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RESEARCH ARTICLE

CYTOTOXICITY OF DECOCTION OF A POLYHERBAL FORMULATION IN BREAST CELL LINE.

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Abstract

The incidence of cancer especially breast cancer is increasing alarmingly world wide with a high percentage of death mainly in developing countries. Recently herbal medicines are coming to play a vital role in the reduction and prevention of cancer. The preliminary studies on a number of promising herbal drugs have anticancer potential. The current study investigates the in vitro cytotoxicity of decoction of a polyherbal formulation [*Punarnava* (*Boerhaavia diffusa Linn*.), *Sunthi* (*Zingiber officinale Rosc*.), *Varuna (Crataeva nurvala Buch.Ham.)*] in breast cell line (MCF-7). The selection of these drugs was based on Malayalam traditional treatment book "Sarvarogachikitsanool" and also from the information that it was practiced by Ayurvedic physicians for breast cancer. The five different concentrations of test sample were used for in vitro cytotoxicity by MTT assay at 24 hours and 48 hours and the result exhibited cytotoxicity in breast cell line. The result were analyzed statistically and found that it was highly significant ($p<0.001$). The IC_{50} value was also calculated and the result obtained was 190µg/ml. The in vitro Lymphocyte viability assay was performed in normal lymphocytes. The result revealed that the test sample showed no significant toxicity in normal cells. Apoptosis was also checked by two staining methods (Acridine orange-Ethidium bromide stain and Hoesct stain), revealed that the test sample showed cytotoxicity by apoptosis. Thus the study showed that the polyherbal formulation possess cytotoxicity in breast cell line (MCF-7) and no toxicity in normal cells. Therefore, this might possess potentiality for the development of novel anticancer medicine

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Introduction:-

Cancer is one of the most dreadful diseases. Many efforts have been taken to find a complete cure but yet success is still far that's why terror of a disease is bigger than the disease. Recent study showed that 7 lakh people die of cancer every year in India. In 2012, WHO released new statistics on cancer incidence, mortality and prevalence worldwide (GLOBACAN 2012) estimates 28 types of cancer in 184 countries in which there is more prevalence of breast cancer. [1]

On analysing the description of neoplasm as *Granthi*, *Arbudam* etc in Ayurvedic classics, it can be assumed that *Arbudam* can be correlated as a malignant neoplasm. [2] Surgery, chemotherapy and radiotherapy are considered as the most common methods of cancer treatment. Although these methods are highly effective methods of cancer treatment, they exerts severe side effect in use. One of the main problems in cancer treatment is gradual resistance of cancer cells against treatment. Therefore modern medical research focuses on finding new anticancer agents in order to reduce the existing resistance mechanisms. Herbs and other natural plant products have become the main source for this purpose. Herbal medicines play a vital role in the prevention and treatment of cancer. Herbal drugs include plants, herbal complexes or even a combination of plants, which were used thousand years before inventing modern drugs.

The Ayurvedic system treasures a host of medicinal formulations that have been shown to possess cytotoxic effects on tumor cell lines. Several medicinal plants have been screened based on the integrative approaches on drug development from Ayurveda. Reference of a poly herbal formulation from three herbal drugs *Punarnava moolam (Boerhaavia diffusa Linn.), Sunthi (Zingiber officinale Rosc.)* and *Varuna moolam (Crateava nurvala Buch.Ham.)* indicated in management of *arbudam* is found in a Malayalam traditional book on treatment "*Sarvarogachikitsanool*". [3] This same combination is also practiced as a traditional medicine since many years specifically for the treatment of breast cancer by *Ayurvedic* physicians. Therefore this combination is selected for the study. Drug action is mainly based on *dravyaprabhava*, *gunaprabhava* or both; when combination, it also produces *samudayaprabhava* (synergetic action). Keeping this in mind, this study is an attempt to evaluate the cytotoxic effect of decoction of the poly herbal formulation in breast cell line.

Materials And Methods:-

Collection of plant materials:-

The plant materials used in this research are mature root of *Punarnava* (*Boerhaavia diffusa L.)*, mature root bark of *Varuna* (*Crataeva nurvala Buch.Ham*) and rhizome of *Sunthi* (*Zingiber officinale Rosc*.) were collected from natural habitat and was shade dried and stored in air tight containers.(Fig 1)(Fig 2)(Fig 3)

Human Breast Cancer Cells (MCF-7) collection

MCF-7 (Human Breast Cancer cells) and normal lymphocytes were obtained from National Centre for Cell Sciences, Pune, India.

Methodology of preparation of drug:-

Step 1 **Decoction preparation [4]:-**

Punarnava, varuna, Sunthi decoction (combination):-18gm of *Punarnava*, 18 gm of *Varuna* and 12gm of *Sunthi* were coarsely powdered and is added with 16 times of water and reduced to 96 ml (1/8 th) and strained through clean white cloth according to *kwatha* preparation procedure mentioned in *Sarngadharasamhitha*.

Step 2 - **Filtration of decoction**

Decoction was again filtered by using Whatman's filter paper of pore size 1

Step 3- **Concentration of Decoction**

1. The decoction was collected after the filtration. It was then transferred to borosil glass beaker and kept over a hot water bath and heated.
2. Heating was continued till almost all water get evaporated from the decoction.
3. It was then stored in petridishes and sealed it carefully and stored in refrigerator.

Of them 0.002gm of test sample was taken and dissolved in 1 ml triple distilled water and was taken up for the study (Fig 4)(Fig 5)

Tissue culture:-

Sterilisationof glasswares:-

All glasswares and filtration apparatus used for tissue culture were soaked in solution of 5% Savlon overnight, cleaned using brush and washed thoroughly under running water. They were then soaked in boiling water for 15 minutes and rinsed in distilled water and dried in a hot air oven. These were then autoclaved at a pressure of about 15lbs for 15 minutes, dried and used for experiments.

Preparation of culture media:-

DMEM (Dulbecos Modified Eagle's medium) was prepared by mixing DMEM powder of about 1.03gm in autoclaved triple distilled water. To this 1.95gm of Herpes buffer, 3.75gm sodium bicarbonate and antibiotics like Penicillin (100µg/ml), Streptomycin(100µg/ml), Amphotericin-B(100µg/ml) were added. This is the amount of drugs should be added in 1000ml of triple distilled water. The pH was confirmed to be 7.2-7.4 using pH meter and adjustments made if needed. It was filtered under negative pressure using 0.22µm cellulose filter. 10% FBS (Foetal bovein serum) was mixed with the medium before used for culture.

Maintenance of adherent breast cell lines:-

Adherent MCF- 7(Michigan Cancer Foundation-7)cells was cultured in tissue culture flasks. The cells were disaggregated by Trypsinization and sub cultured when the monolayer reached about 70% confluency. Cells were also cryopreserved at -80 °C With an inverted microscope, degree of confluency of the cell monolayer was assessed and the absence of bacterial and fungal contaminants was confirmed. Spent medium was removed. Cells were washed with PBS-EDTA (Phosphatebuffered saline-Ethylene diamine tetra acetic acid) for removing all the traces of serum. Trypsin was applied on to the cell monolayer, and the flask was swirled to cover the monolayer with Trypsin. Flask was incubated at 37°C for 2-3 minutes. The Flask was examined under the inverted microscope to ensure uniform detachment of the cells. 1-2 ml of medium was added to the flask as fast as possible to reduce the Trypsin induced stress, and the contents of the flask transferred to a centrifuge tube. Cells were then centrifuged at 1500 rpm to 2000 rpm, for 10 minutes. The supernatant was discarded, and the cells were re-suspended in minimum volume of medium. Cells were counted using a Haemocytometer and used for subculture, storage and experimental purposes.

In vitro Cytotoxicity on MCF-7 cells by MTT method [5]:-

The cells were harvested, counted and seeded (5000 cells/well) in 96 well plate and PBS was added to the outer wells. DMEM is added to all the wells mixed with 10% FBS. After 24 hours of incubation at 37°C in 5% CO_2 incubator to allow cell attachment. Then the media were removed. Cultures were treated with test sample at different concentrations such as 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 800µg/ml. Again medium is added along with 10% FBS. Untreated cancer cells served as negative control. The plates were then incubated at two stages for 24 and 48 hours. On completion of each stage of incubation, media were removed without disturbing the cells and to each well, 100µl of 1mg/ml solution of MTT were added. Plates were then incubated for 2 hours in dark at 37ºC. 100µl of lysis buffer was added to each well and the plates were further incubated for 4 hours in dark in a 37ºC incubator and absorbance was read using ELISA multi plate reader at 570nm. Triplicates were set up for each concentration.

The percentage of growth inhibition was calculated as follows.

$$100 - \frac{\text{Absorbance of the Drug treated cells}}{\text{Absorbance of Untreated control cells}} \times 100$$

Lymphocyte viability assay on normal lymphocytes by MTT Method [6]:-

The *in vitro* response test sample against normal lymphocyte was studied using Lymphocyte viability assay. Normal lymphocytes were harvested, counted and seeded (5×10^3 cells/well in 100µl) in 96 well titre plates. PBS was added to the outer wells (200µl/well). After 24 hours of incubation at 37ºC in CO_2 incubator to allow cell attachment, media were removed. Then Cultures were treated with test sample such at different concentrations such as 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ ml, and 800µg/ml along with medium. The plates were further incubated for 72 hours. On completion of incubation with the test sample, media were removed without disturbing the cells. To each well, 100µl of 5mg/ml stock solution of MTT were added and plates were further incubated for 2 hours in dark at 37ºC in a CO_2 incubator. 100µl of lysis buffer was added to each well and the plates were further incubated for 4 hours in dark in a CO_2 incubator and absorbance was read using ELISA plate reader at 570nm. Three replicates were set up for each concentration.

The percentage of viability was calculated by

$$= \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

Analysis of cell death (Apoptosis) using fluorescence microscopy [7]:-

Analysis of morphological cell death by Acridine Orange and Ethidium Bromide Dual staining:-

Cells were harvested, counted and seeded (5×10^3 cells/well in 100µl) in 96 well titre plate. The morphological study of cultured cells were treated with test sample at different concentrations such as 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ ml, 800µg/ml along with DMEM medium and 10% FBS. The plates were then incubated in a CO_2 incubator at 37ºC for 24 hours. For assessment of apoptosis, the cells were then added immediately with 5µl of Acridine orange- Ethidium bromide dual stain. Plates were then observed under a fluorescent microscope. These dyes stain the DNA and allow visualization of condensed chromatin of apoptotic cells. It was found that untreated cells appeared green fluorescence and drug treated showed orange fluorescence. This is because live cells take up Acridine orange and appears green and the dead cells take up Ethidium bromide and appears orange. Ethidium Bromide stains cells only in late stages of apoptosis and in case of secondary necrosis when membrane integrity has been lost. The number of cells manifesting morphologic features of apoptosis such as chromatin condensation and the loss of nuclear envelope confirms that cell death occur due to apoptosis.

Analysis of morphological cell death by Hoechst stain:-

Cells were harvested, counted and seeded (5×10^3 cells/well in 100µl) in 96 well titre plate. The cultured cells were treated with test samples at different concentrations such as 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ ml, 800µg/ml along with DMEM medium and10% FBS. The plates were then incubated in a CO_2 incubator at 37ºC for 24 hours. For assessment of apoptosis, the cells were then stained with 5 µl Hoechst 33342. Slides were observed under a fluorescence microscope. These dyes stain the DNA by emitting blue/cyan fluorescent light and allow visualisation of condensed chromatin of apoptotic cells. The number of cell manifesting morphologic features of apoptosis such as chromatin condensation and the loss of nuclear envelope confirms that cell death occur due to apoptosis.

Results:-

In vitro cytotoxicity - Screening by MTT method on MCF-7 cell line:-

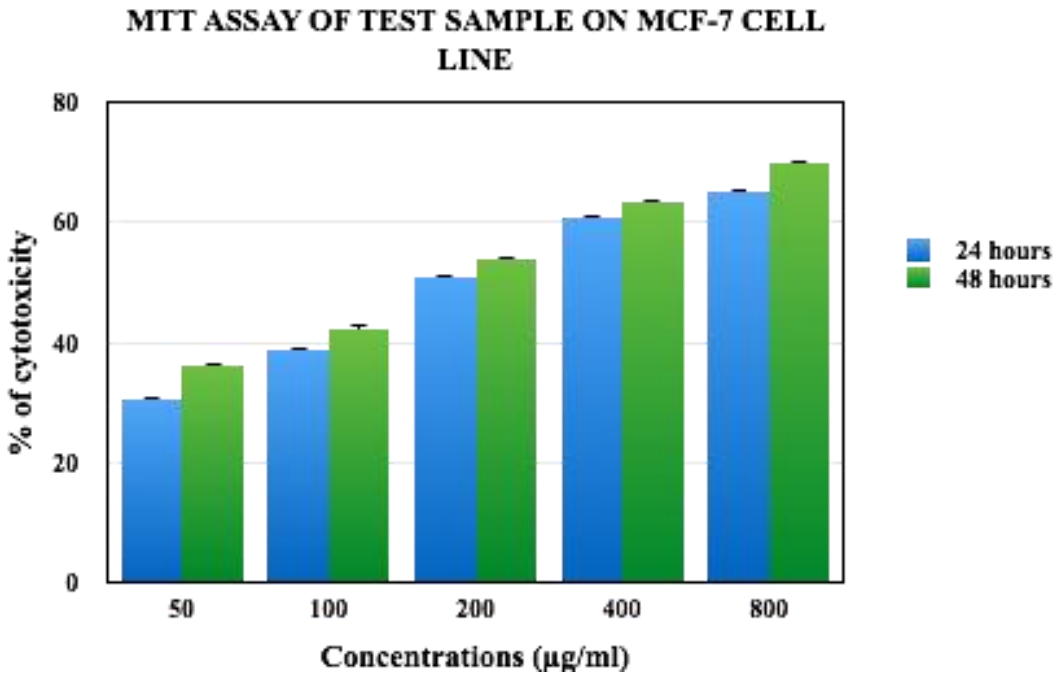
The result of invitro cytotoxicity assay by MTT method on MCF-7 cell lines were as follows

OD value for test control - 1.425

Mean percentage of inhibition of test sample in 24 and 48 hours

During 24 hours, the test sample showed cytotoxicity of about 30.5%, 38.8%, 50.8%, 60.6% and 65% in 50,100,200,400 and 800µg/ml concentrations receptively where as in 48 hours it shows 36%, 42.3%, 53.7%, 63.4% and 69.7% in 50,100,200,400 and 800 µg/ml concentrations receptively

Graph showing the mean percentage of inhibition of test sample in 24 and 48 hours:-



IC_{50} values was also obtained from sigma plot software for the test sample and is about 190 µg/ ml
The mean percentage of inhibition of test sample was statistically evaluated. The results obtained are

Table 1:-Invitro Cytotoxicity of test sample on MCF-7 cell line

Test sample	Concentration µ g/ ml	24 hour			48 hour		
		Mean	SD	p value	Mean	SD	p value
Polyherbal Formulation	50	30.5	0.8	<0.001	36.0	0.5	<0.001
	100	38.8	0.2		42.3	0.4	
	200	50.8	1.3		53.7	0.1	
	400	60.6	0.5		63.4	0.1	
	800	65.0	1.3		69.7	2.3	

In vitro Lymphocyte viability assay - Screening by MTT method on normal lymphocytes;-
The result of in vitro Lymphocyte viability assay by MTT method were as follows
OD value for test control - 0.327

Mean percentage of viability of test sample in 72 hours:-
The test sample shows 96%, 92%, 89%, 87% and 86% viability in 50, 100, 200, 400 and 800µg/ml concentrations receptively.

Graph showing the mean percentage of viability of test sample at 72 hours:-

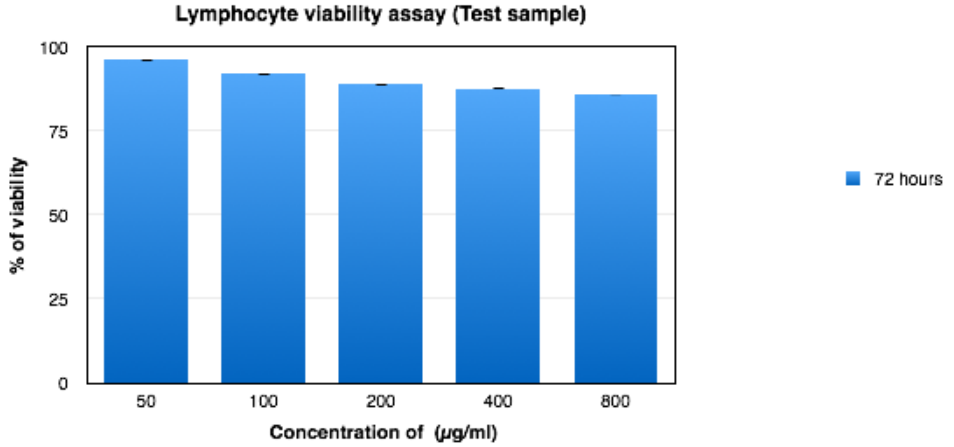


Table 2:-Invitro Lymphocytic viability assay of test sample on MCF-7 cell line

Con: µg/ ml	Test sample (72 hours)		
	Mean	SD	p value
50	96	4.3	0.020
100	92	4.6	
200	88.7	2.4	
400	87.5	1.6	
800	85.9	1.6	

Evaluation of Apoptosis by fluorescent staining:-

3(a) Acridine orange/ Ethidium bromide dual staining:-

The result were as follows

The control (untreated cells) MCF-7 cell line appears green fluorescence indicates that the live MCF-7 cell lines take up the stain Acridine orange and showed the green fluorescence. (Fig 6)

The test sample treated MCF-7 cell line appears orange fluorescence indicates that the cell death happened and it take up the Ethidium bromide stain at 800µgm/ml concentration. The morphological characters of cell such as chromatin condensation and the loss of nuclear envelope confirms that the cell death occurred by means of apoptosis. (Fig 7)

Hoechst staining:-

The result were as follows

The control MCF-7 cell line emit only a little blue/cyan light indicated that these are live MCF-7 cell lines (Untreated cells) (Fig 8)

The test sample treated MCF-7 cell line appears blue/cyan light indicates that the cell death happened and it take up the Hoesct stain at 800µgm/ml concentration. The morphological characters of cell such as chromatin condensation and the loss of nuclear envelope confirm that the cell death occurred by means of apoptosis. (Fig 9)

Discussion:-

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Breast cancer is characterized by the uncontrolled growth of abnormal cells in the milk producing glands of the breast or in the passages that deliver milk to the nipples. Each year 1- 1.5 million new cases of breast cancer are being added all over the world. For the treatment of cancer modern science has developed many drugs but these drug burdened the patient by their cost and drug induced toxic effects. Also there is a common belief that anticancer drugs produce non-selective cell killing of normal as well as cancerous tissues. So in the present era poly herbal formulation/ single herbal drugs has got more importance. Many scientific researchers have drawn attention to anticancer properties of medicinal herbs.

Reference of a poly herbal formulation is found with three herbal drugs *Punarnava moolam*, *Sunthi* and *Varuna moolam* in a traditional book " Sarvaroga chikitsa nool". Ayurvedic physicians also practice this same combination as a traditional medicine since many years specifically for the treatment of breast cancer. But this claim is not scientifically recorded. Therefore this combination is selected for the study. The present study is taken up an attempt to evaluate the cytotoxic effect of decoction of polyherbal formulation in MCF-7 breast cell line.

The test sample showed maximum cytotoxicity of about 69.7 % at 48 hours in 800µg/ml concentration and there is a trend showing that when the concentration and time period increases the percentage of inhibition also increases. Statistically also its significance was calculated and it indicated that the values are highly significant in 50 µg/ ml - 800 µg/ ml concentrations. The lymphocyte viability assay was also performed and found that the test sample has no significant toxicity in normal cells. It proved the safety of drug in normal cells. Treating cells with cytotoxic agents have several fates, either the cell may undergo lysis or it decreases in cell viability or apoptosis will occur. Analysis of cell death by apoptosis was also observed by using acridine orange -ethidium bromide stain and hoesct stain. The results obtained are, for the test sample at 800 µgm/ml concentration the MCF-7 cell line showed apoptic cell death for both acridine orange -ethidium bromide stain and hoesct stain. The cells showed chromatin condensation and loss of nuclear envelope confirms that the cell death occurred by means of apoptosis.

Fig 1:-Dried root of *Boerhaavia diffusa L.*

Fig 2:-Dried rootbark of *Crataeva nurvala Buch.Ham*

Fig 3:-Dried rhizome of *Zingiber officinale rosc.*

Fig 4:-Concentrated decocotion of test sample

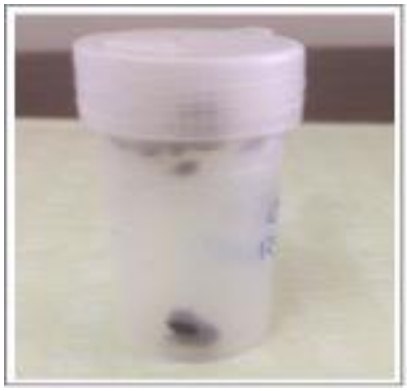


Fig 5:-Concentrated test sample diluted with distilled water

Fig 6:-Control MCF-7 cell line treated with Acridine orange- Ethidium bromide stain

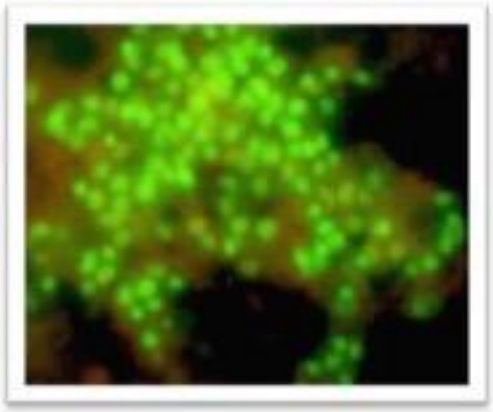


Fig 7:-Test sample treated MCF-7 cell line at 800 µgm/ml

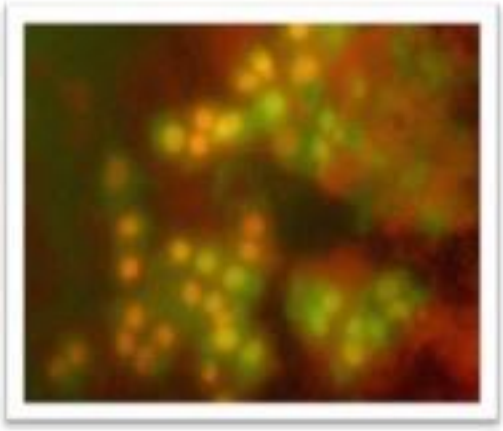


Fig 8:-Control MCF-7 cell line treated with Hoesct stain

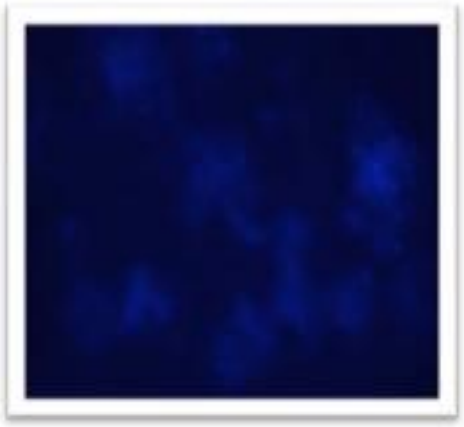
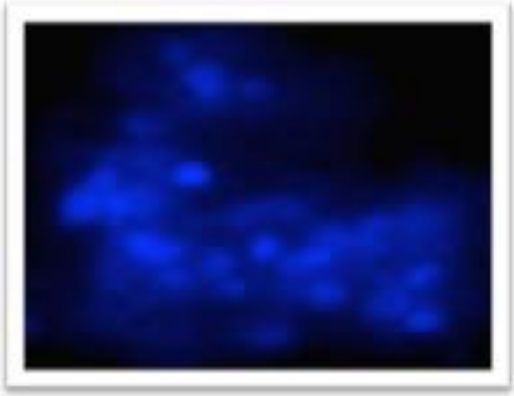


Fig 9:-Test sample treated MCF-7 cell line at 800 µgm/ml



Conclusion:-

The results of present study demonstrated that the polyherbal formulation has got cytotoxicity in MCF-7 cell line. This may be due to the synergetic action of all the three drugs present in the polyherbal formulation. The cytotoxicity activity in MCF-7 cell lines and minimal toxicity in normal cells proved that it can be used as a good anticancer agent. However, in vivo studies have to be carried out to substantiate the in vitro results by employing different in vivo models and clinical trials for their effective utilization as therapeutic agents.

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