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EFFECT OF CURCUMIN (*CURCUMA LONGA)* AGAINST HUMAN PULMONARY CARCINOMA CANCER CELLS: AN IN-VITRO COLONY FORMING AND APOPTOSIS ASSAY

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Abstract

The present study focused the anticancer effect of Curcumin against Pulmonary Carcinoma (NCI-H292) through colony forming and apoptosis assay. In addition, Paraquat act as an inducer. Different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) of Curcumin and Paraquat were used. In colony forming study, colonies were stained with crystal violet and the number of clones in a given area was counted for each condition. But in apoptosis assay, the study was determined by measuring Annexin V activity using Annexin V apoptosis kit. This study witnessed that higher concentration of the curcumin gave a good results against cancer cell lines.

INTRODUCTION

Curcumin (diferuloylmethane), 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 has been shown to interfere with multiple cell signaling pathways, including cell cycle (cyclin D1 and cyclin E), apoptosis (activation of caspases and down-regulation of antiapoptotic gene products), proliferation (HER-2, EGFR and AP-1), survival (PI3K/AKT pathway), invasion (MMP-9 and adhesion molecules), angiogenesis (VEGF), metastasis (CXCR-4) and inflammation (NF-κB, TNF, IL-6, IL-1, COX-2 and 5-LOX). The activity of curcumin reported against leukemia and lymphoma, gastrointestinal cancers, genitourinary cancers, breast cancer, ovarian cancer, head and neck squamous cell carcinoma, lung cancer, melanoma, neurological cancers and sarcoma reflects its ability to affect multiple targets. Thus an "old-age" disease such as cancer requires an "age-old" treatment of using turmeric, popularly called "curry powder".

It is a potent antioxidant, anti-inflammatory, analgesic and anticancer agent used in traditional medicine. It has been shown to suppress cellular transformation, proliferation, invasion, angiogenesis and metastasis through a mechanism not fully understood. A lipooxygenase and cyclooxygenase inhibitor, Blood-brain barrier permeable substance and it exhibits a diverse range of actions including free radical scavenging activity *in vitro* and *in vivo* with cardio and neuroprotective effects (Aggarwal *et al*., 2005). Pure paraquat, when ingested, is highly toxic to mammals, including humans, potentially leading to acute respiratory distress syndrome. In acute toxicity studies using laboratory animals, paraquat has been shown to be highly toxic by the inhalation route and has been placed in Toxicity Category I (the highest of four levels) for acute inhalation effects. The main aim of this study is, to analyze the clonogenic and apoptosis assay on human human pulmonary carcinoma cell line (Caco-2) with Curcumin, Paraquat and two different combinations Curcumin and Paraquat.

MATERIALS AND METHODS

Curcumin and Paraquat

Curcumin is a derivative from *Curcuma longa* and was purchased as a crystalline solid. A stock solution was prepared by dissolving the curcumin in acetone and was diluted with sterile triple distilled water (1, 5, 10, 25, 50 and 100 µg/ml) for making the different concentrations. The herbicide paraquat (Paraquat Dichloride 24% SL) was purchased from HPM Chemicals & Fertilizers Ltd., Coimbatore and Tamil Nadu, and different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) were prepared with addition of aqueous solution.

Growth medium for human pulmonary carcinoma (NCI-H292) cells

The following culture methods were followed for growth, sub-culturing, cryopreserving and thawing of Pulmonary Carcinoma (NCI-H292) (ATCC® CRL-1848™). The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, ATCC 30-2001. To make the complete growth medium, add the following components to the base medium: fetal bovine serum (ATCC 30-2020) to a final concentration of 10%.

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. Remove and discard culture medium. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mMEDTA solution to remove all traces of serum that 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). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 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. Incubate cultures at 37°C. Sub-cultivation Ratio: A sub-cultivation ratio of 1:3 to 1:8 is recommended. Medium Renewal: Every 2 to 3 days.

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

Colony forming assay using pulmonary carcinoma (NCI-H292) with curcumin

Different doses of Curcumin were used (1, 5, 10, 25, 50 and 100µg/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 pulmonary carcinoma (NCI-H292) 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 and 5 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 pulmonary carcinoma (NCI-H292) 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 pulmonary carcinoma (NCI-H292) 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 pulmonary carcinoma (NCI-H292) with curcumin

Different doses of Curcumin were used (1, 5, 10, 25, 50 and 100µg/ml). A control without curcumin was also employed. No significant increase in 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 pulmonary carcinoma (NCI-H292) 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 increase in apoptosis compared to the control was noticed in 1 and 5 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 pulmonary carcinoma (NCI-H292) 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 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 pulmonary carcinoma (NCI-H292) 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).

Curcumin is a dicinnamoylmethane food dye consisting of three principal colouring components. The product consists essentially of curcumins i.e. the colouring principle (1E,6E)-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, with the molecular formula C21H20O6 and molecular weight of 368.39 g/mol and its desmethoxy- and bis-desmethoxy-derivatives with molecular formulas C20H18O5 and C19H16O4 and molecular weights of 338.39 and 308.39 g/mol respectively, in varying proportions. Curcumin is an orange-yellow crystalline powder which is water-insoluble but soluble in ethanol. More than 45 synonyms are mentioned in ChemIDplus. Turmeric Yellow, Kurkum, INS no. 100(i), CI Natural Yellow 3 and diferoylmethane are frequently used synonyms. Minor amounts of oils and resins naturally occurring in turmeric may be present in the final preparation.

According to JECFA (2004) and (1995), only the following solvents may be used in the extraction and purification: acetone, methanol, ethanol, isopropanol, hexane, ethyl acetate. Supercritical carbondioxide may also be used in the extraction. According to Directive 2008/128/EC only the following solvents may be used in the extraction: ethyl acetate, acetone, carbondioxide, dichloromethane, n-butanol, methanol, ethanol and hexane. LD50 values reported upon oral dosing amounted to 2 g/kg bw (test material not specified) and >10 g/kg bw (test material estimated to contain about 79% curcumin) for mouse and to 5 g/kg bw (test material not specified) for rat. The highest dose of 12 000 mg/day was not acceptable to the patients because of the bulky volume of the tablets (Cheng et al., 2001).

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). 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).

This study proved that the curcumin is one of the best herb for anticancer treatment and further study is needed for more development.

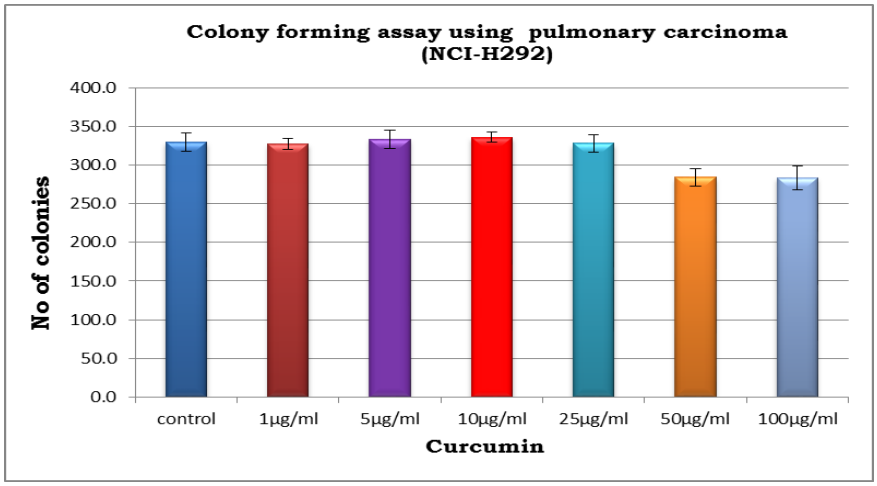

Figure 1. Colony forming assay using Pulmonary Carcinoma (NCI-H292) with curcumin.

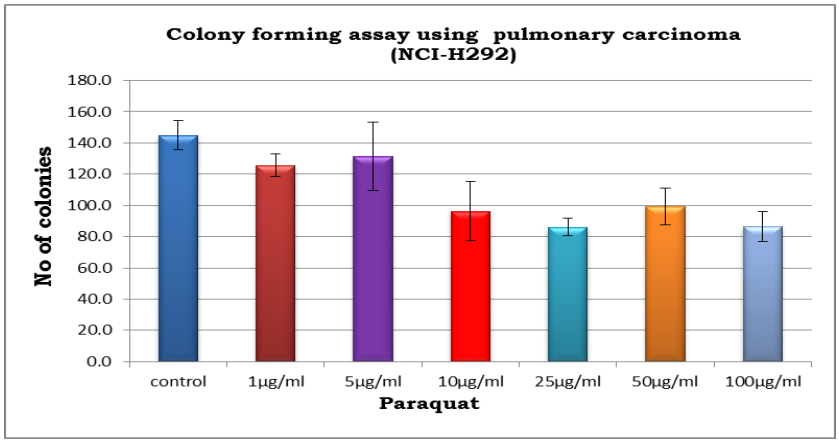

Figure 2. Colony forming assay using Pulmonary Carcinoma (NCI-H292) with paraquat.

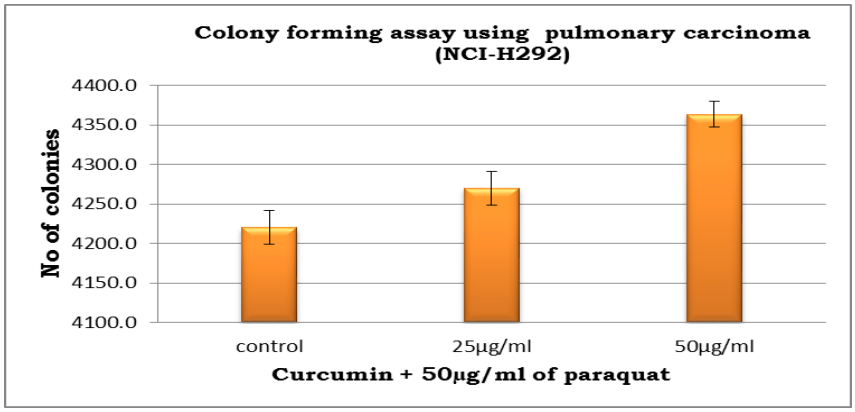

Figure 3. Colony forming assay using Pulmonary Carcinoma (NCI-H292) with curcumin 25 and 50 µg/ml and 50 µg/ml of paraquat.

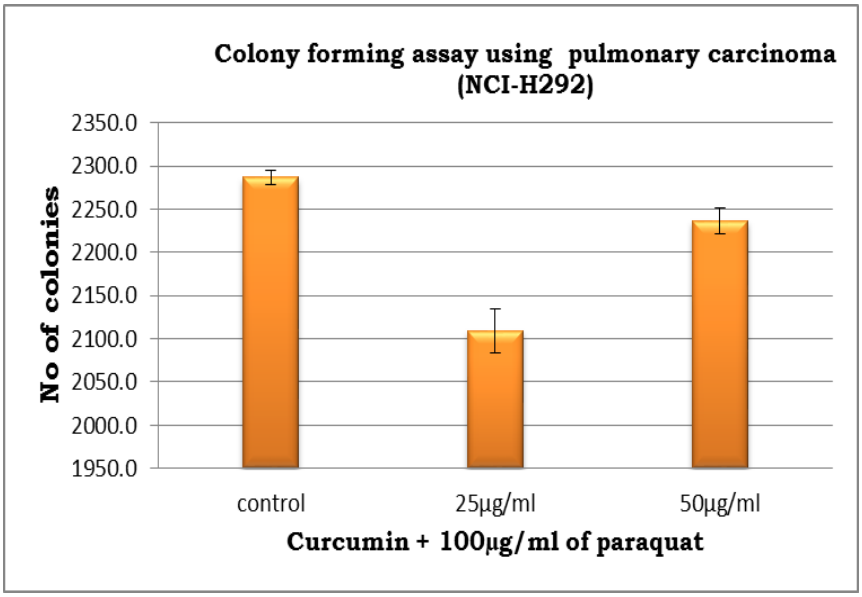

Figure 4. Colony forming assay using Pulmonary Carcinoma (NCI-H292) with curcumin 25 and 50 µg/ml and 100 µg/ml of paraquat.

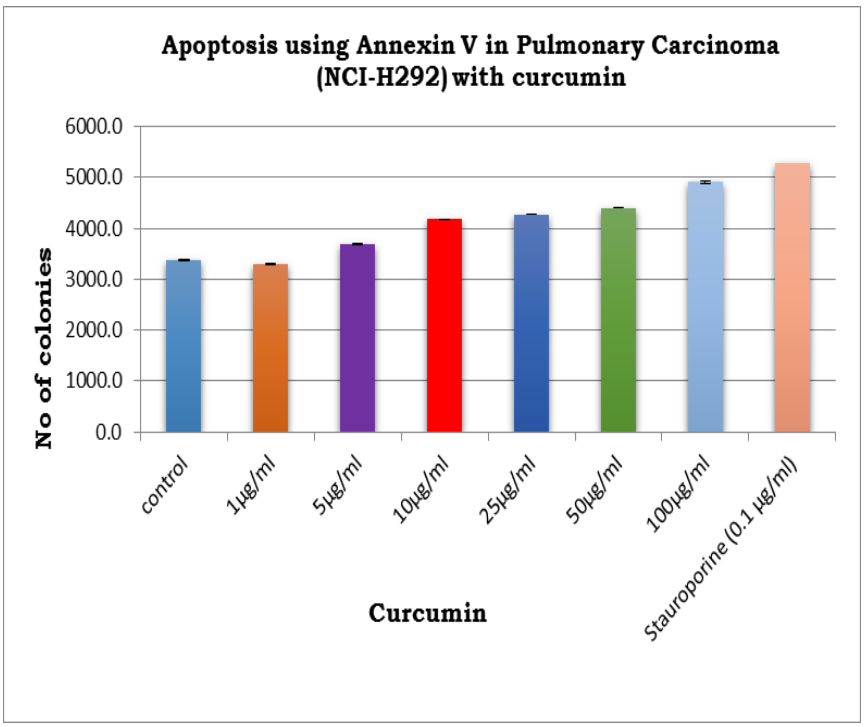

Figure 5. Apoptosis using Annexin V in Pulmonary Carcinoma (NCI-H292) with curcumin.

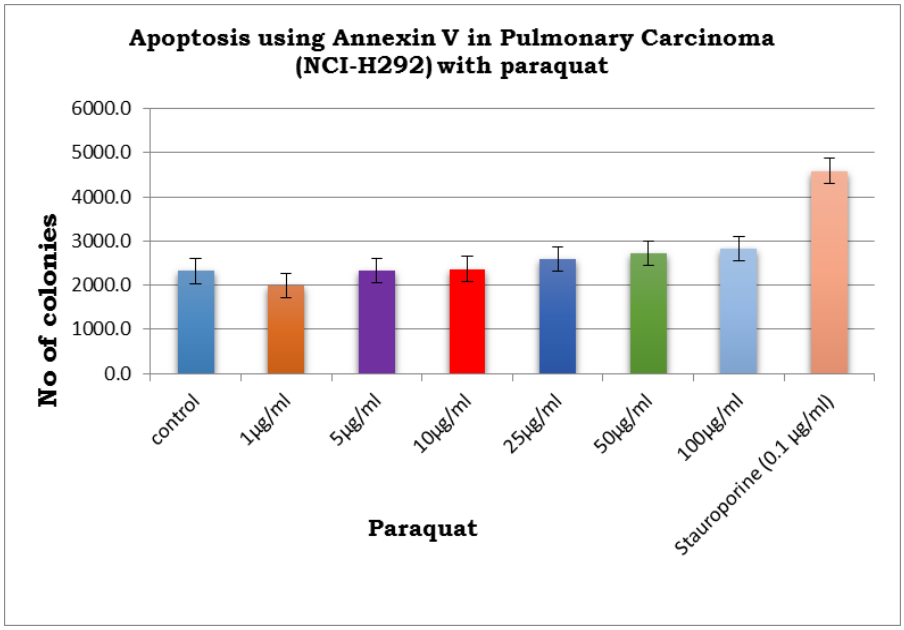

Figure 6. Apoptosis using Annexin V in Pulmonary Carcinoma (NCI-H292) with paraquat.

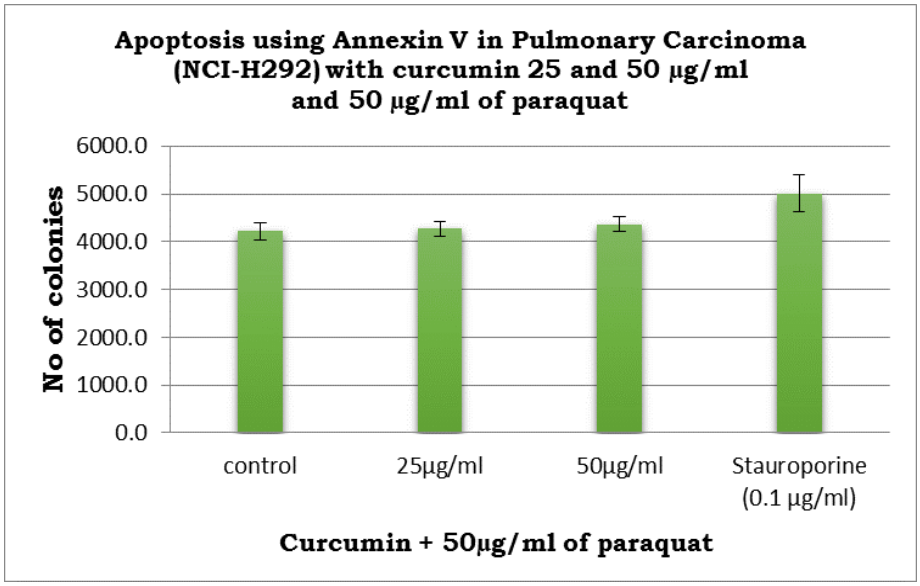

Figure 7. Apoptosis using Annexin V in Pulmonary Carcinoma (NCI-H292) with curcumin 25 and 50 µg/ml and 50 µg/ml of paraquat.

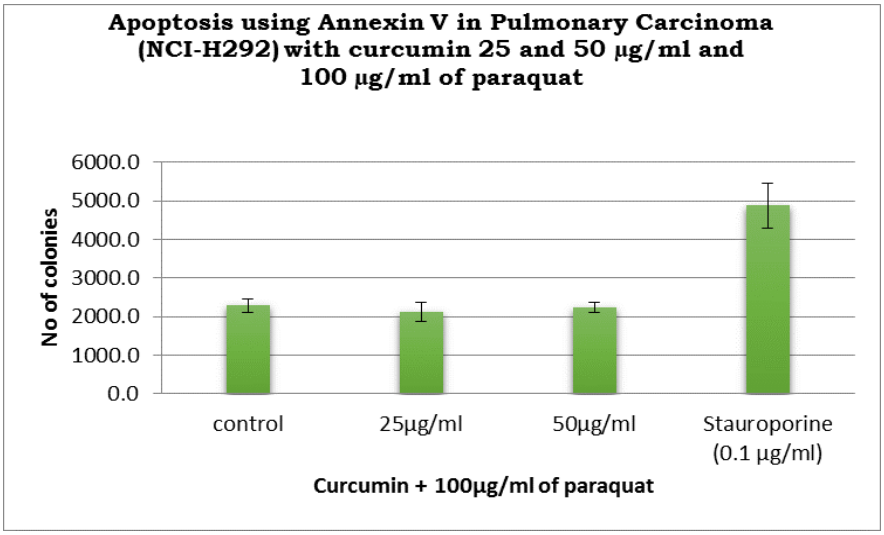

Figure 8. Apoptosis using Annexin V in Pulmonary Carcinoma (NCI-H292) with curcumin 25 and 50 µg/ml and 100 µg/ml of paraquat.

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