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IN-VITRO CLONOGENIC AND APOPTOSIS SCREENING OF CURCUMIN (*CURCUMA LONGA)*, BRUSATOL AND PARAQUAT COMBINATION AGAINST HUMAN COLORECTAL ADENOCARCINOMA [NCI-PBCF-HTB37 (CACO-2)] CANCER CELL LINE

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ABSTRACT

The present study focused on the colony forming and apoptosis assay of Curcumin, Paraquat and brusatol combinations against human colon adenocarcinoma cell line (Caco-2). In this study, different concentrations of Curcumin, brusatol 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. In colony forming assay, increase in colony formation compared to the control was observable in 25 µg/ml dose of curcumin. Considerable reduction in colony formation resulted in 50 µg/ml dose of curcumin in both studies (50 and 100 µg/ml Paraquat). Similar to colony forming assay, considerable increase in apoptosis resulted in 50 µg/ml dose of curcumin in apoptosis assay. In both study, the combination of curcumin and brusatol gave an effective anticancer activity.

KEYWORDS: Clonogenic, Curcumin, Brusatol, Paraquat, Cancer.

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". Most human malignancies are driven by chromosomal translocations or other genetic alterations that directly affect the function of critical cell cycle proteins such as cyclins as well as tumor suppressors, e.g., p53. In this respect, cell cycle regulation and its modulation by curcumin are gaining widespread attention in recent years. The mechanisms implicated are diverse and appear to involve a combination of cell signaling pathways at multiple levels. Curcumin binds to a variety of proteins and inhibits the activity of various kinases. By modulating the activation of various transcription factors, curcumin regulates the expression of inflammatory enzymes, cytokines, adhesion molecules, and cell survival proteins.

Curcumin has been shown to protect against skin, oral, intestinal, and colon carcinogenesis and also to suppress angiogenesis and metastasis in a variety animal tumor models. It also inhibits the proliferation of cancer cells by arresting them in the various phases of the cell cycle and by inducing apoptosis. Moreover, curcumin has a capability to inhibit carcinogen bioactivation via suppression of specific cytochrome P450 isozymes, as well as to induce the activity or expression of phase II carcinogen detoxifying enzymes

(Lee and Surh, 2005).

Brusatol, a quassinoid extracted from *Brucea javanica* (L.) Merr., a medicinal plant distributed widely throughout Asia where its bitter fruits have been used traditionally in medicine for treating various ailments. Pure paraquat, when ingested, is highly toxic to mammals, including humans, potentially leading to acute respiratory distress syndrome. The main aim of this report is, to find the colony forming and apoptosis assay on human colon adenocarcinoma cell line (Caco-2) (while Paraquat used as an inducer) with Curcumin and brusatol in different concentrations.

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.

BRUSATOL

Brusatol (13,20-Epoxy-3,11β,12α-trihydroxy-15β-[(3-methyl-1-oxo-2-butenyl)oxy]-2,16- 72 dioxopicras-3-en-21-oic acid methyl ester) was purchased from Sigma chemicals (St. Louis, MO, USA). Required dilution was prepared using appropriate solvent. 1µg/ml dilution of brusatol was 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 12 d 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, brusatol and Paraquat were used to study Colony forming assay and Apoptosis.

COLONY FORMING ASSAY USING COLORECTAL ADENOCARCINOMA NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 50 µG/ML OF PARAQUAT

Two separate doses of curcumin (25 and 50 µg/ml) were used. Two separate doses of curcumin were used along with 1µg/ml of brusatol and 50 µg/ml of paraquat. Increase in colony formation compared to the control was observable in 25 µg/ml dose of curcumin. Considerable reduction in colony formation resulted in 50 µg/ml dose of curcumin. Three culture plates were used in the experiment for each dose of curcumin (Figure 1).

COLONY FORMING ASSAY USING COLORECTAL ADENOCARCINOMA NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 100 µG/ML OF PARAQUAT

Two separate doses of curcumin (25 and 50 µg/ml) were used. Two separate doses of curcumin were used along with 1µg/ml of brusatol and 100 µg/ml of paraquat. Significant increase in colony formation compared to the control was observable in 25 µg/ml dose of curcumin. Strong increase in colony formation resulted in 50 ug/ml dose of curcumin. Three culture plates were used in the experiment for each dose of curcumin (Figure 2).

APOPTOSIS USING COLORECTAL ADENOCARCINOMA (NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 50 µG/ML OF PARAQUAT

Two separate doses of curcumin (25 and 50 µg/ml) were used. Two separate doses of curcumin were used along with 1µg/ml of brusatol and 50 µg/ml of paraquat. Increase in apoptosis compared to the control was observable in 25 µg/ml dose of curcumin. Considerable increase in apoptosis resulted in 50 µg/ml dose of curcumin. Three culture plates were used in the experiment for each dose of curcumin (Figure 3).

APOPTOSIS USING COLORECTAL ADENOCARCINOMA (NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 100 µG/ML OF PARAQUAT

Two separate doses of curcumin (25 and 50 µg/ml) were used. Two separate doses of curcumin were used along with 1µg/ml of brusatol and 100 µg/ml of paraquat. Significant increase in apoptosis compared to the control was observable in 25 µg/ml dose of curcumin. Strong increase in apoptosis resulted in 50 ug/ml dose of curcumin. Three culture plates were used in the experiment for each dose of curcumin (Figure 4).

Curcumin is obtained by solvent extraction of turmeric i.e. the ground rhizomes of natural strains of *Curcuma longa* L. (*Curcuma domestica* Valeton). In order to obtain a concentrated curcumin powder, the extract is purified by crystallisation. Curcumin may be converted to its corresponding aluminium lake under aqueous conditions by reacting aluminium oxide with the colouring matter. Undried aluminium oxide is usually freshly prepared by reacting aluminium sulphate or aluminium chloride with sodium carbonate, sodium bicarbonate or aqueous ammonia. Following lake formation, the product is filtered, washed with water and dried (JECFA, 2004). The aluminium lake of curcumin has a quite high ratio of aluminium: pigment (2:1) and is to some extent similar to the more conventional lakes made with synthetic colours (based on single supplier data).

For specific foodstuffs, the CIAA provided the Panel with the following typical use levels of curcumin per kg of food. Confectionery products (10-300 mg/kg), decorations and coatings (150-500 mg/kg), fine bakery wares (4-200 mg/kg), edible ices (0.64-150 mg/kg), desserts, including flavoured milk products (1-74 mg/kg), sauces and seasonings (2-500 mg/kg), soups (2.5-33 mg/kg), jams, jellies and marmalades (5-30 mg/kg), extruded or expanded savory snack products (typical value of 12 mg/kg), complete formulae for weight control (typical value of 10 mg/kg), solid food supplements (typical value of 10 mg/kg), sausages, pates and terrines (typical value of 2.5 mg/kg) and for margarine/minarine (typical value of 10 mg/kg). The association for Natural Colours (NATCOL) also provided use level data for the following foodstuffs per kg of food: confectionery (5-300 mg/kg), decorations and coatings (10-500 mg/kg), fine bakery wares (1-200 mg/kg), edible ices (2-150 mg/kg), flavoured processed cheese (50-100 mg/kg), desserts, including flavoured milk products (1-150mg/kg), sauces and seasonings (2-300 mg/kg), smoked fish (2-25 mg/kg), snack products (10-50 mg/kg), edible cheese rind and casings (10-50 mg/kg), jams, jellies and marmalades (5-100 mg/kg), and margarine/minarine (2-40 mg/kg).

Curcumin was tested in ADME studies in mice and humans (Tullberg *et al*., 2004). In the mouse study 20 female and 20 male MF1 mice were dosed by oral gavage at 220 mg/kg bw and 50 male and 50 female adult B6C3F1 mice were dosed at 10 mg/kg bw by oral gavage. In animals dosed at 220 mg/kg bw extremely low plasma concentrations were detected, given the high dose administered. There were statistically significant differences between the sexes ($p<0.05$ by unpaired t-test) suggesting a possible difference in elimination and probably due to more extensive first pass metabolism in males. In the mice dosed at 10 mg/kg bw the results suggested the possibility of non-linear kinetics when

compared to the data at the higher dose of 220 mg/kg bw, although the authors also stated that a clear conclusion could not be reached because of the poor precision of the plasma concentration data at the lower dose. The JECFA evaluated four acute oral toxicity studies. 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. This study proved that the brusatol with curcumin combination effectively act against the human colorectal adenocarcinoma cell line.

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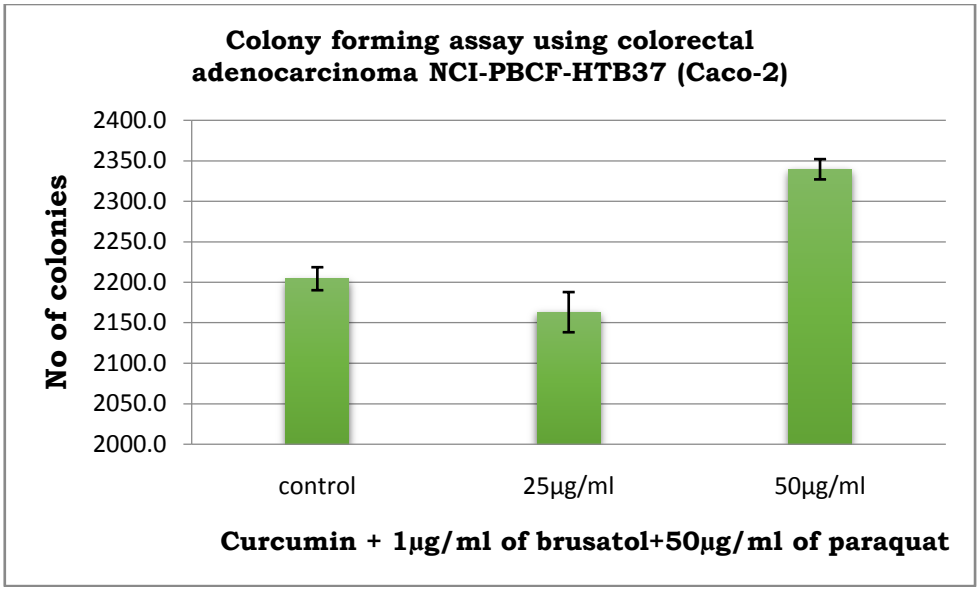

FIGURE 1
COLONY FORMING ASSAY USING COLORECTAL ADENOCARCINOMANCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 50 µG/ML OF PARAQUAT

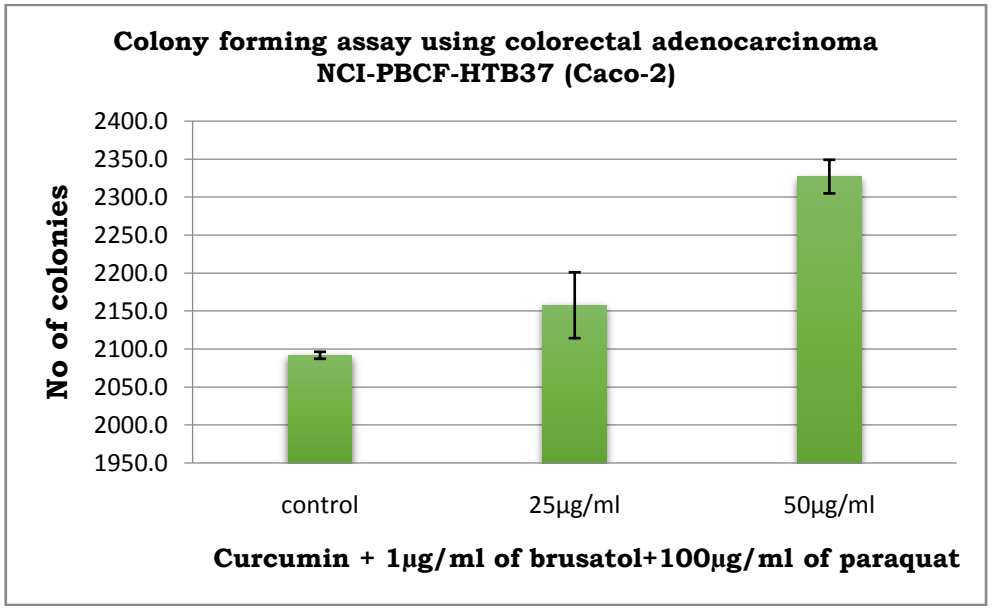

FIGURE 2
COLONY FORMING ASSAY USING COLORECTAL ADENOCARCINOMA NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 100 µG/ML OF PARAQUAT

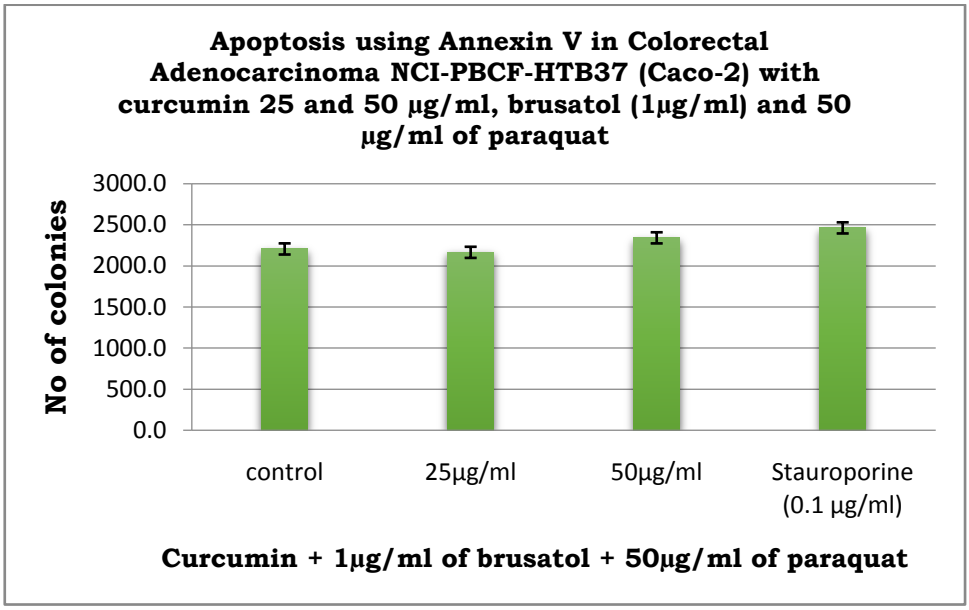

FIGURE 3
APOPTOSIS USING ANNEXIN V IN COLORECTAL ADENOCARCINOMA NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 50 µG/ML OF PARAQUAT

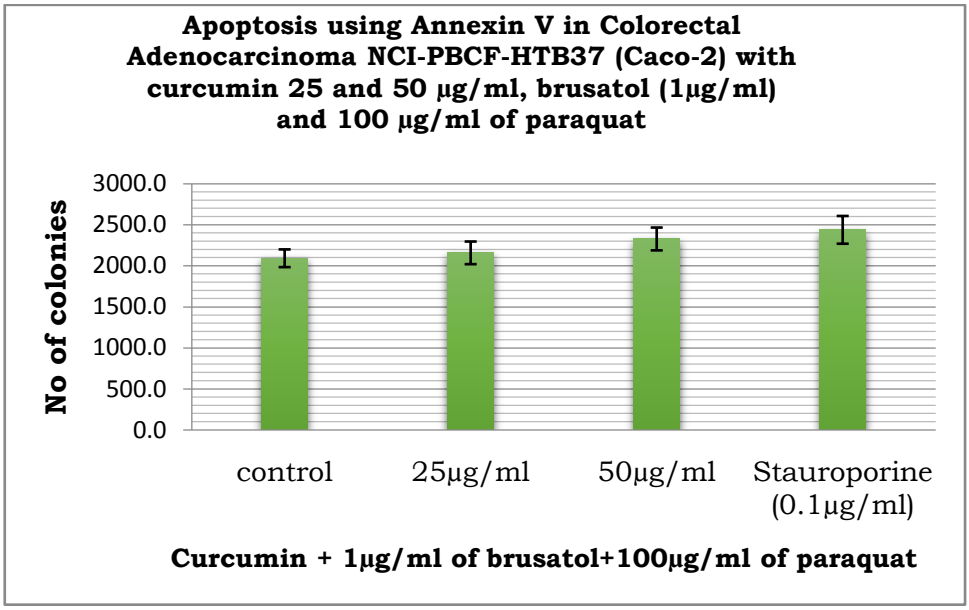

FIGURE 4
APOPTOSIS USING ANNEXIN V IN COLORECTAL ADENOCARCINOMA NCI-PBCF-HTB37 (CACO-2) WITH CURCUMIN 25 AND 50 µG/ML, BRUSATOL (1µG/ML) AND 100 µG/ML OF PARAQUAT