EVALUATION OF IN-*VITRO* CYTOTOXIC ACTIVITY OF *AMARANTHUS SPINOSUS* LINN. ON HUMAN CERVICAL CANCEROUS CELL LINE (HELA- HENRIETTA LACKES) BY MTT ASSAY.

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Abstract:

Back ground: In the present study we are evaluated In-vitro cytotoxic activity of Amaranths spinosus Linn. On Human Cervical Cancerous cell line (HeLa). Material and methods: We were selected Amaranths spinosus for the based on literature survey and extracts with different solvents petroleum ether followed by ethyl acetate and ethanol. The extracts are collected and subjected to evaporation .these extracts are preserved in a dedicator in our lab for further experimental purpose .these extracts are subjected to in-vitro cytotoxic activity by MTT Assay. Results :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. Among the three extracts through MTT assay ethanol extract was found to be more effective. Conclusion: The result shows the Ethanol extract having better in-vitro cytotoxic activity on HeLa cells and further research is required to prove anti-cancer activity of this ethanol extract for commercial use.

***Key words**: Amaranthus spinosus Linn. Human Cervical Cancerous cell line (HeLa) Ethanolic extract, MTT assay, In- Vitro cytotoxic activity.*

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]Worldwide cancer incidence and mortality statistics are taken from the International Agency for Research on Cancer GLOBOCAN database and also the World Health Organization, 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, esophagus, stomach, and larynx. In females, cancers of the cervix, breast, ovary and esophagus 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 behavioral and dietary risks, High BMI, Low fruit and vegetable, Lack of physical activity, Tobacco use and 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 is the most commonly occurring cancer in females. About 70% of cervical cancers occur in the developing countries Worldwide, 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]

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. [17]

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 anti-metabolites. Traditional chemotherapeutic agents act by killing cells that divide rapidly one of the main properties of most cancer cells.[18]

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 tumor 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. [19]

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 cancer tissue leading to cellular death. As with chemotherapy, different cancers respond differently to radiation therapy.[20]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. [21]

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. [22] 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. [23]

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, [24] 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. [25] Today plant based drugs continues 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 [26] 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. [27] 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 forthe presence of novel compounds and investigation of their biological activities. [28] 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. [29] 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. Topside, a semi synthetic derivative of podophyllotoxin a plant glycoside being used in treatment of testicular tumors, lung cancer, bladder cancer. Topotecan and irinotecanare two recently introduced semisynthetic analogues of camptothecin and antitumor principle obtained from Chinese tree. Vincristine, vinblastine, colchicine 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. [30]

Amaranthus spinosus Linn (Amaranthaceous), 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 tribal 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, gonorrhea, 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, [31] Amaranthus gangeticus. [32] 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.

MATERIALS AND METHODS

PLANT COLLECTION

The plant Amaranthus spinosus was collected from collected from Tirumala Hills, Tirupati, and Chittoor district of Andhra Pradesh, near Seshachalam and Tirumala Hills (Rayalaseema region, Andhra Pradesh, India), areas that are geographically located in the South Eastern Ghats, are recognized for their rich flora and fauna. The plant specimen was verified to be of the correct species by Dr. MadhavaSetty, a botanist from the Department of Botany, S. V. University, Tirupati Specimen Voucher no:2168, Preserved for further reference at our laboratory.

Instruments: Soxhlet apparatus, Co2Incubator, Cooling centrifuge Haemocytometer, Inverted microscope, UV- Spectrophotometer, All biochemical investigation was done by using COBAS MIRA PLUS-S Auto analyzer from Roche Switzerland. Hematological 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.

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 colorless. The extraction procedure carried out in A M Reddy Memorial College of pharmacy, narasaraopet, Andhra Pradesh and 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.

METHODOLOGY

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 titer plate, Fully grown/confluence 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), Aluminum foil, Micropipette, Inverted microscope, Reagent bottle, Bio safety cabinet, Tryptan blue, UV- chamber,CO2 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% CO2, 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 hem cytometer and diluted with medium containing 5% FBS to give final density of 1×105cells/ml. One hundred micro liters per well of cell suspension were seeded into 96- well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºC, 5% CO2, 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%CO2, 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 Micro culture tetrazolium 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 (Mossman et al., 1983). Cells (1 × 105/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 were 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 formula: % cell viability = A570 of treated cells / A570 of control cells × 100%.Linear regression graph was plotted between % cell viability and Log concentration. The IC50 was determined by using graphical method

RESULTS

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	2.4
Ethyl acetate	60	4	6.5
Ethanol	60	9.5	15.8

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 2: 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	1:01	0.07	11.11
3	250	1:02	0.12	19.04
4	125	1:04	0.16	25.39
5	62.5	1:08	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	0.08611	0.39	61.9
9	Cell control	-	0.63	100

Table.3: 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	1:01	0.14	22.22
3	250	1:02	0.2	31.74
4	125	1:04	0.25	39.68
5	62.5	1:08	0.31	49.2
6	31.2	1:16	0.36	57.14
7	15.6	1:32	0.41	65.07
8	7.8	0.08611	0.44	69.84
9	Cell control	-	0.63	100

Table.3: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	1:01	0.09	14.28
3	250	1:02	0.15	23.8
4	125	1:04	0.19	30.15
5	62.5	1:08	0.23	36.5
6	31.2	1:16	0.27	42.85
7	15.6	1:32	0.32	50.79
8	7.8	0.08611	0.37	58.73
9	Cell control	-	0.63	100

Anticancer effect of Ethanol extract of *Amaranthus spinosus* on HeLa Cell line

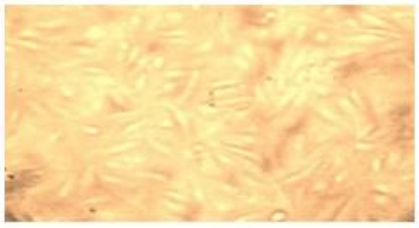


Fig. No: 1a Normal HeLa Cell line

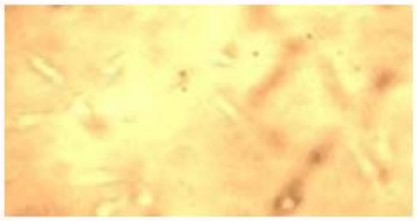


Fig. No: 1b Toxicity-1000µg/ml

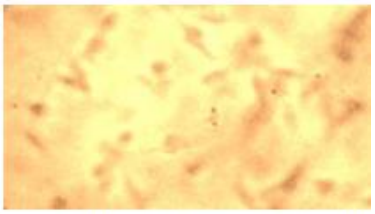


Fig. No: 1c Toxicity- 125µg/ml

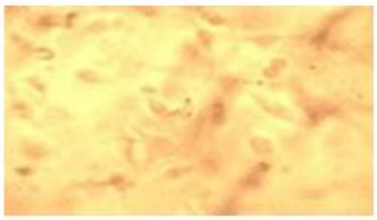


Fig. No: 1d Toxicity-62.5µg/ml

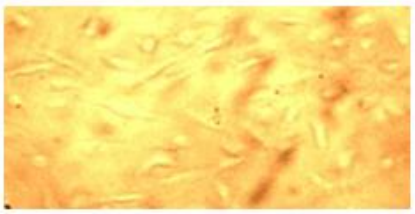


Fig. No: 1e Toxicity- 31.2µg/ml

Anticancer effect of Ethyl acetate extract of *Amaranthus spinosus* on HeLaCell line

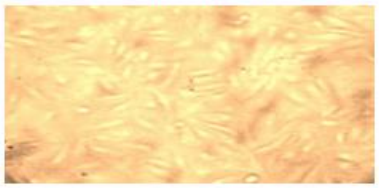


Fig. No: 2a Normal HeLa Cell line

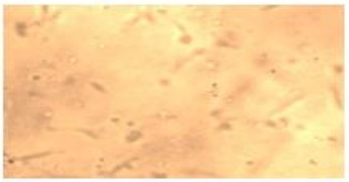
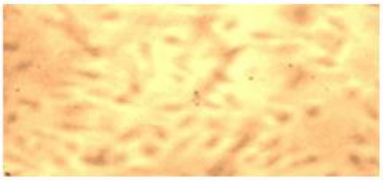


Fig. No: 2b Toxicity-1000µg/ml



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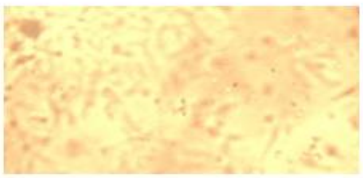

Fig. No: 2d Toxicity-62.5µg/ml

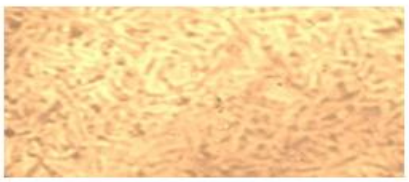

Fig. No: 2e Toxicity- 31.2µg/ml

Anticancer effect of Petroleum Ether extractof *Amaranthus spinosus* on HeLa Cell line

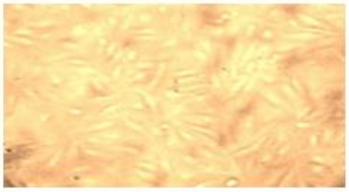

Fig. No: 3a Normal HeLa Cell line

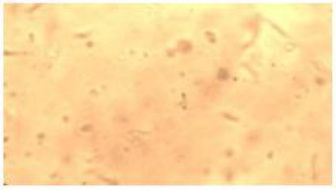

Fig. No: 3b Toxicity-1000µg/ml

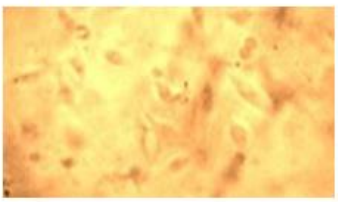

Fig. No: 3c Toxicity-125µg/ml

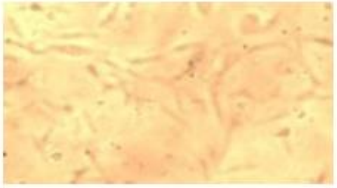

Fig. No: 3d Toxicity-62.5µg/ml

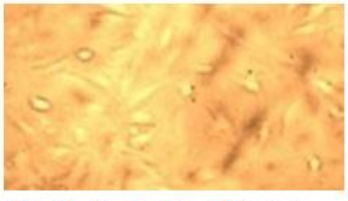

Fig. No: 3e Toxicity- 31.2µg/ml

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.[33] 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. [34]

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 IC50 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.

Conflict of intrest: No conflict of intrest

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