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Cumulative Anticancer Activity Of Some Herbal Extracts On Selected Malignant Cancer Cell Lines †

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Abstract: The cumulative cytotoxic activity of 16 combined extracts aqueous &ethanol extract of Annona reticulata with Allium sativum, Allium fistolisum and Brassica oleraceae were investigated using a (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with two human cancer cell lines, MCF-7and HCT 116. A mitochondrialenzyme in living cells, succinate-dehydrogenase cleaves the tetrazolium ring and converts the MTT to an insoluble purple formazan whose intensity is directly proportional to the presence of viable cells in the microwell plate. Results showed a significant ($p<0.05$) cytotoxic effect of the extract in a dose dependent manner. ComparativeCytotoxicity increased with increase in the concentration of the extract used. IC50 results calculated after MTT test showed the concentration of ethanol extract of Annona reticulata with Allium sativum, Allium fistolisum and Brassica oleraceae required for 50% inhibition of the different cell lines as follows: Sample 1 shows 0.7 µg/ml, Sample 3 shows 3.0 µg/ml Sample 16 shows 0.1 µg/ml showed a significant reduction ($p<0.05$) in the total count of viable cells for MCF-7and HCT 116 cell lines. Analysis for cell cycle and apoptosis levels of HCT116 cells upon extracts treatment was measured by flowcytometry. Comparative results obtained indicate that there is a correlation between the combined extracts adopted in establishing the antiproliferative and cytotoxic activity of aqueous & ethanol extract of Annona reticulata with Allium sativum, Allium fistolisum and Brassica oleraceae obtained in this study..

Keywords: cumulative cytotoxic activity, Annonareticulata, Allium sativum, Allium fistolisum and Brassica oleraceae, human cell lines, MTT

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1. Introduction

Cancer is one of the most dreaded diseases of the 20th century and increasing incidentally in 21st century. It is inevitably one of the most studying, but yet unsolved non-communicable human disease and a group of disease characterized by uncontrolled cell division leading to the abnormal growth of the tissue (Heena VS, Sunil HG, Rama P. et al 2012). It is an idiopathic disease for which doctors and scientist are constantly trying to evolve new effective drug for its treatment. There is no other disease which parallels cancer in diversity, its origin, nature, and treatment. International Agency for Research on Cancer estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and aging of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025. Rise in incidence of lung, breast, oral, and colorectal cancer are a cause of concern. The most commonly diagnosed cancers worldwide were those of the lung cancer (1.8 million, 13.0% of the total), breast cancer (1.7 million, 11.9%), and colorectal cancer (1.4 million, 9.7%). The most common

causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) (IARC, Press Release No. 223). Oral squamous cell carcinoma (SCC) is one of the most common malignant tumors in human; it is the fifth most common malignancy worldwide and a major cause of cancer morbidity and mortality in India representing approximately 40-50% of all cancers. The highest incidence rates have been observed in the Indian subcontinent (Suresh K, Manoharan S, et. al, 2010).

Chemotherapy, being a major treatment modality used for the control of advanced stages of malignancies and as a prophylactic against possible metastasis, exhibits severe toxicity on normal tissues. The side effects of such drugs make it a necessity for a new improved drug. Herbal medicines have been used since the dawn of civilization to maintain health and to treat various diseases. They maintain the health and vitality of individuals and also cure diseases including cancer without causing toxicity (Madhuri S, Pandey G. 2009). Annona reticulata, commonly known as bullock's heart or ramphal plant, is widely distributed all over India. They are rich in annonaceous acetogenins. Acetogenins from the leaves were found to be selectively cytotoxic to certain human tumors (Suresh HM, Shivakumar B, et al, 2011). Leaf contains anonaine, roemerine, norcorydiene, corydine, norisocorydine, dienone isocorydine, norlaureline, glaucine hyperoside, rutin and quercetin, n-hexacosanol, n-octacosanol, n-triacontanol, 16 hentriacontanone, campsterol, stigmasterol, and sitosterol. Leaf also contains essential oils, carvone, linalool, and (+) O-methyl armepavine (Pathak K, Zaman K.et al, 2013). However, species of the Annonaceae family have also been targeted for investigation due to appurtenant substances in the acetogenins, a class that has been isolated from different parts of the plant (Pathak K, Zaman K.et al, 2013).

Allium sativum contains more than 100 biologically useful secondary metabolites, which include alliin, alliinase, allicin, S-allyl cysteine (SAC), diallyldisulphide (DADS), diallyltrisulphide (DATS) and methylallyltrisuphide. The γ-glutamyl-S-alk(en)yl-L-cysteines are the primary sulfur compounds in the intact garlic, which can be hydrolyzed and oxidized to yield S-alkyl(en) yl-L-cysteine sulfoxide (alliin) (Brodnitz MH, Pascale JV, Derslice LV (1971). DATS reduced mitosis in tumors, decreased histone deacetylase activity, increased acetylation of H3 and H4, inhibited cell cycle progression, and decreased pro-tumor markers (survivin, Bcl-2, c-Myc, mTOR, EGFR, VEGF) (Tsubura A, Lai YC, et al, 2011). Garlic components have been found to block covalent binding of carcinogens to DNA, enhance degradation of carcinogens, have anti-oxidative and free radical scavenging properties, and regulate cell proliferation, apoptosis, and immune responses. Ajoene, garlic-derived natural compound, have been shown to induce apoptosis in human leukemic cells via stimulation of peroxide production, activation of caspase-3-like and caspase-8 activity. Garlic synergizes the effect of a breast cancer suppressor, eicosapentaenoic acid, and antagonizes the effect of a breast cancer enhancer, linoleic acid (Gonzalez FJ.,1991).

Allium vegetables, especially garlic intake, are related to decreased risk of prostate cancer.(Zeng YW, Yang JZ., et al, 2013). Quercetin, a principal flavanoid compound in onions, has been shown to possess a wide spectrum of pharmacological properties, including anticancer activities. Quercetin inhibits migration and invasion of SAS human oral cancer cells through inhibition of NF-κB and MMP-2/-9 signaling pathways. (Yu CS, Lai KC., et al, 2010). Quercetin is a member of the flavonoid family and has been previously shown to have a variety of anticancer activities. (Chae JI, Cho JH. et al, 2012). Quercetin shows antiproliferation, cell cycle arrest, and induction of apoptosis of cancer cells. It induces the apoptosis of certain malignant cells mainly on human malignant pleural mesothelioma. (Syed DN, Adhami VM, et al, 2013). Quercetin (3,5,7,3′,4′-pentahydroxylflavone) induces apoptosis in human cancer cell lines, including human leukemia HL-

60 cells. Quercetin stimulates macrophage phagocytosis and promotes natural killer cell activity. Dihydroquercetin (taxifolin) is a potent flavonoid found in onions, dihydroquercetin in major disease states such as cancer, cardiovascular disease and liver disease.

Crucifers are important sources of glucosinolates (GLs) whose de-generated products like isothiocyanates were attributed to chemopreventive activity. Cruciferous vegetables in particular have attracted a great deal of attention since they are rich in aromatic and aliphatic Isothiocyanates. Glucosinolates are anionic, hydrophilic plant secondary metabolites and play an important role in the prevention of cancer and other chronic and degenerative diseases (Fahey et al., 2002; Halkier, 1999).

Brassica oleraceae belongs to the Cruciferae family is inexpensive and is easy to grow, harvest and store (Timberlake, C. F. And Bridle et al, 1973). *Brassica oleraceae* colour can be used as a natural food colour. *Brassica oleraceae* is a rich source of anthocyanins, mainly acylated anthocyanins such as cyanidin 3, 5-diglucoside, cyanidin 3-sophoroside-5-glucoside and cyanidin 3-sophoroside-glucoside acylated with sinapic acid (Wu et al., 2006). In general, acylated anthocyanins contain two sugar molecules (glucose and sophorose) and several aromatic acids (Tanchev 1969; Dyrby et al., 2001; McDoughall et al., 2007). The *Brassica oleraceae* anthocyanins transition from purple-red to pink-red to blue-green between pH levels of 3 and 6 respectively (Hagiwara et al., 2001).

In this study, cumulative cytotoxic activity of aqueous & ethanol extract of Annona reticulata with Allium sativum, Allium fistolisum and Brassica oleraceae,total 16 samples were investigated was tested. The antitumoral activity of this plant extract was tested on two human cancer cell lines: MCF-7 (breast carcinoma cells), and HCT 116 (Colorectal carcinoma cells). Cytotoxicity tests implored include MTT (3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for cell count along with Cell Cycle and Apoptosis Analysis was carried out.

Materials and Methods:

Plant materials

The leaves of A. reticulate, Allium sativum (bulbs), A. fistolisum and B. Oleraceae were collected from sahydri regions of Karjat Dist-Raigad, Maharashtra, India. All Plant materials were authenticated at “The Blatter Herbarium” St. Xavier’s College, Mumbai.

Preparation of extract stock and working solution

After identification and authentication of the plant, leaves of the plant were collected for the experimental process. The leaves were shade dried, made into coarse powder and the powdered material was initially defatted with petroleum ether and then subjected to cold maceration process for 72-h using 1:1 mixture of methanol and water as solvent to prepare hydro-alcoholic extract of Annona reticulata leave (percentage yield 20.5% w/w

with respect to dried powder). The extract was filtered and concentrated by rotary evaporator. For the preparation of different fractions method was used (Zhishen,J.;Mengcheng,T.;Jianming,W.1999, Bondet,V.; Brand Williams,W.; Berset,C.1997 and Brand-Williams,W.; Cuvelier,M.E.; Berset,C.1999).

The sun dried and powdered leaves (76 g) of A. reticulata were successively extracted in a Soxhlet extractor at elevated temperature using 200 mL of distilled n-hexane (40–60)°C which was followed by petroleum ether, methanol, and chloroform. All extracts were filtered individually through filter paper and poured on petri dishes to evaporate the liquid solvents from the extract to get dry extracts. The dry crude extracts were weighed and stored in air-tight container with necessary markings for identification and kept in a refrigerator for future investigations.

Ten grams of spring onion's leaves or bulb was soaked in 100 mL of methanol and water, respectively. The prepared samples were shake using orbital shaker for 7 hrs followed by centrifugation for 15 min at 7000 rpm. The extracts were then filtered using vacuum filtration assembly.

Red cabbage leaves were shade dried followed by hot air oven drying at 50° centigrade and then ground to a fine powder and stored in air tight container for analysis. Fresh red cabbage leaves were grinded in the mixer for the collection of juice. The coarse powder and juice of red cabbage were extracted with methanol and water. The extracts of red cabbage powder and juice were collected separately and filtered using Whatman filter paper. All the extracts were concentrated and the excessive solvents were evaporated under vacuum.

Sample No.	Name of the sample
1	Control
2	Standard
3	Annona reticulata (Alcoholic extracts)
4	Annona reticulata (Aqueous extract)
5	Allium sativum (Alcoholic extracts)
6	Allium sativum (Aqueous extract)
7	Allium fistolisum (Alcoholic extracts)
8	Allium fistolisum (Aqueous extract)
9	Brassica oleraceae (Alcoholic extracts)
10	Brassica oleraceae (Aqueous extract)

11	Allium sativum and Annona reticulate (Alcoholic extracts) (1:1)
12	Allium sativum and Annona reticulate (Aqueous extract) (1:1)
13	Allium fistolisum and Annona reticulate (Alcoholic extracts) (1:1)
14	Allium fistolisum and Annona reticulate (Aqueous extract) (1:1)
15	Brassica oleraceae and Annona reticulate (Alcoholic extracts) (1:1)
16	Brassica oleraceae and Annona reticulata (Aqueous extract) (1:1)

Different concentrations were prepared using simple aqueous and alcoholic extract portions of plant materials. Likewise 16 samples were prepared in aqueous and alcoholic solutions.

Cytotoxicity screening

Growth inhibitory assays

MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amount of MTT. Thus the amount of MTT cleaved is directly proportional to the number of viable cells present, which is quantified by colorimetric methods. This assay was performed at Deshpande Laboratories, Bhopal using the standard operating procedures. Briefly the compounds were dissolved in DMSO and serially diluted with complete medium to get the concentrations a range of test concentration. DMSO concentration was kept < 0.1% in all the samples HCT 116 colon cancer, MCF7 breast cancer cells maintained in appropriate conditions were seeded in 96 well plates and treated with different concentrations of the test samples and incubated at 37 °C, 5% CO2 for 96 hours. MTT reagent was added to the wells and incubated for 4 hours; the dark blue formazan product formed by the cells was dissolved in DMSO under a safety cabinet and read at 550nm. Percentage inhibitions were calculated and plotted with the concentrations used to calculate the IC50 values.

Cell Cycle and Apoptosis Analysis

HCT116 colon cancer cells were grown in DMEM, FCS 10% in a T25 flask. Cells were seeded in a 6 well plate and serum starved over night. Cells were provided with serum containing medium and test sample 10ug/ml, untreated cells were treated with PBS. 24h post treatment, cells were gently trypsinized and washed with PBS twice. Cells were fixed in methanol and stained with 0.1% propidium iodine (SIGMA) and anaylzed for cell cycle and apoptosis on flow cytometer (BD FACS calibur). For cellular uptake

studies, cells were treated with FITC tagged samples and incubated for 5 hours. The cells were washed with PBS and fixed in methanol and observed under fluorescent microscope.

RESULTS AND DISCUSSIONS

MTT Assay

Concentrations of 0.001 to 10 µg/mL of extracts showed an increase ($p<0.05$) in cytotoxicity activity on MCF-7 and HCT 116 as compared to the untreated control cells (Figures 1 to 5).

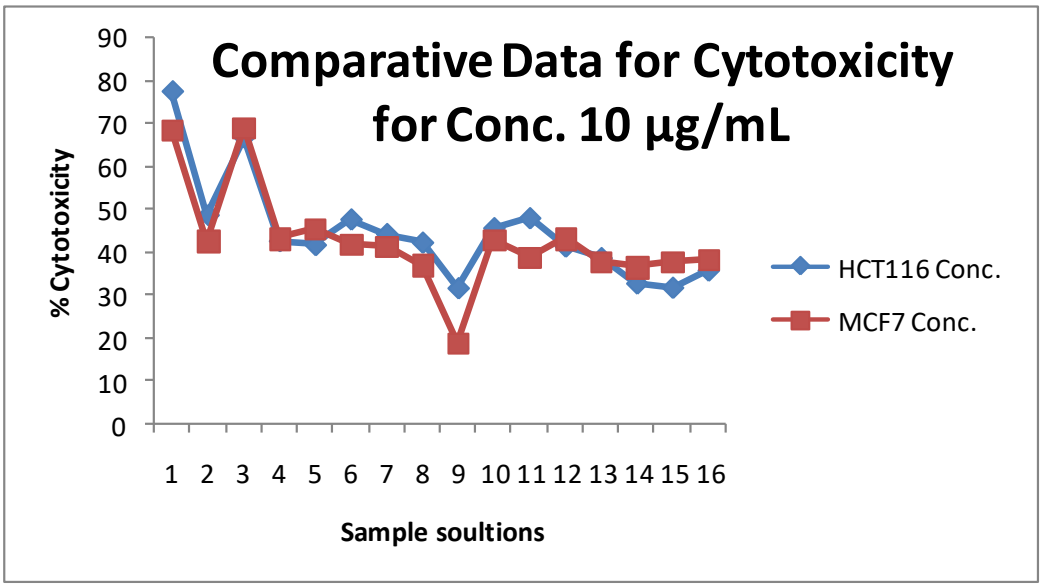

Figure. 1 In vitro anti-cancer cytotoxicity of 16 test samples for Conc. 10 µg/mL against MCF-7) cell line and HCT 116 cell lines

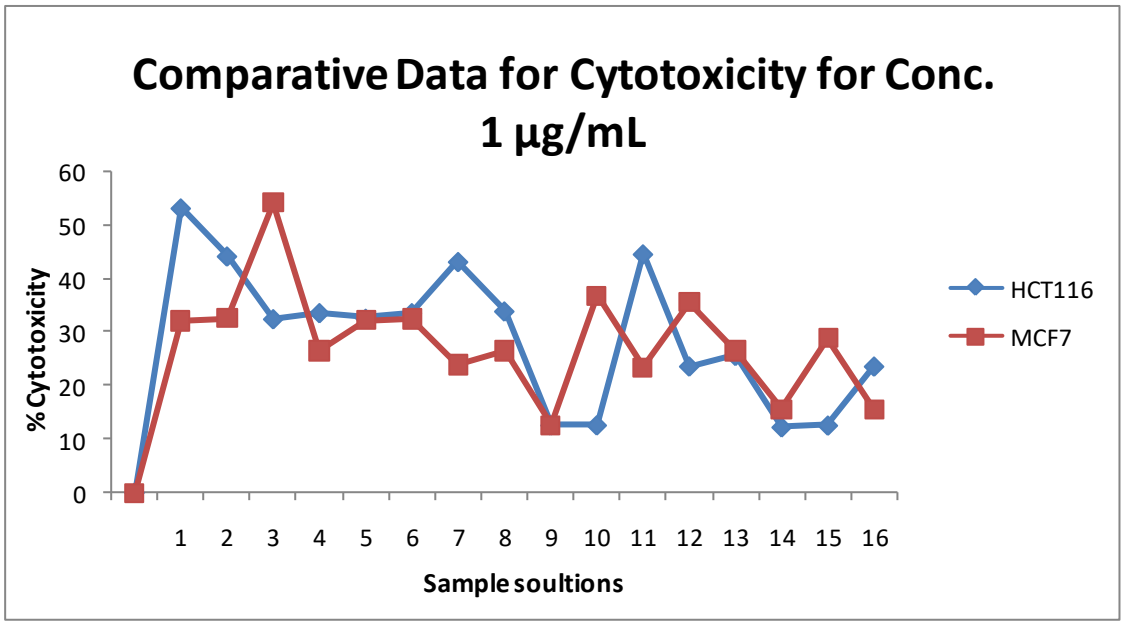

Figure. 2 In vitro anti-cancer cytotoxicity of 16 test samples for Conc. 1 µg/mL against MCF-7) cell line and HCT 116 cell lines

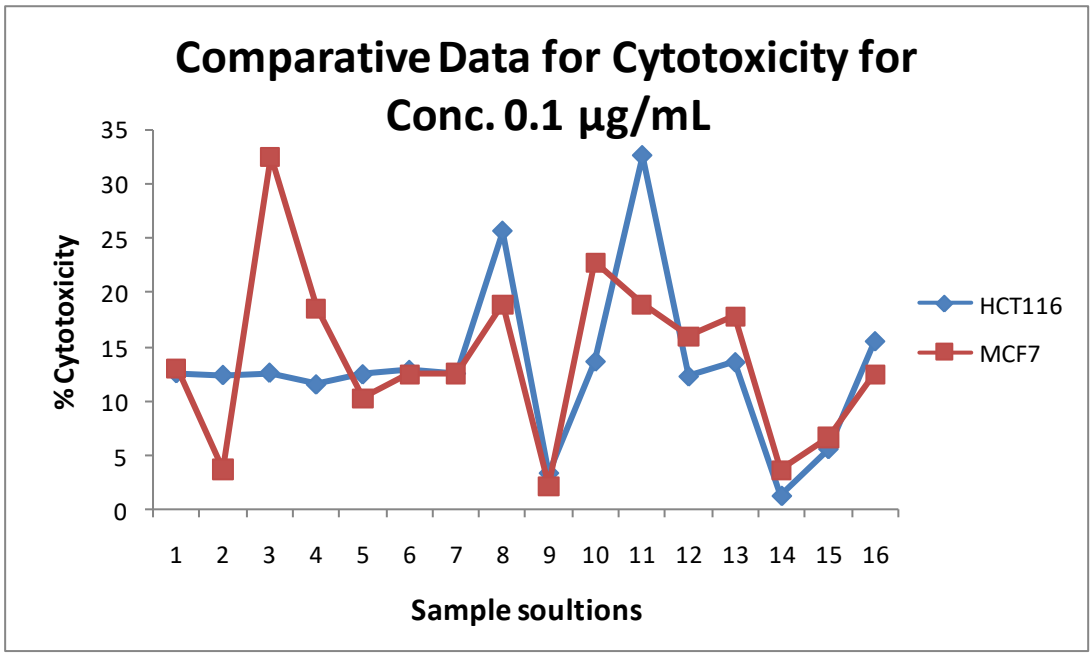

Figure. 3 In vitro anti-cancer cytotoxicity of 16 test samples for Conc. 0.1 µg/mL against MCF-7) cell line and HCT 116 cell lines

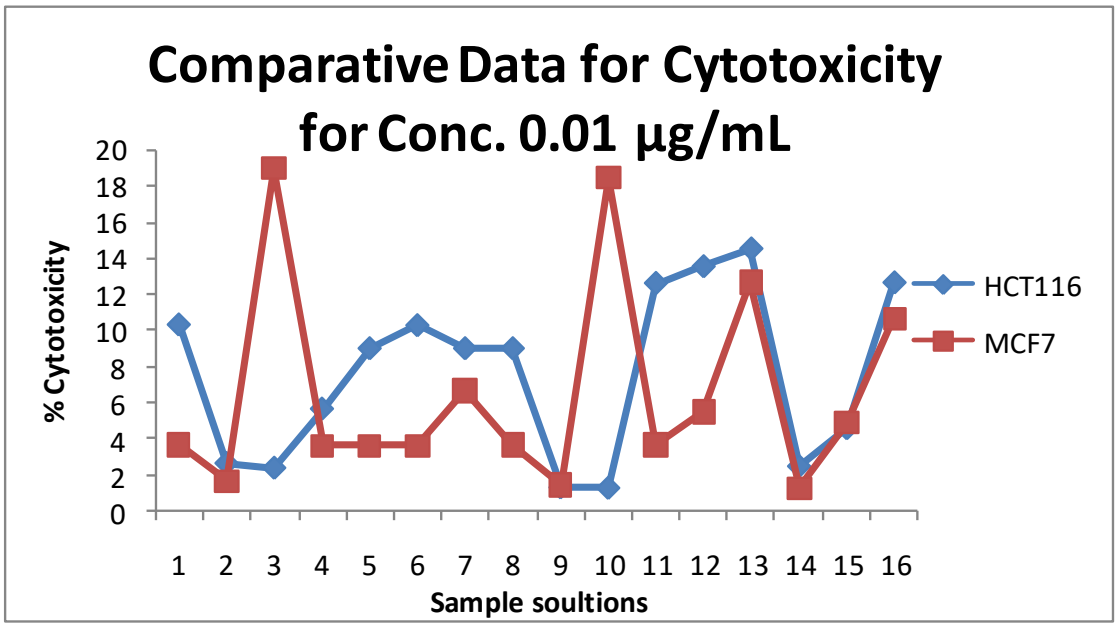

Figure. 4 In vitro anti-cancer cytotoxicity of 16 test samples for Conc. 0.01 µg/mL against MCF-7) cell line and HCT 116 cell lines

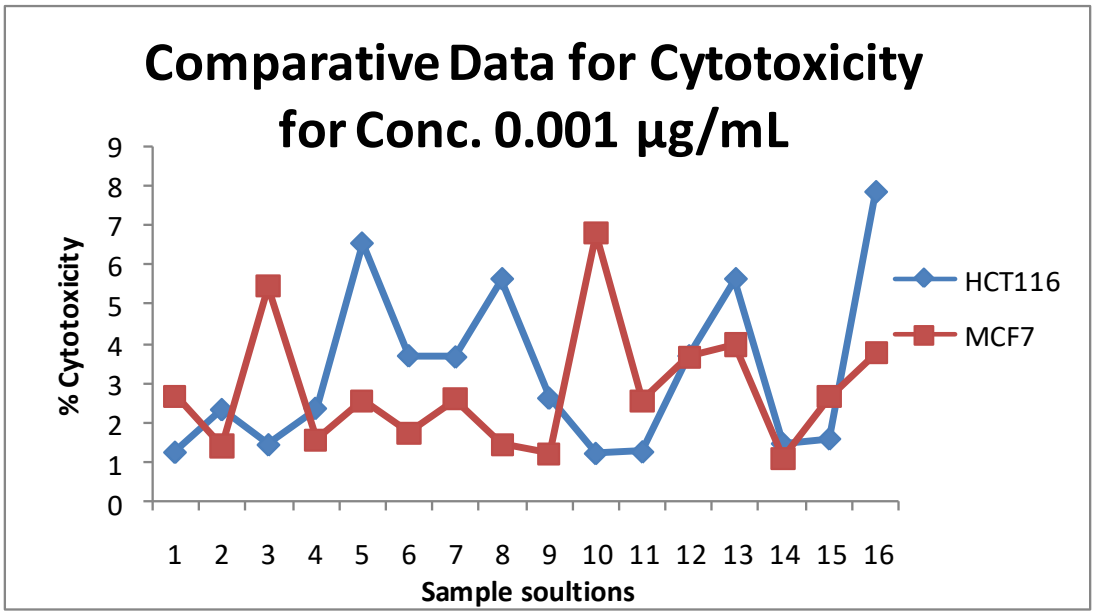

Figure. 5 In vitro anti-cancer cytotoxicity of 16 test samples for Conc. 0.001 µg/mL against MCF-7) cell line and HCT 116 cell lines

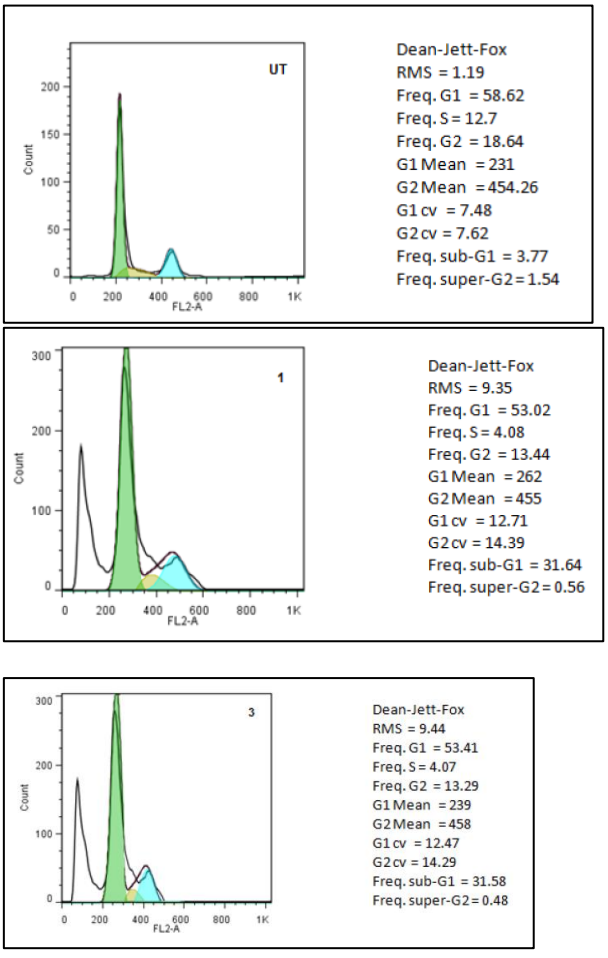

Figure 6. Cell cycle analysis on HCT116 cell lines for untreated sample comparison with sample 1 and 3

MTT test showed the concentration of aqueous and ethanol extract of extracts required for 50% inhibition of the different cell lines as follows: Sample 1 shows of MCF-7 and and HCT 116 = 0.7 μg/ml, Sample 3 shows of MCF-7 and and HCT 116 = 3 μg/ml and Sample 16 shows of MCF-7 and and HCT 116 = 0.1 μg/ml as compared to standard sample Staurosporine 0.6uM. However, according to the criteria of the American National Cancer Institute, the IC50 limit to consider a crude extract promising for further purification is lower than 30 μg/ml.

In the present study, the treatment with the standard suppressed the cell viability up to 77.45% at 1 μg/ml 66.74% at 10 μg/ml concentrations when compared to the untreated cells. The profile of cell growth after treated with is presented in Figure-1and Figure-2 respectively.

From the Figure-2, it was found that showed that in 1 μg/ml and 10 μg/ml a significant reduction in the number of viable cells at the concentration higher than 0.1 and 0.001 μg/ml against MCF-7 and HCT 116 cell lines. Therefore, the Sample 0.001 μg/ml shows 1.25% and 2.68%. The killing activity was specific toward tumor cells, as the compounds had no effect on primary cultures of healthy human cells. Cell death caused by samples was via apoptosis. Varieties of chemical compounds have been reported to protect against chemical carcinogenesis and thus, are considered to be cancer chemo preventive agents.

Sample No. 10 and 11 on HCT116 cell line were shows on 0.001 μg/ml shows1.22% and 1.26% respectively. Sample No. 9 and 14 on MCF-7 cell line were shows on 0.001 μg/ml shows1.22% and 1.11% respectively.

Besides the antiproliferative property, effects on the cell cycle profile were additionally characterized by Flow cytometry. Flow cytometry revealed that sample 1 and 3 exerted effects on the cell cycle distribution. The exposure of HCT116 cells to sample 1 and 3 resulted in an increased proportion of cells in the G2 phase and a decreased proportion of cells in the G1/M phase as compared with the control. To establish the change in the cell cycle, the quotient of S to G2/M was calculated, which increased in a dose-dependent manner.

CONCLUSION

Comparing impacts of four plant extracts tested in this research show efficacy of aqueous and alcoholic extracts for the cytotoxicity towards MCF-7 and HCT 116 cell lines with lower IC50 values compared to others (Fig. 1-5), so it can be considered as potential for cancer treatment. However according to US NCI plant screening program, crude extracts and pure compounds can be considered as cytotoxic agents against carcinoma cells if after 48-72 h incubation, they show IC50 value less than 30. So extract tested in this study shows significant cytotoxicity against MCF-7 and HCT 116 cell line, because MTT results showed that IC50 values of most of extracts are less than 30 μg/ml.

The cell cycle analysis revealed that its distribution changes in aqueous and alcoholic extracts treated MCF-7 and HCT 116 cell line. The proportion of the G2 phase increased, while the G1/M phase decreased, leading to an increase in the quotient. The change in this parameter was significant in the cases of compounds sample 1 and 3. These data suggested a perturbation of the cell cycle, blocking the transition from the S to the G2 phase. The apoptosis-induction capacity rather than necrosis induction is accepted as a key feature as a potential antitumour drug. Accordingly, in the given set of experiments, the apoptotic potentials of the tested samples were investigated.

Results indicate that there is a correlation between the this method adopted in establishing the antiproliferative and cytotoxic activity of aqueous and alcoholic extracts extract of Annona reticulata with Allium sativum, Allium fistolisum and Brassica oleraceae obtained in this study. However, this plants has shown pronounced cytotoxic activity against MCF-7 and HCT 116 cell line and will be evaluated further for the possible isolation of active anticancer compounds.

Conflict of Interests

The authors declare that they have no conflict of interests.

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