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Effect of Apoptosis on Human Breast Cancer Cell Line (MCF-7 and MDA-MB231) Using *Curcuma longa*

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Abstract
Cancer cells usually have increased cell proliferation; have ability to survive in unique environment and decreased apoptosis. Decreased apoptosis gives the cancer cells a survival advantage. All cells showed a small base line apoptotic level. Treatment of cancer cells with curcumin significantly increased apoptosis by Caspase 3/7 assay, Annexin IV assay and tunel assay suggesting that both early and late apoptotic events are triggered by curcumin. Increased apoptosis by curcumin may be used to kill the cancer cells and thereby help in treatment of cancer. The overall results obtained in the study point out that the active molecule present in *Curcuma longa* have considerable consequence on the survival of human breast cancer cell lines. Finally, it is concluded that curcuminoids are a group of phenolic compounds isolated from the rhizome of *Curcuma longa* has various pharmacological properties. They exhibit growth inhibitory effects on a broad range of tumors and act as potent anticancer, anti-inflammatory and analgesic agent and more research should be carried out and this data should be made accessible for both health care providers and patients for safe anticancer treatments.

Keywords
Curcumin, cancer, apoptosis, therapy.

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INTRODUCTION
Curcumin is the medicinal extract of a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae bearing many rhizomes on its root system which are the source of its culinary spice known as turmeric. The plant belongs to the genus: Curcuma and species: *longa*. Its scientific name is *Curcuma longa* Linnaeus and it is native in southeast India. It has gained access to many other parts of the world as an exotic variety. Curcumin (diferuloylmethane) is a polyphenol derived from the rhizome of the turmeric plant, *Curcuma longa*. It is a non-nutritive food chemical used as a flavouring, coloring agent and as a food preservative. It has been consumed for centuries as a dietary spice regularly at a reasonable amount by people in Asian countries. Modern therapist attention started to revolve round the turmeric species for its wide use in traditional medicine as a effective antioxidant, anti-inflammatory, analgesic and anticancer agent.

Several pilot studies showing suppression in cellular transformation, proliferation, invasion, angiogenesis, and metastasis have further kindled their interest. Being a blood-brain barrier permeable substance exhibiting a diverse range of actions including free radical scavenging activity *in vitro* and *invivo* with cardio and neuro-protective effects added strength to the world-wide attention.

Cancer is an abnormal growth and proliferation of cells. It is a fearsome disease because the patient suffers pain, disfigurement and loss of many physiological processes ending in fatality in most cases. The alarming facet of cancer is that it may occur at any time at any age in any part of the body. It is caused by a complex, poorly understood interplay of genetic and environmental factors. It continues to represent the largest cause of mortality in the world and kills annually about 3500 per million populations around the world. Although more anticancer drugs are in active development with many of them under clinical trials, there is a pressing necessity to develop much more efficient and less toxic drugs especially from the plant kingdom to offer a cure for cancer patients. The overall aims and objectives of the current study is to observe the following end results: Apoptosis effect of curcumin using MCF-7 and MDA-MB231breast cancer cell line and Caspase 3/7 assay, Annexin IV assay and Tunel assay. Curcumin increases breast cancer cell sensitivity to cisplatin by decreasing FEN1 expression (Jiao Zou *et al*., 2018).

MATERIALS AND METHODS

Cancer cell lines

Human breast cancer cell lines (MCF-7andMDA-MB-231) and CRL-714 (normal breast cell) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Literature supplied along with these cell lines state that these cells were characterized by mycoplasma detection, DNA –Fingerprinting, isoenzyme analysis and cell vitality detection. These cells were maintained in cell culture media and conditions as per the recommendations of American Type Culture Collection centre. MCF-7 cells were grown in DMEM medium containing 10% (V/V) FBS without antibiotics at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. MDA-MB-231 cells were grown in L-15 medium containing 10% (V/V) FBS without antibiotics at 37°C.

Cells and cell culture

Human breast carcinoma cells, MCF-7, were cultured in RPMI1640 medium supplemented with 0.22% sodium bicarbonate, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin and incubated at 37°C in 5% CO_2. Curcumin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mM and was diluted to the required concentration with RPM I1640medium immediately before use. Cells grown in medium containing an equivalent final volume of DMSO (final concentration <0.01%, V: V) served as control.

Curcumin treatment

Curcumin could be purified from crude curcumin (a mixture of curcuminoid) by column chromatography. It is a technology that uses different organics such as activated carbon, activated clay or silica with mixtures of solvent like dichloromethane/ acetic acid or methanol/chloroform/dichloromethane, and ethanol/methanol mixtures as eluents to yield fractions. Among these, macroporous resin column chromatography is widely adopted. Macroporous resin was invented in 1964, with holes of 100-1000 nm distributed on the surface area. Combined with styrol and propionate, phenylethylene forms a porous polymer with a screening function (Wang *et al*., 2015). A recent paper introduced a novel method for curcumin preconcentration. The molecularly imprinted polymers (MIPs) based on magnetic multiwalled carbon nanotubes possessed excellent selectivity toward curcumin. Other advanced techniques based on functional comonomers, such as thermo responsive magnetic molecularly imprinted polymers (TMMIPs), have also been developed with high reproducibility and stability for selective curcumin extraction (Wulandari *et al*, 2015 and Zhang *et al*., 2015).

The test cell lines were grown to a density of approximately 75% and were then treated with test substance curcumin at different concentrations for the indicated times. The control cell lines were incubated with DMSO without curcumin at the same final concentration. Treatment of cancer cells with curcumin significantly increased apoptosis by Annexin V, Caspase 3/7 and tunnel assay suggesting that both early and late apoptotic events are triggered by curcumin. Increased apoptosis by curcumin may be used to kill the cancer cells and thereby help in treatment of cancer.

RESULTS

Apoptosis:

Cancer cells usually have increased cell proliferation; have ability to survive in unique environment and decreased apoptosis. Decreased apoptosis gives the cancer cells a survival advantage. All cells showed a small base line apoptotic level.

Caspase 3/7 assay

Apoptosis, or programmed cell death detection was done on two human breast cancer cell lines (MCF-7 and MDA-MB231) with reference to normal cell CRL-714 (Breast cell) using Caspase 3/7 assay. The result shows that curcumin increases the apoptotic activity and it results in treating the cancer cell.

Annexin V assay

The apoptotic activity of two human breast cancer cell lines (MCF-7 and MDA-MB231) with reference to normal cell CRL-714 (Breast cell) using fluorochrome-labelled Annexin V after 3 hrs exposure in 40 µg/ml concentrations. The result suggests that both early and late apoptotic events are triggered by curcumin and as a result it reduces the cancer intensity and helps to recover.

Tunel assay

The study was conducted to evaluate the apoptotic activity by Tunel assay in two human breast cancer cell lines (MCF-7 and MDA-MB231) with reference to normal cell CRL-714 (Breast cell). In this assay it is evident that increased apoptosis by curcumin may be used to kill the cancer cells and thereby help in treatment of cancer.

DISCUSSION

Breast cancer is among the most common malignant tumors. It is the second leading cause of cancer mortality among women in the United States. Curcumin, an active derivative from turmeric, has been reported to have anticancer and chemoprevention effects on breast cancer. Curcumin exerts its anticancer effect through a complicated molecular signaling network, involving proliferation, estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2) pathways. Experimental evidence has shown that curcumin also regulates apoptosis and cell phase–related genes and microRNA in breast cancer cells. Yiwei Wang *et al*., 2016 reviewed the recent research efforts in understanding the molecular targets and anticancer mechanisms of curcumin in breast cancer. As a cancer chemosensitizing agent, curcumin can effectively eliminate resistance to many chemotherapy drugs, including cisplatin, mitomycin C and paclitaxel, in a wide variety of tumor cell types (Lu *et al*., 2017 and Kumar *et al*., 2017).

Apoptosis:

The process of apoptosis is highly complex and sophisticated, involving an energy-dependent cascade of molecular events. So far, research directs that there are two key apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). Apoptotic cells also show several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition that together result in the characteristic structural pathology (Hengartner, 2000). Caspases are generally expressed in an inactive proenzyme form in most cells and once activated can often trigger other procaspases, allowing initiation of a protease cascade. Some procaspases can also aggregate and autoactivate. This proteolytic cascade, in which one caspase can activate other caspases, upsurges the apoptotic signaling pathway and thus leads to rapid cell death (Elmore, 2007). Curcumin inhibits intracellular fatty acid synthase and induces apoptosis in human breast cancer MDA-MB-231 cells (Huijin Fan *et al*., 2016).

Caspase 3/7 assay

Caspases are a family of endoproteases that play a vital role in retaining homeostasis through regulating cell death and they are found to have proteolytic activity which is able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighbouring amino acids. Once caspases are activated, there seems to be an irretrievable commitment towards cell death. At present, ten major caspases have been identified and generally categorized into initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) reported by Teiten *et al.,* 2010 and Li *et al.,* 2014. Watson *et al.,* (2010) indicated that curcumin exhibited time- and dose-dependent cytotoxicity against monolayer cultures of ovarian carcinoma cell lines with the activation of caspase-3 and caspase-8 system. Wu *et al.,* (2011) observed that curcumin increased the expression of Caspase-7 and caspases-9 mRNA, but not caspase-8, indicating that curcumin induces apoptosis through the intrinsic pathway and Caspase-3 activation, rather than the extrinsic pathways in the study on the effects of curcumin on cell growth and apoptosis in the human NPC cell line CNE-2z. Involvement of caspase-3 was further confirmed by using a caspase-3 specific inhibitor.

Gogada *et al.,* (2011) demonstrated that curcumin treatment of cancer cells caused dose- and time-dependent caspase 3 activation, which is required for apoptosis and was confirmed using the pan-caspase inhibitor. Analyses of curcumin-treated THP-1 cells using caspase-3/7 activity and propidium iodide staining revealed that curcumin induced THP-1 cell death via apoptotic pathway. The results showed the activation of caspases by curcumin started at 3 hours post-treatment, followed by the degradation of PARP-1.The data suggest that curcumin concentration-dependently induces THP-1 cell apoptosis through both the extrinsic and intrinsic apoptotic pathways (Yang *et al.,* 2012). The apoptosis was confirmed by caspase-3 activity study (Kaushik *et al.,* 2012). MCF-7 cells lack caspase-3 which is the main executioner caspase of cell death. Caspase-7 is similar to caspase-3 which works on most of the substrates of caspase-3. In absence ofcaspase-3, caspase-7 is known to take over the function of caspase-3 (Singh *et al.,* 2013). Curcumin acting as pro-oxidant, effectively raised the cell's oxidative status beyond a threshold limit inducing apoptosis in leukemic cells. Increase in caspase 9 and caspase 3 activities in post curcumin treatments as compared to untreated control cells were noticed in JURKAT cells (Gopal *et al.,* 2014).

Annexin V assay

Annexin V is a recombinant phosphatidylserine-binding protein that interacts powerfully and precisely with phosphatidylserine residues and can be used for the revealing of apoptosis (Arur *et al*., 2003). Loss of plasma membrane symmetry is one of the earliest features of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, there by exposing PS to the external cellular environment. Annexin V is a 35–36 kDaCa2+-dependent phospholipid-binding protein with high affinity for PS, and binds to exposed apoptotic cell surface PS. Annexin V can be conjugated to fluorochromes while retaining its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis (Casciola-Rosen *et al.,* 1996; Van Engeland *et al.,* 1996; Vermes *et al.,* 1995). PS translocation precedes the loss of membrane integrity, which accompanies the later stages of cell death. Therefore, staining with Annexin V is typically used for identification of apoptotic cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, cells that are considered viable are Annexin V negative, while cells that are in early and late apoptosis are Annexin V positive (Hingorani *et al.,* 2011).

Tunel assay

The study of DNA damage holds an extensive interest within both basic and applied fields of research. Revealing the mechanisms pertaining to the generation of DNA damage, and the consequences of this damage, will have a huge impact on multiple fields of scientific research and will eventually lead to a better understanding of human disease and treatment. One of the most commonly used methods for detecting DNA damage *in situ* is TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining. Initially, this method was described as a method for staining cells that have undergone programmed cell death, or apoptosis, and exhibit the biochemical hallmark of apoptosis – inter nucleosomal DNA fragmentation. Now TUNEL staining has nearly universally been adopted as the method of choice for detecting apoptosis *in situ* as the method relies on the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate labelled UTP into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single stranded DNA (Gavrieli *et al.,* 1992; Arends *et al.,*1990; Bortner *et al.,*1995; Kerr *et al.,*1972; Loo and Rillema,1998; Ansari *et al.,* 1993; Loo, 2002, Wyllie, 1980).

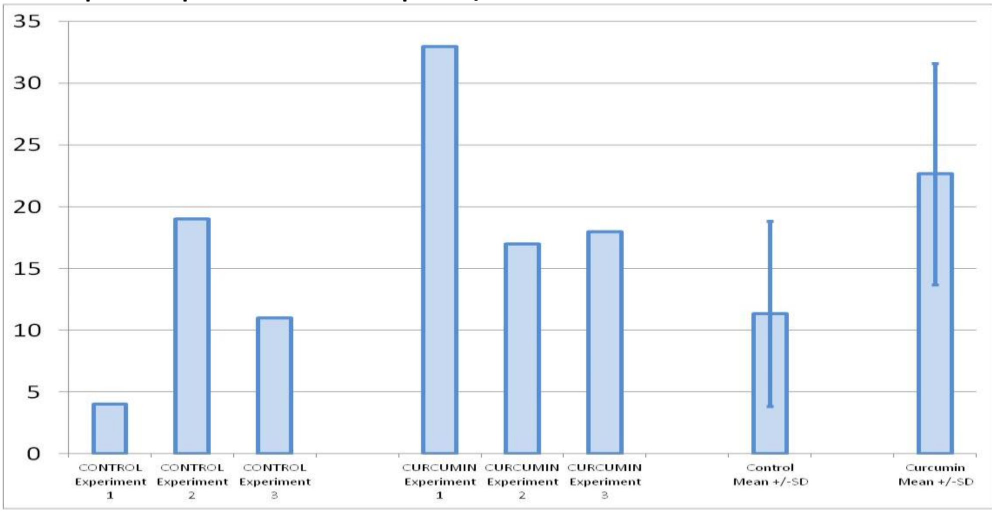
CONCLUSION

Treatment of cancer cells with curcumin significantly increased apoptosis by Annexin V, Caspase 3/7 and tunel assay suggesting that both early and late apoptotic events are triggered by curcumin. Increased apoptosis by curcumin may be used to kill the cancer cells and thereby help in treatment of cancer. Finally, it is concluded that curcuminoids are a group of phenolic compounds isolated from the rhizome of *Curcuma longa* has various pharmacological properties. They exhibit growth inhibitory effects on a broad range of tumors and have recently been shown to act as potent anticancer, anti-inflammatory and analgestic agent.

Caspase 3/7 measurement results of breast cancer cell lines and normal breast cell

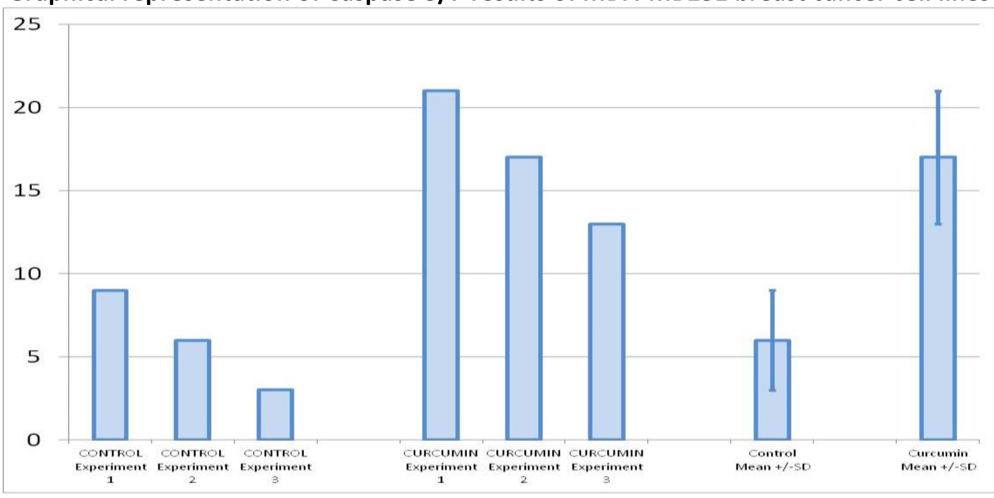
		Percent cells with Caspase 3/7 of breast cancer cell lines (MCF7 and MDA-MB231)		Percent cells with Caspase 3/7 of normal breast cell (CRL-714)
Control	Experiment 1	4	9	11
	Experiment 2	19	6	12
	Experiment 3	11	3	8
	Mean	11.33	6.00	10.33
	SD	7.51	3.00	2.08
Curcumin (40 µM; 3 hours)	Experiment 1	33	21	30
	Experiment 2	17	17	18
	Experiment 3	18	13	17
	mean	22.67	17.00	21.67
	SD	8.96	4.00	7.23

Graphical representation of Caspase 3/7 results of MCF 7 breast cancer cell lines



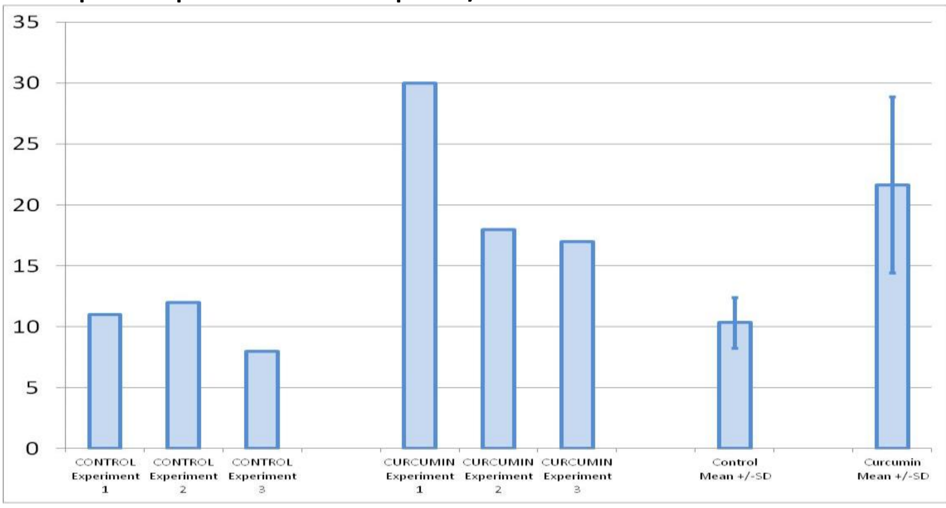
Y axis: Percent cells with Caspase 3/7 expression
X axis: Treatment groups

Graphical representation of Caspase 3/7 results of MDA-MB231 breast cancer cell lines



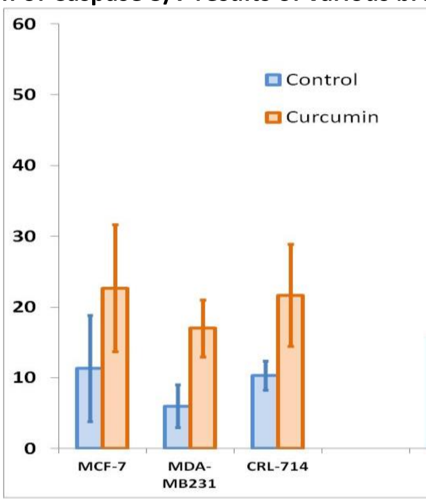
Y axis: Percent cells with Caspase 3/7 expression
X axis: Treatment groups

Graphical representation of Caspase 3/7 results of CRL-714 normal breast cell



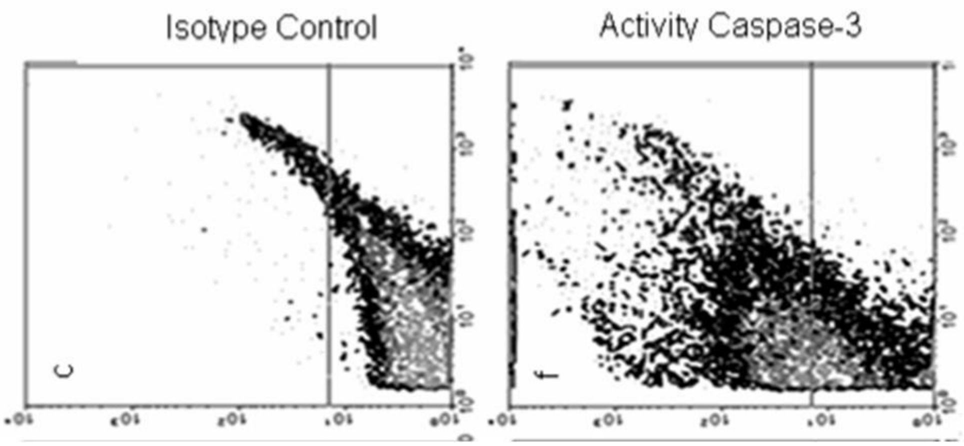
Y axis: Percent cells with Caspase 3/7 expression
X axis: Treatment groups

Graphical comparison of Caspase 3/7 results of various breast cancer cell lines.



Y axis: Percent cells with Caspase 3/7 expression
X axis: Treatment groups

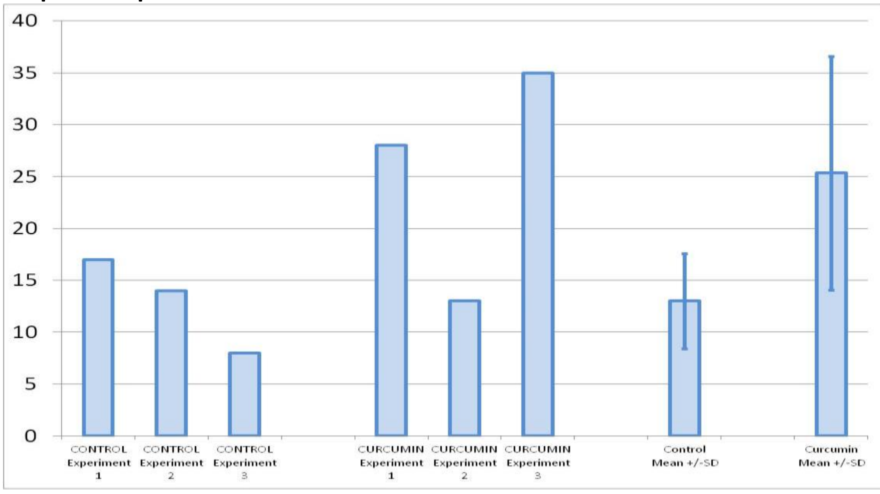
Representative Caspase 3/7 measurements by Flow cytometry in breast cancer cell lines



Annexin V measurement results of breast cancer cell lines and normal breast cell

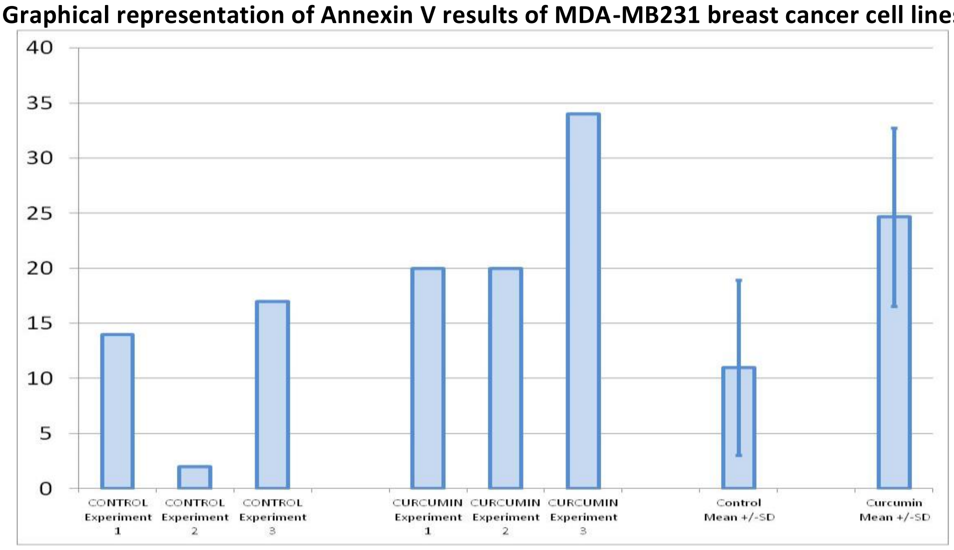
		Percent cells with Annexin V of breast cancer cell lines (MCF7 and MDA-MB231)		Percent cells with Annexin V of normal breast cell (CRL-714)
Control	Experiment 1	17	14	7
	Experiment 2	14	2	5
	Experiment 3	8	17	4
	Mean	13.00	11.00	5.33
	SD	4.58	7.94	1.53
Curcumin (40 µM; 3 hours)	Experiment 1	28	20	14
	Experiment 2	13	20	31
	Experiment 3	35	34	23
	mean	25.33	24.67	22.67
	SD	11.24	8.08	8.50

Graphical representation of Annexin V results of MCF 7 breast cancer cell lines



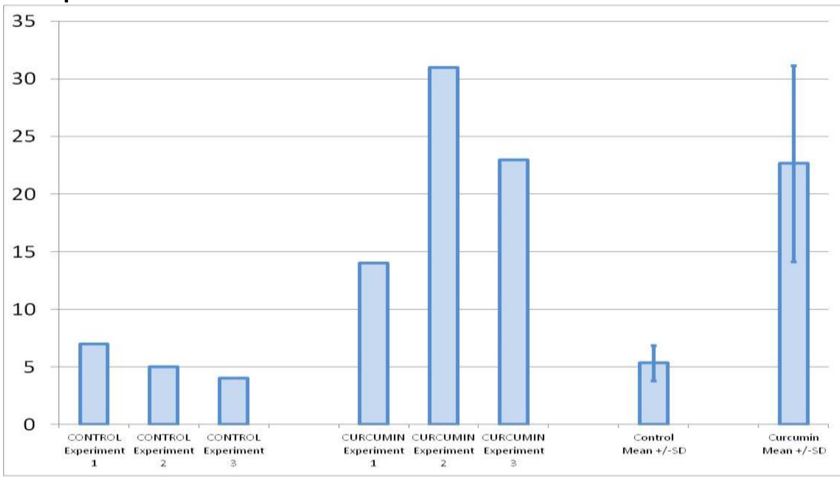
Y axis: Percent cells with Annexin V expression
X axis: Treatment groups

Graphical representation of Annexin V results of MDA-MB231 breast cancer cell lines



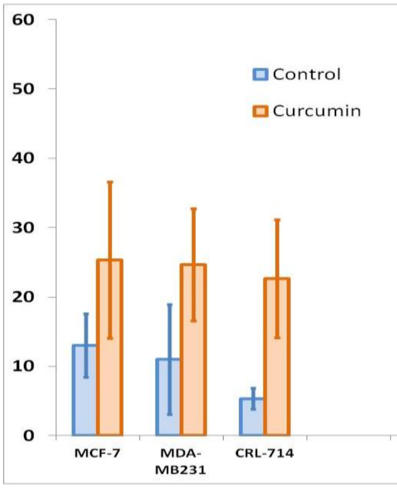
Y axis: Percent cells with Annexin V expression
X axis: Treatment groups

Graphical representation of Annexin V results of CRL-714 normal breast cancer cell lines



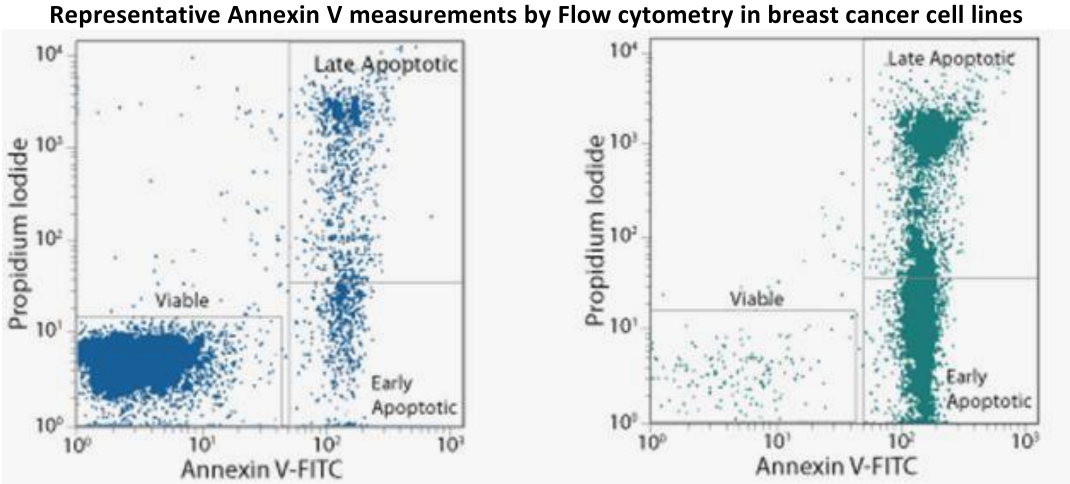
Y axis: Percent cells with Annexin V expression
X axis: Treatment groups

Graphical comparison of Annexin V results of various breast cancer cell lines.



Y axis: Percent cells with Annexin V expression
X axis: Treatment groups

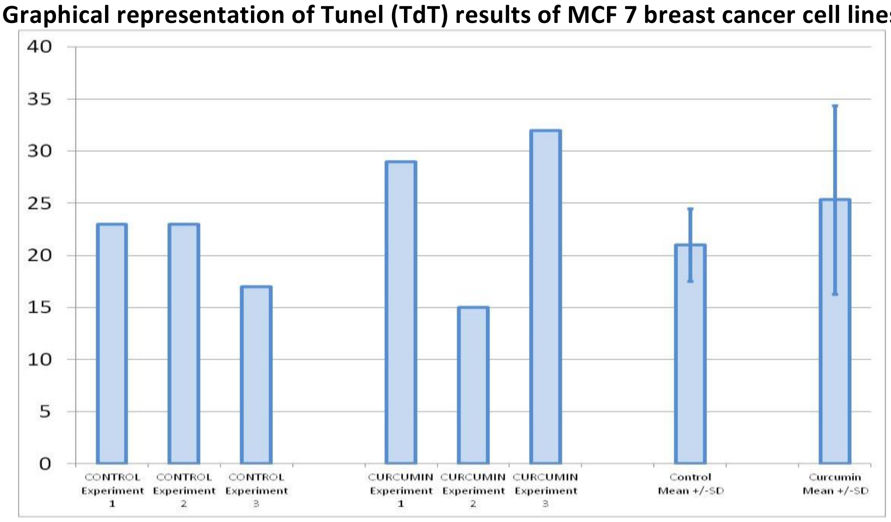
Representative Annexin V measurements by Flow cytometry in breast cancer cell lines



Tunel (TdT) measurement results of breast cancer cell lines and normal breast cell

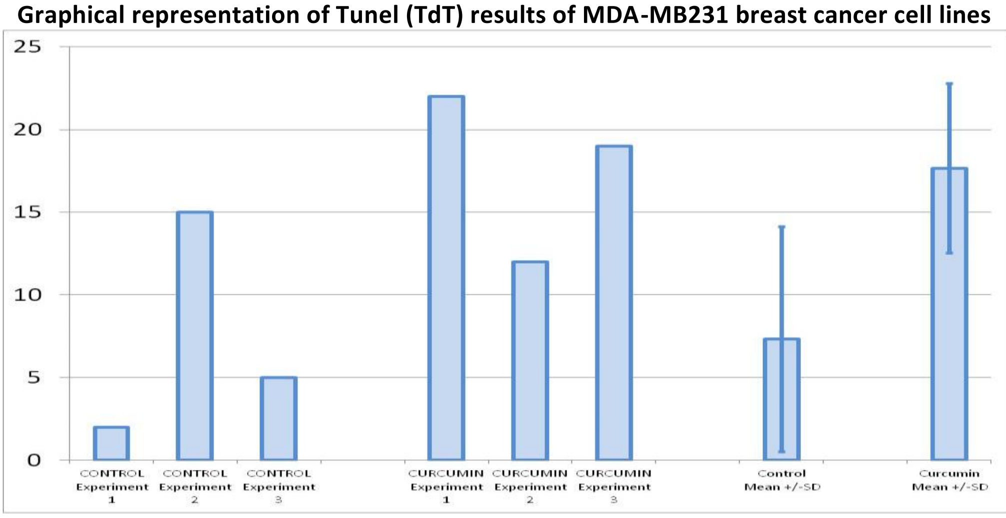
		Percent cells with Tunel (TdT) of breast cancer cell lines (MCF7 and MDA-MB231)		Percent cells with Tunel (TdT) of normal breast cell (CRL-714)
Control	Experiment 1	23	2	6
	Experiment 2	23	15	13
	Experiment 3	17	5	2
	Mean	21.00	7.33	7.00
	SD	3.46	6.81	5.57
Curcumin (40 µM; 3 hours)	Experiment 1	29	22	19
	Experiment 2	15	12	24
	Experiment 3	32	19	32
	mean	25.33	17.67	25.00
	SD	9.07	5.13	6.56

Graphical representation of Tunel (TdT) results of MCF 7 breast cancer cell lines



Y axis: Percent cells with Tunel (TdT) expression
X axis: Treatment groups

Graphical representation of Tunel (TdT) results of MDA-MB231 breast cancer cell lines



Y axis: Percent cells with Tunel (TdT) expression
X axis: Treatment groups

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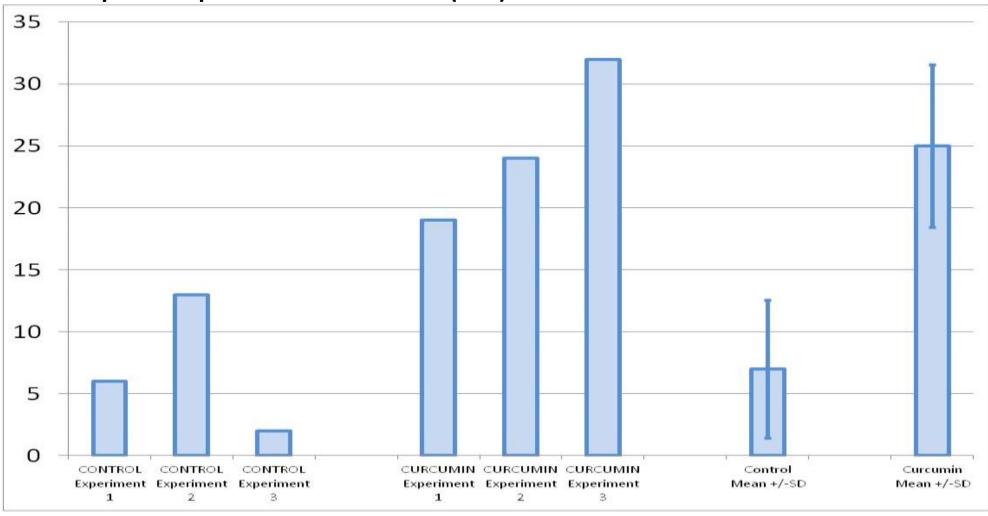
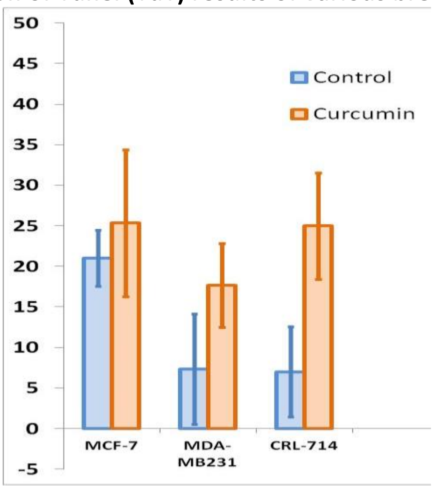
Graphical representation of Tunel (TdT) results of CRL-714 normal breast cell



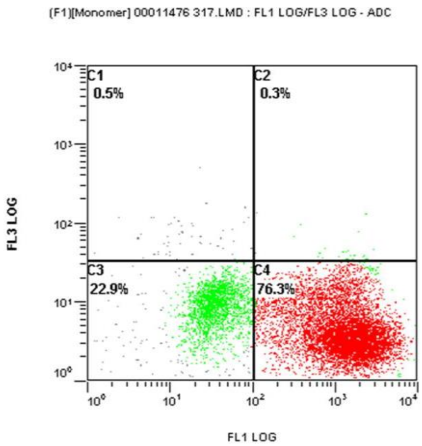
Y axis: Percent cells with Tunel (TdT) expression
X axis: Treatment groups

Graphical comparison of Tunel (TdT) results of various breast cancer cell lines.



Y axis: Percent cells with Tunel (TdT) expression
X axis: Treatment groups

Representative Tunel (TdT) measurements by Flow cytometry in breast cancer cell lines



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