C. inerme* and doxorubicin in a dose dependent manner. As shown in figure 1 the IC₅₀ values were 259.5 µg/ml and 88.05 µg/ml for hydro-alcoholic extract of *C. inerme* and doxorubicin, respectively when treated for a period of 24 hrs. Since we observed the marked effect on treatment, therefore the results demonstrated that hydro-alcoholic extract of *C. inerme* has time and dose dependent antiproliferative activity on lung cancer cell lines, which showed the sensitivity of cells to plant extract, that showing the strongly

support its promising results to cancer treatment by Ayurvedic physicians.

Change in cellular morphology. A-549 lung cancer cells were seeded in 24 well plate and observed for cellular morphological changes using inverted microscope at 40x after 24 hrs treatments of untreated and treated with plant extract and standard drug doxorubicin at IC_{50} values. The change in cellular morphology is presented in figure 2 in which (A) represents the untreated normal morphology of A-549 lung cancer cells, (B) treated cells with IC_{50} of

hydro alcoholic extract of *C. inermis* plant extract and (C) treated with standard drug doxorubicin treated cells. The different morphology, which showed the state of cancer cells, floating and rounded dead cells, detached from the surface of 24 well plate. Also attached cells loosing the growth and changing the shape of cells and the proliferation, which can be easily understood by microscopic visualization. When cells were treated with anticancer drug, results showed similar effect that support the previous study pattern.²²

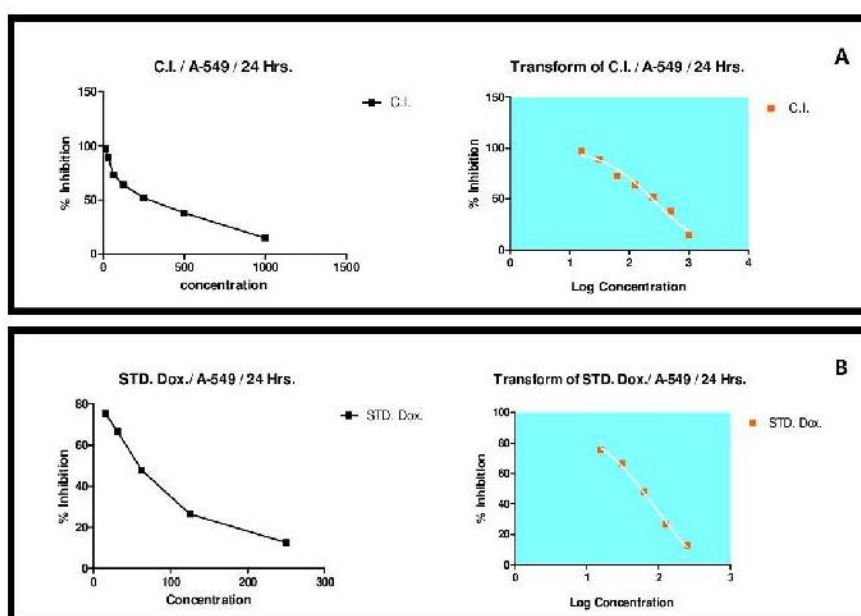


Figure 1. Cytotoxic effect on human lung cancer cell line A-549, graphs represents the percentage inhibition at different concentration (A) treatment with *C. inermis*, (B) treatment with doxorubicin, data were expressed as mean \pm standard deviation.

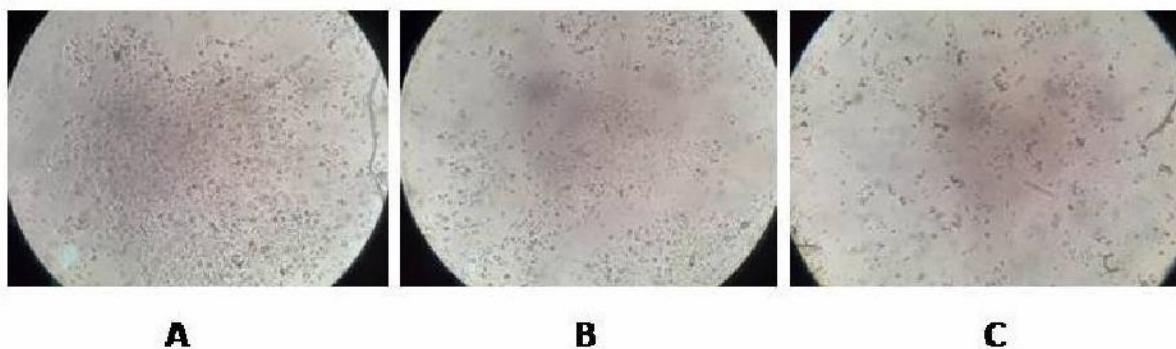


Figure 2. Represents the cellular morphology of A-549 lung cancer cells (A) Represents the untreated normal morphology of cells, (B) Cells were treated with IC_{50} concentration of *C. inermis* plant extract (C) Cells were treated with IC_{50} concentration of standard drug doxorubicin for 24 hrs.

Toxicity analysis of plant extract on red blood cells. The interaction study of plant extract and doxorubicin with human blood components are highly desirable, a few numbers of studies that have been conducted so far indicate the potential for adverse effects at the cellular level, with susceptibilities in the circulatory and dermal systems.²³ This can explain the damage on red blood cells (RBCs) and its interactions. In this investigation, it has assessed at the cellular level, the impact of plant extract on red blood cells (RBCs) for future application of the plant extract, which can be

helpful for further investigations. In this method, the cellular impact of plant extract on red blood cells (RBCs) has been evaluated. The standard drug doxorubicin treatment at IC_{50} , which does not course a significant aggregation of erythrocytes upon 1 h incubation at $37^{\circ}C$, whereas the plant extract have showed less effect with the erythrocytes (Figure 3). These results clearly revealed that the plant extract elicit no toxic response as cellular clumping (aggregation), when compare with the standard drug treatment.²⁴

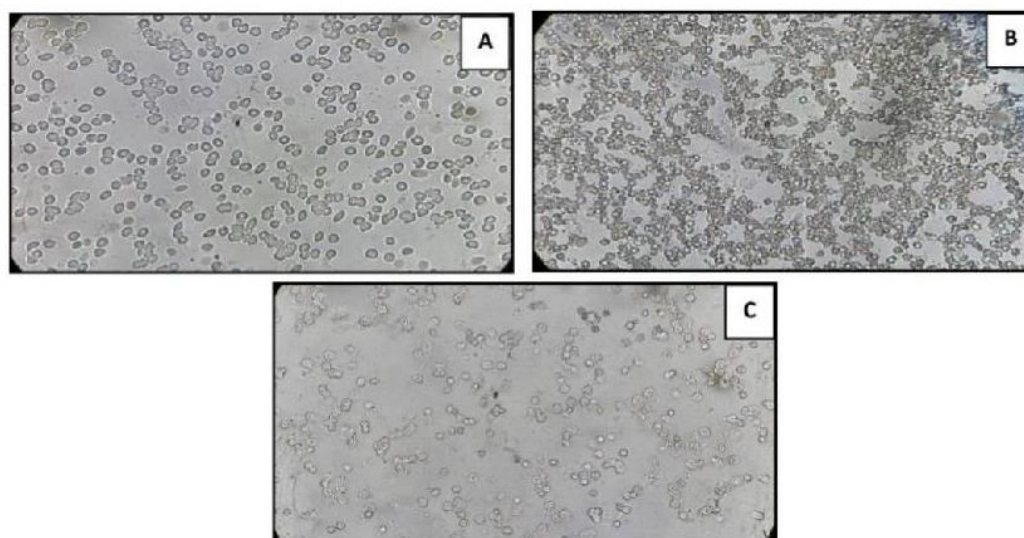


Figure 3. Microphotograph of RBC aggregation at 40X, (A) Untreated cells, (B) Treated with doxorubicin IC_{50} value, (C) Treated with plant extract at IC_{50} value.

CONCLUSION

C. inerme is an indigenous plant which has been used in the folk medicine for many years. This study provides the scientific validation to its traditional medicinal claims. Cell based assays were used to determine the effectiveness of the test extract on cell proliferation to reveal the direct cytotoxic effect which lead to cell death. MTT assay helped to measure the binding to receptors and associated with a variety of signal transduction events which involved in various expression processes. The results cytotoxicity of *C. inerme* confirms the antiproliferative potential which reflects

cytocompatibility with RBC, trypan blue dye exclusion assay, MTT assay, cellular morphological changes/ These confirms the previous study findings on the antiproliferative potential of the plant extract.

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Annexure-V

Best Oral / Poster Presentation Award

1. Best Poster Presentation award on “Evaluation of *Achyranthes coynei* (Amaranthaceae) on A-549 Lung Cancer Cell Line” at International conference of International Society of Ethnopharmacology at Dhaka University, Dhaka, Bangladesh, held on January 14, 2018.
2. Best Oral Presentation award on " Anticancer potential of *Achyranthes coynei* on HT-29 cells" at International conference of Society of Ethnopharmacology at Mae Fah Luang University, Chiang Rai, Thailand held on December 6-7, 2018.

Conference/ Workshop Certificates

1. Attended ICMR sponsored training programme on good laboratory practices, organized by KLE University’s J. N. Medical College, Belagavi held from 15th December 2014 to 02nd January 2015.
2. National level field workshop on medicinal plants in Western Ghats on Affordable quality healthcare: Herbs to medicines, held on organized by Regional Medical Research Centre: Indian Council for Medical Research (RMRC-ICMR) Belagavi in association with KLE College of Pharmacy, B.M.K. Ayurveda Mahavidhyalaya and Dr. Prabhakar Kore Basic Science Research Centre, Belagavi
3. Workshop on B.L.S. and A.C.L.S., conducted on 20th June 2015 organized by KLE University College of Pharmacy
4. One day workshop on total parenteral nutrition held on 12th august 2015 at KLEU’s J.N. Medical College & KLE Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.

5. Specific symposia in the 33rd annual conference of Indian society for medical statistics organized by KLE University Department of Epidemiology & Biostatistics, Belagavi on 13th December 2015.
6. International CME on Research Methodology & Communication on 19th November 2016, organized by Research Unit, USM-KLE, IMP Belagavi.
7. 5th Field workshop on medicinal plants in Western Ghats on Herbals to Phytopharmaceuticals: Approaches and Applications, organized by ICMR-Regional Medical Research Centre and KLE University, Belagavi on 23rd to 26th November at Nature camp- Kali Tiger Reserve, Kulgi, Dandeli, Karnataka.
8. AICTE sponsored two weeks quality improvement programme on Newer trends in pharmaceutical technology and research from 30th October to 11th November 2017 organized by Department of pharmaceuticals, as a resource person.
9. 4th international congress of the society for ethnopharmacology, India: Healthcare in 21st century perspectives of ethnopharmacology and medicinal plant research, held during 23rd to 25th February 2017, at Uka Tarsadia University, Bardoli, Gujrat, India.
10. Workshop on effective scientific writing skills on 22nd Sept. 2017, organized by KLEU's BSRC, Belagavi.
11. 5 days hands on training programme on Laboratory Procedures on 21st November 2017 to 25th November 2017, organized by KLEU's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.
12. One day seminar on Intellectual Property Rights IPR on 24th November 2017 at College of Pharmacy, KLE University, Belagavi.
13. NFD-2017 scientific conclave, on 23rd December 2017 organized by KLES Dr. Prabhakar Kore Hospital & Medical Research Centre, Belagavi.
14. Workshop on Analytical techniques on 24-25 January 2018 organized by KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.

15. 6th National level field workshop on medicinal plants in Western Ghats on Globalizing traditional medicinal knowledge, 31st January- 3rd February, 2018 at Kulgi nature camp, Kulgi, Dandeli, Uttara Kannada, organized by KLE Academy of Higher Education and Research, ICMR- National Institute of Traditional Medicine, Belagavi, Karnataka and Kokum foundation, Goa in association with the society for ethnopharmacology and society of pharmacognosy, India.
16. Workshop on Regulatory compliance: Laboratory practice on 11th April 2018 organized by KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.
17. Workshop on Ethical issues in research involving human subject on 30th April 2018 organized by J.N. Medical College, Belagavi.
18. Workshop on Fostering publication integrity and plagiarism awareness on 21st August 2018 organized by KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.
19. Traditional Healers Meet-2018, as Resorce Person on 24th November, 2018 organized by KLE Academy of Higher Education and Research, Shri BMK Ayurveda Mahavidhyalaya, ICMR-- National institute of Traditional Medicine, Belagavi, Karnataka in association with Society of Ethnopharmacology, INDIA.
20. Workshop on Laboratory procedures on 18-22 December 2018 organized by KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.
21. 6th International congress of society of ethnopharmacology on Medicinal plants and traditional medicine- Ethnopharmacology at the interface and global nodes on 8-10 February 2019 at Manipal college of Pharmaceutical Sciences, Centre for integrative medicine and research, Manipal.
22. 23rd National convention of society of pharmacognosy and International conference on new age opportunities and challenges for quality, safety and GMPs in herbal drug development on 22-23 February 2019 organized by CSIR-National Botanical Research institute, Lucknow.

23. International symposium prostate cancer on 16th March 2019 at KLES Dr. Prabhakar Kore Hospital & Medical Research Centre, Belagavi.
24. Workshop on Regulatory compliance: Good laboratory practice on 11 April 2019 organized by KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.
25. Workshop on Integrity and ethics in clinical research & publication as Organizer on 8th November, 2019 organized by ICMR- National institute of Traditional Medicine, Research Integrity Unit (RIU)- ICMR New Delhi & KLE Academy of Higher Education and Research, Belagavi, Karnataka.



Best Oral Presentation award at International conference of Society of Ethnopharmacology at Mae Fah Luang University, Chiang Rai, Thailand.





**ICMR Sponsored Training Program on
Good Laboratory Practices**

**Organised by
KLE University's J. N. Medical College, Belagavi**

Certificate of Participation

Dr./Mr./Mrs./Miss **MAHENDRA KUMAR CHOUDHAN** *hias*

Participated as Delegate in a ICMR DHR Sponsored Training Program on "GOOD LABORATORY PRACTICES"
conducted by **KLE University's J. N. Medical College, Belagavi, Karnataka. vide letter No. V-25011/25/2013-HR**
held from *15/12/2014* to *02/01/2015*.....

[Signature]
Dr. Ganga S. Pilli
Training Program Co-ordinator
J N Medical College, Belagavi

[Signature]
Dr. N.S. Mahantshetti
Principal
J N Medical College, Belagavi

[Signature]
Dr. V. D. Patil
Registrar
KLE University, Belagavi






KLE UNIVERSITY
 Accredited 'A' Grade by NAAC

JAWAHARLAL NEHRU MEDICAL COLLEGE & KLES DR. PRABHAKAR KORE HOSPITAL
CLINICAL SKILLS LAB

CERTIFICATE

This is to certify that Mahendra Kumar Chouhan was a
Resource Person / Participant for workshop on BLS
 and ACLS conducted on 20th June 2015




DR. A. S. GODHI Chairman	DR. S. C. METGUD Member	DR. ANIL S. HARUGOP Member	DR. M. G. DHORIGOL Member-Secretary
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DR. (Mrs) N. S. MAHANTSHETTI
 Principal, J. N. Medical College
 Professor Dept. of Paediatrics
 KLE University, Belgaum

DR. M. G. DHORIGOL
 Member - Secretary, Clinical Skills Lab
 Professor Dept. of Anaesthesiology
 J. N. Medical College, KLE University, Belgaum



KLEU's College of Pharmacy Belagavi

One Day Workshop on "Total Parenteral Nutrition"

12th August 2015



This is to certify that Mr./Miss./Mrs./Dr./Prof. MAHENDRA KUMAR CHOUHAN has participated as Delegate/faculty in "One day work shop on Total Parenteral Nutrition" held on 12th August 2015 at KLEU's College of Pharmacy, Belagavi.

Prof. (Dr.) V.P. Rasal
Principal
(Chief Co-ordinator)

Prof. (Dr.) M. S. Ganachari
Head - Dept. of Pharmacy Practice
(Programme co-ordinator)



**33rd Annual Conference of Indian Society for Medical Statistics
as a part of Centenary Celebrations of KLE Society**

ISMS CONFERENCE 2015

Certificate

This is to Certify that Ms. MAHENDRA KUMAR CHOUHAN
has Participated in Pre Conference Workshop / Discipline Specific Symposia in the
33rd Annual Conference of Indian Society for Medical Statistics organized by
KLE University Department of Epidemiology & Biostatistics, Belagavi
on 13th October 2015

N K Tyagi
Dr. N. K. Tyagi
Organizing Secretary & Convener

Dr. B. S. Kodkany
Dr. B. S. Kodkany
Organizing Chairman

Dr. A. Indrayan
Dr. A. Indrayan
President ISMS

Dr. V. D. Patil
Dr. V. D. Patil
Registrar



**33rd Annual Conference of Indian Society for Medical Statistics
as a part of Centenary Celebrations of KLE Society**

ISMS CONFERENCE 2015


Certificate

This is to Certify that MA. MAHENDRA KUMAR CHOUHAN
has Chaired a Session / Delivered-Invited-Talk / Paper-Presentation / Poster-Presentation /
Delegate / Member of Organizing Committee in the 33rd Annual Conference of Indian Society
for Medical Statistics organized by KLE University Department of Epidemiology &
Biostatistics, Belagavi during 14th to 16th October 2015

NK Tyagi
Dr. N. K. Tyagi
Organizing Secretary & Convener


Dr. B. S. Kodkany
Organizing Chairman


Dr. A. Indrayan
President ISMS


Dr. V. D. Patil
Registrar



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BELAGAVI

K.L.E. UNIVERSITY'S

COLLEGE OF PHARMACY, BELAGAVI

20th NATIONAL CONVENTION OF SOCIETY OF PHARMACOGNOSY

Certificate

This is to certify that Mr./Ms./Dr./Prof. MAHENDRA KUMAR CHOUHAN

has participated as Delegate / in Poster Presentation at **20th National Convention of Society of Pharmacognosy**

on **"NEWER APPROACHES FOR DEVELOPMENT AND PROMOTION OF NATURAL PRODUCTS"**

held on 26th and 27th March 2016 organized by KLE University's College of Pharmacy, Belagavi, Karnataka.



Dr. V. P. RASAL
Chairman LOC



Dr. S. S. JALALPURE
Organizing Secretary



This is to certify that

Dr. / MR. / MRS. MAHENDRA KUMAR CHOUHAN

has Participated in the INTERNATIONAL CME On

'RESEARCH METHODOLOGY & COMMUNICATION'

On 19th November 2016,

Organized by Research Unit, USM-KLE, IMP Belagavi,
as a Delegate / Resource Person / Organizing Committee member.

Asma Ismail

Prof Datuk Dr Asma Ismail
Vice-Chancellor
USM Penang, Malaysia

H. B. Rajasekhar

Dr. H. B. Rajasekhar
Director
USM-KLE-IMP, Belagavi

Sadanand B. Patil

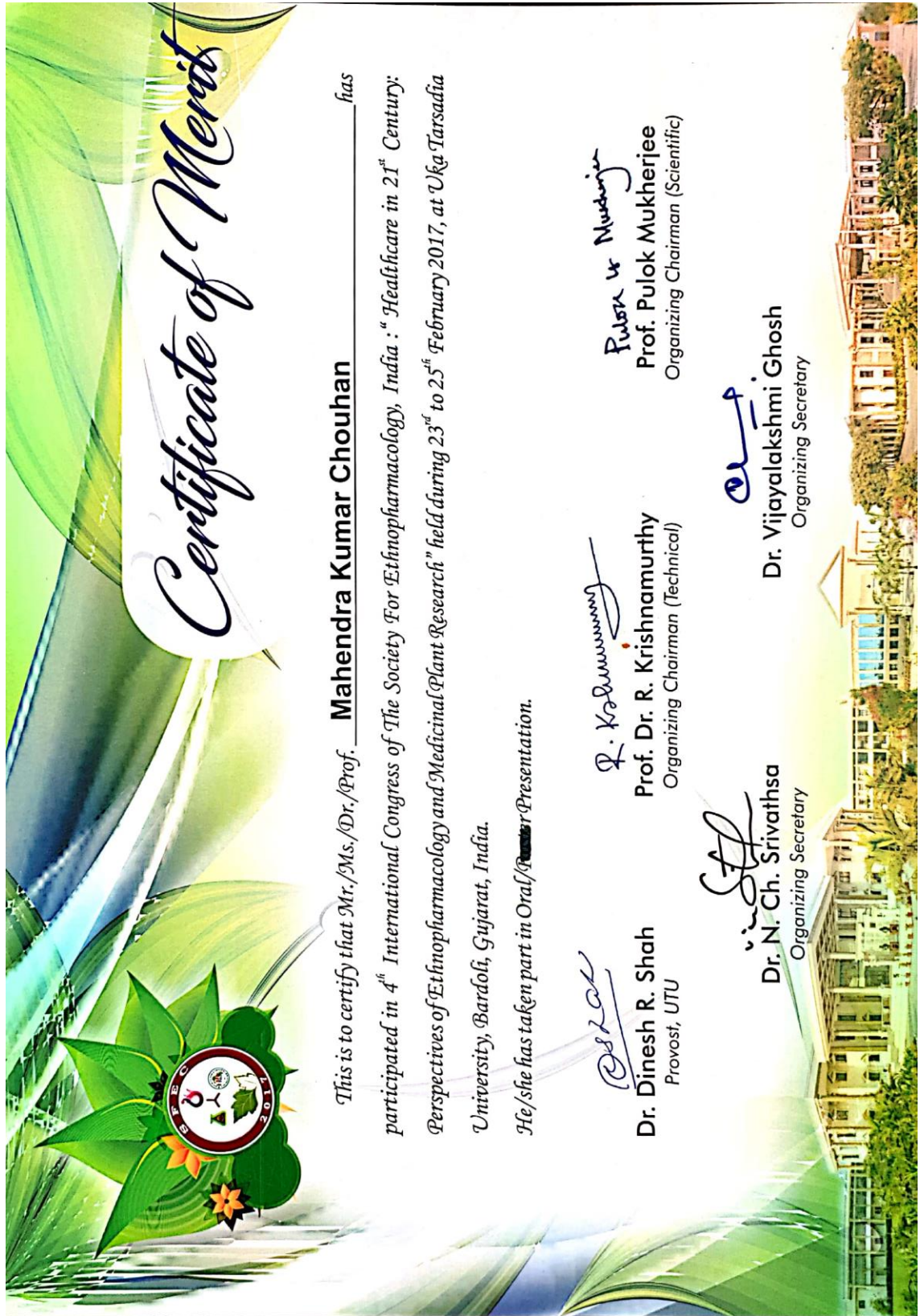
Dr. Sadanand B. Patil
Organizing Secretary
USM-KLE-IMP, Belagavi

Kamarudin Jaalain

Dr. Kamarudin Jaalain
Organizing Chairman
USM-KLE-IMP, Belagavi







KLE UNIVERSITY

(Formerly known as KLE Academy of Higher Education & Research, Belgaum)

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956, vide Government of India Notification No. F.9-19/2000-U3(A)]

Placed in **Category 'A'** by MHRD, Govt. of India Accredited '**A'** Grade by NAAC

Nehru Nagar, Belgaum - 590 010, Karnataka State, India

Ph. : 0831-2444444 FAX : 0831-2493777 Web: <http://www.kleuniversity.edu.in> E-mail: info@kleuniversity.edu.in

UNIVERSITY DEPARTMENT OF EDUCATION FOR HEALTH PROFESSIONALS

This is to certify that

Dr./Mr./MRS. MAHENDRA KUMAR CHOUHAN

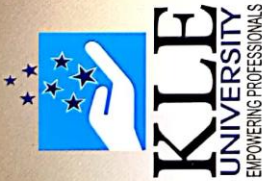
has participated in the Workshop entitled

"EFFECTIVE SCIENTIFIC WRITING SKILLS"

On 22nd Sept. 2017 organised by KLEU's -BSRC : B6M
_____ as a Delegate / Resource Person.

Prashant
Dr. PADMAJA WALVEKAR
DIRECTOR, UDEHP

V.D. Patil
Dr. V.D. PATIL
REGISTRAR







Certificate of Participation

KLE UNIVERSITY SPONSORED ONE DAY SEMINAR ON
“INTELLECTUAL PROPERTY RIGHTS (IPR)”

[Friday, 24th November 2017]

This is to certify that Prof. / Dr. / Mr. / Ms. Mohendra Kumar Chouhan has

participated as delegate/resource person in the Seminar on **“INTELLECTUAL PROPERTY RIGHTS”** on

Friday, 24th November 2017 at the KLE University, Belagavi (India) organized by College of Pharmacy, Belagavi.

Dr. V. D. Patil
 Registrar
 KLE University, Belagavi

Dr. B. M. Patil
 Principal - COP, Belagavi
 Chief Coordinator

Dr. Sunil S. Jalalpure
 Professor - COP, Belagavi
 Co-ordinator

 <p>Integration of Omics Data Key to Personal Medicine</p>	<p>New Frontiers in DIABETES NFD -2017</p>	 	 	 
<h2>Certificate of Participation</h2>				
<p>This is to certify that</p>				
<p>Dr./Mr./Mrs./Ms. <i>Mahendra Kumar Chohan</i></p>				
<p>has attended and participated in the <i>NFD-2017 Scientific Conclave</i>, as Orator / Faculty / Delegate /</p>				
<p>Member in Organising Committee held on 23rd of December, 2017 organised by the</p>				
<p>KLES Dr Prabhakar Kore Hospital & Medical Research Centre, Belagavi - 590010</p>				
	<p>Dr. (Mrs), Rekha Patil MD. Organizing Secretary HOD - Dept. of Medicine, JNMC</p>		<p>Dr. M.V. Jali, MD, FRCP (London) Organizing Chairman MD & CEO, KLES Dr Prabhakar Kore Hospital & MRC</p>	





6th National Level Field Workshop on Medicinal Plants in Western Ghats

Theme: Globalizing Traditional Medicinal Knowledge


31st January- 3rd February, 2018

Kulgi Nature Camp, Kulgi, Dandeli, Uttara Kannada

Grant this **CERTIFICATE** of achievement to

Mahendra Kumar Chouhan

In recognition of his / her participation as Resource person / Delegate / Organizer in the field workshop organized by- KLE Academy of Higher Education and Research, Indian Council for Medical Research- National Institute of Traditional Medicine, Belagavi, Karnataka and Kokum Foundation, Goa, in association with the Society for Ethnopharmacology and Society of Pharmacognosy, India.


Dr. S. L. Hoti
Director-In-Charge
ICMR-NITM, Belagavi


Prof. (Dr.) V. D. Patil
Registrar
KAHER, Belagavi


Dr. Pramod J. Hurkadale
Organizing Secretary
6th NLFW-2018, Belagavi

KLE ACADEMY OF HIGHER EDUCATION & RESEARCH

(Formerly known as KLE University, Belagavi)

[Declared as Deemed-to-be-University us 3 of the UGC Act, 1956, vide Government of India Notification No. F.9-19/2000-U3(A)]
 Placed in Category 'A' by MHRD, Govt. of India Accredited 'A' Grade by NAAC 2nd Cycle
 Nehru Nagar, Belagavi - 590 010, Karnataka State, India
 Ph. : 0831-2444444 FAX : 0831-2493777 Web: <http://www.kledeemeduniversity.edu.in> E-mail: info@kledeemeduniversity.edu.in

UNIVERSITY DEPARTMENT OF EDUCATION FOR HEALTH PROFESSIONALS

This is to certify that

Dr/Mr./MRS. Mahendra Kumar Chouhan

has participated in the Workshop entitled

“Regulatory Compliance: Laboratory Practice”

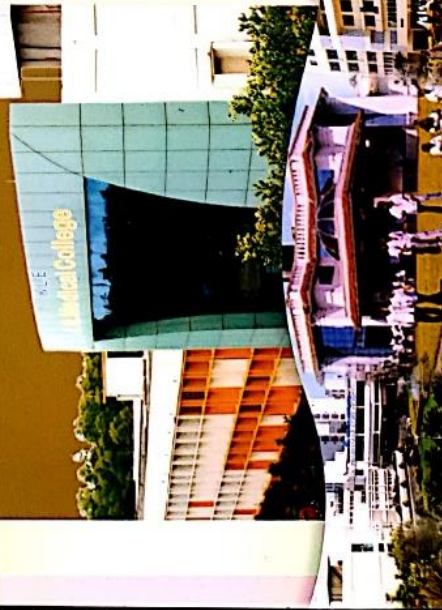
on 11th April, 2018 organised by Dr. Prabhatkar Kase

Basic Science Research Center as a Delegate / Resource Person.
 [B.S.R.C.]

Prabhatkar

Dr. PADMAJA WALVEKAR
DIRECTOR, UDEHP

[Signature]
Dr. V. D. PATIL
REGISTRAR





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KLE ACADEMY OF HIGHER EDUCATION & RESEARCH
(Formerly known as KLE University, Belagavi)

[Declared as Deemed-to-be-University u/s 3 of the UGC Act, 1956, vide Government of India Notification No. F.9-19/2000-U3(A)]
Placed in Category 'A' by MHRD, Govt. of India Accredited 'A' Grade by NAAC 2nd Cycle
Neluru Nagar, Belagavi - 590 010, Karnataka State, India
Ph. : 0831-2444444 FAX : 0831-2493777 Web: <http://www.kledeemeduniversity.edu.in> E-mail: info@kledeemeduniversity.edu.in

**UNIVERSITY DEPARTMENT OF
EDUCATION FOR HEALTH PROFESSIONALS**

This is to certify that

DR.Mr.Mrs. Mahendra Chohan

has participated in the Workshop entitled

"Ethical Issues in Research Involving Human Subjects"

on 30th April 2018 organised by J.N. Medical
College, Belagavi as a Delegate / Resource Person.

Prashant
Dr. PADMAJA WALVEKAR
DIRECTOR, UDEHP

V. D. Patil
Dr. V. D. PATIL
REGISTRAR





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KLE ACADEMY OF HIGHER EDUCATION & RESEARCH
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Nehru Nagar, Belagavi - 590 010, Karnataka State, India

Ph. : 0831-2444444 FAX : 0831-2493777 Web: <http://www.kledeemeduniversity.edu.in> E-mail: info@kledeemeduniversity.edu.in



CERTIFICATE

**UNIVERSITY DEPARTMENT OF
EDUCATION FOR HEALTH PROFESSIONALS**

This is to certify that

DR./MRS. MAHENDRA KUMAR CHOUHAN

has participated in the Workshop entitled

FOSTERING PUBLICATION INTEGRITY AND PLAGIARISM AWARENESS

on 21st August 2018 organised by KAHER's - BSRC
BELAGAVI _____ as a Delegate / Resource Person.



Dr. PADMAJA WALVEKAR
DIRECTOR, UDEHP



Dr. V. D. PATIL
REGISTRAR





Accredited 'A' Grade by NAAC Category 'A' by MHRD (GOI)

Traditional Healers Meet – 2018

Saturday, 24th November 2018

Grant this CERTIFICATE of achievement to

Mahendra Kumar Chouhan

In recognition of his / her participation as Delegate/ Resource Person/ Speaker

in the Traditional Healers Meet -2018 organized by KLE Academy of Higher Education and Research,

Shri B M K Ayurveda Mahavidyalaya, ICMR-National Institute of Traditional Medicine, Belagavi, Karnataka

in association with Society for Ethnopharmacology, India

Dr. S.L. Hoti
Director In-charge
ICMR-NITM, Belagavi

Chouhan
Prof. (Dr.) B.S. Prasad
Principal & Dean
SBMKAM, Belagavi

Pramod
Prof. (Dr.) Pramod H. J.
Organizing Secretary
THM-2018, Belagavi





**Manipal College of Pharmaceutical Sciences
Centre for Integrative Medicine and Research**
Manipal Academy of Higher Education, Manipal

in association with
Society for Ethnopharmacology - India, Saktigarh, Jadavpur, Kolkata



**6th INTERNATIONAL CONGRESS OF
SOCIETY FOR
ETHNOPHARMACOLOGY, INDIA**



February 8 -10, 2019

**Medicinal Plants and Traditional Medicine -
Ethnopharmacology at the Interface of Local and Global Needs**

CERTIFICATE OF PARTICIPATION

This is to certify that

MAHENDRA KUMAR CHOUHAN

has participated as Delegate in the

6th International Congress of Society for Ethnopharmacology, India

held at Manipal College of Pharmaceutical Sciences, MAHE, Manipal between February 8 and 10, 2019

Dr. N Udupa
Organizing Chairman
SFEC 2019

Dr. Pulok K Mukherjee
Secretary
SFE India, Kolkata

Dr. Saleemulla Khan
Organizing Secretary
SFEC 2019



**Manipal College of Pharmaceutical Sciences
Centre for Integrative Medicine and Research**
Manipal Academy of Higher Education, Manipal

in association with
Society for Ethnopharmacology - India, Saktigarh, Jadavpur, Kolkata



**6th INTERNATIONAL CONGRESS OF
SOCIETY FOR
ETHNOPHARMACOLOGY, INDIA**



February 8 -10, 2019

**Medicinal Plants and Traditional Medicine -
Ethnopharmacology at the Interface of Local and Global Needs**

CERTIFICATE

This certificate is awarded to

MAHENDRA KUMAR CHOUHAN

as Oral/Poster Presenter in the

6th International Congress of Society for Ethnopharmacology, India

held at Manipal College of Pharmaceutical Sciences, MAHE, Manipal between February 8 and 10, 2019

Dr. N Udupa
Organizing Chairman
SFEC 2019

Dr. Pulok K Mukherjee
Secretary
SFE India, Kolkata

Dr. Saleemulla Khan
Organizing Secretary
SFEC 2019



CSIR-NBRI
National Botanical Research Institute
Lucknow



ISP
SOCIETY OF PHARMACOGNOSY
International Society of Pharmacognosy



Ministry of Health & Family Welfare
Government of India

23rd National Convention of Society of Pharmacognosy
and
International Conference on New Age Opportunities and
Challenges for Quality, Safety and GMPs in Herbal Drug Development

February 22-23, 2019

Certificate

This is to certify that Prof./Dr./Mr./Mrs./Ms. *Mahendra Kumar Chauhan*..... has participated as a
speaker/resource person/oral/poster/exhibitor/participant at 23rd National Convention of Society of
Pharmacognosy and International Conference on "New Age Opportunities and Challenges for Quality, Safety
and GMP's in Herbal Drug Development", held during 22nd & 23rd February 2019, organized by CSIR- National
Botanical Research Institute, Lucknow (India).


Prof. S. K. Barik
Chairman

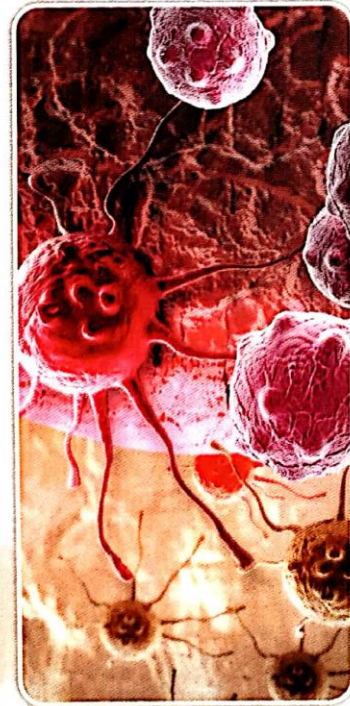

Dr. Sharad Srivastava
Organizing secretary


Dr. Umesh K. Patil
General Secretary ISP


Dr. N.R. Sheth
President ISP

Supported By:





INTERNATIONAL SYMPOSIUM ON DIAGNOSIS OF PROSTATE CANCER 2019

CERTIFICATE

This is to certify that

Dr./Mr./Mrs./Miss. Mahendra Kumar Chauhan.....

has participated as Delegate / Faculty,

*in the International Symposium on Diagnosis of Prostate Cancer, held on 16th March 2019,
at KLES Dr Prabhakar Kore Hospital & Medical Research Centre, Belagavi.*

Girish Nelivigi
Dr. Girish Nelivigi
President
Karnataka Urology Association

[Signature]
Dr. R. B. Nerli
Organising Chairman

[Signature]
Dr. Madhukar Thakur
Professor of Radiology, Radiation Oncology & Urology,
Thomas Jefferson University,
Philadelphia, USA

[Signature]
Dr. Suvarna V. Pai
Organising Secretary

KLE ACADEMY OF HIGHER EDUCATION & RESEARCH

(Formerly known as KLE University, Belagavi)

[Declared as Deemed-to-be-University us 3 of the UGC Act, 1956, vide Government of India Notification No. F.9-19/2000-U3(A)]

Placed in Category 'A' by MHRD, Govt. of India Accredited 'A' Grade by NAAC 2nd Cycle

Nehru Nagar, Belagavi - 590 010, Karnataka State, India

Ph. : 0831-2444444 FAX : 0831-2493777 Web: <http://www.kledeemeduniversity.edu.in> E-mail: info@kledeemeduniversity.edu.in

UNIVERSITY DEPARTMENT OF EDUCATION FOR HEALTH PROFESSIONALS

This is to certify that

Dr./Mr./Mrs. Mahendra Kumar Chouhan

has participated in the Workshop entitled

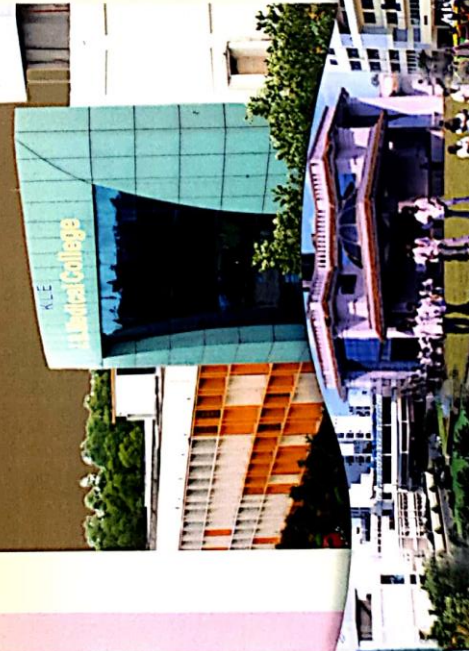
Regulatory Compliance: Good Laboratory Practice

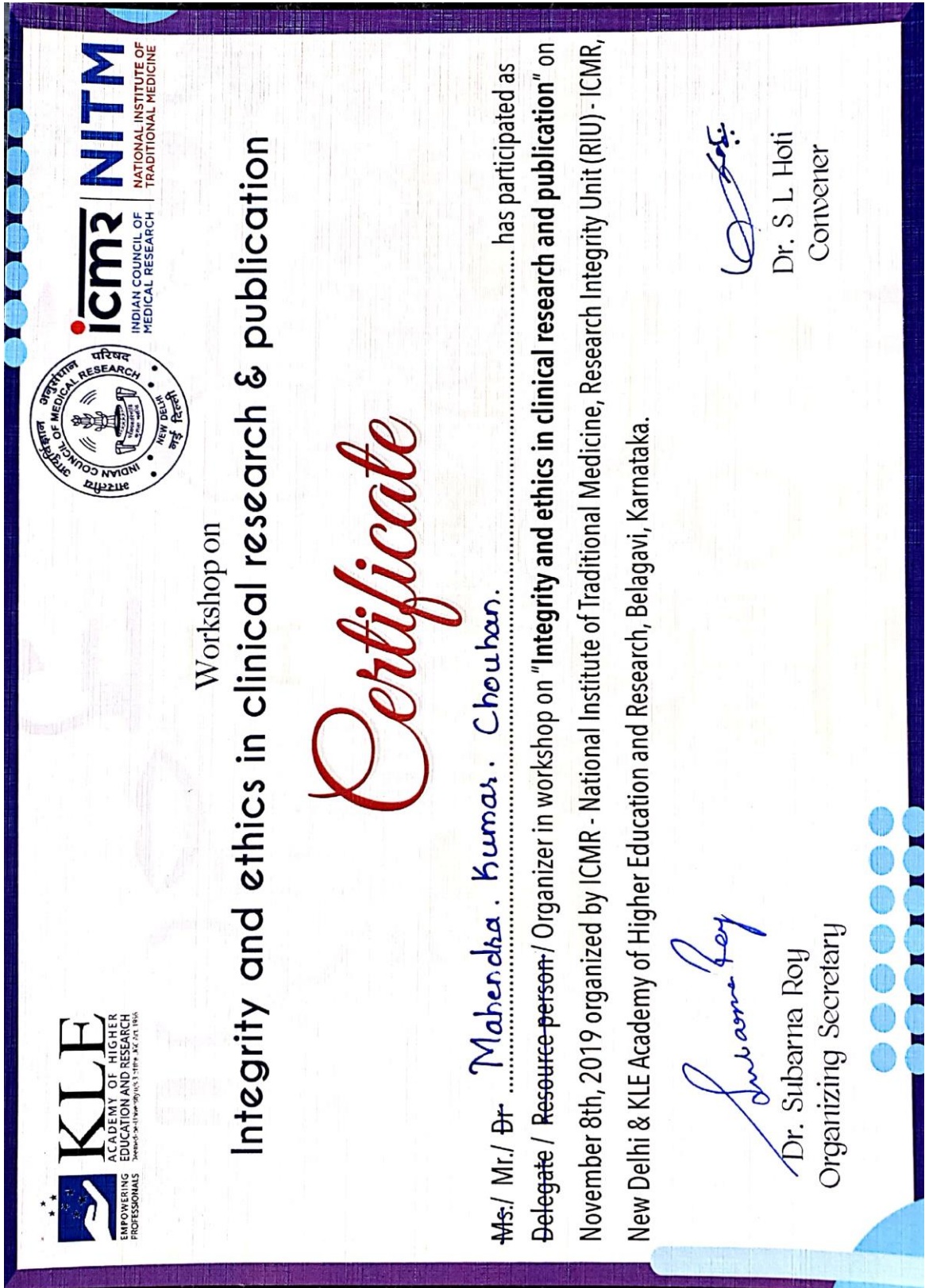
on 11th April, 2019 organised by KAHER Basic Science

Research Center, as a Delegate / Resource Person.
Belagavi.

R. Padmaja
Dr. PADMAJA WALVEKAR
DIRECTOR, UDEHP

V. D. Patil
Dr. V. D. PATIL
REGISTRAR





Annexure-VI

Scientific Body Membership

1. Member, Society for Ethnopharmacology (SFE), India.
2. Life Member, Society for Pharmacognosy (ISP), India.
3. Life Member, Society for Infectious Disease (ISID), USA.



**SOCIETY FOR ETHNOPHARMACOLOGY
(SFE-INDIA)**

www.ethnopharmacology.in

This is to certify that

Mahendra Kumar Chouhan

BELGAUM, KARNATAKA

*Has been accepted as a member of the
Society for Ethnopharmacology, Kolkata, India (SFE-INDIA).*

*In witness whereof this certificate has been signed and
presented on December 01, 2017.*

Membership No: SFE/17/I-1232

Valid till: November 30, 2021

Pratim Banerji

President

Pulok K. Mukherjee

Secretary

SOCIETY FOR ETHNOPHARMACOLOGY
(A Registered Society under West Bengal Society Registration Act 1961)
Affiliated to International Society for Ethnopharmacology
23/3 Saktigarh, Jadavpur, Kolkata 700032, India
sfeindian@gmail.com



SOCIETY OF PHARMACOGNOSY

(Formerly Indian Society of Pharmacognosy)

by approval of the Executive Council, has admitted

.....*Mr. Mahendra Kumar Chouhan*.....

.....*LM-KA/C-045*.....

as LIFE MEMBER

of the society and is entitled to all the privileges

conferred by the constitution of the society.

.....*January 2020*.....

elnoth
President



Pr...
Secretary

No.: Bhopal *12.11.2020* C-045

EVALUATION OF ANTICANCER ACTIVITIES OF ETHNOMEDICINAL PLANTS FROM WESTERN GHATS

An Errata submitted to

KLE Academy of Higher Education and Research, Belagavi

Accredited 'A' Grade by NAAC (2nd Cycle)

[Placed in Category 'A' by MHRD (GoI)]

[Deemed-to-be-University u/s 3 of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]



Under the Guidance of

Dr. Pramod J. Hurkadale

**Professor and Head
Department of Pharmaceutical Biotechnology,
KLE College of Pharmacy, Belagavi**

***In the Faculty of
Pharmacy***

By

Mr. Mahendra Kumar Chouhan

Full Time Ph.D. Research Scholar

(Registration No: KLEU/Ph.D./14-15/DO1214013)

Feb - 2021

Errata submission of the thesis:

Examiner:

S. No.	Observations	Clarifications / corrected as	Corrected thesis copy page number
1.	Table 14- Dose selection: 1/10 th of 2000 mg/kg = 200 mg/kg is considered as low dose. How come 1/20 th of 2000 mg/kg = 400 mg/kg high dose?? It is supposed to be 100 instead of 400 (half of low dose). Entire dose is questionable	Typographical error, It should read as “2/10 th of 2000 mg/kg i.e. = 400 mg/kg” is correct (as per OECD 423). Incorporated in the thesis.	page no. 74, 106
2.	Page no 42 (2.7.6 Group distribution for cancer model in mice- Daltons Ascitic Lymphoma model) Here Doxorubicin given IP, whereas in Fibrosarcoma model IV route was selected. Justify.	<p>In - Daltons Ascitic Lymphoma model, where Doxorubicin was given I.P. for better bioavaibility, because the site of tumor was in peritoneal cavity [1].</p> <p>In Fibrosarcoma model I.V. route of administration is selected due to high concentrations of doxorubicin are localized around blood vessels in several experimental tumors which is the best selected route of administration for anticancer drug [1, 2].</p> <p><u>References</u></p> <p>[1]. Reddy LH. Drug delivery to tumours: recent strategies. Journal of Pharmacy and Pharmacology. 2005 Oct;57(10):1231-42.</p> <p>[2]. Trédan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. Journal of the National Cancer Institute. 2007 Oct 3;99(19):1441-54.</p>	----Nil----

3.	Page no 115 Figure no 37. You have presumed that ROS is responsible for cancer, why did you not try to study antioxidant activities?	<p>I appreciate the concern raised/noticed; however, the main objective was to evaluate the anticancer activity of the selected ethnomedicinal plants <i>Achyranthes coynei</i> Sant. and <i>Clerodendrum inerme</i> (L.) Gaertn. by <i>in-vitro</i> assay methods using various cell lines and <i>in-vivo</i> models, such as Daltons Ascitic Lymphoma and Fibrosarcoma modes in swiss mice.</p> <p>Hence, as per the literature review the anti-oxidant activity was previously performed by other researchers [Upadhyaya V et al., and Khan SA et al.] which has been included as supporting study for the anticancer activities [3,4].</p> <p><u>References</u></p> <p>[3]. Upadhyaya V, Pai SR, Ankad G, Hurkadale PJ, Hegde HV. Phenolic contents and antioxidant properties from aerial parts of <i>Achyranthes coynei</i> Sant. Indian Journal of Pharmaceutical Sciences. 2013 Jul;75(4):483.</p> <p>[4]. Khan SA, Rasool N, Riaz M, Nadeem R, Rashid U, Rizwan K, Zubair M, Bukhari IH, Gulzar T. Evaluation of antioxidant and cytotoxicity studies of <i>Clerodendrum inerme</i>. Asian J Chem. 2013 Oct 1;25(13):7457-62.</p>	-----Nil-----
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**Name & Signature of
Research Scholar**

Name & Signature of Guide

Mr. Mahendra Kumar Chouhan

Dr. Pramod J. Hurkadale

(Registration No: KLEU/Ph.D./14-15/DO1214013)

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Date: