EVALUATION OF *IN-VITRO* AND *IN-VIVO* ANTICANCER ACTIVITY OF LEAF EXTRACTS OF *Amaranthus spinosus* Linn.

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Submitted by

Reg.No. 261426066

Under the guidance of

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APRIL-2016

CERTIFICATE

This is to certify that this dissertation work entitled **"EVALUATION OF *IN-VITRO* AND *IN-VIVO* ANTICANCER ACTIVITY OF VARIOUS EXTRACTS OF *Amaranthus spinosus* Linn."** submitted by **Reg.No: 261426066** in partial fulfilment of the requirements for the award of the degree in **MASTER OF PHARMACY IN PHARMACOLOGY** by the Tamil Nadu Dr. M.G.R. Medical University, Chennai is a bonafide record of the work done by him in the Institute of Pharmacology, Madras Medical College, Chennai, during the academic year 2015-2016 under the guidance of **Mrs. R. INDUMATHY, M. Pharm., Asst. Professor,** Institute of Pharmacology, Madras Medical College, Chennai-600003.

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LIST OF ABBREVIATION

5-FU	5-Fluorouracil
ALP	Alkaline Phosphates
ALT	Alanine amino Transferase
AST	Aspartate amino Transferase
BMI	Body Mass Index
DLA	Dalton's Lymphoma Ascites
DMSO	DiMethyl Sulfoxide
DMBA	Dimethoxybenzaldehyde
DPPH	Diphenyl Picryl Hydrazile
DNA	Deoxyribo Nucleic Acid
EAC	Ehrlich Ascites Carcinoma
EDTA	Ethylene Diamine Tetra Acetic acid
FBS	Foetal Bovine Serum
Hb	Haemoglobin
HCT-116	Hematopoietic Cell Transplantation
HeLa	Henrietta Lacks
HPV	Human Papilloma Virus
IC_{50}	Median Inhibition Concentration
IL	Interleukin
ILS	Increased Life Span
MCF-7	Michigan Cancer Foundation

MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
NCCS	National Centre for Cell Science
OD	Optical Density
PCV	Packed Cell Volume
PLT	Platelet
RBC	Red Blood Corpuscle
RNA	Ribose Nucleic Acid
rpm	revolution per minute
STZ	Streptozotocin
TG	Triglycerides
TC	Total Cholestrol
TNF	Tumor Necrosis Factor
TPVG	Trypsin, PBS, Versense/EDTA, Glucose
WHO	World Health Organization
WBC	White Blood Corpuscle

1. INTRODUCTION

Cancer is an enormous global health burden, touching every region and socioeconomic group. Tobacco use is a major cause of the increasing global burden of cancer as the number of smokers worldwide continues to grow[1].

World wide cancer incidence and mortalitystatistics are taken from the International Agency for Research on Cancer GLOBOCAN database and also the World Health Organisation, Global Health Observatory and the United Nations World Population Prospects report[2].

- In 2008 approximately 12.7million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers). In 2010 nearly 13.98 million cancers were diagnosed. In 2012, an estimated 14.1 million new cases of cancer occurred worldwide. More than half of cancers occurring worldwide are in less developed regions.

- The four most common cancers occurring worldwide are lung, female breast, bowel and prostate cancer. These four cancers account for around 4 in 10 of all cancers diagnosed worldwide.

- Lung cancer is the most common cancer in men worldwide. More than 1 in 10 of all cancers diagnosed in men is lung cancers. It accounts 1.4 million deaths in worldwide.

- In 2010 nearly 7.98 million people died with cancer. In 2012, estimated 8.1 million people died from cancer worldwide. More than 6 in 10 cancer deaths worldwide occur in less developed regions of the world [3].

The Society's Global Health and Intramural Research departments are raising awareness about the growing global cancer burden and promoting evidence-based cancer and tobacco control programs.

The Society has established key focus areas help to reduce the global burden of cancer, including global grassroots policy and awareness, tobacco control, cancer screening and vaccination for breast and cervical cancers, access to pain relief and the support of cancer registration in low and middle-income countries [4,5].

Cancer in India

Cancer rates in India are considerably lower than those in more developed countries such as the United States data from population based cancer registries in India show that the most frequently reported cancer sites in males are lung, oesophagus, stomach, and larynx. In females, cancers of the cervix, breast, ovary and oesophagus are the most commonly encountered [6].

- India officially recorded over half a million deaths due to cancer in 2011 – 5.35 lakhs as against 5.24 lakhs in 2010 and 5.14 lakhs in 2009.

- The estimated number of new cancers in India per year is about 7 lakhs and over 3.5 lakhs people die of cancer each year. Out of these 7 lakhs new cancers about 2.3 lakhs (33%) cancers are tobacco related.

- ❖ In India, which has nearly three million patients suffering from the disease. Annually, nearly 500,000 people die of cancer in India. The WHO said this number is expected to rise to 700,000 by 2015[7,8]

As per WHO,

- ❖ Cancer is the leading cause of death worldwide, accounting for 7.6 million deaths in 2008.
- ❖ Lung, stomach, liver, colon, breast cancer cause the most cancer deaths each year.
- ❖ About 30% of cancer deaths are due to the behavioural and dietary risks,
    - High BMI
    - Low fruit and vegetable
    - Lack of physical activity
    - Tobacco use
    - Alcohol use
- ❖ Death from cancer worldwide are projected to continue rising with an estimated 13.1million deaths in 2030.
- ❖ The risk of developing cancer generally increases with age and mass lifestyle changes occur in the developing world [9, 10].

Cervical Cancer

Cervical cancer is the most commonly occurring cancer in females. About 70% of cervical cancers occur in developing countriesWorldwide, cervical cancer is both the fourth-most common cause of cancer and the fourth-most common cause of death from

cancer in women. In 2012, an estimated 528,000 cases of cervical cancer occurred, with 266,000 deaths. This is about 8% of the total cases and total deaths from cancer.

In India, the numbers of people with uterine cervix cancer are rising, but overall the age-adjusted rates are decreasing. Improvement of education in the female population has improved the survival of women with cancers of uterine cervix[11].

Cervical cancer is a cancer arising from the cervix. It is due to the abnormal growth of cells that have the ability to invade or spread to other parts of the body. Early on, typically no symptoms are seen. Later symptoms may include abnormal vaginal bleeding, pelvic pain, or pain during sexual intercourse. While bleeding after sex may not be serious, it may also indicate the presence of cervical cancer[12].

Human papilloma virus (HPV) infection appears to be involved in the development of more than 90% of cases. Cervical cancer may be benign or malignant. Benign tumor is not life threatening and non- invasive but, malignant tumor is life threatening and invasive[13].

Causes of Cervical cancer

In most cases, cells infected with the HPV virus heal on their own. In some cases, however, the virus continues to spread and becomes an invasive cancer.

High-risk HPV types may cause cervical cell abnormalities or cancer. More than 70% of cervical cancer cases can be attributed to two types of the virus, HPV-16 and HPV-18, often referred to as high-risk HPV types.

A woman with a persistent HPV infection is at greater risk of developing cervical cell abnormalities and cancer than a woman whose infection resolves on its own. Certain

types of this virus are able to transform normal cervical cells into abnormal ones, these abnormal cells develop in to cervical cancer [14].

Symptoms of Cervical Cancer

Precancerous cervical cell changes and early cancers of the cervix generally do not cause symptoms. For this reason, regular screening through Pap and HPV tests can help catch precancerous cell changes early and prevent the development of cervical cancer [15].

Possible symptoms of more advanced disease may include abnormal or irregular vaginal bleeding, pain during sex, or vaginal discharge. Notify your healthcare provider if you experience:

- Abnormal bleeding, such as
    - Bleeding between regular menstrual periods
    - Bleeding after sexual intercourse
    - Bleeding after douching
    - Bleeding after a pelvic exam
    - Bleeding after menopause
- Pelvic pain not related to your menstrual cycle
- Increased urinary frequency
- Heavy or unusual discharge that may be watery, thick, and possibly have a foul odour
- Pain during urination

These symptoms could also be signs of other health problems, not related to cervical cancer. If you experience any of the symptoms above, talk to a healthcare provider[16].

Dalton's lymphoma ascites

Experimental tumor models have a wide role in anticancer drug discovery. A Dalton's lymphoma ascites (DLA) tumorigenesis model in Swiss albino mice provides a convenient model system to study antitumor activity within a short time.

The Dalton's tumor has been used as a transplantable tumor model to investigate the anti-neoplastic effects of several molecules and compounds. Following intraperitonial inoculation of Dalton's tumor cells, the ascetic volume and number of tumor cells increase progressively (Vincent and Nicholls, 1967). Ascitics is probably formed in consequence of tumor-induced inflammation due to the increase in peritoneal vascular permeability (Fastaia and Dumont, 1979). Another hypothesis argues that ascitis may be formed as a consequence of the impaired peritoneal lymphatic vessels drainage. Mice bearing the ascetic tumor, die after a short period of time due to several factors. They are

- Mechanical pressure exerted by the progressive increase of ascetic fluid.
- Intraperitoneal haemorrhage.
- Endotoxemia (Hartveit, 1965) [17].

Treatment

Many treatment are available for cancer exist, with the primary once including chemotherapy, surgery, hormonal therapy, radiation therapy, targeted therapy and

palliative care. Which treatments are used depends upon the type, location and grade of the cancer as well as the person's health and wishes. The treatment intent may be curative or not curative [18].

Chemotherapy

Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs (chemotherapeutic agents) as part of a standardized regimen. The term encompasses any of a large variety of different anticancer drugs, which are divided into broad categories such as alkylating agents and antimetabolites. Traditional chemotherapeutic agents act by killing cells that divide rapidly one of the main properties of most cancer cells[19].

Surgery

Surgery is the primary method of treatment of most isolated solid cancers and may play a role in palliation and prolongation of survival. It is typically an important part of making the definite diagnosis and staging the tumour as biopsies are usually required. In localized cancer surgery typically attempts to remove the entire mass along with, in certain cases, lymph nodes in the area. For some types of cancer this is all that is needed to eliminate the cancer [20].

Radiation

Radiation therapy involves the use of ionizing radiation in an attempt to either cure or improve the symptoms of cancer. It works by damaging the DNA of cancerous tissue leading to cellular death. As with chemotherapy, different cancers respond differently to radiation therapy[21].

- External Beam Radiation , which is well tested, long lasting treatment option

- Internal Beam Radiation (implantation of radioactive seeds), which is recently developed, shorter treatment interval, focused to the affected area [22].

Palliative care

Palliative care refers to treatment which attempts to make the person feel better and may or may not be combined with an attempt to treat the cancer. Palliative care includes action to reduce the physical, emotional, spiritual and psycho-social distress experienced by people with cancer [23].

People at all stages of cancer treatment should have some kind of palliative care to provide comfort. In some cases, medical especially professional organizations recommend that people and physicians respond to cancer only with palliative care and not with cure-directed therapy [24].

Hormonal Therapy

It is given for the patients with hormone receptor-positive cancers. It is used to reduce the amount of estrogen or block its action to reduce the risk of recurrence at the early stage of the disease and to shrink or slow down the growth of existing tumor at the stage of the disease. Hormone therapy includes,

- Aromatase inhibitors - Latrazole, Anastrazole
- Selective estrogen receptor modulators - Tamoxifen, Tormifene[25]

Immunotherapy

A variety of therapies using immunotherapy, stimulating or helping the immune system to fight cancer, have come into use since 1997, and this continues to be an area of very active research [26].

Today plant based drugs continue to play an essential role in health care. It has been estimated by the World Health Organization that 80% of the population of the world rely mainly on traditional medicines for their primary health care. Natural products also play an important role in the health care of the remaining 20% people of the world, who mainly reside in developed countries. Currently at least 119 chemicals, derived from 90 plant species, can be considered as important drugs in one or more countries. Studies in 1993 showed that plant-derived drugs represent about 25% of the American prescription drug market and over 50% of the most prescribed drugs in the US had a natural product either as the drug or as the starting point in the synthesis or design of the agent [27].

There are more than 250,000 species of higher plants in the world, and almost every plant species has a unique collection of secondary constituents distributed throughout its tissues. A proportion of these metabolites are likely to respond positively to an appropriate bioassay, however only a small percentage of them have been investigated for their potential value as drugs. In addition, much of the marine and microbial world is still unexplored, and there are plenty of bioactive compounds awaiting discovery in these two worlds. Besides their direct medicinal application, natural products can also serve as pharmacophores for the design, synthesis or semi-synthesis of novel substances for medical uses. The discovery of natural products is also important as a means to further refine systems of plant classification [28].

India is one of the earliest civilizations that have recognized the importance of herbal products for disease management, nutrition and beauty enhancement. With the discovery of several new molecules from herbs for treating diseases like cancer and the

relative safety of these products, the global demand for medicinal plant products has increased in recent years.

More than 30% of the pharmaceutical preparations are based on plants. An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from medicinal plants. Searching for new drugs in plants implies the screening the plants for the presence of novel compounds and investigation of their biological activities [29].

Many medicinal plants contain large amount of chemical components having broad spectrum of pharmacological activities. Anticancer activities are mainly due to phenolic acids, flavonoids and phenolic diterpenes. **Natural products** have long been a rich source of cure for cancer, which has been the major cause of death in the past decades [30].

- **Taxol,** one of the most outstanding drug used for the treatment of metastatic ovarian, breast carcinoma and small cell lung cancer have been obtained from bark of western yew tree.
- **Etoposide ,**a semi synthetic derivative of podophyllotoxin a plant glycoside being used in treatment of testicular tumours, lung cancer, bladder cancer.
- **Topotecan** and **irinotecan**are two recently introduced semisynthetic analogues of camptothecin and antitumour principle obtained from Chinese tree.
- **Vincristine, vinblastine, colchicines** and **ellipticine** are other important molecules from plant source.

Considering the toxicities which arise from cytotoxic drugs like bone marrow suppression, alopecia, lymphocytopenia and occurrence of secondary cancers like

leukemia and lymphomas. The search further intensifies for the toxicity free herbal remedy for cancer, which acts by without interfering with the body's natural healing process [31].

***Amaranthus spinosus* Linn** (Amaranthaceae), commonly known as ' Spiny amaranth' in India, Africa and Southeast Asia, is an important medicinal plant employed for different ailments in India traditionally.

Juice of *Amaranthus spinosus* is used by tribals of Kerala, to prevent swelling around stomach. The leaves are boiled without salt and consumed for 2-3 days to cure jaundice and also employed to cure some kind of rheumatic pain. The leaves and roots are applied as poultice to relief bruises, abscesses, burns, wound, inflammation, menorrhagia, gonorrhoea, eczema and inflammatory swelling. It is used as a sudorific, febrifuge, an antidote to snake poison and as a Galactagogue. It is also used in nutritional deficiency disorders and various other diseases in many parts of Africa.

Amaranthus spinosus having medicinal properties like **anti-diabetic, anti-microbial, anthelmintic, antioxidant, antihyperlipidemic, immunomodulatory, diuretic, analgesic and anti- inflammatory properties.**

Recent discovery of anti-cancer activity carried out by *Amaranthus* species plants (*Amaranthus viridis* **[32]**, *Amaranthus gangeticus* **[33]**). But there was no report for the evaluation of its anticancer activity in plant extract of *Amaranthus spinosus*.

Hence the present study is carried out to evaluate *in vivo* and *in vitro* anticancer activity of leaf extract of *Amaranthus spinosus* Linn.

2 AIM AND OBJECTIVE

- Successive extraction of leaf of *Amaranthus spinosus* by hot percolation method using the different solvents.

- To evaluate the safety of the effective extract of *Amaranthus spinosus* by acute toxicity studies in Swiss Albino mice.

- To evaluate the *In-vitro* cytotoxic activity of petroleum ether, ethyl acetate and ethanol extract of *Amaranthus spinosus* Linn. in Human Cervical Cancerous cell line (HeLa- Henrietta Lackes) by MTT assay.

- To evaluate the *In-vivo* anticancer activity of effective extract of *Amaranthus spinosus* Linn. by DLA on Swiss Albino mice.

3 REVIEW OF LITERATURE

Review related to *Amaranthus spinosus*

Antioxidant and hepatoprotective activity

B.S. Ashok Kumar *et al.*., (2010) was evaluated the hepatoprotective and antioxidant activity of methanolic extract of whole plant of *Amaranthus spinosus* (MEAS), against paracetamol (3g/kg p.o.) induced-liver damage in Wistar rats at dose of 200 and 400 mg/kg respectively. In this method the hepatoprotective activity is determined by measuring liver marker serum enzymatic levels.The presence of amino acids, flavonoids and phenolic compounds in the MEAS might be responsible for its marked chemo protective and antioxidant activities in paracetamol induced-liver damage in Wistar rats [34].

Hussain Zeashan *et al.*, (2008) has been showed hepatoprotective and antioxidant activity of ethanolic extract of whole plant of *Amaranthus spinosus* (EEAS), against carbon tetrachloride (CCl_4) induced hepatic damage in rats. The results of this study strongly indicate that whole plants of *A. Spinosus* have potent hepatoprotective activity. Their study suggested that possible, hepatoprotective activity may be due to the presence of flavonoids and phenolics compound present in the EEAS.

They were also reported that antioxidant activity of *Amaranthus spinosus* Linn. by non-enzymatic haemoglycosylation. They investigated the antioxidant activity of different extracts by estimating the degree of non-enzymatic haemoglobin glycosylation, measured colorimetrically. They have been reported that rutin and quercetin showed the inhibition of haemoglycosylation as maximum 42% and 52%, respectively.They also

reported that methanolic extract of *Amaranthus spinosus* leaves showed antioxidant activities [35].

Antidiabetic activity

B.S. Ashok Kumar *et al.*, (2010) was evaluated the alpha amylase and the antioxidant potential of methanol extract of *A. spinosus*(MEAS) which exhibited *in vitro* alpha amylase enzyme inhibition by CNPG3 (2-chloro-4-nitrophenol a-D-maltotrioside) and *in vivo* antioxidant potential of malondialdehyde (MDA), glutathione (GSH), catalase (CAT) and total thiols (TT) in alloxan induced diabetic rats. This study provided evidence that the methanolic extract of *A. Spinosus* has potent anti-diabetic activity [36].

Shanti Bhushan Mishra *et al.*, (2012) was evaluated anti diabetic effects of *Amaranthus spinosus* leaf extract (ASEt) against Streptozotocin-nicotinamide oxidative stress in albino rats. Experimental diabetes was induced by a single dose STZ (60 mg/kg) administered by intraperitoneal way after the administration of nicotinamide (120mg/kg).Results clearly indicate that *Amaranthus spinosus* treatment attenuate hyperglycemia by decreasing oxidative stress and pancreatic cells damage which may be attributed to its antioxidative potential[37].

Antipyretic and antioxidant activity

B.S. Ashok Kumar *et al.*, (2010) was reported the methanolic extract of *Amaranthus spinosus* leaves was screened for antioxidant and antipyretic activities. Antipyretic activity of methanolic extract of *A. Spinosus* was measured by yeast induced pyrexia method at concentration of 200 and 400 mg/kg using paracetamol as standard drug. Antioxidant activity was measured by 1,1-diphenyl-2-picryl-hydrazile (DPPH) free

radical scavenging, superoxide anion radical scavenging, hydroxyl free radical scavenging, nitric oxide radical scavenging and total phenolic content was also determined[38].

Anti-depressant activity

B.S. Ashok Kumar *et al.*, (2008) was reported the antidepressant activity of methanolic extract of *Amaranthus spinosus* (MEAS) was investigated by using Forced swimming test (FST) and Tail suspension test (TST) models. Escitalopam and Imipramine were used as reference standards. It has been observed from this study the MEAS at higher concentration showed significant ($p<0.01$) reduction in immobility in tail suspension and forced swim model of depression comparable to Escitalopam and Imipramine [39].

Anthelmintic activity

ManikBaral *et al.*, (2010) was evaluated water extracts of whole plant of *Amaranthus spinosus* Linn for anthelmintic on adult Indian earthworms (*Pheriti maposthuma*) and *Tubifex tubifex*, using piperazine citrate as reference standard. Aqueous extract showed anthelmintic activity in dose dependent manner giving shortest time of paralysis (P) and death (D) with 50 mg/ml concentration, for both the worms[40].

Antimalarial activity

A. Hilou *et al.*, (2006) was reported *in vivo* anti-malarial activities of extracts from *Amaranthus spinosus* L. in mice. The plant extracts showed significant anti-malarial activities in the 4^{th}-day of suppressive anti-malarial assay in mice inoculated with red blood cells parasitized with Plasmodium *Berghei berghei* [41].

Anti-nociceptive activity

Hussain Zeashana *et al.*, (2009) was studied the anti-nociceptive and anti-inflammatory activities of 50% ethanol extract (ASE) of *Amaranthus spinosus* (whole plant). *Amaranthus spinosus* possessed significant and dose dependant anti-inflammatory activity, it has also central and peripheral analgesic activity [42].

Anti-inflammatory activity

Olumayokun A. Olajide *et al.*, (2004), wasreported that methanol extract of *Amaranthus spinosus* L. leaves exhibited anti-inflammatory activities in different animal models. The effect of the plant extract was also studied on castor oil–induced diarrhoea and gastric mucosal integrity. These results demonstrate the anti-inflammatory properties of the leaf extract of *A. spinosus.* They suggested that the plant extract probably acts by the inhibition of prostaglandin biosynthesis[43].

Heamatologic activity

Olufemi B.E *et al.*, (2003) has been reported that haematological effects of ethanol extract of *Amaranthus spinosus* leaf (EEAL) when administered orally to growing pigs to determine its effects onpacked cell volume (PCV), red blood cell (RBC) and white blood cell (WBC) counts, andhaemoglobin (HB) concentration. The EEAL significantly reduced the PCV, RBC and Hb of pigs albeit temporarily. The final weight and average weight gains of the pigs weresignificantly improved with the administration of EEAL[44].

Ankitasrivastava *et al.*, (2011) has been showed that alteration in hematocellular components of albino rats due to methanolic extract of *Amaranthus*

spinosus. The study was carried out by single and daily administration *Amaranthus spinosus* for 5, 7 & 14 days. Results revealed that the RBC and WBC count as well as Hb% was significantly altered due to administration of methanolic extract of *Amaranthus spinosus* [45].

Anti-diarrheal and antiulcer activity

Zeashan Hussain *et al.*, (2009) was showed that the ethanol extract (50%) of the whole plant of *Amaranthus spinosus* Linn. (ASE) significantly inhibited travel of a charcoal meal at three different doses of ASE, but when 400mg/kg of ASE was repeated in the presence of yohimbine, intestinal propulsive inhibition decreased, while morphine reversed the activity. Lipid peroxidation was also associated with a concomitant decrease in ulcer index[46].

Antigenic and allergenic activity

Anand B. Singh *et al.*, (2008) was reported that *Amaranthus spinosus* is an important aeroallergen in India and grows commonly in different parts of the country, specially significance in Type I hypersensitivity disorders. Investigated antigenic and allergenic properties of 5 pollen samples of *Amaranthus spinosus* collected from the Delhi area at fortnightly intervals. The observations will be helpful in standardizing pollen antigens for diagnosis and immunotherapy in India[47].

Antimicrobial activity

Z. C. Maiyo *et al.*, (2010) was investigated the phytochemical constituents and antimicrobial activity of hexane, ethyl acetate, dichloromethane and methanol leaf extracts of *Amaranthus spinosus.* The leaf extracts showed a broad spectrum anti-

microbial activity against *Staphylococcus aureus* and *Bacillus* spp, the gram-negative *Escherichia coli, Salmonella typhi* and a pathogenic fungus *Candida albicans*. It shows significant antimicrobial activity[48].

Antibacterial activity

Harsha Vardhana S (2011) was investigated the ethanol and aqueous extracts of roots of *Amaranthus spinosus* for their antibacterial activity against ten bacterial strains including Gram-positive andGram-negative bacteria using the agar-well diffusion method. The ethanol extract presented thebest results while the aqueous extract showed moderate inhibition of the microbial growth[49].

Anti-hyperlipidemic activity

Girija K and Lakshman K. (2011) was investigated antihyperlipidemic activity of methanol extracts of *Amaranthus spinosus.* The antihyperlipidemic effects investigated by using normal and triton-WR 1339 induced rats at the dose of 200, 300 and 400mg/kg p.o. The serum harvested was analyzed for total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein. It was found that all the three plants at 400mg/kg dose showed significant anti-hyperlipidemic effect, whereas 300mg/kg dose is less significant in the entire parameters used for evaluation of anti hyperlipidemic effect[50].

Diuretic activity

Amuthan A, Chogtu B, Bairy KL, Sudhakar and Prakash M. (2012) was evaluated the diuretic activity of *Amaranthus spinosus* Linn. In this study different concentrations of aqueous extract of *Amaranthus spinosus* (200, 500, 1000, 1500mg/kg),

thiazide (10mg/kg) and vehicle were orally administered to rats (n=6 animals per group) and their urine output was collected after 24h. Volume, pH, Na+, K+ and Cl- concentrations of urine were estimated. ASAE produced increase in Na+, K+, and Cl- excretion, caused alkalinization of urine, and showed strong saluretic activity and carbonic anhydrase inhibition activity. These effects were observed predominantly at 500mg/kg dose and suggested that the *A. Spinosus* is acting as a thiazide like diuretic [51].

Review related to Method-MTT assay

Kiranmayi. Gali *et al.*, (2011) was determined the effective IC_{50} concentration of methanol extract of leaves of *Argemone mexicana* Linn, on HeLa and MCF-7 cell lines by MTT assay. The absorbance was read at 595nm using micro plate reader[52].

T.Vithya*et al.*, (2012) was reported the cytotoxicity of methanolic extract of *Sophora interrupta* against HeLa and HePG2 cell lines by MTT assay. The result showed an IC50 value of 211.5g/ml and158.2g/ml respectively in HeLa and HePG2 cell lines. The MTT assay results confirmed the cytotoxicity of the plant [53].

Sanjay Patel *et al.*, (2009) was evaluated the cytotoxicity of methanolic extract of *Solanum nigrum* against HeLa and Vero cell lines by MTT assay. *Solanum nigrum* methanolic extract showed that inhibitory action on *HeLa* cell line in concentration range between 10mg/ml to 0.0196mg/ml by using MTT assay. From the performed assay, methanolic extract of these drug shows greater activity on HeLa cell line that mean *Solanum nigrum* possses anticancer activity[54].

Kanimozhi D *et al.*, (2012) was evaluated the cytotoxicity of ethanolic extract of *Cynodon dactylon* Hela, HEP-2 and MCF-7 cell lines by MTT assay. Anticancer activity of ethanolic extract of *Cynodon dactylon* on HEP-2, HELA and MCF-7 cancer cell lines showed potent cytotoxic activity. The inhibition percentage with regard to cytotoxicity was found to be 93.5%, 88.5% and 79.2% at 10mg/ml, which was comparable to the control Cyclophosphamide. From the results shows *Cynodon dactylon* can possess anticancer activity [55].

SreejamoleK L *et al.*, (2013) was studied the cytotoxicity of ethyl acetate extract of Indian green mussel *Perna viridis* against HCT 116 and MCF 7 cell lines by MTT assay. The absorbance was read at 595nm using micro plate reader and the percentage growth inhibition was calculated [56].

SankaraAditya J *et al.*, (2014) was studied the cytotoxicity of methanol extract of *Durantaser ratifolia* against MCF-7 and HT-29 cell line by MTT assay. To determine the IC_{50} the absorbance was taken at 592nm in a micro plate reader. Percentage of residual cell viability was determined [57].

Review related to Method-DLA method

M. Devi *et al.*, (2013) was evaluated the antitumor activity of methanolic extract of *Decalepisha miltonii* was evaluated using Dalton's Lymphoma Ascites (DLA) tumor model in Swiss albino mice. The result showed that methanolic extract of *Decalepisha miltonii* root increased the mean survival time and percentage increase in life span and also decreased the body weight of the DLA tumor bearing mice [58].

Manokarankalaiselvi *et al.*, (2012) reported the anticancer effect of *Jasminum sambac* against Dalton's lymphoma ascites (DLA) induced Swiss albino mice in *In vitro* and *In vivo* model. The tumor cell proliferation inhibitory activity of methanolic extract showed dose dependent in both HeLa and mouse fibroblast cells. At concentrations 25-400µg/ml, the percentage of cell inhibition concentration of normal and cancer cells was found to be 123.3 and 93.8µg/ml respectively.Thus it could be concluded that the methanolic extract of *J. sambac* possesses significant anticancer properties [59].

Ritika Prasad and Biplob Koch (2014) was studied the anticancer property of the ethanolic extract of *Dendrobium formosum* on Dalton's lymphoma. The IC_{50} value of ethanolic extract was obtained at 350*µ*g/mL in Dalton's lymphoma cells. The *in vivo* anticancer activity study illustrates significant increase in the survival time of Dalton's lymphoma bearing mice on treatment with ethanolic extract when compared to control. These results substantiate the antitumor properties of ethanolic extract of *Dendrobium formosum* and suggest an alternative in treatment of cancer [60].

G. Anand *et al.*, (2013) was evaluated the antitumor activity of Hydroalcoholic Extract of *Ipomoea carnea* (HAEIC) for studying anticancer activity by using both *in vitro* and *in-vivo* method. The *In vitro* anticancer activity of HAEIC was evaluated by the MTT assay method using Ehrlich Ascites Carcinoma (EAC) cell lines. Later the extract subjected to *in-vivo* anticancer therapy using EAC tumor model. Oral administration of HAEIC at the dose of 250 and 500mg/kg, significantly (p<0.001) increased the survival time, nonviable cell count and decrease in body weight, food intake and viable cell count of the tumor bearing mice. The results indicate that HAEIC possess significant antitumor activity on dose dependent manner[61].

Ramesh HA and Dinesh B Shenoy (2013) was studied the anticancer property of the methanolic extract of leaves of *Careya arborea* Roxb was on DLA model in rats was evaluated. Evaluation of the effect of drug response was made by the study of tumor growth response including increase in life span, study of hematological parameters, biochemical estimations. Experimental results revealed that *Careya arborea* Roxb extract possesses significant anticancer activity [62].

B. Samuel Thavamani, *et al.*, (2014) was evaluated the anticancer activity of the methanol extract of *Cissampelo spareira* for *in vitro* cytotoxicity and *in vivo* antitumor activity against Dalton's Lymphoma Ascites (DLA) cells in Swiss mice. The antitumor effect was assessed by evaluating the packed cell volume, viable tumor cell count, increase in body weight, and increase in life-span. Experimental results showed the methanol extract of*Cissampelo spareira* possesses significant anticancer agent [63].

Gopika Gopinath *et al.*, (2015) was reported the Cytotoxic and antitumor activities of ethyl acetate extract of leaves of *Phyllanthus acidus* against Hep G2 and DLA cell lines. The antitumor activity of the extract was determined by using DLA cell line induced solid tumor model in Swiss albino mice and its comparison with anticancer drug cyclophosphamide. There was significant reduction of tumor volume in *P. Acidus* treated animals**.** The results shows ethyl acetate extract of leaves of *Phyllanthus acidus* possesses significant anticancer activity [64].

Purushoth Prabhu. T *et al.*, (2011) was studied the anticancer property of the ethanolic extracts of *Canthium parviflorum* for *in vitro* cytotoxicity and *in vivo*

antitumor activity against Dalton's Lymphoma Ascites (DLA) cells in Swiss mice. In this study shows a significant increase in the life span and a decrease in the cancer cell number & tumour weight were noted in the tumor induced mice after treatment with *Canthium parviflorum.* Experimental results revealed that *Canthium parviflorum* extract possesses significant anticancer activity [65].

PLANT PROFILE

Figure No: 1 *Amaranthus spinosus* Linn.

Figure No: 2 Leaves of *Amaranthus spinosus Linn.*

***Amaranthus spinosus* Linn.**

Synonym **:** Spiny amaranth

Biological source **:** *Amaranthus spinosus*

Family **:**Amaranthaceae

Regional names [66]

English	**:**	Prickly Amaranth, Spiny amaranth, Thorny amaranth
Hindi	**:**	Kantachaulai,
Tamil	**:**	Mullukkeerai
Gujarati	**:**	Kantalodhimdo, Kantanudant
Malayalam	**:**	Kattumullenkeera
Telugu	**:**	Mullatotakura
Kannada	**:**	Mulluharivesoppu
Bengali	**:**	Kantanotya
Oriya	**:**	Kantaneutia
Sanskrit	**:**	Tanduliuyah

Taxonomy

Kingdom	**:**	Plantae
Division	**:**	Magnoliophyta
Class	**:**	Magnoliopsida
Order	**:**	Caryophyllales
Family	**:**	Amaranthaceae
Genus	**:**	*Amaranthus*
Species	**:**	*spinosus*

Origin and Distribution

Amaranthus spinosus has spread through tropical and subtropical latitudes around the world. It is an annual plant growing to 0.6 m that is widely distributed in the humid zone. The plant grows wild in cultivated fields, waste places, roadsides, garbage heaps and abandoned fields. It will grow both in wet or dry sites, but grows best when soil moisture levels are below field capacity. It is being commercially cultivated in West Bengal, Maharashtra and Tamil Nadu.

Description of the Plant

A. spinosus is an erect, multi-branched, smooth, herbaceous annual growing to 120cm. Stems angled or with longitudinal lines or ridges, green or brown, leaves alternate, broadly lanceolate to ovate, discolorous, conspicuously veined beneath, up to 7cm long, 4cm wide, margins entire, the base tapering to the slender petiole up to 7cm long, with a pair of straight spines up to 1cm long at the base. Inflorescences long, slender, terminal, with ancillary spikes in clusters, greenish; flowers unisexual, straw-coloured, perianth segments fine, acuminate, upper third of spike being male with five stamens each, lower two-thirds female with three, rarely two, styles to the ovary, fruit one-seeded, opening by a line around the centre, seeds reddish-brown, lens-shaped, shiny.The striated, often reddish, stem with two sharp, long spines at the base of the petioles, and the fruit which opens by a line around the centre are distinguishing characteristics of this species[67].

Chemical constituents

The plant has been reported to contains Glycosides, Flavonoids, Sterols and triterpenoids.Leaves and stems contain n-alkanes, hentriacontane, octacosanoate, sterols including a-spinasterol, fatty acids, free alcohols, proteins and mixture of saponins,

composed of oleanolic acid, D-glucose and D-glucoronic acid. It contains 7-p-coumaroylapigenin 4-O- beta-D-glucopyranoside, a new coumaroyl flavone glycoside called spinoside. It is a good source of calcium, also contains phosphorous, iron, nicotinin acid, ascorbic acid and protein. Roots contain a-spinasterol, octacosanoate and a number of saponins, ß-sitosterol, stigmasterol, campesterol and cholesterol, stearic, oleic and linoleic acids, quercetin and rutin also isolated from the plant [68].

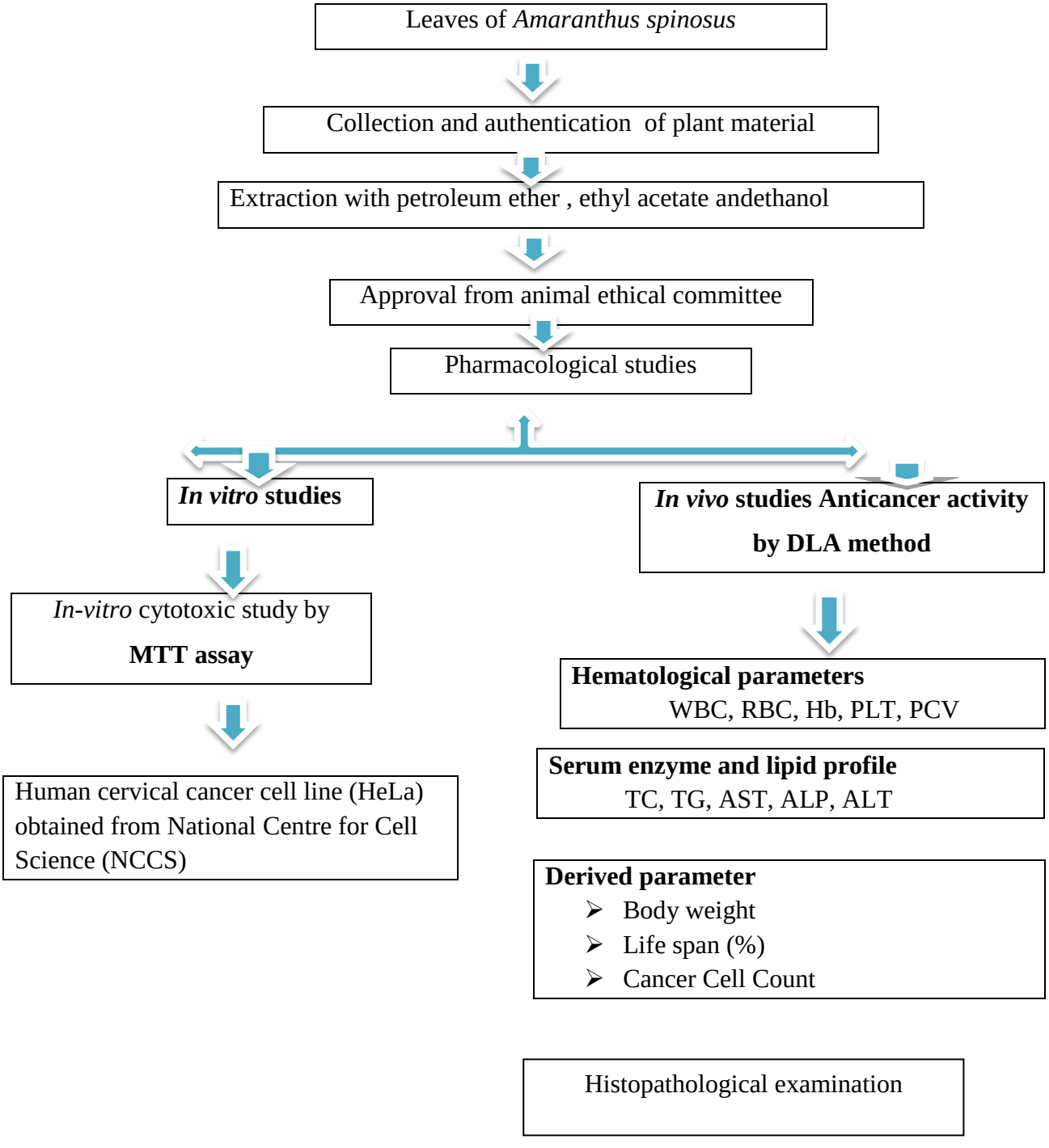
Uses

- Cosmetics, Dyes and Colouring agent.
- Yellow and green dyes can be obtained from the whole plant.
- A red pigment obtained from the plant is used as colouring in foods and medicines.

Medicinal uses

- *Amaranthus spinosus* has many medicinal properties like astringent, diaphoretic, diuretic, emollient, febrifuge, galactogogue etc.
- *Amaranthus spinosus* used in the treatment of internal bleeding, diarrhoea, excessive menstruation
- It is also used in the treatment of snake bites.
- Externally, it is used to treat ulcerated mouths, nosebleeds and wounds
- The root is emmenagogue and galactogogue. A paste of the root is used in the treatment of menorrhagia, gonorrhoea, eczema and colic
- The juice of the root is used to treat fevers, urinary troubles, diarrhoea and dysentery[69].

4. PLAN OF WORK



5 MATERIALS AND METHODS

5.1 PLANT COLLECTION

The plant *Amaranthus spinosus* was collected from Institute of Siddha College, Thirunelveli, in November 2015. The plant material was identified and authenticated by **Mr**. **V. Chelladurai,** Research Officer -Botany (retd.), Central Council for Research in Ayurveda & Siddha, Govt. of India.

Instruments used during the course of study

- Soxhlet apparatus
- Co_2 Incubator
- Cooling centrifuge
- Haemocytometer
- Inverted microscope
- UV- Spectrophotometer
- All biochemical investigation was done by using COBAS MIRA PLUS-S Auto analyzer from Roche Switzerland.
- Haematological tests are carried out in COBAS MICROS OT 18 from Roche
- Newly added Hi-Tech instruments MAX MAT used for an auto analyzer for all biochemistry investigations in blood sample.

5.2 Preparation of extract

The plant was shade dried at room temperature and was subjected to size reduction to a coarse powder by using dry grinder. 60grams of this coarse powder was packed in to soxhlet apparatus and was subjected to extraction sequentially with 500ml of petroleum ether followed by ethyl acetate and ethanol.

The extraction was continued until the colour of the solvent in the siphon tube become colourless. Extraction procedure was carried out in Institute of Pharmacology, Madras Medical College, Chennai-03. Extracts of pet ether, ethyl acetate and ethanol were subjected to evaporation by Rotary evaporator at below 60ºc. The percentage yield from the *Amaranthus spinosus* using different solvents is given as below **Table 1.**

Table No 1: Percentage yield from the plant *Amaranthus spinosus* using different solvents.

Extracts	Plant material used for Extraction (g)	Yield (g)	Percentage Yield (%)
Petroleum ether	60	1.5	2.40
Ethyl acetate	60	4.0	6.50
Ethanol	60	9.5	15.80

METHODOLOGY

5.3 *IN-VITRO* ANTICANCER ACTIVITY

The anticancer activity of *Amaranthus spinosus* Linn was evaluated by MTT assay against Human cervical cancer cell line (HeLa).

Materials required

- 96 well micro titre plate
- Fully grown/confluency reached cell in culture flask
- Minimum essential medium with 10% FBS
- TPVG solution
- Plant extract- Ethanol, Ethyl acetate, Petroleum ether

- MTT (5mg/ml in PBS-pH7.4)
- DMSO solution (0.1% v/v)
- Aluminium foil
- Micropipette
- Inverted microscope
- Reagent bottle
- Bio safety cabinet
- Tryptan blue
- UV- chamber
- CO_2 incubator

Cell line

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Essential Medium containing 10% fetal bovine serum (FBS).

The cells were maintained at 37ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passages weekly and the culture medium was changed twice a week.

Cell treatment procedure

- The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspension and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1×10^5cells/ml.

- One hundred micro litres per well of cell suspension were seeded into 96-wellplates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºC, 5% CO_2, 95% air and 100% relative humidity.

- After 24hr the cells were treated with serial concentration of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium.

- Additionally four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100µl of these different sample dilutions were added to the appropriate wells already containing 100µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48hr at 37ºC, 5%CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

It is otherwise called as tetrazolium salt assay or Microculturetetrazolium test. MTT assay is an *in-vitro* method for screening, which has been internationally accepted. 3-[4,5- dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an

insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

The cytotoxicity of samples on Chang Liver cells was determined by the MTT assay (*Mosmann et al.*,1983). Cells (1×10^5/well) were plated in 5ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence samples for 24 - 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 1ml/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-- tetrazolium bromide cells(MTT) phosphate- buffered saline solution was added. After 4h incubation, 0.04M HCl/ isopropanol was added.

Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The absorbance at 570 nm was measured with a UV-Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of Chang Liver cells was expressed as the % cell viability, using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100%

Linear regression graph was plotted between % cell viability and Log concentration. The IC_{50} was determined by using graphical method.

5.4 *IN- VIVO* ANTICANCER ACTIVITY

The anticancer activity of *Amaranthus spinosus* Linn was evaluated in Swiss albino mice. Tumor was induced by intra-peritoneal injection of ($1x10^6$) Dalton's Lymphoma Ascites (DLA) cells. The anticancer effect of plant extract was compared with standard drug 5- fluorouracil at 20mg/kg body weight.

Materials required

- Dalton's Lymphoma ascites (DLA) cells
- Mice (20-25gm)
- Ethanolic extract of *Amaranthus spinosus* (EEAS)
- Tryphan blue
- Sterile Phosphate Buffer Solution
- Ice cold Normal saline
- 5-flurouracil (20mg/kg)
- Cooling centrifuge
- Insulin syringe
- 5% formaldehyde
- Haematoxylin
- Micro nylon boxes

Experimental procedure

Selection, Grouping and Acclimatization of Laboratory Animal[70]

Male Swiss albino mice (20-25gm) were produced from animal experimental laboratory and used throughout the study .They were housed in micro

nylon boxes in a control environment (temp 25+2ºC) and 12 hr dark/light cycle with standard laboratory diet and water *ad libitum*.

All animal procedures were performed after approval from the Institutional ethical committee. The experimental protocol has been approved by Institutional ethical committee, Madras Medical College, Chennai. IAEC/MMC/07/243/CPCSEA/2015-16. As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Technique for Inducing Tumor

Various technique for induction of cancer in animals via, chemically induced (using DMBA/croton oil, etc)[71] virus induced, cell line induced (sarcoma-180, ULCA fibro sarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Lymphoma Ascitic (DLA), Ehrlich Ascites Carcinoma (EAC)[72,73,74] methods have been used in experimental studies of anticancer activity.

In the present study, cell lines induced cancer in mice was used to evaluate the anticancer activity of ethanolic extract of *Amaranthus spinosus* Linn. (EEAS).

EVALUATION OF ANTICANCER ACTIVITY

Induction of cancer using DLA cells

Dalton's Lymphoma Ascitic (DLA) cells were supplied by Amala cancer research centre, Trissur, Kerala, India. The cells are maintained in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline.

The cell counts were done and further dilutions were made so that total cell should be 1×10^6, this dilution was given intraperitoneally. Let the tumor grown in the mice for minimum seven days before starting treatments.

Treatment Protocol [75]

Swiss Albino mice were divided into five group of six each. All the animals in four groups were injected with DLA cells (1×10^6cells per mouse) intraperitoneally and the remaining one group is normal control group.

Table No: 2 Grouping of Animals and Treatment Schedule

S.NO	GROUP	TREATMENT SCHEDULE
1	Group – 1	Served as the normal control
2	Group – 2	Served as the tumor control. Group 1 and 2 receives normal diet and water.
3	Group –3	Served as the positive control, was treated with injection of 5-fluorouracil at 20mg/kg body weight, intraperitoneally.
4	Group –4	Served as treatment control received (200mg/kg.bw) EEAS administered through orally.
5	Group – 5	Served as treatment control received (400mg/kg.bw) EEAS administered through orally.

Treatment

In this study, drug treatment was given after the 24hr of inoculation, once daily for 14 days. On day 14, after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital puncture blood collecting method. From the collected blood haematological parameters were checked, the remaining blood was centrifuged and serum was used for the estimation of biochemical parameters.

Haematological parameters

- WBC count
- RBC count
- Hb content
- Platelet count
- Packed cell volume

Serum enzyme and lipid profile

- Total Cholesterol (TC)
- Triglycerides (TG)
- Aspartate amino Transferase (AST)
- Alanine amino Transferase (ALT)
- Alkaline Phosphatase (ALP)

Derived parameters

- Body weight
- Life span (%)
- Cancer Cell Count

Determination of percentage Increase of Life Span (ILS)[76]

In this study, drug treatment was given after the 24hr of inoculation, once daily for 14 days. The animals were monitored daily twice for 30 days. Antitumor effect of *Amaranthus spinosus* was determined by monitoring the death pattern of animals due to tumor burden and calculating the percentage increase in life span (%ILS). The percentage increase in life span was calculated using the following formula:

$$\mathbf{ILS}(\%) = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \times 100$$

Determination of Average Body weight changes

The body weight (BW) of the control and treated group animals were measured from 0th day to 30th day interval up to 30 days.

Cancer cell count

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8ml of ice cold normal saline or sterile Phosphate buffer Solution and 0.1ml of tryphan blue (0.1mg/ml) and total numbers of the living cells were counted using haemocytometer.

Cell count = No. of cells Dilution/Area × Thickness of liquid film

Histopathological analysis

A small portion of liver was taken and fixed in to 5% formaldehyde, after several treatments for dehydration in alcohol, sections having 4µm thickness were cut and strained with haematoxylin and eosin and histopathological analysis was carried out for the treated as well as control group of mice.

Statistical analysis

Values are expressed as mean (±SEM). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett test using Graph pad In Stat version 3.0, Graph Pad Software, San Diego, California< USA. P-Values (i.e., *$p<0.05$, **$P<0.01$) were considered statistically significant compared to DLA tumor control.

6 RESULTS

MTT assay

MTT assay carried out with petroleum ether, ethyl acetate and ethanol extract of *Amaranthus spinosus* and the results was shown in the following tables.

Table No 3: IC_{50} concentration and % cell viability of ethanolic extract of *Amaranthus spinosus*

S. No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.02	3.17
2	500	1:1	0.07	11.11
3	250	1:2	0.12	19.04
4	125	1:4	0.16	25.39
5	62.5	1:8	0.22	34.92
6	31.2	1:16	0.29	46.03
7	15.6	1:32	0.33	52.38
8	7.8	1:64	0.39	61.90
9	Cell control	-	0.63	100

Fig. No 3: % Cell viability Vs Concentration in µg/ml of ethanolic extract of *Amaranthus spinosus*

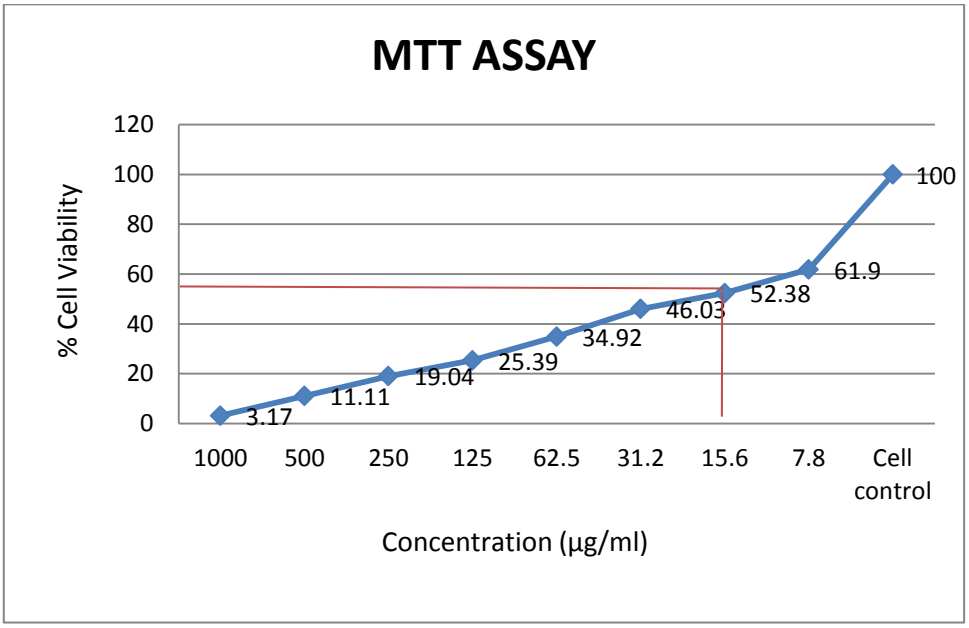


Table No 4: IC_{50} concentration and % cell viability of Ethyl acetate extract of *Amaranthus spinosus*

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.08	12.69
2	500	1:1	0.14	22.22
3	250	1:2	0.20	31.74
4	125	1:4	0.25	39.68
5	62.5	1:8	0.31	49.20
6	31.2	1:16	0.36	57.14
7	15.6	1:32	0.41	65.07
8	7.8	1:64	0.44	69.84
9	Cell control	-	0.63	100

Fig. No 4: % Cell viability Vs Concentration in µg/ml of Ethyl acetate extracts of *Amaranthus spinosus*

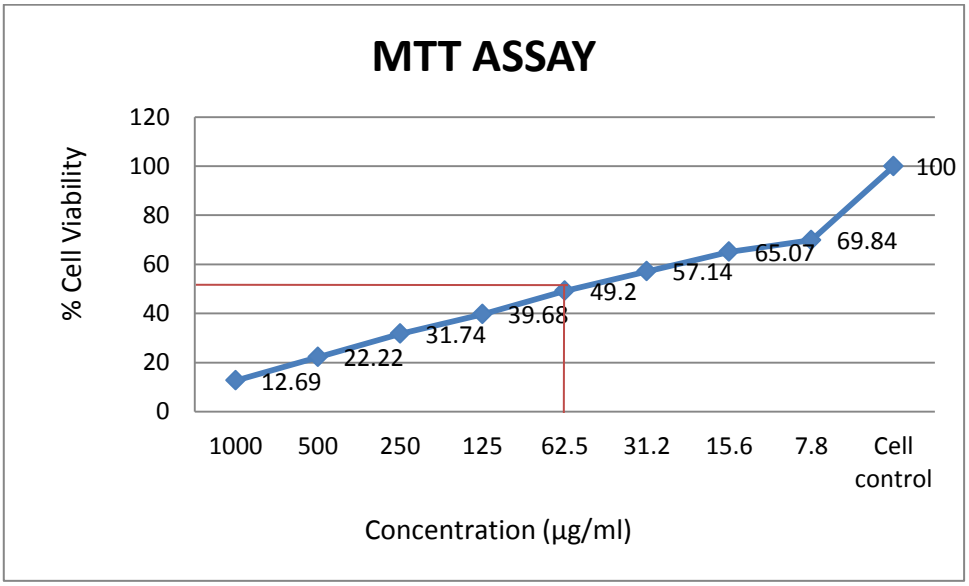
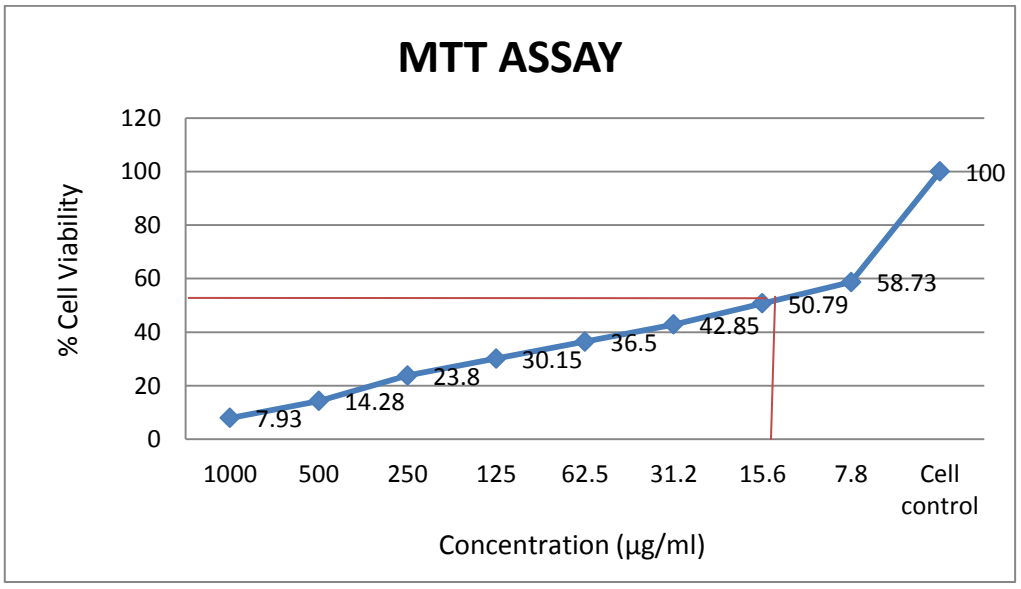


Table No 5: IC_{50} concentration and % cell viability of Petroleum ether extract of *Amaranthus spinosus*

S. No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.05	7.93
2	500	1:1	0.09	14.28
3	250	1:2	0.15	23.80
4	125	1:4	0.19	30.15
5	62.5	1:8	0.23	36.50
6	31.2	1:16	0.27	42.85
7	15.6	1:32	0.32	50.79
8	7.8	1:64	0.37	58.73
9	Cell control	-	0.63	100

Fig. No 5: % Cell viability Vs Concentration in µg/ml of Petroleum ether extract of *Amaranthus spinosus*



In- vitro* cytotoxicity study of *Amaranthus spinosus

Anticancer effect of Ethanol extract on *HeLa* Cell line

Fig. No: 6a Normal HeLa Cell line

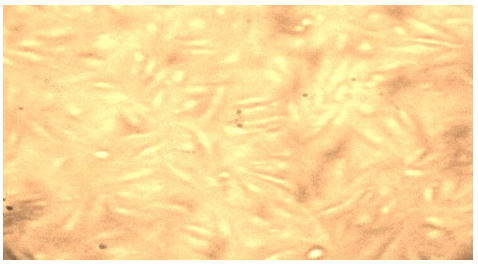


Fig. No: 6b Toxicity-1000µg/ml

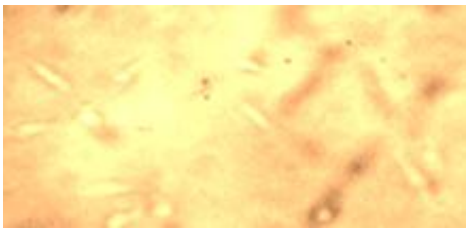


Fig. No: 6c Toxicity- 125µg/ml

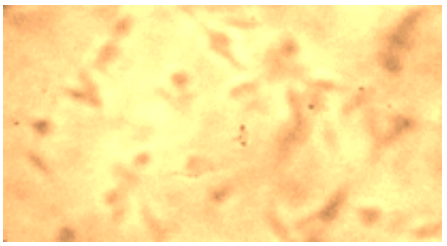


Fig. No: 6d Toxicity-62.5µg/ml

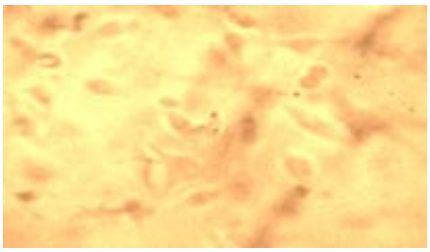
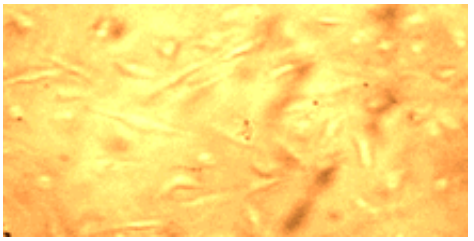


Fig. No: 6e Toxicity- 31.2µg/ml



Anticancer effect of Ethyl acetate extract on *HeLa* Cell line

Fig. No: 7a Normal HeLa Cell line

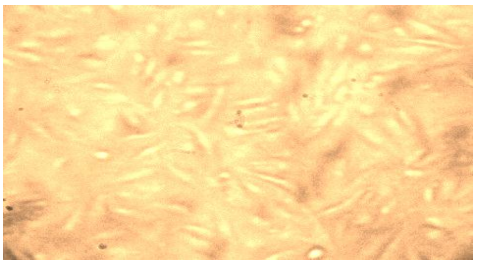


Fig. No: 7b Toxicity-1000µg/ml

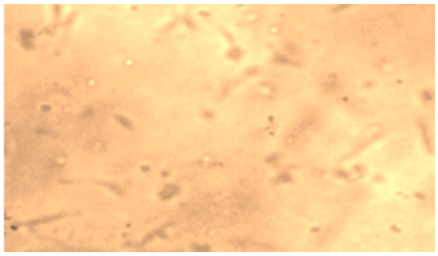


Fig. No: 7c Toxicity-125µg/ml

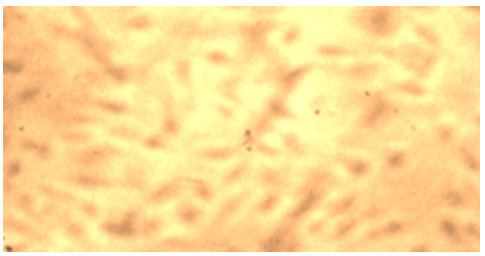


Fig. No: 7d Toxicity-62.5µg/ml

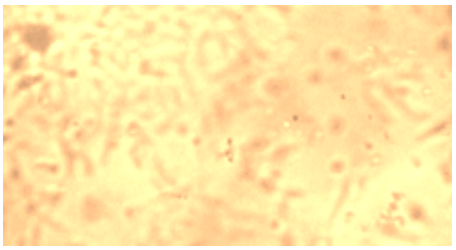
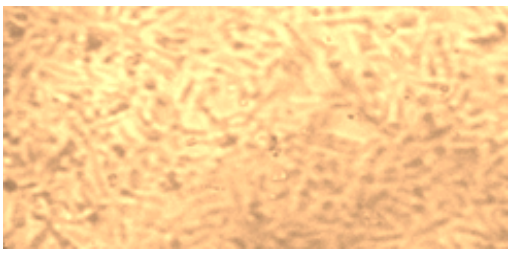


Fig. No: 7e Toxicity- 31.2µg/ml



Anticancer effect of Petroleum Ether extract on *HeLa* Cell line

Fig. No: 8a Normal HeLa Cell line

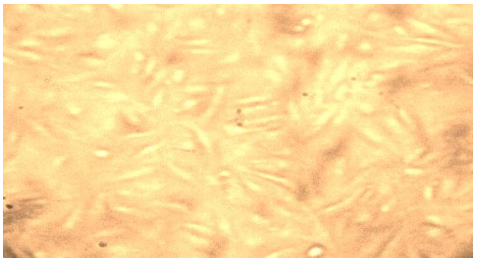


Fig. No: 8b Toxicity-1000µg/ml

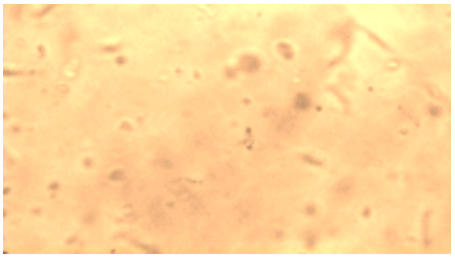


Fig. No: 8c Toxicity-125µg/ml

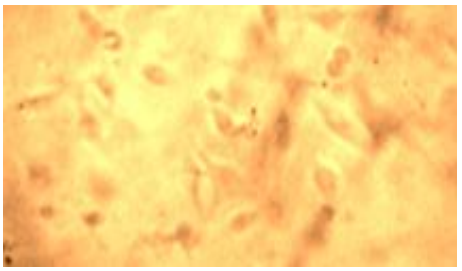


Fig. No: 8d Toxicity-62.5µg/ml

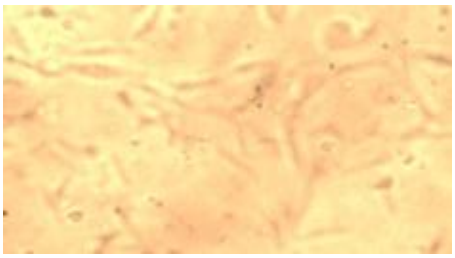
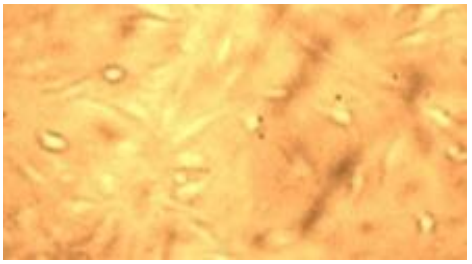


Fig. No: 8e Toxicity- 31.2µg/ml



IN- VIVO ANTICANCER ACTIVITY

Evaluation of clinical parameters

- **Hematological parameters**

i) WBC count
ii) RBC count
iii) Platelet count
iv) Haemoglobin
v) Packed Cell Volume

i) WBC count

The total WBC count was found to be increased in cancer control, when compared with normal and treated tumor-bearing mice. [77]

ii) RBC and Hb

RBC and Hb content decreases with tumor bearing mice when compared with Normal control mice.

iii) Platelets

In Hodgkin lymphoma, increased in platelet count often reported in laboratory finding[78].

iv) Packed cell volume

In any case of anaemia the packed cell volume is decreases.

Table No 6: Effect of EEAS on Hematological parameters

Treatment	Total WBC Cells /mlx10^3	RBC Count Mill/cum	Hb Gm/dl	PCV %	Platelets Lakhs/cum
G1	10.30 ±1.26	4.32±0.90	12.35 ±1.30	14.20±2.40	3.28±0.75
G2	16.20 ±2.98^{a**}	2.25±0.50^{a**}	6.15 ±0.90^{a**}	36.30±3.30^{a**}	1.02±0.55^{a**}
G3	13.25 ±1.95^{b**}	3.95±1.40^{b**}	11.27±1.50^{b**}	16.45±1.45^{b**}	2.98±0.92^{b**}
G4	14.30 ±1.80^{b**}	4.03±0.62^{b**}	12.09±1.35^{b**}	20.62±2.35^{b**}	3.02 ±0.66^{b**}
G5	14.20±2.01^{b**}	4.15±0.35^{b**}	12.25±1.65^{b**}	18.70±2.30^{b**}	3.25±0.75^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control (200mg/kg) G5 – Treatment control (400mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.
****a – Values are significantly different from normal control (G1) at P < 0.001**
****b – Values are significantly different from cancer control (G2) at P < 0.01**

SERUM ENZYME AND LIPID PROFILE

The serum was analyzed for the following parameters

(a) Aspartate amino Transferase (AST)
(b) Alanine amino Transferase (ALT)
(c) Alkaline Phosphatase (ALP)
(d) Total Cholesterol (TC)
(e) Triglyceride (TG)

1. TOTAL CHOLESTEROL AND TRIGLYCERIDE (lipid profile)

Abnormal blood lipid profile has been associated with cancer. In Hodgkin lymphoma, high cholesterol level and low triglyceride level has been reported [79]

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1. LIVER ENZYMES (AST, ALT, ALP)

Abnormal liver function seen in patient with Hodgkin lymphoma that these liver enzyme levels markedly increase in tumor bearing mice. ALP is an enzyme mainly derived from the liver, bones and in lesser amount from intestines, placenta, kidneys and leukocytes. An increase in ALP levels in the serum is frequently associated with the variety of disease. [80] ALP comprises a group of enzyme that catalyzes the phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate.

Markedly elevated serum ALP, hyperalakline-phosphatasemia, is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma and sarcoidosis[81].

Table No7: Effect of EEAS on serum Enzymes and lipid proteins

Treatment	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G_1	110.05±3.45	134.80±2.50	38.45 ±1.62	33.30 ±1.40	130.30 ±2.35
G_2	142.90±4.25^{a**}	228.30±4.45^{a**}	74.4±2.70^{a**}	60.30±2.65^{a**}	258.35±4.30^{a**}
G_3	128.32±3.80^{b**}	162.10±2.62^{b**}	46.45 ±1.70^{b**}	32.50±1.65^{b**}	175.40±2.36^{b**}
G_4	114.25±2.60^{b**}	170.35±2.50^{b**}	48.40±1.95^{b**}	38.38 ±1.70^{b**}	194.35±2.50^{b**}
G_5	112.20±3.02^{b**}	166.60±2.70^{b**}	40.50 ±2.10^{b**}	35.22±1.95^{b**}	196.32±2.12^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control (200mg/kg) G5 – Treatment control (400mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.
****a – Values are significantly different from control (G1) at P < 0.001**
****b – Values are significantly different from cancer control (G2) at P < 0.001**

DERIVED PARAMETERS

1. Body weight

All the mice were weighed, from the beginning to 15th day of the study. Average increase in body weight on the 15th day was determined.

2. Percentage increase in life span (ILS)

% ILS was calculated by the following formula

$$\%ILS = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \times 100$$

Table No 8: Effect of EEAS on the life span, body weight and cancer cell count of tumor induced mice.

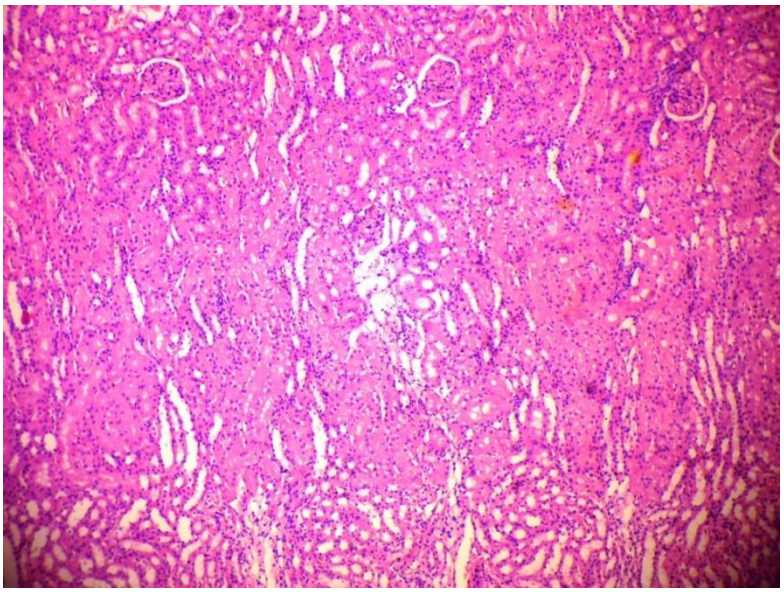
Treatment	Number of animals	% ILS Life span	Increase in Body weight grams	Cancer cell count ml X 10^6
G_1	6	>>30 days	2.20±0.55	-
G_2	6	48%	9.60±0.90^{a**}	2.70±0.40^{a**}
G_3	6	94%	5.85±0.50^{b**}	1.15±0.35^{b**}
G_4	6	79%	6.40±0.72^{b**}	1.50±0.45^{b**}
G_5	6	86%	5.95±0.88^{b**}	1.42±0.28^{b*}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control (200mg/kg) G5 – Treatment control (400mg/kg)

All values are expressed as mean ± SEM for 6 animals in each group.
****a – Values are significantly different from control (G_1) at P < 0.001**
****b – Values are significantly different from cancer control (G_2) at P < 0.001**

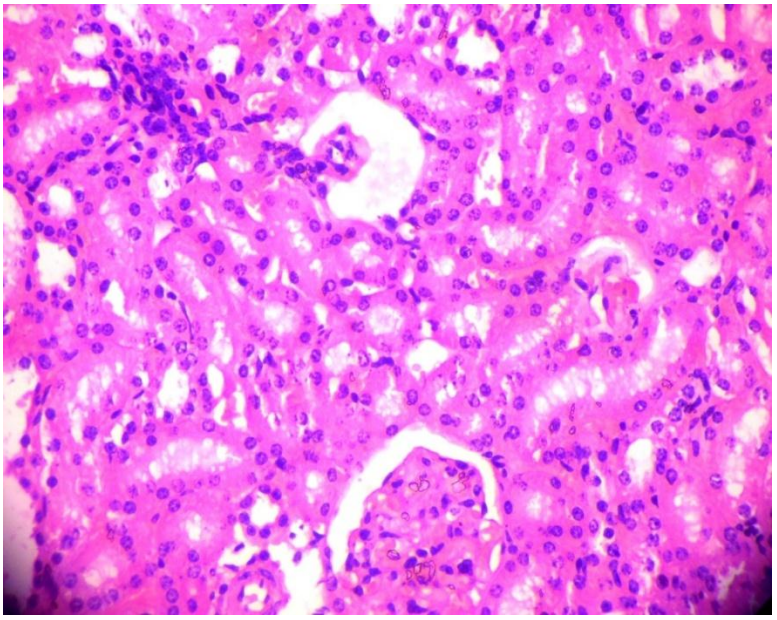
HISTOPATHOLOGICAL STUDIES

Fig. No 9: Normal control group treated with 10ml/kg of normal saline



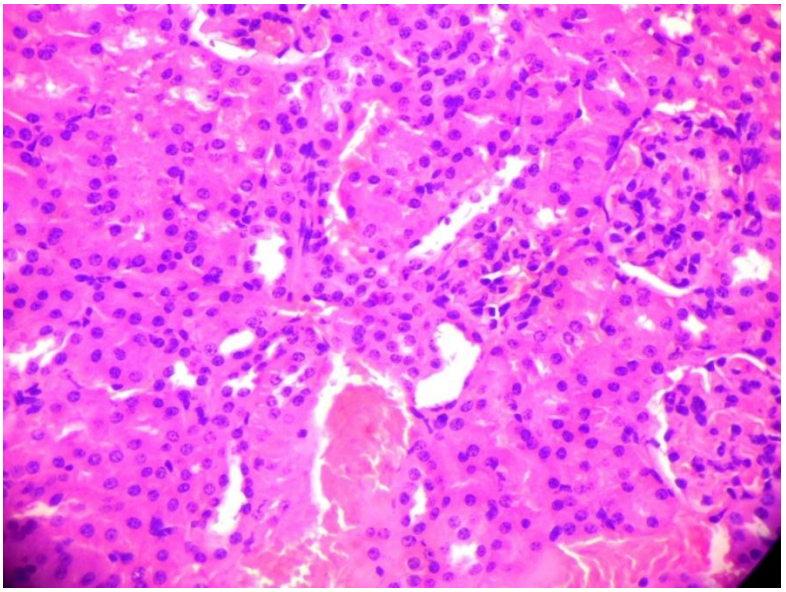
Section shows Structure of Liver with sheets of Hepatocytes Separated by Sinusoids Cartial Vein and Portal tract appear normal.

Fig. No: 10 Cancer control group (Tumor control)



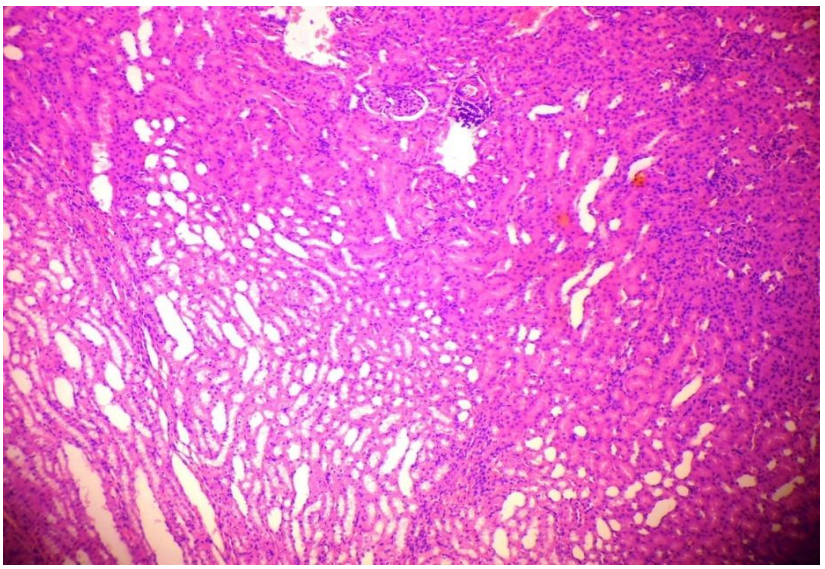
Section shows structure of liver with cords of hepatocytes and small area of lymphmatous cells.

Fig. No: 11 Positive control (5-fluorouracil)



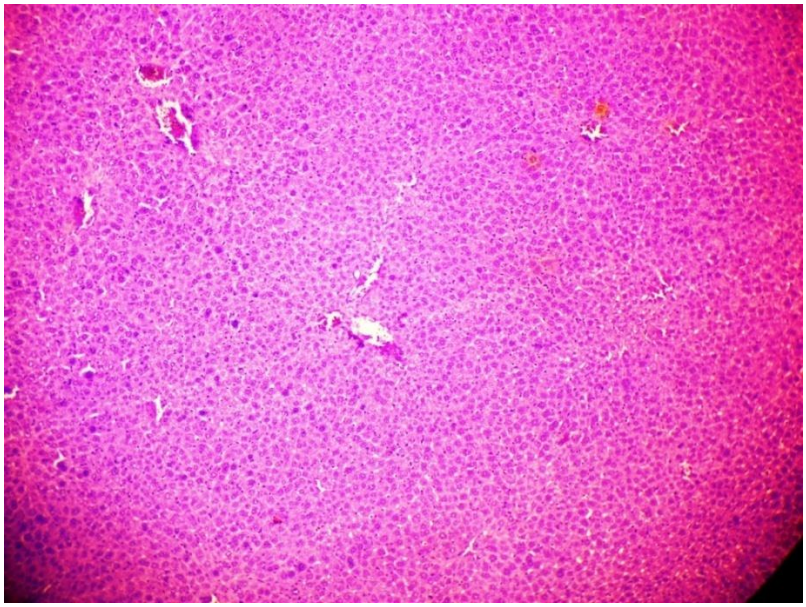
Structure of liver with cords of hepatocytes with reduced lymphmatous cells.

Fig. No: 12 Treatment control received 200mg/kg of EEAS



A section shows liver parenchyma with decreased inflammatory cells.

Fig.No: 13 Treatment control 400mg/kg of EEAS



A section shows liver parenchyma with cords of hepatocytes, No lymphmatous cells.

HISTO PATHOLOGICAL OBSERVATIONS[82]

Histology of liver sections of normal control animals **(Group I)** showed normal liver architecture with were brought out central vein, were preserved cytoplasm and prominent nucleus and nucleolus (**Fig no: 9).** The liver sections of DAL treated animals (Group II) showed hepatic cells with serum toxicity characterized by inflammatory cell collection, scattered inflammation across liver parenchyma, focal necrosis and swelling up of vascular endothelial cells **(Fig no: 10).**

5FU (Group-III) exhibited protection from DAL cell line induced changes in the liver **(Fig no: 11).** EEAS pretreatment at a dose of 200mg and 400mg/kg (group IV and V) appeared to significantly prevent the DAL toxicity as revealed by the hepatic cells with were preserved cytoplasm. EEAS pretreatment also caused marked decrease in inflammatory cells **(Fig no: 12 and 13).**

7 DISCUSSION

Cancer is considered as a serious health problem worldwide. Tumor is a mass of tissues which proliferative, spread throughout the body and may eventually cause death of the host[83]. With increase in mortality rates among patients suffering from cancer with limited success being achieved in clinical therapies including radiation, chemotherapy, immune modulation and surgery in treating cancer patients, there arises a need for new way for cancer management.

Natural phytochemicals derived from medicinal plants have attained a greater significance in potential management of several diseases including cancer. Several researches have been carried carcinogenic causes [84].

Discovery of very effective herbs and elucidation of their underlying mechanisms could lead to development of an alternative and complimentary method for cancer prevention and treatment. Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries around the world. Currently more than 3000 plants around the world have been reported to possess anticancer property [85].

Screening of different plant components in search of anticancer drugs is one of the main research activities throughout the world. Vinca alkaloids and cytotoxic podophyllotoxins were discovered in the 1950s as first anticancer agents from plants [86].

Amaranthus spinosus are traditionally used as cosmetics, dyes and colouring agent and medicinally used to treat diabetes mellitus, diuretic, internal bleeding, anthelmintic, anti-pyretic and anti- inflammatory etc.

The Phytochemical analysis of ethanolic extract of leaves of *Amaranthus spinosus* revealed the presence of flavonoids, terpenoids, glycosides, tannins, steroids and carbohydrates. The ethyl acetate and petroleum ether extracts didn't show the

presence of flavonoids and glycosides. The ethanolic extracts revealed the presence of flavonoids and glycosides which shows that it may anticancer activity and destroy cancer cells.

In-vitro cytotoxicity assay

Dried leaf part of *Amaranthus spinosus* were extracted with solvents like Petroleum ether, Ethyl acetate and Ethanol. *In-vitro* cytotoxic activity was carried out in Human Cervical Cancer cell line (HeLa) with extracts of Petroleum ether, Ethyl acetate and Ethanol. Test for cytotoxicity was carried out by MTT assay. Among the three extracts evaluated, the effective extract was found to be Ethanol extract with IC_{50} value of 15.6µg/ml followed by ethyl acetate and petroleum ether with IC_{50} value of 62.5µg/ml and 15.6µg/ml respectively. Linearity was expressed with help of graph plotted in Microsoft excel.

By carrying out MTT assay ethanol extract was found to be more effective of all three extracts and further studies carried out with ethanol extract. Acute toxicity studies were carried out by using Swiss albino mice at dose level up to 2000mg/kg.

In-vivo anticancer activity

In-vivo anticancer study was carried out by using Dalton's Ascetic Lymphoma cells inducing cancer in Swiss albino mice.

Effect on Tumor Growth

In the DLA tumor control group, the average life span of animal was found to be 48% whereas ethanolic extract of *Amaranthus spinosus* Linn. at a dose of 200mg/kg and 400mg/kg body weight increase the life span to 79%, and 86% respectively. These values were significant. However the average life span of 5-FU treatment was found to be 94%, indicating its potent antitumor nature. The antitumor nature of ethanolic extract of

Amaranthus spinosus Linn. at a dose of 200 and 400 mg/kgwas evidenced by the significant reduction in percent increase in body weight of animal treated with ethanolic extract of *Amaranthus spinous* Linn. at a dose of 200 and 400 mg/kg body weight when compared to DLA tumor bearing mice.

It was also supported by the significant reduction in packed cell volume and viable Tumor cell count in ethanolic extract of *Amaranthus spinosus* Linn at a dose of 200mg/kg and 400mg/kg treatments when compared to the DLA tumor control. **(Table No:8).**

Effect on Hematological Parameters

As shown in **(Table No: 6)** RBC, Hb, Platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with ethanolic extract of *Amaranthus spinosus* Linn. at a dose of 200 and 400 mg/kg significantly increases the Hb content, RBC, Platelets and significantly decreased the WBC count to normal level. All these results suggested the anticancer nature of the ethanolic extract of *Amaranthus spinosus* Linn. at a dose of 200 and 400 mg/kg. However, the standard 5FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

Effect on Biochemical Parameters

The inoculation of DLA cellscaused significantly increase in the level of Total Cholesterol, Aspartate amino Transferase, Alanine amino Transferase, Alkaline Phosphatase in the tumor control animals(G_2), when compared to the normal group. The treatment with ethanolic extract of *Amaranthus spinosus* Linn. at a dose of 200 and 400 mg/kg body weight reversed these changes towards the normal level. **(Table No: 7**) All

the value was found to be significant. The treatment with standard 5-FU also gave similar results.

Effect on Histopathology

The histopathological observation of liver section of normal, DLA tumor control, standard drug 5-fluorouracil and extract treated animals collected at the end of the experimental periods (i.e., Day 30 after DLA intraperitoneal injection) were shown in **Fig. No: 13**. Normal untreated animals section shows structure of Liver with sheets of Hepatocytes separated by Sinusoids Cartial Vein and Portal tract appear normal. DLA induced Section shows structure of liver with cords of hepatocytes and small area of lymphomatous cells. However, mice treated with *Amaranthus spinosus* and 5-fluorouracil showed no lymphomatous cells. Histopathological examination showed a protective effect of *Amaranthus spinosus* on hepatotoxicity.

In DLA tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells[87].

Treatment with *Amaranthus spinosus* inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals[88].

8. CONCLUSION

- *In-vitro*studies by MTT assay, the ethanol extract of *Amaranthus spinosus* L. was found to be effective, which shows viability 50% of cell growth at concentration in 15.6µg/ml.
- The ethanolic extract of *Amaranthus spinosus* L. (200mg/kg and 400mg/kg) exhibited significant reduction in packed cell volume and viable Tumor cell count and also increase the life span of cells in DLA induced tumor mice.
- *In- vivo* studies of *Amaranthus spinosus* L. ethanolic extract revealed by enhancing the survivability of the tumor bearing mice. Thus it was concluded that the ethanol extract of *Amaranthus spinosus* L. has antitumor activity.

On the basis of the above result it was suggested that, the *In-vitro* and *In-vivo* anticancer activity of ethanolic extract of *Amaranthus spinosus* leaves possessed significant anticancer effect. This may probably due to the presence of phytochemicals such as phenols, terpenoids and flavonoids. Further isolation and purification of bioactive compound from *Amaranthus spinosus* may reveal the presence of potent novel anticancer agent and also to unveil the molecular mechanism behind its therapeutic action.

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