WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 15, 1173-1183. Research Article ISSN 2277– 7105

CLONOGENIC AND APOPTOSIS SCREENING OF CURCUMIN (*CURCUMA LONGA)* ON PARAQUAT INDUCED TOXICITY IN HUMAN COLORECTAL ADENOCARCINOMA [NCI-PBCF-HTB37 (CACO-2)] CANCER CELL LINE

Dilla Jose[1] and P. Senthilkumaar[2]

[1]Department of Zoology, St. George's College, Aruvithura, Erattupetta, Kerala, India.
[2]School of Enzymology and Environmental Toxicology, P.G. and Research Department of Zoology, Sir Theagaraya College, Chennai, Tamil Nadu, India.

Article Received on
29 Sep. 2017,
Revised on 19 Oct. 2017,
Accepted on 09 Nov. 2017
DOI: 10.20959/wjpr201715-10195

***Corresponding Author**
Dilla Jose
Department of Zoology, St. Georges College,
Aruvithura, Erattupetta,
Kerala, India.
sr.dillajose@gmail.com

ABSTRACT

The present study focused on the colony forming and apoptosis assay on human colon adenocarcinoma cell line (Caco-2) with Curcumin, Paraquat and two different combinations Curcumin and Paraquat. In this study, different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) of Curcumin and Paraquat were used. The test cancer lines (NCI-PBCF-HTB37 (Caco-2) - Human Colorectal Adenocarcinoma, (ATCC® HTB-37™) were procured from American Type Culture Collection (ATCC) and were subculture with suitable culture medium and stored in cryo preservation. The apoptosis induction was determined by measuring Annexin V activity using Annexin V apoptosis kit. In Clonogenic assay, 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 colonies were stained with crystal violet and the number of clones in a given area was counted for each condition. In both study, the higher concentration of the curcumin gave a good results and it indicated that the secondary bioactive compounds of curcumin have good anticancer activity.

INTRODUCTION

Curcumin (diferuloylmethane), is a polyphenol 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 (Shishodia *et al*., 2003). It has been consumed for centuries as a dietary spice at the rate of up to 100 mg/day by people in Asian countries (Ammon and Wahl, 1991). Curcumin acts as a potent anticarcinogenic compound and induction of apoptosis plays an important role in its anticarcinogenic effect. It induces apoptosis and inhibits cell-cycle progression, both of which are instrumental in preventing cancerous cell growth in rat aortic smooth muscle cells (Chattopadhyay *et al*., 2004). Curcumin also down regulates cyclin D1, cyclin E and MDM2; and upregulates p21, p27 and p53. Various preclinical cell culture and animal studies suggest that curcumin has potential as an antiproliferative, anti-invasive and antiangiogenic agent. Other clinical trials suggest a potential therapeutic role for curcumin in diseases such as colon and pancreatic cancer (Goel *et al*., 2008).

Lai *et al*., (2012) studied the potential utility of curcumin in the treatment of HER-2-overexpressed breast cancer and showed that curcumin could reduce the cell viability of both HER-2-overexpressed herceptin-sensitive BT-474 cells and herceptin-resistant SK-BR-3-hr breast cancer cells. In the BT-474 xenograft model, though not as much as herceptin, curcumin did effectively decrease the tumor size. The combination of curcumin with herceptin was not better than herceptin alone; however, the combination of taxol and curcumin had an antitumor effect comparable with taxol and herceptin. The results, both *in vitro* and *in vivo*, suggested that curcumin has the treatment potential for HER-2-overexpressed breast cancer. Paraquat (PQ, 1,1’-dimethyl-4-4’-bipyridinium dichloride) is a bipyridyl herbicide. It is a highly potent systemic poison and this herbicide is used throughout the world as contact herbicide and as crop desiccants in agriculture. Bipyridyl means that the structure contains two pyridine rings, aromatic rings in which one carbon atom is replaced by a nitrogen atom joined by an ethylene group. Paraquat is usually manufactured as a salt with chloride ion. Paraquat is absorbed through the skin, gastrointestinal and respiratory tracts.

The main objective of this study is, to analyze the colony forming assay and apoptosis assay on Human colon adenocarcinoma cell line (Caco-2) with Curcumin, Paraquat and two different combinations Curcumin and Paraquat.

MATERIALS AND METHODS

Curcumin

Curcumin is a derivative from *Curcuma longa*. Curcumin powder was obtained from Sigma chemicals (St. Louis, MO, USA) and stored at room temperature. It was purchased as a crystalline solid. A stock solution was made by dissolving the curcumin in acetone. Further

dilutions of stock solution into aqueous solution were made prior to performing experiments. Different doses of Curcumin were used (1, 5, 10, 25, 50 and 100 µg/ml).

Paraquat

The herbicide paraquat (Paraquat Dichloride 24% SL) was purchased from HPM Chemicals & Fertilizers Ltd., Coimbatore and Tamil Nadu. Different doses of paraquat were used (1, 5, 10, 25, 50 and 100 µg/ml). Serial dilutions of the test herbicide were made as per the requirements in the present investigation.

Cancer cell lines

The test cancer lines (NCI-PBCF-HTB37 (Caco-2) - Human Colorectal Adenocarcinoma, (ATCC® HTB-37™) were procured from American Type Culture Collection (ATCC), Manassas, VA 20108 USA.

Complete growth medium

The following culture methods were followed for growth, sub-culturing, cryopreserving and thawing of Colorectal Adenocarcinoma (NCI-PBCF-HTB37 (Caco-2) (ATCC® HTB-37™). The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the fetal bovine serum to a final concentration of 20%.

Volumes are given for a 75 cm^2 flask. Increase or decrease the amount of dissociation medium needed proportionally for culture vessels of other sizes. Corning® T-75 flasks (catalog #430641) are recommended for sub culturing this product.

Remove and discard culture medium. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor. Add 2.0 to 3.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting. Add appropriate aliquots of the cell suspension to new culture vessels. The recommended inoculum is $1x10^4$ viable cells/cm2. Subculture cells when they are about 80% confluent, at a cell concentration between 8×10^4 and 1×10^5 cell/cm2. Incubate cultures at 37°C.

Clonogenic assay

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 was 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.

Apoptosis

Apoptosis induction was determined by measuring Annexin V activity using Annexin V apoptosis kit. The kit was sourced from Promega corporation, Southapton, UK and consisted of Annexin V buffer and lyophilized Annexin V substrate. Cells were seeded at the concentration of 10^4 cells per well in 96 well microtitre plate and incubated for 48 hours. Aliquot of 100 µL of fresh media containing appropriate concentration of extracts were transferred to the assigned respective wells. Stauroporine 0.1 µg/mL was used as positive control and untreated wells were treated as negative control. Total of six wells were assigned for each treatment. The plate was allowed to equilibrate to room temperature after 24 hours of incubation prior to performing the assay. 100 µL of Annexin V reagent was added to each well and mixed for 60 seconds and incubated for further 1 hour at room temperature. A aliquot of 100 µL of contents from each well was transferred to white-walled 96 well plate. The light emitted was measured by Packard lumicount microplate luminometer and measurement was recorded using THERMOmax^{TM} plate reader linked to a computer using SoftMax Pro software (Muñoz-Alonso *et al.*, 2008). The results are depicted in the tabular form.

Statistical analysis

The mean and standard deviation were performed for the observed results by ORIGIN 8.0 software.

RESULTS AND DISCUSSIONS

The selected cancer line is human Colorectal Adenocarcinoma (NCI-PBCF-HTB37 (Caco-2). Curcumin and Paraquat were used to study Colony forming assay and Apoptosis.

Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin

Different doses of Curcumin were used (1, 5, 10, 25, 50 and 100 µg/ml). A control without curcumin was also employed. No significant reduction in colony formation compared to the control was noticed in 1, 5, 10 and 25µg/ml dose of curcumin. A slight reduction in colony formation compared to the control was visible in 50 and 100µg/ml dose of curcumin. Three culture plates were used in the experiment for each dose of curcumin. Mean and SD were calculated for statistical analysis (Figure 1).

Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with paraquat

Different doses of paraquat were used (1, 5, 10, 25, 50 and 100 µg/ml). A control without paraquat was also employed. No significant reduction in colony formation compared to the control was noticed in 1 µg/ml dose of paraquat. Significant reduction in colony formation compared to the control was visible in 10, 25, 50 and 100µg/ml dose of paraquat. Three culture plates were used in the experiment for each dose of paraquat. Mean and SD were calculated for statistical analysis (Figure 2).

Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 50 µg/ml of paraquat

Two separate doses of curcumin (25 and 50 µg/ml) were used. Increase in colony formation compared to the control was observable in both the doses of curcumin. Three culture plates were used in the experiment for each dose of curcumin. Mean and SD were calculated for statistical analysis (Figure 3).

Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 100 µg/ml of paraquat

Two separate doses of curcumin (25 and 50 µg/ml) were used. Increase in colony formation compared to the control was observable in both the doses of curcumin. Three culture plates were used in the experiment for each dose of curcumin. Mean and SD were calculated for statistical analysis (Figure 4).

Apoptosis using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin

Different doses of Curcumin were used (1, 5, 10, 25, 50 and 100 µg/ml). A control without curcumin was also employed. No significant apoptosis compared to the control was noticed in 1, 5, 10 and 25µg/ml dose of curcumin. A slight increase in apoptosis compared to the control was visible in 50 and 100µg/ml dose of curcumin. Three culture plates were used in the experiment for each dose of curcumin. Mean and SD were calculated for statistical analysis (Figure 5).

Apoptosis using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with paraquat

Different doses of paraquat were used (1, 5, 10, 25, 50 and 100 µg/ml). A control without paraquat was also employed. No significant reduction in apoptosis compared to the control was noticed in 1 µg/ml dose of paraquat. Significant increase in apoptosis compared to the control was visible in 10, 25, 50 and 100µg/ml dose of paraquat. Three culture plates were used in the experiment for each dose of paraquat. Mean and SD were calculated for statistical analysis (Figure 6).

Apoptosis using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 50 µg/ml of paraquat

Two separate doses of curcumin (25 and 50 µg/ml) were used. Decrease in apoptosis compared to the control was observable in both the doses of curcumin. Three culture plates were used in the experiment for each dose of curcumin. Mean and SD were calculated for statistical analysis (Figure 7).

Apoptosis using Colorectal Adenocarcinoma (NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 100 µg/ml of paraquat

Two separate doses of curcumin (25 and 50 µg/ml) were used. Increase in apoptosis compared to the control was observable in both the doses of curcumin. Three culture plates were used in the experiment for each dose of curcumin. Mean and SD were calculated for statistical analysis (Figure 8).

Kim *et al*., (2012), studied lung tumour promotion by curcumin in a transgenic mouse model for lung cancer that expresses the human Ki-ras (G12C) allele in a doxycycline (DOX) inducible and lung specific manner. The effects of curcumin were compared with the lung

tumour promoter, butylated hydroxytoluene (BHT) and the lung cancer chemopreventive agent, sulindac. DOX was given in the drinking water (500 g/ml) beginning 8 weeks after birth. BHT was administered 1 week after initiation of DOX treatment and consisted of six weekly intraperitoneal injections of 150 mg/kg of BHT in olive oil. Control mice received olive oil vehicle at a dose of 0.5 ml/25g. Separate groups of mice were fed either the chemopreventive agent sulindac at a dose of 80 mg/kg diet or 4000 mg/kg diet (the latter being equivalent to 600 mg/kg bw/day) of curcumin starting 2 days after the initiation of DOX.

Treatment of DOX-induced mice with dietary curcumin increased tumour multiplicity (36.3 +/- 0.9 versus 24.3 +/- 0.2) and progression to later stage lesions, results which were similar to animals that were co-treated with DOX/BHT. Microscopic examination showed that the percentage of lung lesions that were adenomas and adenocarcinomas increased to 66% in DOX/BHT, 66% in DOX/curcumin and 49% in DOX/BHT/curcumin-treated groups relative to DOX only treated mice (19%). Immunohistochemical analysis also showed increased evidence of inflammation in DOX/BHT, DOX/curcumin and DOX/BHT/curcumin mice relative to DOX only treated mice. In contrast, co-treatment of DOX/BHT mice with 80 mg/kg ppm of sulindac inhibited the progression of lung lesions and reduced the inflammation. Lung tissue from DOX/curcumin-treated mice demonstrated a significant increase (33%, P = 0.01) in oxidative damage, as assessed by the levels of carbonyl protein formation, relative to DOX-treated control mice after 1 week on the curcumin diet.

These results suggest that curcuminmay exhibit organ-specific effects to enhance reactive oxygen species formation in the damaged lung epithelium of smokers and ex-smokers. Also studies on antioxidant/prooxidant activity of curcumin in *in vitro* models with various cells have been reported (Banerjee *et al*., 2008; Kunwar *et al*., 2008; McNally *et al*., 2007) but these studies do not add to the re-evaluation of the safety of curcumin as an additive. This study is an witnessed for anticancer effect of Curcumin against cancer cell line.

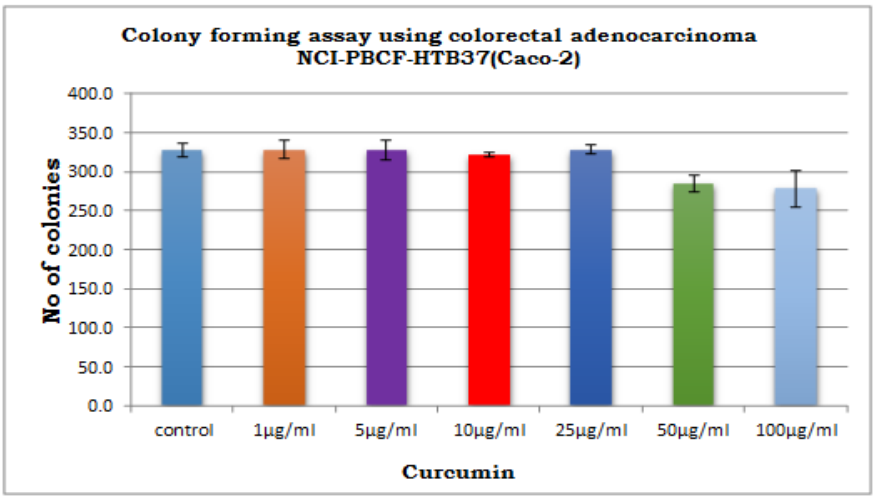

Figure 1. Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin.

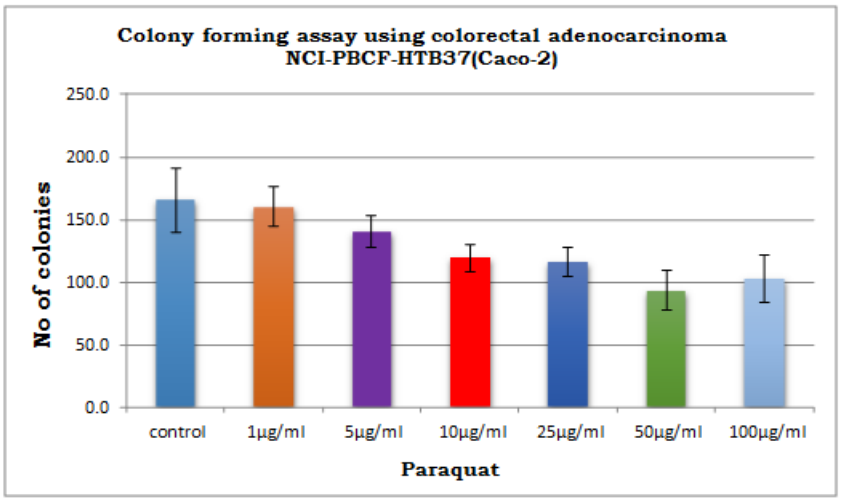

Figure 2. Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with paraquat.

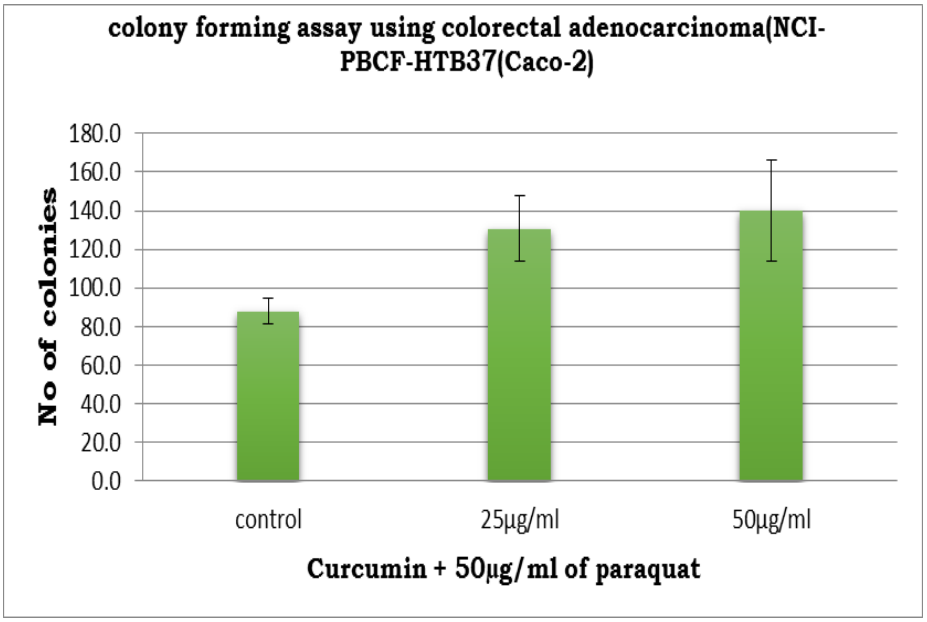

Figure 3. Colony forming assay using colorectal adenocarcinomaNCI-PBCF-HTB37(Caco-2) with curcumin 25 and 50 µg/ml and 50 µg/ml of paraquat.

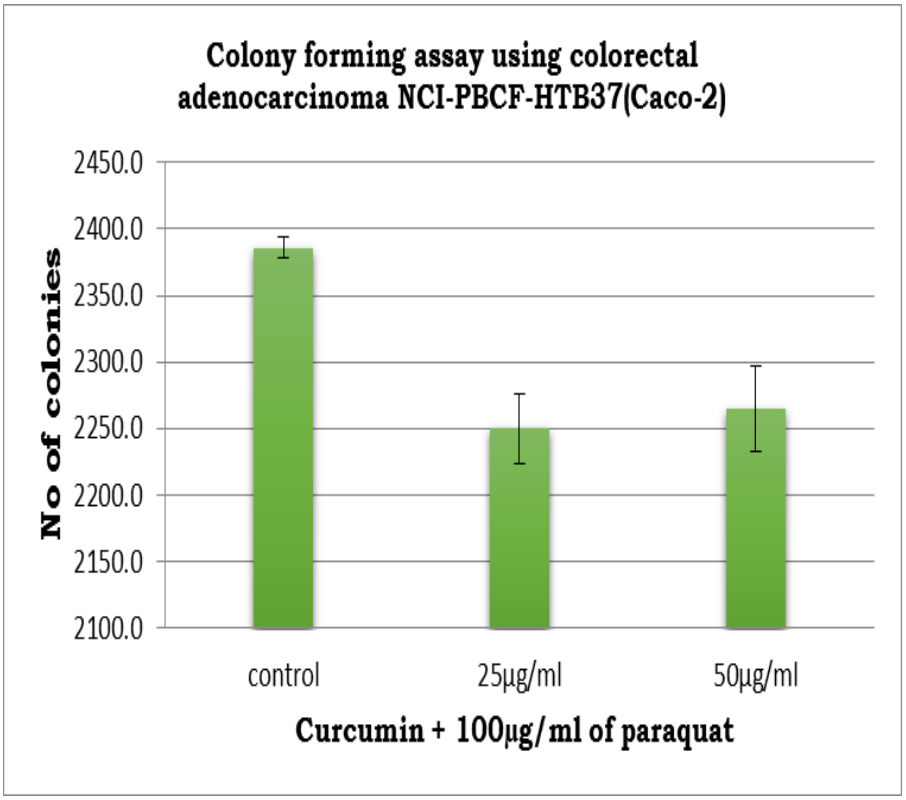

Figure 4. Colony forming assay using Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 100 µg/ml of paraquat.

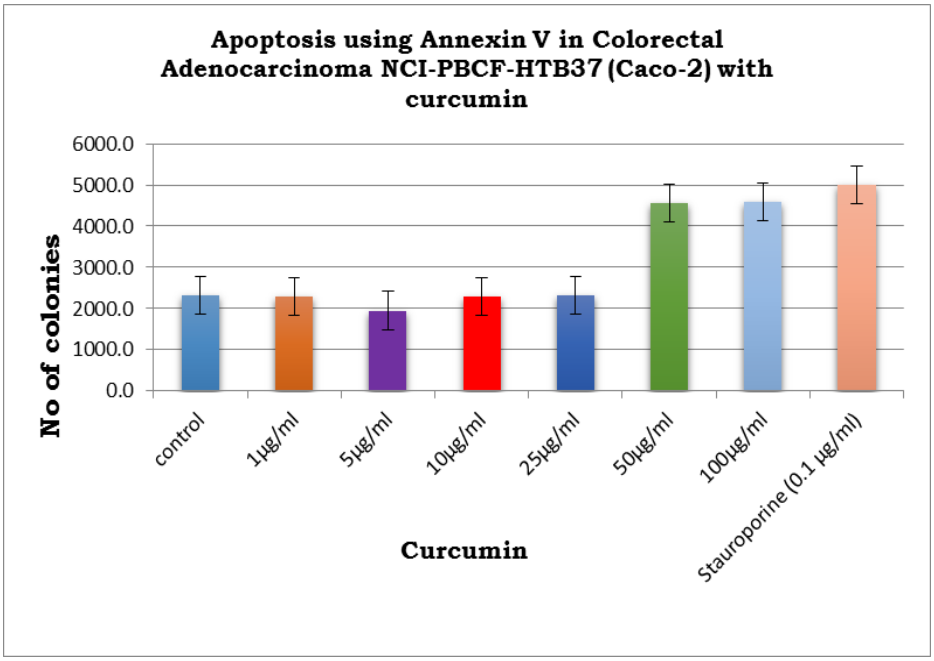

Figure 5. Apoptosis using Annexin V in Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin.

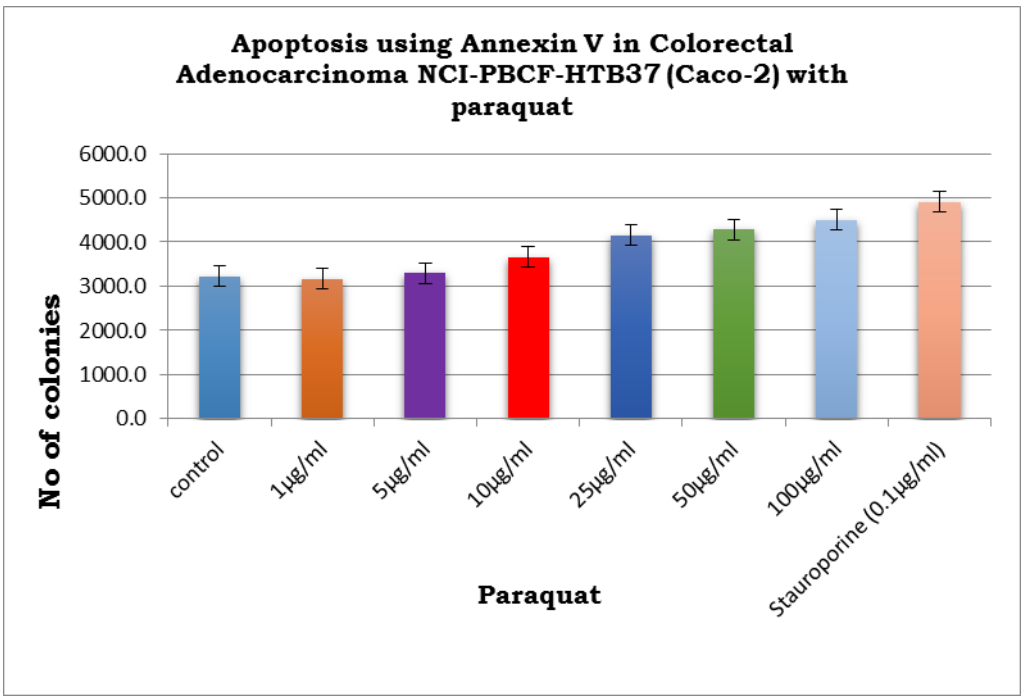

Figure 6. Apoptosis using Annexin V in Colorectal AdenocarcinomaNCI-PBCF-HTB37 (Caco-2) with paraquat.

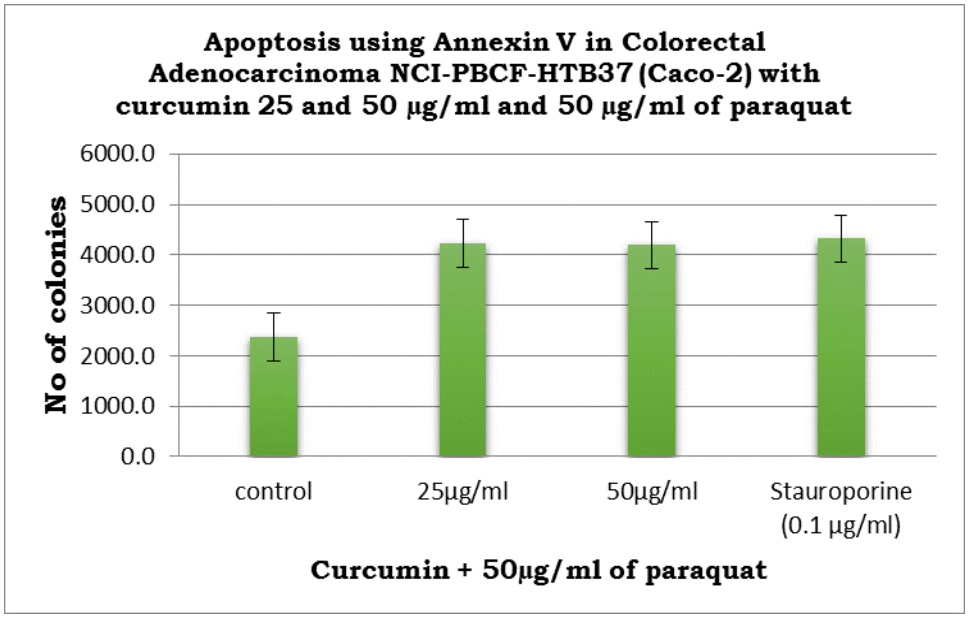

Figure 7. Apoptosis using Annexin V in Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 50 µg/ml of paraquat.

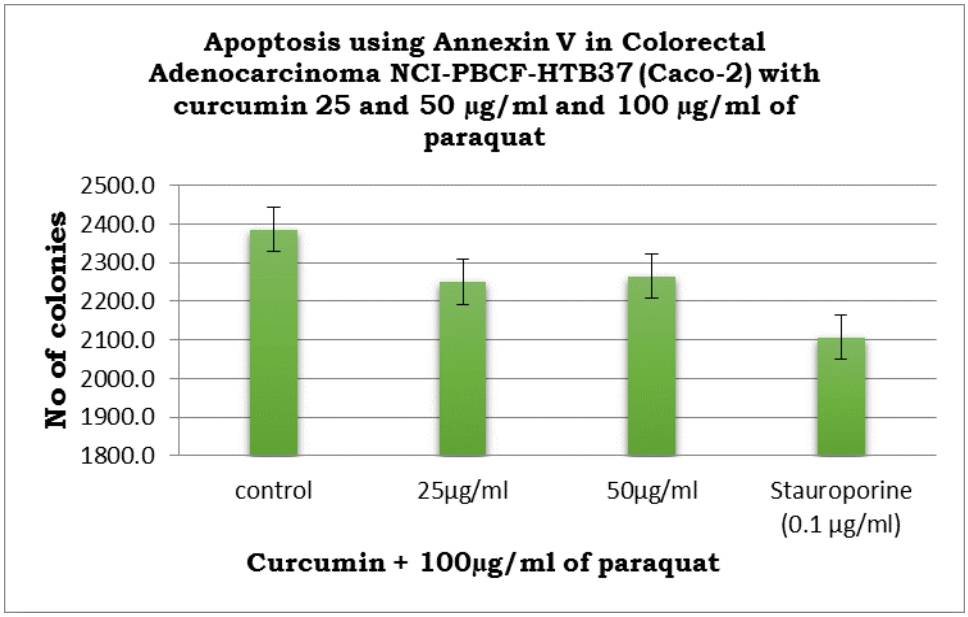

Figure 8. Apoptosis using Annexin V in Colorectal Adenocarcinoma NCI-PBCF-HTB37 (Caco-2) with curcumin 25 and 50 µg/ml and 100 µg/ml of paraquat.

REFERENCES

1. Ammon, H.P. and M.A. Wahl. (1991) Pharmacology of Curcuma longa. Planta Med., 57(1): 1-7.
2. Chattopadhyay, I., K. Biswas, U. Bandopadhyay and R.K. Banerjee. (2004) Turmeric and curcumin. Current Science., 87(10): 44-53.

3. Goel, A., B. Ajaikumar, Kunnumakkara, B. Bharat and Aggarwal. (2008) Biochemical Pharmacology., 75(4): 787–809.
4. Kim, J. Y., Tae Jin Cho, Bok Hee Woo, Kyung Un Choi, Chang Hun Lee, Mi Heon Ryu and Hae Ryoun Park. (2012) Curcumin-induced autophagy contributes to the decreased survival of oral cancer cells. Archives of Oral Biology., 57: 1018-1025.
5. Lai, H.W., Su. Yu Chien, Shou-Jen Kuo, Ling-Ming Tseng, Hui-Yi Lin, Chin-Wen Chi and Dar-Ren Chen. (2012) The Potential Utility of Curcumin in the Treatment of HER-2-Overexpressed Breast Cancer: An In Vitro and In Vivo Comparison Study with Herceptin. Evidence-Based Complementary and Alternative Medicine., pp.1-12.
6. Shishodia, S., Pravin Potdar, C. Gary Gairola and Bharat B. Aggarwal. (2003) Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF-kB activation through inhibition of IkBa kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. Carcinogenesis., 24(7): 1269-1279.