INHIBITORY MECHANISM OF *SOLANUM XANTHOCARPUM* FRUIT IN MCF-7 BREAST CANCER CELL LINE

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ABSTRACT:

Medicinal plants from a large group of economically important plants that provide the basic raw materials for indigenous pharmaceuticals. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body. The cytotoxicity studies using different dyes showed great anticancer potential of the fruit extract of *Solanum xanthocarpum*. Viability of cells measured by MTT assay, SRB assay and nuclear fragmentation. The percentage viability of MCF-7 line cells was considerably decreased with the increasing concentration of the extracts in MTT assay, SRB assay and nuclear fragmentation. These results imply that higher contents of bioactive compound and antioxidant potential of *Solanum xanthocarpum* fruit extracts can possibly act as chemopreventive agents with respect to inhibition of the growth of breast cancer cells through the induction of apoptosis. In our study , caspase 3 activity of ethonolic extract of *Solanum xanthocarpum* revealed that in the expression of caspase 3, the drug induced only about 12% to 15% caspase 3 expression when compared to the expression rate in untreated cells.

KEY WORDS: Solanum *Xanthocarpum*, MTT assay, SRB assay, Caspase 3,

Nuclear Fragmentation.

INTRODUCTION

Cancer is the third leading cause of death worldwide, preceded by cardiovascular and infectious diseases. In India, during the year 2011, it was estimated that 0.44 million died owing to cancer .This estimated cancer mortality can increase to 0.51 million by the year 2026 as a result of changes in the size and composition of the population [1].

Solanum xanthocarpum fruit yield solanocarpidine and a sterol carpesterol. Root is one of the constituents of Dasmulasava. Seed are used diuretic. Juices of berries are reported to be useful in sore throat. A decotion of the plant is used in Gnorrhea and it is also said to promote conception in females. Kantakari is reported to be useful in Kasa rog (cough) and also in Tamakwasa (Bronchail asthama).Along with these it has antifertility, antipyretic, anticancer, anti-allergic properties as well. It is considered as a most valuable herb by traditional healers in treatment of over 100 common diseases alone or in combination with other herbs. According to Ayurveda it is a bitter appetizer, laxative, antihelminthic, and useful in fever, lumbago, pain, piles, thirst, urinary and heart disease [2]. Extract of dried fruits of *S. xanthocarpum* and its combination with extract of dried fruits of *Cassia fistula* are reported to possess anti-inflammatory activity [3]. The herb is beneficial in the treatment of cardiac diseases associated with edema, since it is a stimulant to the heart and a blood purifier. It may provide an effective treatment in the prevention of post menopausal symptoms [4].

MATERIALS AND METHODS:

Collection and Extraction of *Solanum xanthocarpum* fruit

Solanum xanthocarpum was collected from Sathyamangalam hills, Coimbatore, Tamil Nadu. Authentication of plant material was carried out at the herbarium centre of Botanical Survey of India; Coimbatore, Tamil nadu, India. After collection, fruits were weighed, washed with water, sliced and dried for 20 days. The dried fruit material was ground coarsely and stored for further use. Using soxlet extractor the dried fruit material was separately extracted with ethanol for 48 hours. The filtrate was evaporated to dryness at 40˚C for further use.

MTT ASSAY: This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

Procedure: Plate cells (104 – 106 cells) in 200 ml PBS in 96-well (flat bottom).Add 20 ml of MTT solution, mix well and Incubate for 4h in 37˚C in dark. Remove aliquot for analysis; add 200 ml acidic

isopropanol and mix well. Incubate additional 1h in 37˚C in dark. Read plate in ELISA Reader – measure OD in 570nm (background wavelength is 630nm)

SRB assay: The monolayer cell culture was trypsinized and the cell count adjusted to $1.0x10^5$ cells/ml using growth medium. To each well of a 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells/well) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once and 100µl of drug dilution prepared in maintenance media was added per well in microtitre plates. The plates were then incubated at 37ºC for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, 25µl of 50% trichloro-acetic acid was added to the wells gently such that it forms a thin layer over the drug dilutions to form a overall concentration of 10%. The plates were then incubated at 4ºC for one hour. The plates were flicked; culture was washed five times with tap water to remove traces of medium, drug and serum, and was then air-dried. The air-dried plates were stained with SRB for 30 minutes. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100µl of 10mM Tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 490nm.

Determination of Apoptotic Nuclei Using Hoechast Stain: Seed $1x10^5$ cells/well in a 6 well plate having sterile cover slip and the total volume of media made upto 2 mL per well. This was incubated at 37^0C for 12 hours in a CO_2 incubator. The MCF-7 line cells were treated with test drug (250 ug/mL) and Berberine (40 uM) and incubated at 37^0C, in a CO_2 incubator for 24 hrs. The nuclear stain (Hoechst stain) was prepared by mixing 10mg/mL stock in distilled water. Working solution of 1:2000epared in PBS. The spent media is discarded from all the wells and washed with D-PBS (500 uL). Add 2% PFA to the wells and incubate at RT for 10 min. Discard the spent PFA and wash the cells with D-PBS (500 uL), twice. Discard D-PBS and add staining solution onto the cover slips. Incubate for 5-10mins in dark. Prepare permanent slides, using glycerine. Observe for the cells under fluorescence microscope.

Caspase 3 Activity:

Active Caspase-3 Staining Protocol

Determine total amount of experimental samples (tests) and calculate the amount of BD Perm/Wash™ buffer (1X) and antibody you will need so that each test will have 100 µl BD Perm/Wash™ buffer (1X) and 20 µl antibody. Dilute the needed amount of BD Perm/Wash™ buffer (10X) 1:10 in distilled water prior to use. Note: Precipitate may be occasionally observable with the BD Perm/Wash™ buffer (10X) which will not affect performance of the buffer. The precipitate may be removed by filtering the 1X solution through a 0.45 µm filter. Wash cells twice with cold 1X PBS, then resuspend cells in BD Cytofix/Cytoperm™ solution at a concentration of 1x10^6 cells/0.5 ml. Incubate cells for 20 min on ice. Pellet cells, aspirate, and discard BD Cytofix/Cytoperm™ solution; wash twice with BD Perm/Wash™

buffer (1X) at a volume of 0.5 ml buffer/1x10^6 cells at room temperature. Resuspend cells in the above calculated BD Perm/Wash™ buffer (1X) plus antibody and incubate for 30 min at room temperature. Wash each test in 1.0 ml BD Perm/Wash™ buffer (1X), then resuspend the test in 0.5 ml BD Perm/Wash™ buffer (1X) and analyze by flow cytometry.

RESULT AND DISCUSSION

Invitro cytotoxic effect of *Solanum xanthocarpum* fruit extract against MCF-7 cell line

The relationship between apoptosis and cancer has been over and again stated, suggesting that the related processes of neoplastic transformation, progression and metastasis involve the alteration of normal apoptotic pathways. Apoptosis can provide a number of clues with respect to effective anticancer therapy, and many chemotherapeutic agents reportedly exert their antitumor effects by inducing apoptosis in cancer cells. Apoptosis is a strictly regulated pathway responsible for the ordered removal of superfluous, aged, and damaged cells. It not only plays an important role in the development and maintenance of tissue homeostasis, but it also represents an effective mechanism by which harmful cells can be eliminated. Morphological hallmarks of this process includes loss of cell volume, hyperactivity of the plasma membrane, and condensation of peripheral heterochromatin, followed by cleavage of the nucleus and cytoplasm into multiple membrane - enclosed bodies containing chromatin fragments [5].

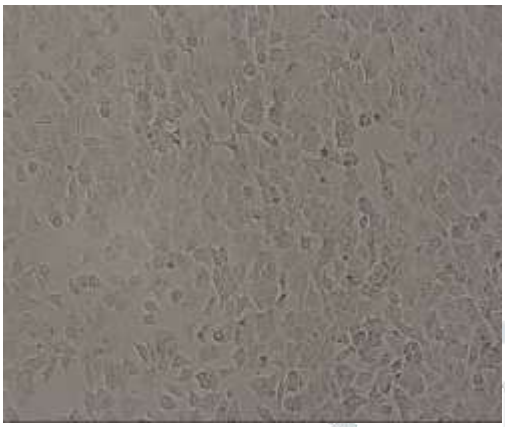
Cytotoxic evaluation of the ethanolic fruit extract by inducing apoptosis in MCF 7 cells and then studying their percentage decreased viability was conducted using different dyes like 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; Sulforhodamine B, Hoechast in MCF-7 cell line. The results of the same have been tabulated in **table 1.**

Table No: 1 MTT Assay in MCF-7 cell line treated with *Solanum xanthocarpum* fruit extract

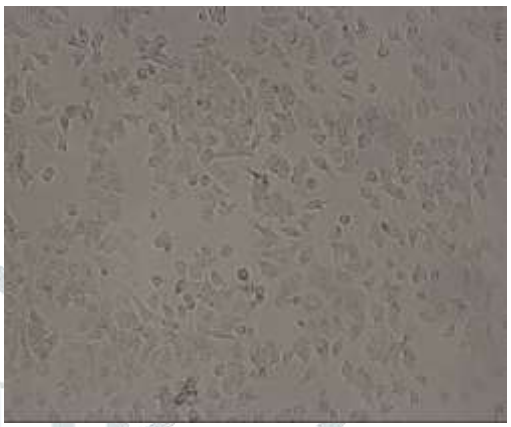
Concentration of the ethanolic fruit extract (µg/ml)	% cell viability	IC $_{50}$ value (µg/ml)
50	90.20	197.71µg/ml
150	58.90	
250	31.18	
350	9.55	
450	0.23	

The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth [6].

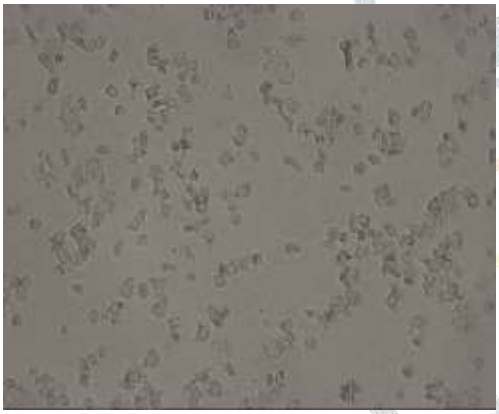
Figure No: 1 MTT assay in MCF – 7 cell line treated with different concentration of fruit extract.



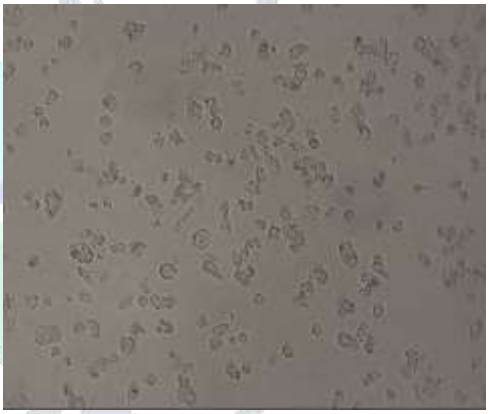
Untreated cell



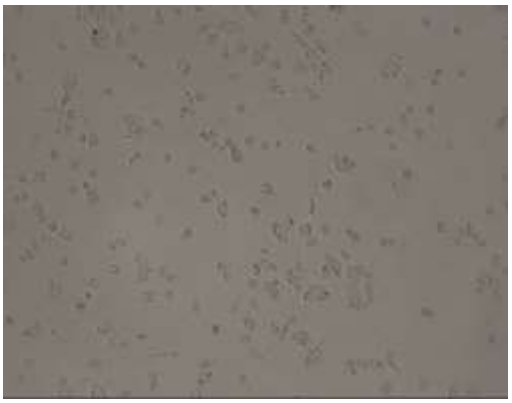
50µM Extract treated cells



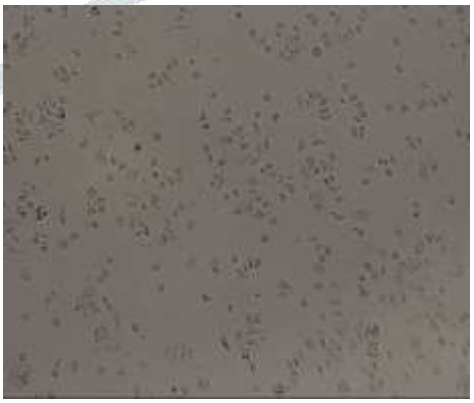
150 µM Extract treated cells



250µM Extracttreated cells



350 µM Extract treated cells



450µM Extract treated cells

We can easily see from the **figure 1** where a comparative percentage inhibition is shown between the varying concentration of the plant extract. The assay with MTT dye showed an IC_{50} value of

197.710µg/ which indicates that at a concentration of around 200µg/ml the viability of cells of the cancerous MCF-7cell line was more than 50% decreased showing the high potency of the fruit extract. The percentage growth inhibition was found to be increasing with increasing concentration.

SULFORDAMINE B ASSAY

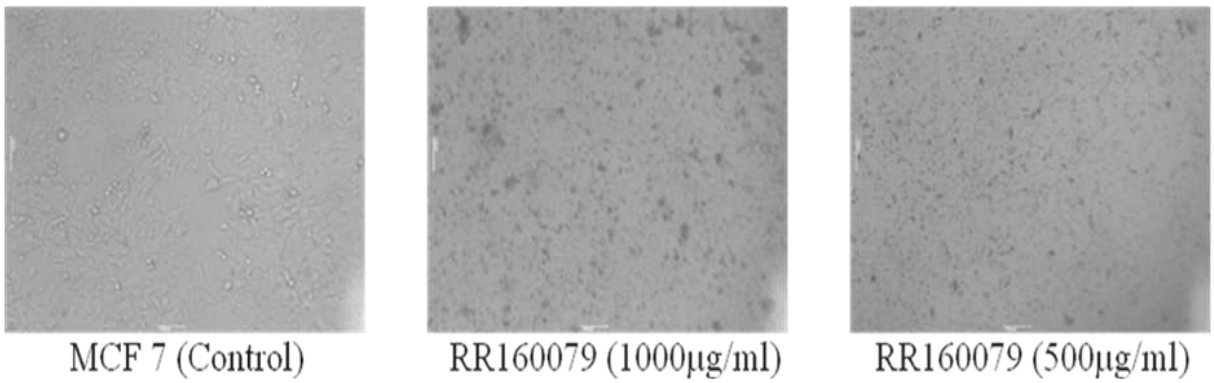
Cell toxicity study result using MTT dye was validated using Sulfordamine (SRB) dye. The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content [7]. It has been found to be useful and a sensitive method for measuring drug cytotoxicity in culture. The results obtained have been shown in table 2.

Table No: 2 Cytotoxic effect of fruit extract against MCF-7 cell line

Conc. of the fruit extract (µg/ml)	% cytotoxicity	IC_{50} Value (µg/ml)
1000	73.54 ± 1.9	117.88 ± 4.6
500	68.77 ± 2.6	
250	58.08 ± 1.2	
125	55.09 ± 3.5	
62.5	12.28 ± 2.2	

Just like in the MTT assay even SRB assay showed cytotoxicity of the MCF-7 cell line when treated with the ethanolic fruit extract. The IC_{50} Value was 117.88 and with increasing concentraion the percentage of viability of the MCF-7 cells dropped considerably. This is in consistence with the results obtained in other studies [8].

Figure No:2 SRB assay of MCF-7 cell line treated with *Solanum xanthocarpum* fruit extract.



MCF-7 Control **1000µg/ml Treated cell** **500µg/ml Treated cells**

Determination of Apoptotic nuclei using Hoechst dye

To further confirm the cytotoxic effect of the fruit extract, cytotoxic assay using another stain – Hoechast stain was used to study the nuclear fragmentation when MCF-7 cell are treated with the *Solanum xanthocarpum* fruit extract. Hoechast stain was taken instead of DAPI because Hoechast stain is considered to be much less toxic than DAPI[9]. Other than this the additional ethyl group of the Hoechast dyes renders them more cell-permeable.

Apoptosis is a form of programmed cell death characterized by cytoplasmic condensation, plasma membrane blebbing and nuclear pycnosis, leading to nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal size fragments. The detection of apoptosis in cultured cells relies heavily on techniques involving the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis. Many methods have been developed in order to detect apoptosis in vitro. In this method the staining procedure is performed with fluorescent substances like Hoechst dye. As florescent dyes are bound to DNA, the chromatin, and as such, the nucleus of the cell becomes visible. Fluorescence systems are considerably more expensive than light microscopy, but if used in cellular culture studies, they may be beneficial in differentiating live cells from dead cells. The basic principle of this method in identifying viability is to determine whether the plasma cellular membrane is intact or not. Fragmented DNA remains in the supernatant and can be used directly for quantitation using the fluorescent dye Hoechst 33258 [10].

Figure 3 shows untreated MCF 7 cells where regular apoptosis is seen . Figure 4 shows MCF-7 cell line treated with 200µg/ml of the fruit extract. Few cells with nuclear fragmentation is seen showing enhanced apoptosis. Figure 5 shows MCF-7 cell line treated with a higher concentration of 250µg/ml of the fruit extract. This higher concentration causes more cells to fragment, around 7-8 cells . The fragmented cells have been marked with an arrow for ease in identification.

Figure No.3 MCF-7 cell line Untreated cell

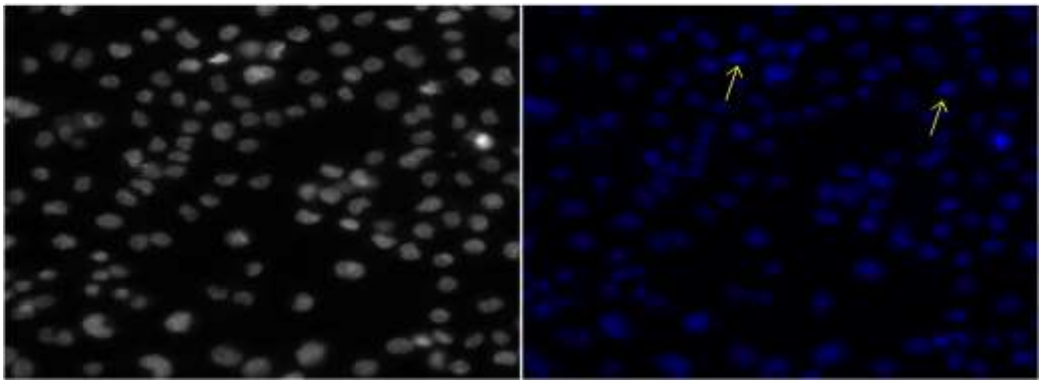


Figure 4: Apoptosis in MCF-7 Cell line treated with 200µg/ml of *Solanum xanthocarpum*fruit extract

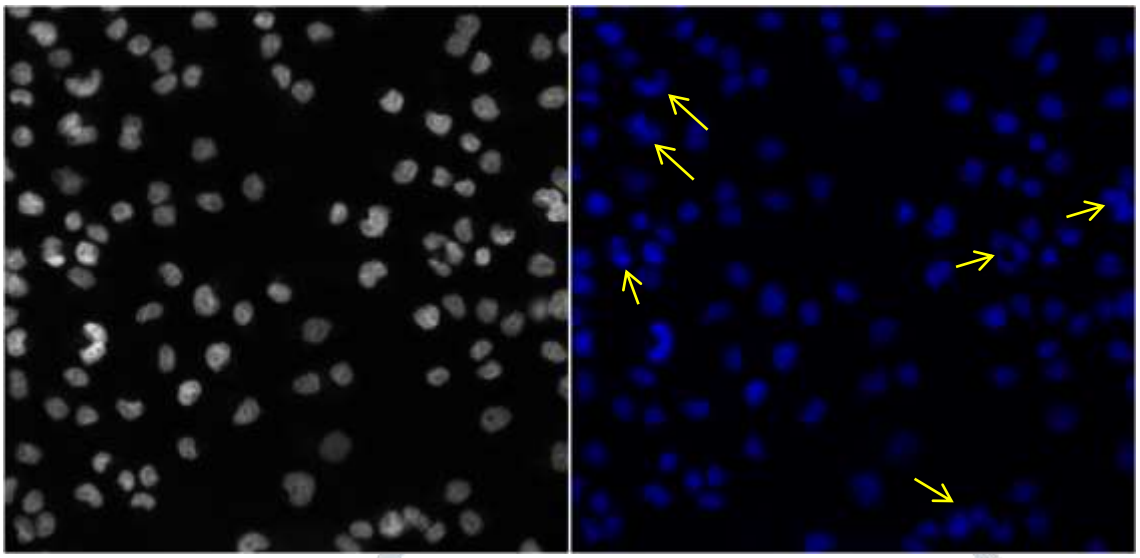
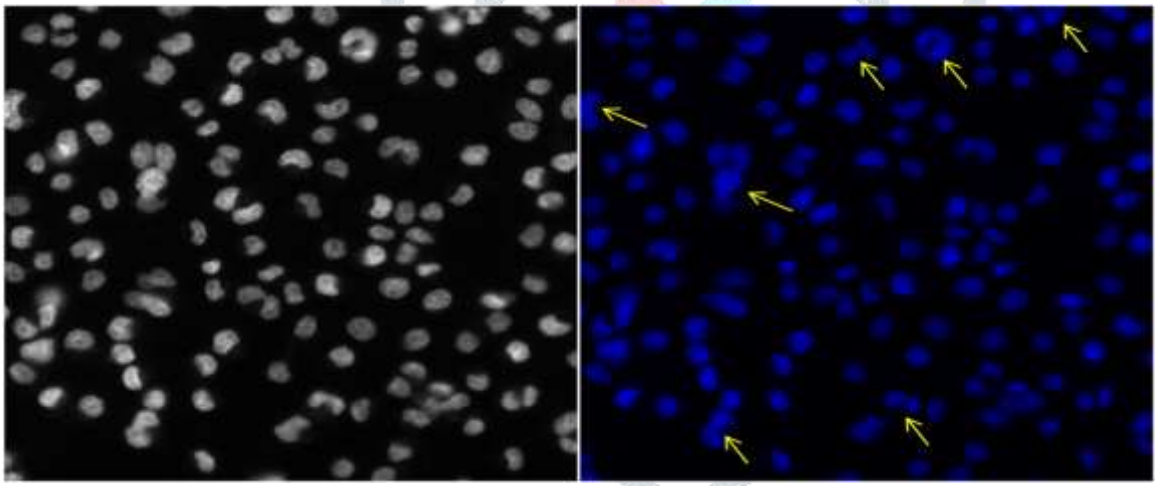


Figure No: 5- Apoptosis in MCF-7 Cell line treated with 250µg/ml of *Solanum xanthocarpum* fruit extract



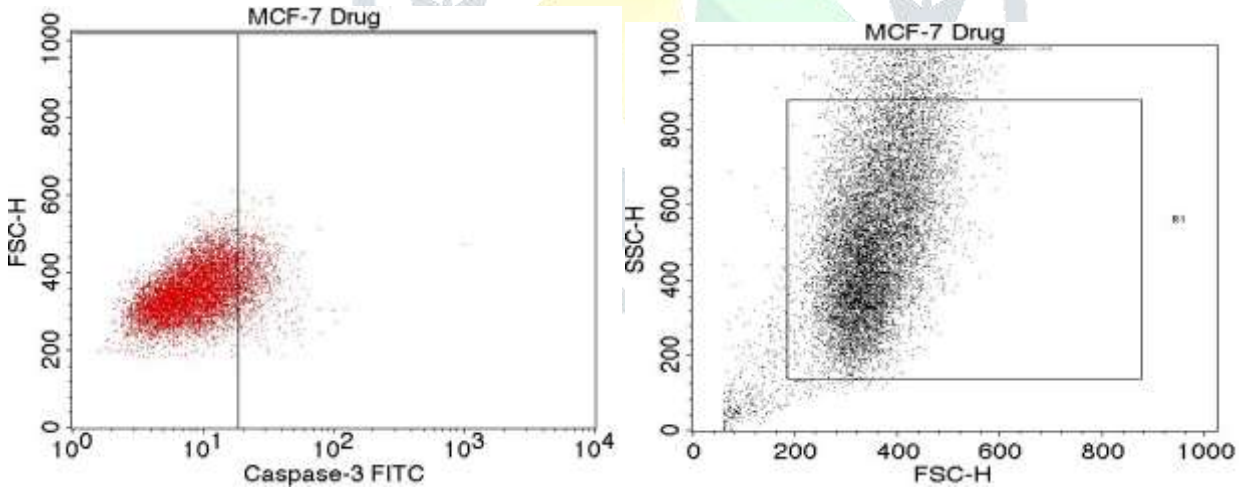
MCF-7 cell treated with *Solanum xanthocarpum* fruit extract showed cytotoxicity at a concentration of 250µM with some cells showing plasma membrane breakage which have been marked with an arrow. Even at a lower concentration of 200µM also some of the cells showed nuclear fragmentation suggesting apoptosis can be induced with the help of *Solanum xanthocarpum* fruit extract. The results got using Hoechst stain has been shown in figure 3,4,5 and shows a positive corellation between the concentration of the fruit extract used and nuclear fragmentation. It supports our initial hypothesis that *Solanum xanthocarpum* fruit extract can induce apoptosis in breast cancer cells and hence can be used for further drug evaluation.

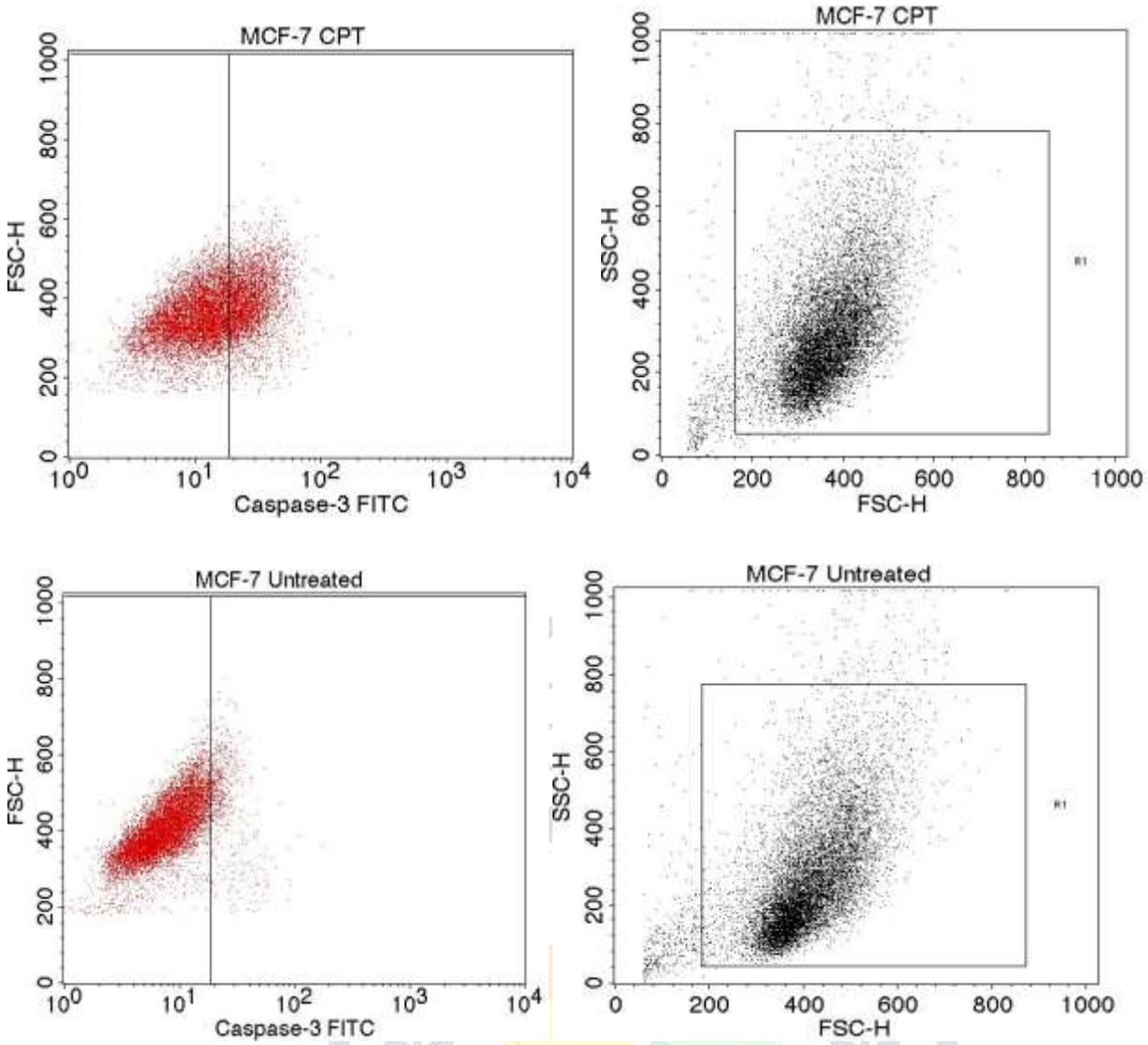
CASPASE 3 ACTIVITY:

The caspase family of cysteine proteases plays a key role in apoptosis and inflammation. Caspase-3 is a key protease that is activated during the early stages of apoptosis and, like other members of the caspase family, is synthesized as an inactive pro-enzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. The processed forms of caspases consist of large (17-22 kDa) and small (10-12 kDa) subunits which associate to form an active enzyme. Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which is derived from the 32 kDa pro-enzyme. Active caspase-3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm, e.g., D4-GDI and Bcl-2, and in the nucleus (e.g. PARP). This antibody has been reported to specifically recognize the active form of caspase-3 in human and mouse cells. It has not been reported to recognize the pro-enzyme form of caspase-3[11].

Flow cytometric analysis of apoptotic and non-apoptotic populations for active caspase-3. MCF-7 cell line were left untreated (left panel) or treated for 4 hr with camptothecin (right panel) to induce apoptosis. Cells were permeabilized, fixed, and stained for active caspase-3. Cells were then analyzed by flow cytometry. Untreated cells (left panel, M1) were primarily negative for the presence of active caspase-3, whereas greater than one third of the treated cells were positive for active caspase-3 staining (right panel, M2), which shown in figure 6.

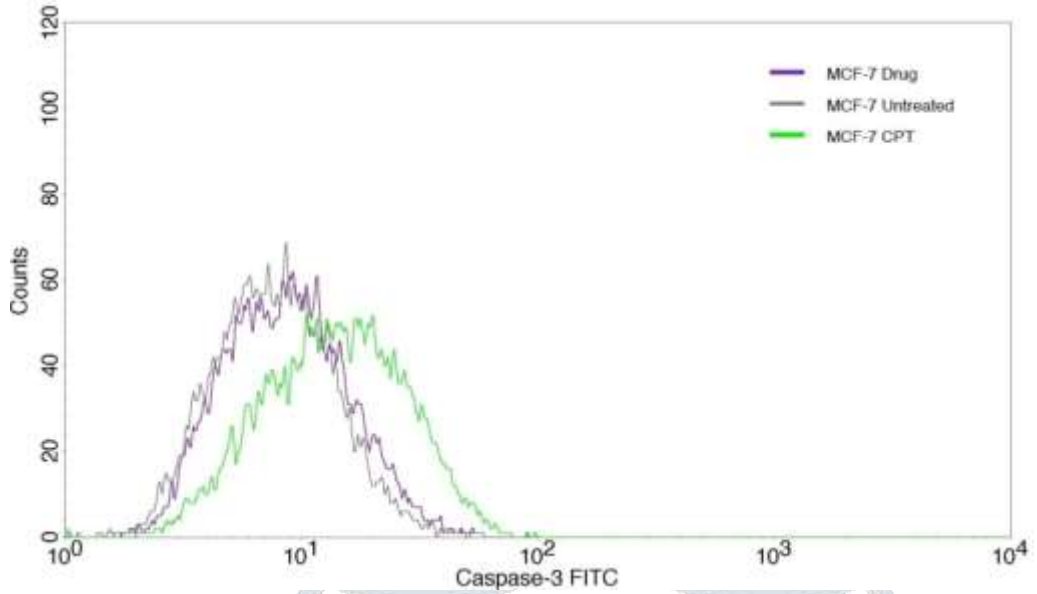
Figure 22: Histogram of caspase 3 expression





The expression of caspase 3 is compared with the control and drug (*Solanum xanthocarpum)* with different peaks through flouresent intensity. The caspase 3 expression was compared to the expression rate in untreated cells. This difference could be found in the histogram statistical data of each sample. The result was interpreted by comparing the cell count and concentration of drug in figure 7.

Figure 7: Overlay of Expression of caspase 3



Activation of the caspase-3 pathway is a hallmark of apoptosis and can be used in cellular assays to quantify activators and inhibitors of the "death cascade." The response is both time and concentration dependent,suggesting that multiple pathways play a role in triggering the caspase 3 activation.

CONCLUSION:

In the present study caspase 3 activity of ethanolic extract of *Solanum xanthocarpum* revealed that in the expression of caspase 3, the drug induced only about 12% to 15% caspase 3 expression when compared to the expression rate in untreated cells. In conclusion, *Solanum xanthocarpum* fruit may exhibit an antiproliferative effect by induction of apoptosis. As apoptosis has become a new therapeutic target in cancer research,these results confirm the potential of *Solanum xanthocarpum* as an agent of chemotherapeutic and cytotoxic activity in human breast cancer cells.

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