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Induction of Apoptosis in MCF-7 Cell Lines by Various Ethanol Extracts

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ABSTRACT

Cancer is most dreadful disease despite of advanced medicine and treatment options. Breast cancer is one of those cancers that affect most of female patients. Radiation therapy, chemotherapy and other conventional treatment options are effective but have significant side effects and sometimes ineffective too. Thus focus was kept on discovering the alternative sources of treatment and thus herbs posed themselves as best alternatives in terms of safety and effectiveness. In this research ethanol extracts of Yams of Dioscoreavillosa, leaves of Occimumgratissimum and Annonamuricata were investigated for their anti-proliferative activity *invitro*against MCF-7 cell lines using MTT assay. The results showed that extracts had a cytotoxic activity in a dose dependent manner and Dioscorea showed a potent activity with IC-50 of nearly 35 µg/ml. The extracts were also investigated for apoptosis inducing activity against MCF-7 cell lines using flow cytometer. Results suggest that the extracts were effective in induction of apoptosis in cell lines and the extract of Annona was proven best in apoptosis induction. Overall it was proven that the extracts can lead to active principle that can be considered as potential candidates for treatment of breast cancer.

Keywords:

Breast cancer, Dioscorea, Occimum, Annona, Apoptosis, MCF-7 cell lines

1.Introduction

Cancer especially breast cancer is the most notable causes of death and depression in woman worldwide (Jemal et al., 2011). It is prevalent in approximately12 million patients and causes around 7 million deaths annually and stands in second place to heart diseases in causing mortality(Siegal et al., 2015). Almost 25% of the cancer patients are suffering from breast cancer and contributes to about 50% cancers in woman(Taghavi et al., 2012).Traditional medical treatment of breast cancer utilizes options like chemotherapy, radiotherapy and surgical resection. But due to some serious adverse effects and drug resistance, these treatment strategies have not shown any successful and standard improvementin the breast cancer patients(Waxman & Schwartz, 2003). So there were always investigations on alternative therapies and treatment methods to effectively treat breast cancers. So it demands for safer and more effective alternatives to existing synthetic drugs.

The growth and rapid multiplication of cancer cells is due to the manipulations and alterations in the genetic programs leading to death of cells otherwise called as cell apoptosis. Usually apoptosis process is delayed due to genetic alterations which result in enhanced cell growth and delay in maturation and death. The current treatment options concentrate on induction of apoptosis to the cancer cells. Chemotherapeutic agents also target the induction of apoptosis that destroys the cancers and regulates the uncontrolled multiplication(Chu &Sartorelli, 2004). Herbal products were always considered safe and posed themselves as

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viable alternatives for the existing chemotherapy. Medicinal produces various types of chemical constituents like coumarins, xanthanes, alkaloids, and carotenoids etc. which have high potency to fight and cure cancer invitro and invivo models. The secondary metabolites were established as potent sources for the synthesis of new drugs that are used for treatment of cancer(Fulda&Efferth, 2015).

Dioscoreaceae, a family of medicinal plants is rich in titerpenoid and steroidal saponins(Tabopda et al., 2014). The chemical constituents from dioscorea were potent againt cancer cells which had been investigated in various models and were found effective against different types of cancers like cholangiocarcinoma, CNS and prostatecancer(Hu&Yao, 2002), acute myeloid leukemia(Hu et al., 1996), melanoma, lung cancer (Guohua et al., 2003) etc. Apoptotic induction was effective in breast cancer MCF7, nasopharyngeal cancer CNF2 (Chan& Ng, 2013), sarcoma-180 tumour (He et al., 2012).

Ocimum genus plants naturally possess anticancer activity and polar and non-polar fractions of Ocimumgratissimum were investigated for anticancer activity against breast cancer celllines MCF10AT1 and MCF10AT1-E118 (Nangia-Makker et al., 2013). Aqueous extract of Ocimumgratissimuminhibited the morphogenesis, migration, and proliferation and COX-2 induction in breast cancer cells. The extract also lowered the tumor size and neo-genesis in human ductal carcinoma in-situ (Nangia-Makker et al., 2007). Purified fractions of ethanol and aqueous extracts of Ocimumgratissimumexhibited anti-proliferative effect against prostate adenocarcinoma, PC-3 cells(Stephen et al., 2010). Induction of apoptotic signaling was observed in the pulmonary adenocarcinoma cell lines, A549 with the treatment of aqueous extracts of Ocimumgratissimum(Chen et al., 2011).

The crude extract of *Annonamuricata*was investigated and proven effective against breast cancer cell lines (MCF-7, MDA-MB-231 and 4T1) and in-vivo experiments showed anti-cancer activity against 4T1 induced breast cancer in mice (Najmuddin et al., 2016). Experiments were conducted on extracted fractions of various parts of *Annonamuricata*to prove their anticancer properties. Ethyl acetate and methanol fractions were proven effective while apoptosis and karyokinesis were hypothesized mechanism behind the anti-cancer activity (Agu et al, 2018).

2.Objective

The current research focussed on fractionation and purification of the extracts of Dioscoreavillosa, Occimumgratissimum and Annonamuricata and determination of cytotoxicity of the isolated fractions on breast cancer cell lines (MCF-7) using MTT assay. The apoptotic activity of the fractions was investigated using flow cytometer.

3. Materials and methods

3.1. Chemicals

MTT assay kit and dimethyl sulfoxide, Dulbecco's modified eagles (DME) media were purchased from HiMedia, Mumbai, Fetal Bovine Serum (FBS), Trypsin in Phosphate Buffered Saline (PBS) and EDTA were supplid from Invitrogen, India. Fluorescein Isothiocyanate (FITC)Muse™ Annexin V & Dead Cell Reagent kit procured from Sigma-Aldrich, India. All the reagents used in the research otherwise specified were procured from SD Fine Chem, India and were of analytical grade.

3.2. Cell Lines

Human breast cancer (MCF-7) cell lines were purchased from NCCS Pune were maintained in DMEM enriched with FBS (10%w/w)along with 1% streptomycin and incubated in a CO_2

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incubator at 37°c in 5 % CO_2. The culture was trypsinized using 500µl of 0.025% Trypsin suspended in PBS and 0.5mM EDTA solution for 2min after attaining the confluence. The cultures were centrifuged at 1000rpm for 5min and re-suspended in culture medium. This cell cultures in log growth phase were used in further study.

3.3. Extraction and Fractionation

Yams of Dioscoreavillosa, leaves of Occimumgratissimum and Annonamuricata(non-pesticide) were dried under shade and crushed into fine powder passed through sieve 40. The herb powder (500g) was separately extracted with ethanol (90%v/v)using soxhlet apparatus. The resultant was filtered off and the filtrate was concentrated using a rotary vacuum evaporator at 65^0C to yield extracts (Dioscoreavillosa (DV)-16.5%w/w; Occimumgratissimum (OG)-21.6%w/w; Annonamuricata (AM)-22.4%w/w). The extracts were defatted using petroleum ether and separatedwith double distilled water. The aqueous solution of the extract was then partitioned with ethyl acetate and n-butanol saturated using distilled water. The ethyl acetate fractions of the extracts were then concentrated to yield dry triterpenoid fractions of three extracts (Dioscoreavillosa (EADV)-5.6g; Occimumgratissimum (EAOG)-8.5g; Annonamuricata (EAAM)-9.2g)).

3.4. Determination of *Invitro* Anti-Proliferative Activity on MCF-7 Cell Lines

The cultured cell lines mentioned in section 3.2 were used to determine the anti-proliferative effect of extracts. EADV, EAOG and EAAM were added to the isolated cell cultures($1x10^4$ per well) in various concentrations (6.25, 12.5, 25, 50 and 100 µg/mlin acetone). The well plates were incubated again for 24hr and the percentage viability was measured using MTT assay(Sylvester).After incubation of the cells for 24hr, the cells were washed with PBS and 30µl MTT (2mg/ml) is added. The culture was incubated for 3hr at 37°c after which MTT was removed by washing with PBS. 200µl DMSO was added to the cell lines and incubated again for 30min. The medium was centrifuged for 2min at 2500rpm. The supernatant liquid was collected and absorbance was measured at 540nm against DMSO control using a micro plate reader (Elisa scan, Erba). The measurements were done in triplets and the IC_{50} was calculated using graph pad prism.

3.5. Detection of Morphological Changes

The cell cultures of untreated and treated cells with various extracts were visualized under an inverted microscope coupled with a camera setup (Nikon, Japan).

3.6. Determinaitonof Apoptotic Activity using Flow Cytometry

Apoptosis of MCF-7 cells were measured using Annexin V & Dead Cell Reagent(Propidium iodide) Kit (Jamalzadeh et al., 2017; Silva et al., 2016) by following the user's instructions. Cultured MCF-7 Cell lines ($2x10^5$) from section 3.2 were seeded in 6-well plates and treated with extract fractions at IC_{50} and were incubated for 24hr. The culture was harvested and washed with PBS. 100µl of Muse™ Annexin V & Dead Cell Reagent was added to each tube. The mixture was thoroughly shaken for 3min and incubated for 20min at 37°c in dark. The cells were passed through a flow cytometer to analyse for apoptosis using Muse FCS 3.0 against untreated cells as control. The Muse™ Annexin V & Dead Cell Reagent uses the Annexin V to determine the phosphatidylserine (PS)on the plasma membrane of the cells in the apoptotic stage.The apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence in the line of argon-ion laser excitation beam.

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3.7. Statistical Analysis

Graphpad Prism 5 (Version 5.04) installed in Windows 10 was used to perform the statistical analysis. The values were represented as means and their respective standard deviations. Comparison between groups was done using ANOVA following Dunnett's test and values with $p<0.001$ were and $p<0.05$ were considered as significantly different.

4.Results

4.1. Anti-Proliferative Effect of Extracts on MCF-7 Cell Lines

The anti-proliferative activity of the extracts on MCF-7 cell lines was estimated *invitro*by testing the cytotoxicity against the cell lines. MCF-7 cell lines were treated with various concentrations of the extracts, 6.25, 12.5, 25, 50 and 100 µg/ml. Figure 1 shows the comparison of the inhibitory activity of the extracts at various concentrations. Their respective IC-50 values were also indicated in the figure.The IC-50 value of ethanol extract of Dioscoreavillosa (EADV) is 34.64 µg/ml, Occimumgratissimum (EAOG)-43.81 µg/ml and Annonamuricata (EAAM)-85.95 µg/ml. this indicates that the extract of Dioscorea are more potent in inhibiting the proliferation of the MCF-7 cells. The trend line in figure 1 shows better activity of EADV followed by EAOG and EAAM.

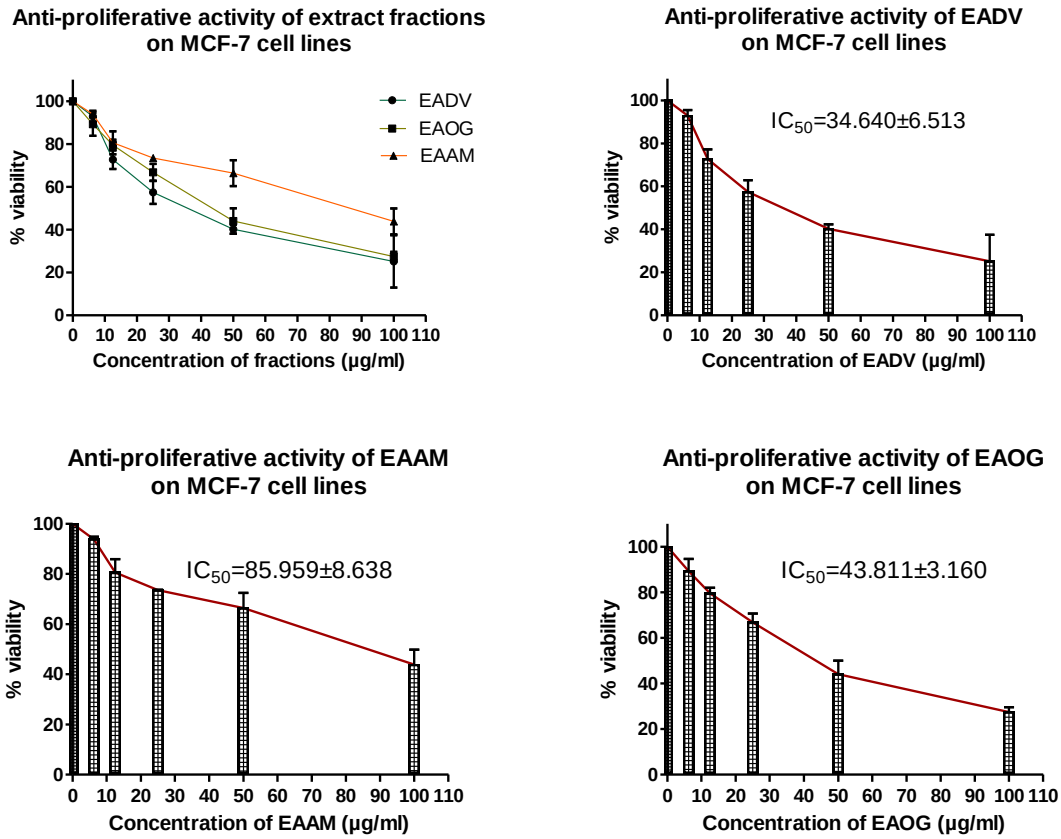

Figure 1. Anti-proliferative activity of extracts against MCF-7 cell lines

EADV- Ethanol Extract of Dioscoreavillosa, EAOG- Ethanol Extract of Occimumgratissimum, EAAM- Ethanol Extract of Annonamuricata

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4.2. Effect of Extracts on Cell Morphology

Inverted microscopic images of the cell cultures showed that the extracts had significantly inhibited the proliferation of cells compared to the control image in figure 2. There is a clear clustering of the cell lines and low cell counts in the cultures treated with extracts.

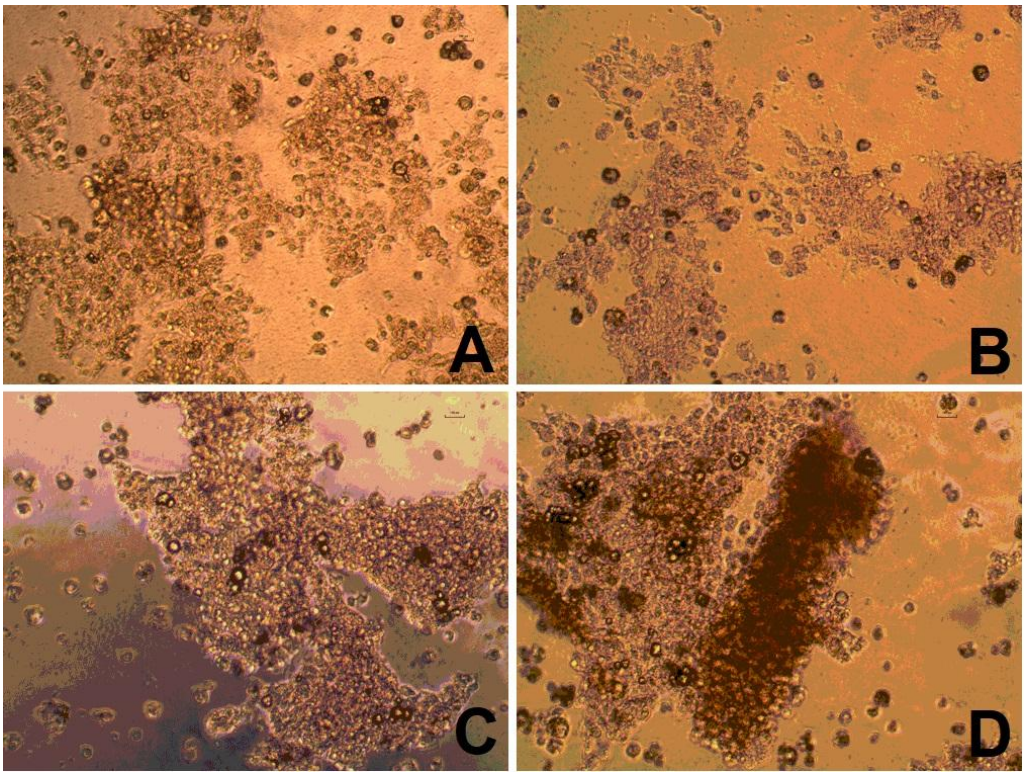

Figure 2. Inverted microscopic images of MCF-7 cell lines exposed to extracts

A-Control group; B-EADV- Ethanol Extract of Dioscoreavillosa; C-EAOG- Ethanol Extract of Occimumgratissimum; D-EAAM- Ethanol Extract of Annonamuricata

4.3. Effect of Extracts on the Apoptosis of MCF-7 Cell Lines

Figure 3 and 4 shows the apoptotic activity of extracts on MCF cell lies. Apoptotic activity was investigated by using flow cytometry as in section 3.6. Stained cells were passed through flow cytometer and analysed for their cell cycle stage and the results suggested that the exposure to the extracts induced apoptosis in the cell lines. The percentage of cells in the apoptotic stage was not less than 97% in control group indicating that the cells were proliferating well and is a viable culture. The cultures exposed to EADV showed live cells of 75% and apoptotic cells at 25% whereas for EAOG live cells ranged approximately at 51% and EEAM resulted in the apoptotic cells of nearly 70%. This indicates that EAAM showed a better apoptosis induction in MCF-7 cell lines.

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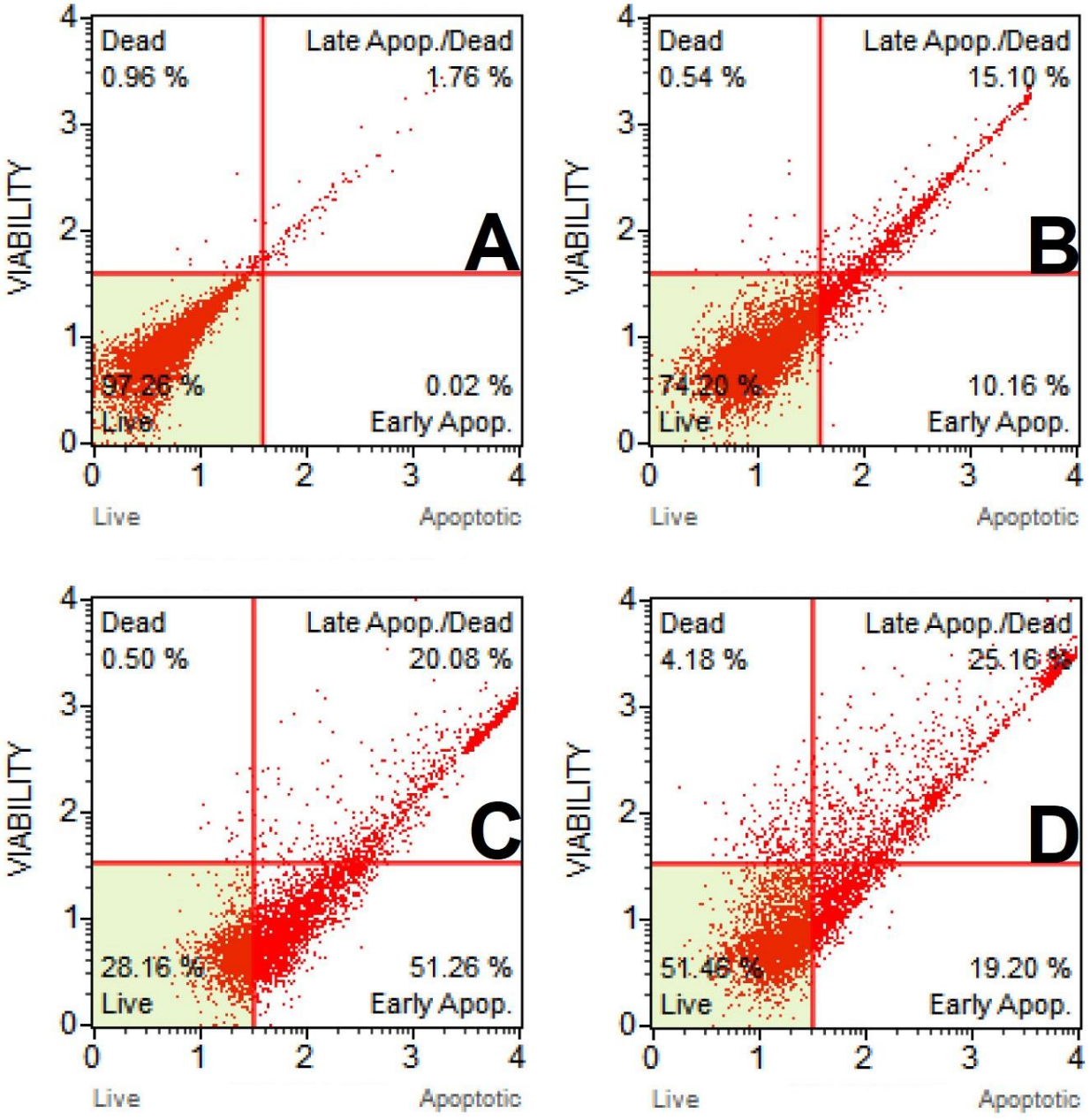

Figure 3.Apoptosis inductionin MCF-7 cell lines exposed to extracts

A-Control group; B-EADV- Ethanol Extract of Dioscoreavillosa; C-EAOG- Ethanol Extract of Occimumgratissimum; D-EAAM- Ethanol Extract of Annonamuricata

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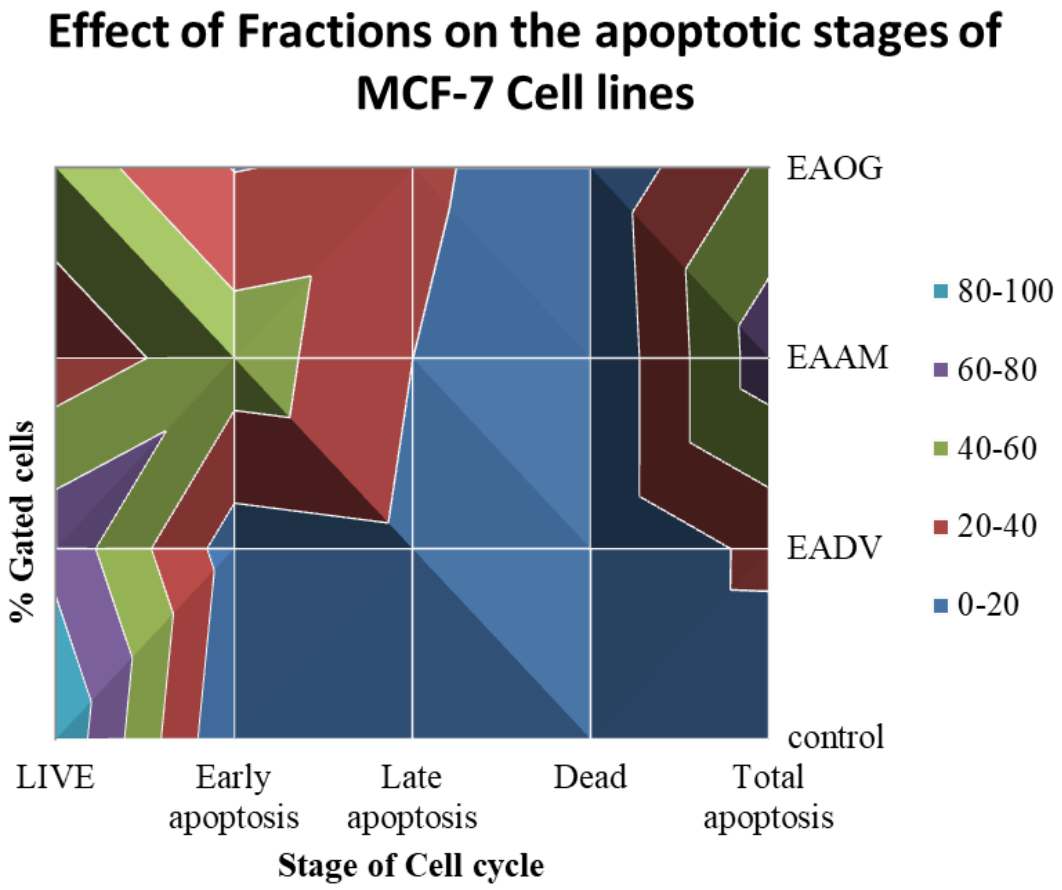

Figure 4.Comparison of apoptosis inductionin MCF-7 cell lines exposed to extracts

EADV- Ethanol Extract of Dioscoreavillosa; EAOG- Ethanol Extract of Occimumgratissimum; EAAM- Ethanol Extract of Annonamuricata

The cell counts in various apoptotic stages after exposure to the extracts was indicated in table 1. Live cells in control group were nearly 35 million compared to EADV of 7.5 million, EAOG-3.3 million and of EEAM is just 2.8 million. Interestingly EADV and EAAM caused death to most number of cells of 5500 and 5000 respectively. But, EAAM induced overall better apoptosis to the cell lines which indicates better activity.

Table 1. Apoptotic activity of extracts against MCF-7 cell lines using flow cytometer

Stage of cells	**Cell count (cells/ml)**			
	Control	**EADV**	**EAAM**	**EAOG**
Live cells	$3.58x10^{6}$	$7.66x10^{5}$	$2.82x10^{5}$	$3.32x10^{5}$
Early apoptotic cells	$7.37x10^{2}$	$1.05x10^{5*}$	$5.14x10^{5}$	$1.24x10^{5*}$
Late apoptotic cells	$6.48x10^{4}$	$1.56x10^{5*}$	$2.01x10^{5}$	$1.62x10^{5}$
Total apoptotic cells	$6.56x10^{4}$	$2.61x10^{5*}$	$7.15x10^{5}$	$2.86x10^{5}$
Dead cells	$3.56x10^{4}$	$5.57x10^{3*}$	$5.01x10^{3*}$	$2.70x10^{4}$

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*P<0.05 indicates significant difference in comparison to control group. EADV- Ethanol Extract of Dioscoreavillosa, EAOG- Ethanol Extract of Occimumgratissimum, EAAM- Ethanol Extract of Annonamuricata

5.Discussion

Breast cancer takes the majority of the share in the cancers that occur in female patients(Siegel et al., 2015). Despite of various treatments, it still remains as an un-curable problem. So there was urgent need to investigate for newer effective drugs but with the concern of side effects that arise with existing synthetic drugs and treatments(Polyak, 2007), focus had been shifted toward investigating herbal sources of anti-cancer drugs. Ethanol extracts of Yams of Dioscoreavillosa, leaves of Occimumgratissimum and Annonamuricata were investigated for the anti-proliferative *invitro*using MTT assay and apoptotic activity against MCF-7 cell lines using flow cytometer. The results suggested that all the extracts possessed significant anti-proliferative activity with IC-50 values less than 100 µg/ml. Out of the three extracts Dioscoreavillosa showed a significantly better anti-proliferative activity compared to other herbs. The inhibition of proliferation was dependent on the dose of the extract that is used to treat the cell lines. The concentration dependent activity was consistent in all the extracts.

The induction of apoptosis was seen in the cells cultures exposed to the extracts. Based on the percentages it is clear that the extract of Annonamuricata caused the apoptosis to more number of cells compared to other extracts. EADV showed a better activity in killing the cells which might be due to the potent cytotoxic activity of the extract. EAAM and EAOG induced apoptosis to cell lines were high concentration of cells lied in the apoptotic stages of cell cycles. Contrarily EAAM also significantly induced cytotoxicity to more number of cells. Overall, the results of this research suggests and provides enough support to the anti-cancer activity of the extracts of Yams of Dioscoreavillosa, leaves of Occimumgratissimum and Annonamuricata and can be used in treatment of breast cancer. In consideration of the long term use of the drugs for cancer, herbal drugs have a better advantage towards the safety of treatment and better activity of the extract.

6. Conclusion

It can be concluded that the extracts of Dioscoreavillosa had a potent anti-proliferative activity and Annonamuricata induced apoptosis in MCF-7 cell lines effectively. Standardization of the extracts to isolate the active principles and to ensure the replication of activity could enable them as potential candidates for the treatment of breast cancer cases effectively. The promise of the treatment lies in the safety of the extract and this research provides evidence of potent activity of herbs as alternative treatment options for stubborn and incurable cancer conditions.

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8.Conflict of interest

Authors had no conflict of interest to declare.

9.Funding support

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