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CELL PROLIFERATION STUDIES ON HUMAN BREAST CANCER CELLS (MCF-7 AND MDA-MB231) USING CURCUMIN

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ABSTRACT

Curcumin, a major yellow pigment and spice in turmeric and curry, is a powerful anti-cancer agent. The anti-tumour activities of curcumin include inhibition of tumour proliferation, angiogenesis, invasion and metastasis, induction of tumour apoptosis, increase of chemotherapy sensitivity, and regulation of cell cycle and cancer stem cell, indicating that curcumin maybe a strong therapeutic potential through modulating various cancer progression. It has been reported that the anticancer effect of Curcumin against human breast cancer cell line through Proliferating Cell Nuclear Antigen (PCNA) assay and colony forming assay. Different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) of Curcumin were used. In PCNA assay, curcumin slows cancer cell proliferation and in colony forming study, colonies were stained with crystal violet and the number of clones in a given area was counted for each condition. This study witnessed that higher concentration of the curcumin gave a good results against cancer cell lines.

KEYWORDS: Breast cancer cell lines, curcumin, PCNA assay, colony formation.

INTRODUCTION

Curcumin (diferuloyl methane), the major polyphenol in dietary spice, is a potent chemopreventive agent that inhibits proliferation of cancer cells by arresting them at various phases of the cell cycle depending upon the cell type. It is derived from the rhizome of the turmeric plant (*Curcuma longa*) is a non-nutritive food chemical used as a flavouring, coloring agent and as a food preservative and has been shown to possess powerful antioxidant, antitumor promoting and anti-inflammatory properties *in vitro* and *in vivo*. Studies relating to curcumin protection against proliferation of various cancer cell lines, cytoprotective effect in oxidative damage and activation of anti-cancer pathways were

scanned in the previous research literature and are provided in the succeeding passages. Curcumin exerts its anticancer effect through a complicated molecular signalling 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.

Breast cancer is one of the major cancers in women both in the developed and the developing world. The incidence of breast cancer is growing in the developing world due to increase life expectancy, increase urbanization and embracing of western lifestyles. Although some risk reduction might be attained with prevention, these strategies cannot eradicate the majority of breast cancers that develop in low- and middle-income countries where breast cancer is diagnosed in very late stages World Health Organisation (WHO). Remarkable improvements in our understanding of the biology of breast cancer have been progressed over the last few decades using breast cancer cell lines. With this data background, the study must now move beyond the 'one marker, one cell line' studies of the past and use knowledge gained to use cell lines or cell line panels more efficiently as experimental models to study specific subgroups of breast cancer, since this is expected to have the greatest influence on improving outcome for breast cancer patients. Siying Zhou *et al*., 2017 focused on the inhibitory effect of curcumin on cancer progression by regulating expressions of multiple miRNAs and also curcumin could bring a new insight into molecular targeting treatment of malignant tumors. Jiao Zou *et al*., 2018 provided new mechanisms for the research on cisplatin resistance and curcumin as a cisplatin-sensitizing agent in breast cancer cells. FEN1 could be a potential therapeutic target for the treatment of cisplatin resistance in breast cancer. The overall aims and objectives of the current study is to observe the following end results:

Anti-proliferation studies

1. Anti-proliferative effect of curcumin using MCF-7 breast cancer cell line and Proliferating Cell Nuclear Antigen (PCNA) assay.
2. Anti-proliferative effect of curcumin using MDA-MB231breast cancer cell line and Proliferating Cell Nuclear Antigen (PCNA) assay.
3. Anti-proliferative effect of curcumin using MCF-7 breast cancer cell line and colony forming assay.

4. Anti-proliferative effect of curcumin using MDA-MB231breast cancer cell line and colony forming assay.

MATERIALS AND METHODS

Curcumin, with a purity ≥95%, was obtained from Unilever R&D (Sanjivani Phytopharma Pvt. Ltd, India). Curcumin was dissolved in DMSO to a stock concentration of 20 mM in a dark coloured bottle and stored at 10°C and was diluted to the necessary concentration with medium when required. Human breast cancer cell lines (MCF-7and MDA-MB-231) (Fig 3 and 4) and CRL-714 (normal breast cell) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). 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. 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% CO2. Curcumin was dissolved in dimethylsulfoxide (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.

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. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) uptake method. After the cells were seeded (5×103/well) in 200 µl of DMEM medium into 96-well plates and cultured overnight, curcumin (0–70 µM) was added to the cells and further cultured for 24 h. Then, MTT reagent (5 mg/ml) was added and incubation was continued for an additional 4 h. The reaction was terminated with 150 µl dimethyl sulfoxide (DMSO, Sigma) per well. Absorbance values were determined using an MRX Revelation 96-well multiscanner (Dynex Technologies, Chantilly, VA, USA). The cells cultured in DMEM served as the control group. The cell viability index was calculated according to the following formula: Experimental OD value/Control OD value. The

experiments were repeated 3 times. The cancer cells were plated in six-well plates overnight and treated with 40 μM curcumin for a period of 3 or 6 h. After the removal of the drug-containing medium, the cells were washed using PBS, trypsinized and plated at a low density (2000 cells/ well in six-well plates). The cells were then incubated with an equivalent amount of DMSO without curcumin, which served as a control. The cells were cultivated for 7 or 12d and the medium were refreshed every two days. The colonies were stained with crystal violet (Sigma Chemical Co, St. Louis, MO). The number of clones in a given area was counted for each condition.

Anchorage-independent growth is one of the hallmarks of malignant transformation of cells. The Soft Agar Assay for Colony Formation is an anchorage independent growth assay, which is considered the most stringent assay for measuring proliferation of cells in a semisolid culture media and its sensitivity towards carcinogenic inhibitors. The soft agar plate provides a 3D system which mimics our cellular environment. Standard soft agar assays are usually performed in 35mm dishes, cells pretreated with carcinogenic inhibitors or drugs are cultured with appropriate controls in soft agar medium for 14-21 days. Following this incubation period, formed colonies can either be analyzed morphologically using cell stain and quantifying the number of colonies formed per well.

RESULTS

In this investigation the inhibitory effect of curcumin from *Curcuma longa* on PCNA assay after 3 hrs of exposure in 40 μg/ml concentration. PCNA gives an indication of the cancer cells ability to multiply in the body. More PCNA%, more quickly the cancer can proliferate and spread. All cancer cell lines showed PCNA ranging from 16-33%, indicating that all cancer cell lines are actively proliferating. MCF 7 was the most highly proliferating prostate and breast cancer cell lines. CRL-741 had the least proliferating breast cancer cell lines, not surprisingly because of near normal karyotype. Treatment of the 2 cell lines with curcumin significantly decreased the cell proliferation, suggesting that curcumin could slow down cancer cell proliferation. Curcumin slows cancer cell proliferation, in general and more so in prostate cancers. The study was conducted to evaluate the inhibitory effect of curcumin from *Curcuma longa* on colony forming assay in human breast cancer cell line (MCF-7 and MDA-MB231) with reference to normal cell CRL-714 (Breast cell). In this study, it is concluded that colony forming assay indicated that all breast cancer cell lines formed 60-70 colonies plate. Curcumin treatment reduced the colonies formed by nearly 50% in all 2 cell lines.

Colony forming assay results give an indication of cancer cells effectiveness in surviving in the body. Curcumin treatment prevents cancer cells to grow.

DISCUSSION

Curcumin inhibited cell proliferation in a dose-dependent and time-dependent manner ($p<0.05$) and resulted in significant cell cycle arrest in G1 phase after 72 h of treatment at concentrations of over 10 and 20 μM in MCF-7 cells. As biomarkers of apoptosis induction, caspase-3 activity and caspase-9 activity were increased by curcumin in MCF-7 cells (Li *et al.,* 2012). The clonogenic ability of MCF-7 and MDA-MB-231 cells differs following curcumin treatment. To further examine the cytotoxic effects of curcumin over a prolonged period of time, clonogenic assays were performed on both of the cell lines 7 or 12 days after treatment with 40 mm curcumin for 3 or 6 h, respectively. DNA analyses of the stained colonies revealed that curcumin treatment for both 3 and 6 h thoroughly abrogated the clonogenic ability of MDA-MB-231 cells. In MCF-7 cells, however, the same concentration of curcumin did not significantly affect the capacity of the cells to form viable colonies after 3 h of treatment and only caused a slight decrease in the number of colonies after 6 h of treatment. The results were consistent with the findings in the cell viability assays and further confirmed the differential responses of MCF-7 and MDA-MB-231 cells to curcumin (Jia *et al.*, 2014).

Curcumin has been shown to inhibit the proliferation of a wide variety of tumour cells, including B-cell, T-cell leukemia, colon carcinoma and epidermoid carcinoma cells. It has also been shown to suppress the proliferation of various breast carcinoma celllines in culture. Also growth of the breast tumour cell lines BT20, SKBR3,MCF-7, T47D, and ZR75-1 is completely inhibited by curcumin, as indicated by MTT dye uptake,[3H] thymidine incorporation, and clonogenic assay (Agarwal *et al.*,2005).Effects of curcumin on survival and proliferation of different cell types have been studied. It is found to inhibit proliferation of breast tumour cells, induction of apoptosis in H-ras transformed MCF10A cells, induces apoptosis of AK-5 cells, inhibits proliferation of colon cancer (HT29; HCT-15) cells, induces apoptosis in colon (LoVo) cancer cells, induces growth arrest and apoptosis of B cell lymphoma, induces apoptosis in HL-60 cells, induces apoptosis of myeloid (HL-60) cells, induces apoptosis of basal cell carcinoma cells, inhibits proliferation of prostate cancer cells, inhibits proliferation of oral epithelial cells, induces apoptosis of T lymphocytes, induces

apoptosis of osteoclasts, induces apoptosis in VSMC and induces apoptosis in hepatocytes (Agarwal *et al.,* 2003).

Curcumin suppresses the proliferation of a wide variety of tumour cells, including breast carcinoma, colon carcinoma, renal cell carcinoma, hepatocellular carcinoma, T cell leukaemia, B cell lymphoma, acute myelogenousleukemia, basal cell carcinoma, melanoma and prostate carcinoma (Bhaumik *et al.*, 1999; Elattar and Virji, 2000; Bielak-Zmijewska *et al.,* 2000; Cipriani *et al.,* 2001; Pan *et al.*, 2001; Morin *et al.,* 2001; Moragoda *et al.,* 2001; Mukhopadhyay *et al.,* 2001 and Pal *et al.,* 2001). Additionally curcumin suppresses the proliferation of certain normal cells such as hepatocytes (Gomez-Lechon *et al.,* 2002), epithelial cells (Khafif *et al.,* 1998), human vascular endothelial cells (HVEC) (Sup Shim *et al.,* 2002), human vascular smooth muscle cells (HVSMC), osteoclasts (Ozaki *et al.,* 2000), peripheral blood mononuclear cells (PBMC) and T lymphocytes (Gomez-Lechon *et al.,* 2002; Ozaki *et al.,* 2000, Khafif *et al.,* 1998). Kim *et al.,* 2012 have shown that curcumin has anticancer activity against oral squamous cell carcinoma (OSCC). Induction of autophagy, marked by autophagic vacuoles formation, was detected by acridine orange staining and monodansylcadaverine (MDC) dye after exposure to curcumin. Conversion of LC3-Ito LC3-II, a marker of active autophago some formation, was also detectable by Western blot following curcumin treatment. They also observed that curcumin induced autophagic vacuoles.

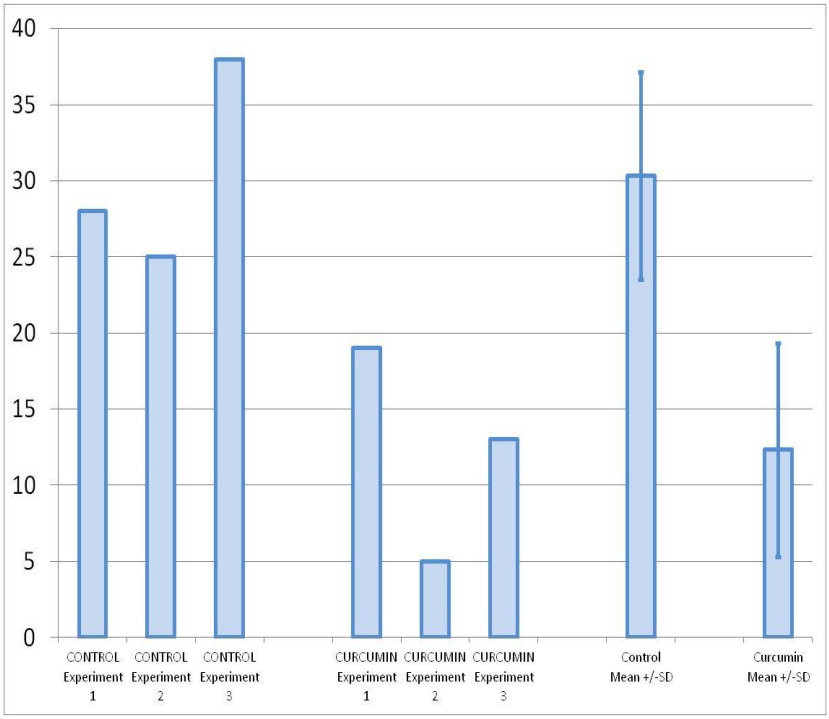
Di *et al.,* (2003) analysed the anti-proliferation effect of curcumin on human breast cancer cells and its mechanism and reported that curcumin inhibited the proliferation in both estrogen receptor (ER) positive MCF-7 cells and ER negative MDA-MB-231 breast carcinoma cells. Shao *et al.,* (2002) presented experimental evidence suggesting that curcumin exerts multiple different suppressive effects on human breast carcinoma cells *in vitro* and demonstrated that curcumin's anti-proliferative effects are estrogen dependent in ER (estrogen receptor)-positive MCF-7 cells, being more pronounced in estrogen-containing media and in the presence of exogenous 17-beta estradiol. Also curcumin inhibits the expression of ER downstream genes including pS2 and TGF-beta (transforming growth factor) in ER-positive MCF-7 cells, and this inhibition is also dependent on the presence of estrogen. Chen *et al.,* (2014) demonstrated that curcumin inhibited Fen1-dependent proliferation of MCF-7 cells and significantly induced Nrf2 protein expression while inhibiting Fen1 protein expression. Curcumin could down-regulate Fen1 gene expression in

an Nrf2-dependent manner. Further investigation revealed that curcumin could lead to Nrf2 translocation from the cytoplasm to the nucleus and decrease Fen1 promoter activity by decreasing the recruitment of Nrf2 to the Fen1 promoter. Curcumin and its derivatives can inhibit the proliferation of triple negative breast cancer cell lines, MDA-MB-231 and MDA-MB-435 human breast cancer cells (Rowe *et al.*, 2009; Yodkeeree *et al.*, 2010, Hua *et al.*, 2010).

PCNA measurement results of MCF7 breast cancer cell line.

		Percent cells with PCNA
Control	Experiment 1	28
	Experiment 2	25
	Experiment 3	38
	Mean	30.33
	SD	6.81
Curcumin (40 µM; 3 hours)	Experiment 1	19
	Experiment 2	5
	Experiment 3	13
	mean	12.33
	SD	7.02

Graphical representations of PCNA measurements of MCF 7 breast cancer cell line



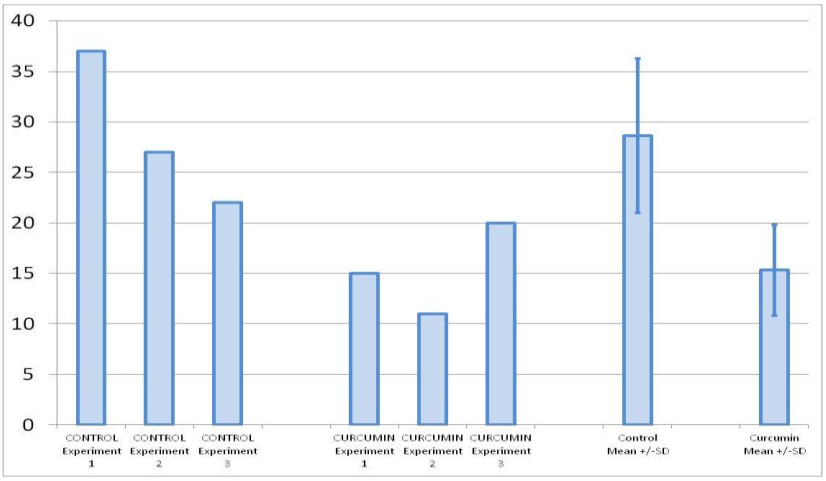
Y axis: Percent cells with PCNA

X axis: Treatment groups

PCNA measurement results of MDA-MB231 breast cancer cell line.

		Percent cells with PCNA
Control	Experiment 1	37
	Experiment 2	27
	Experiment 3	22
	Mean	28.67
	SD	7.64
Curcumin (40 µM; 3 hours)	Experiment 1	15
	Experiment 2	11
	Experiment 3	20
	mean	15.33
	SD	4.51

Graphical representations of PCNA measurements of MDA-MB231 breast cancer cell line



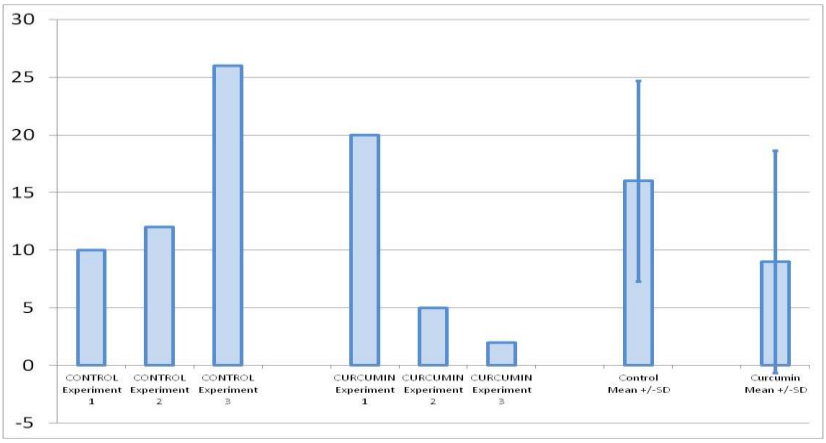
Y axis: Percent cells with PCNA

X axis: Treatment groups

PCNA measurement results of CRL-714 normal breast cell.

		Percent cells with PCNA
Control	Experiment 1	10
	Experiment 2	12
	Experiment 3	26
	Mean	16.00
	SD	8.72
Curcumin (40 µM; 3 hours)	Experiment 1	20
	Experiment 2	5
	Experiment 3	2
	mean	9.00
	SD	9.64

Graphical representations of PCNA measurements of CRL-714 normal breast cell



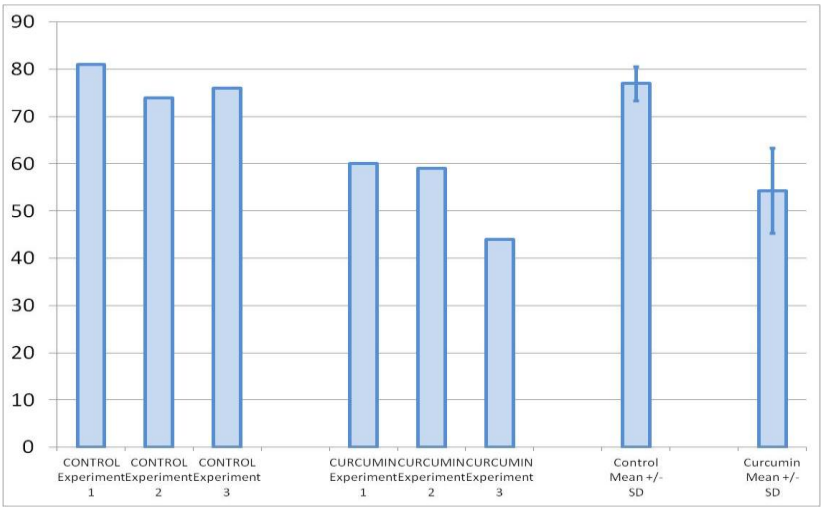
Y axis: Percent cells with PCNA

X axis: Treatment groups

Colony formation assay results of MCF7 breast cancer cell line.

		Number of colonies
Control	Experiment 1	81
	Experiment 2	74
	Experiment 3	76
	Mean	77.00
	SD	3.61
Curcumin (40 µM; 3 hours)	Experiment 1	60
	Experiment 2	59
	Experiment 3	44
	mean	54.33
	SD	8.96

Graphical representation of Colony formation assay of MCF 7 breast cancer cell

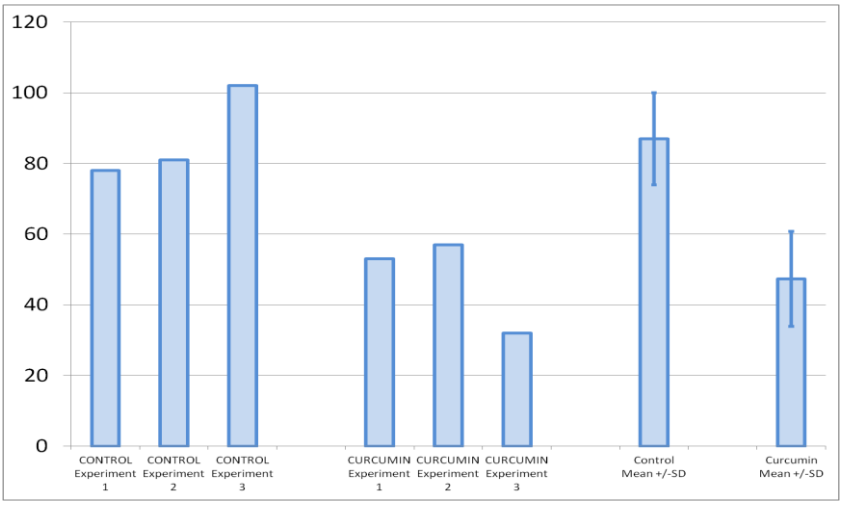


Y axis: Number of colonies
X axis: Treatment group

Colony formation assay results of MDA-MB231 breast cancer cell line.

		Number of colonies
Control	Experiment 1	78
	Experiment 2	81
	Experiment 3	102
	Mean	87.00
	SD	13.08
Curcumin (40 µM; 3 hours)	Experiment 1	53
	Experiment 2	57
	Experiment 3	32
	mean	47.33
	SD	13.43

Graphical representation of Colony formation assay of MDA-MB231 breast cancer cell line



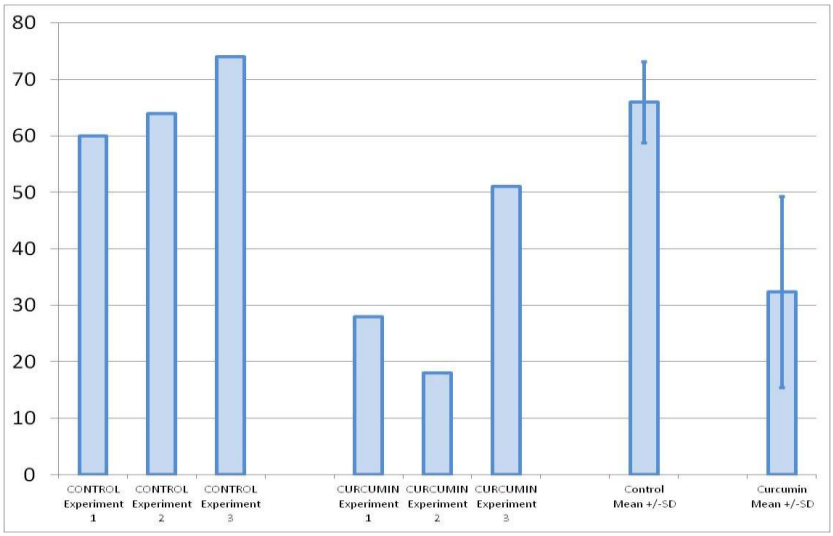
Y axis: Number of colonies

X axis: Treatment groups

Colony formation assay results of CRL-714 normal breast cell

		Number of colonies
Control	Experiment 1	60
	Experiment 2	64
	Experiment 3	74
	Mean	66.00
	SD	7.21
Curcumin (40 µM; 3 hours)	Experiment 1	28
	Experiment 2	18
	Experiment 3	51
	mean	32.33
	SD	13.43

Graphical representation of Colony formation assay of CRL-714 normal breast cell



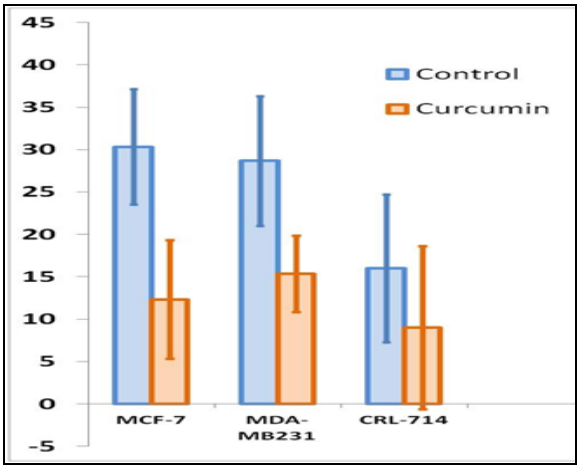
Y axis: Number of colonies

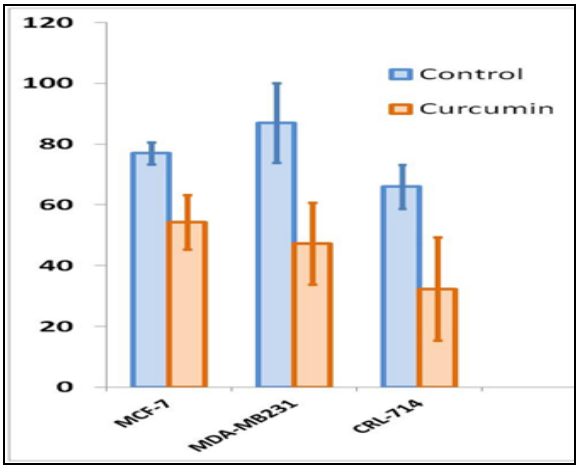
X axis: Treatment groups

Comparison of PCNA measurements and Colony formation assay of breast cancer cell lines

Cell lines	**Cell proliferation by PCNA and colony forming assay**	
Breast cancer cells	**Control**	**Curcumin**
MCF-7	Normal	Decreased
MDA-MB231	Normal	Decreased
CRL-714 (normal breast cell)	Normal	Normal

Graphical comparison of PCNA measurements and Colony formation assay of breast cancer cell lines





Y axis: Percent cells with PCNA

X axis: Cell lines

Y axis: Number of colonies

X axis: Cell lines

CONCLUSION

The study highlighted that extracts on *Curcuma longa* reduced cell proliferation in human breast cancer cell lines (MCF-7 and MDA-MB231) compared to CRL-714 normal human breast cells. The overall results, showed that the active molecule present in *Curcuma longa* have considerable consequence on the survival of human breast cancer. In this scenario, scientific and systematic studies of age-old and household molecule Curcumin (Turmeric), a culinary spice believed to have curative and anticancer properties should be considered 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 was concluded.

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