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Asian Journal of Plant Science and Research, 2014, 4(6):25-31

ISSN : 2249-7412
CODEN (USA): AJPSKY

Anticancerous and antiproliferative/cytotoxic activity of curcuma pseudomontana (hill turmeric) collected from the sub Himalayan region of Uttrakhand, India

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ABSTRACT

In this study we determineanticancerous and cytotoxic/antiproliferative activity of Curcuma pseudomontana. The tuber with rhizomes of Curcuma pseudomontanawere collected, shade dried and then extracted with distill water, methanol and ethanol using soxhlet extraction procedure. Anticancer activity of different plant samples is determined by cytopathic effect of plant extract on BHk-21 cell line, antiproliferative and cytotoxic activity is determined by using MTT assay against BHK-21 cell line. From this study we determine that the active phytocompounds inhibit the growth of BHK-21 cell line, this is confirmed after cytopathic effect determination. Between 2-20 mg is IC_{50} value of plant extract for BHK-21 cell line; at this concentration 50% cells are died. Observations show that antiproliferative/cytotoxic activity of all extractsincreases with increasing the concentration of plant extract.

Key words: *Curcuma pseudomontana*, anticancer, cytotoxic/antiproliferativeactivity, MTT assay, tryphan blue assay.

INTRODUCTION

Cancer is the second leading cause of death in the world [1]. Plants play an important role as a source of effective anti-cancer agents. Currently over 60% anti-cancer agents are derived from natural sources, including plants, marine organisms, and micro-organisms [2]

Curcumin is a primary active compound of all curcuma plants, it is responsible for yellow color of curcuma [3], older investigations shows that curcumin has antimicrobial[4,5,6] anti-inflammatory [7], dyspepsia and gastric ulcer [8] ,irritable bowel syndrome[9,10,11], pancreatitis [12,13] ,rheumatoid arthritis [12,13],osteoarthritis[14], irritable bowel syndrome[9,10,11], anti-inflammatory and anti-oxidant [15] .

Different other species of curcuma show different types of medicinal property, therefore the present study is investigated for determination of Anticancerous and cytotoxic/antiproliferative activity.

MATERIALS AND METHODS

Collection of plant sample
Curcuma pseudomontana (tubers) collected from the forest of IBT Patwadanger Nainital in winter session, when plant is fully mature and then shade dried. The shade dried tubers are then powderedby using mortar and pestle. The powder is then stored in air tight bottles at 4℃.

Preparation of plant extract
The shade dried (40g) powder of each plant materialwere filled separately in the thimble and extracted withmethanol, ethanol and distill water using a Soxhlet extractor followed bydistillation and evaporation. The plant powder is placedin a cellulose thimble. This extract containing thimble is then placed in an extraction chamber, whichis placed on top of a collecting flask beneath a refluxcondenser. Solvent is added to the flask, and the setup is heated under reflux. When a certain level ofcondensed solvent has accumulated in the thimble, it issiphoned into the flask beneath. Nearly 15 cycles arerepeated for 72 hours for all the solvents. Afterextraction the solvent is removed by rotary evaporator, yielding the extracted compound [16].

Cell line-For cytotoxic and cytopathic study we use BHK-21(baby hamster kidney fibroblastic cell) cell line. These are obtained from IVRI muktheswar.These cells are properly revived and sub cultured for proper growth in 48 hour interval.

Determination of cell viability by tryphan blue method-viability of cells determined by tryphan blue method.[17,18]cells are seeded in 96 well plate with the total viable cells (%) were calculated by using haematocytometer as-

Total cells/ml= (total cell count/5) × (dilution factor)×10^4

Viable cells/ml= (viable cell/5) × (dilution factor)×10^4

Percentage (%) of viable cells=100× (viable cell count/total cell count)

Tryphan blue is a diazo dye, which stains dead cells and dead cells become blue in colour.

Cytopathic study of plant sample for anti cancerous activity-CPE of plant extract determined by as per Goncalves *et al., 2005* with slight modification. In this study CPE used for study of morphological changes on BHK-21 cells after plant extract addition. [19]

Procedure- For this firstly we maintain the cell growth and cell no. (Viability of cells3.0 × 10^4 cells/ well) determined by tryphan blue method. After proper growth we add plant sample on cultured cell and incubate these cells in 5% CO_2 incubator at 37℃ . After 4hour interval we see the changes on cells till 48 hours.

Cytotoxic activity of plant active compounds-cytotoxic effect of plant active compounds is determined after IC_{50} value calculation. For calculation of IC_{50} value MTT assay is used [20, 21].

MTT assay-This is a colorimetric assay that measures the percentage of metabolicly active cells.

Principle -MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. Reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. This formazan production is directly proportional to the viable cell no and inversely proportional to the degree of cytotoxicity.

The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viable cells. . The metabolic activity of each well was determined by the (MTT) assay and compared to those of untreated cells.

Procedure-
MTT (5 mg/ml) was dissolved in PBS. This solution was filtered through a 0.2 μm filter and stored at 2 - 8°C. Cells were cultured in 96-well plates (3.0×10^4 cells/ well, this cell no. calculated by using tryphan blue method) containing 100 μl medium, incubate this plate in CO_2incubator (5% CO_2) at 37°C for 4 hrs. Diluted plant extract

solutions were freshly prepared in DMSO prior to each experiment and then applied.After removal of 100 μl medium, MTT dye solution was added and the plates were incubated at 37°C for 3 hrs in a humidified 5% CO2 incubator. After that, 100μl of DMSO were added to each well, and mixed thoroughly to dissolve the dye crystals. The absorbance was measured using an ELISA plate reader at 570 nm with a reference wavelength of 630 nm. High optical density readings corresponded to a high intensity of dye color, that is, to a high number of viable cells able tometabolize MTT salts. The fractional absorbance was calculated by the following formula:

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. The 50% inhibition concentration (IC50) of the active substances was determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture. An IC50 less than 20 µg/ml of crude extract were considered as an active compound against cancer cells, following the standard National Cancer Institute (NCI) criteria.

RESULTS AND DISCUSSION

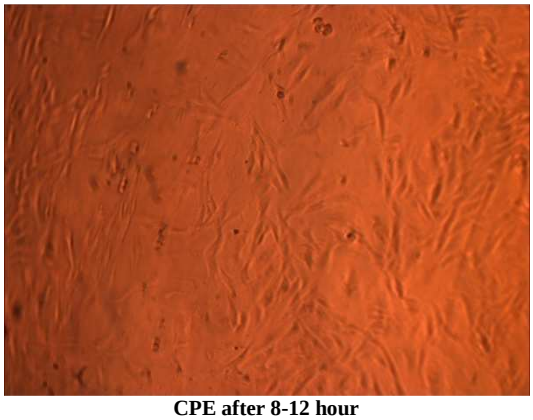
Viability and characterization of cell lines-cell lines are free from any type of bacterial and fungal contamination and viable when sub cultured.

Result for Cytopathic Effect (CPE) of plant extract-cytopathic effect of hill turmeric shown in fig-1. These effects areshown by plant active compounds on BHK-21 cells.In CPE cell structure continuously changed and at the end these cells are died.

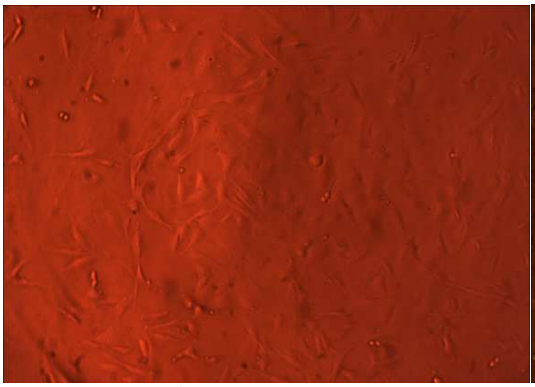
After addition of hill turmeric extract in microtiter culture plate, the structure of BHK-21 cells continuously changed. After 8-12 hours total destruction of cells (all cell's of monolayer rapidly shrink, becomedense and become detach from the surface), after 24 hours swelling and clumping of cell (cells are greatly enlarge and clump together) and after 48 hours cell fusion/syncytium formation (fusion of plasma membranes of four and more cells with four and more nuclei) occur.

Changes in morphology of cell line due to presence of secondary metabolites of plants. Curcumin is biologically active component of all turmeric tubers, this compound show many types of biological activity. Curcumin show anticancer activity by cell cycle arrest and induction of apoptosis [22]. Apoptosis followed by nucleus and DNA fragmentation and small apoptotic body formation.

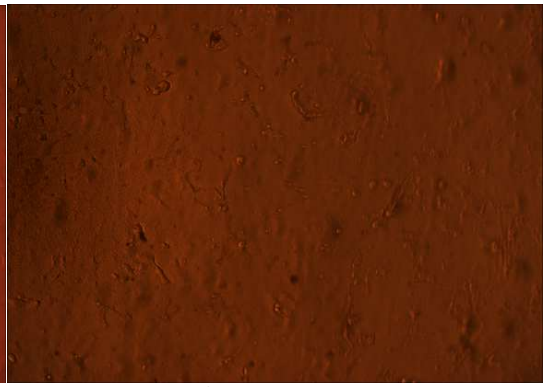
BHK21 Cells
Cell without plant extract.



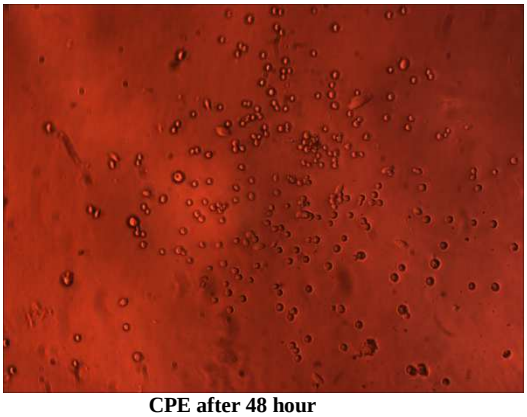
CPE after 8-12 hour
Total destruction of cell's



CPE after 24 hour
Swelling & clumping of cell's



CPE after 28-32 hour
Cell fusion (syncytium formation)



CPE after 48 hour
Focal degeneration of cell's

Fig 1. Effect of tuber extract on BHK21 cell line with time

Table no.1-Percentage of cell viability for various plants extracts (master sample20mg/5ml)

Plant sample	20mg	2mg	0.01 (200µgm)	0.001 (20µgm)	0.0001 (200Pico gm)	0.00001 (20 Pico gm)	0.000001 (200ngm)	0.0000001 (20ngm)
aqueous	44.87	54.87	58.46	59.35	60.38	62.3	64.16	67.82
ethanolic	46.15	57.43	58.97	59.23	59.61	60.51	62.02	74.23
methanolic	46.15	57.43	58.97	59.23	59.61	60.51	62.05	74.23

Determination of cytotoxicity: Cytotoxicity of different plant extracts are determined by MTT assay. Toxicity of plant extract against cells is measured in terms of IC_{50} (inhibitory concentration at which 50% cells are died).Below IC_{50} range the plant extract is toxic for mammalian cells. DMSO used as a standard. Aqueous extract show maximum toxicity against cells, methanolic and ethanolic plant extract show low toxicity and maximum cell survival rate. At concentration 10^{-6}(20ngm) maximum cells survived, so when plant extract is diluted survival rate of cells is increased.

For aqueous,ethanolic and methanolic extract IC_{50} value comes between 2 mg-20 mg,above this concentration the cell mortality rate is increases and below this the cell survival rate is higher.

Cytotoxicity is dose depended with increasing dose cytotoxicity also increases. Cytotoxicity is due to the cell cycle inhibition at the time of cell cycle progression and by apoptosis [23, 24, 25].

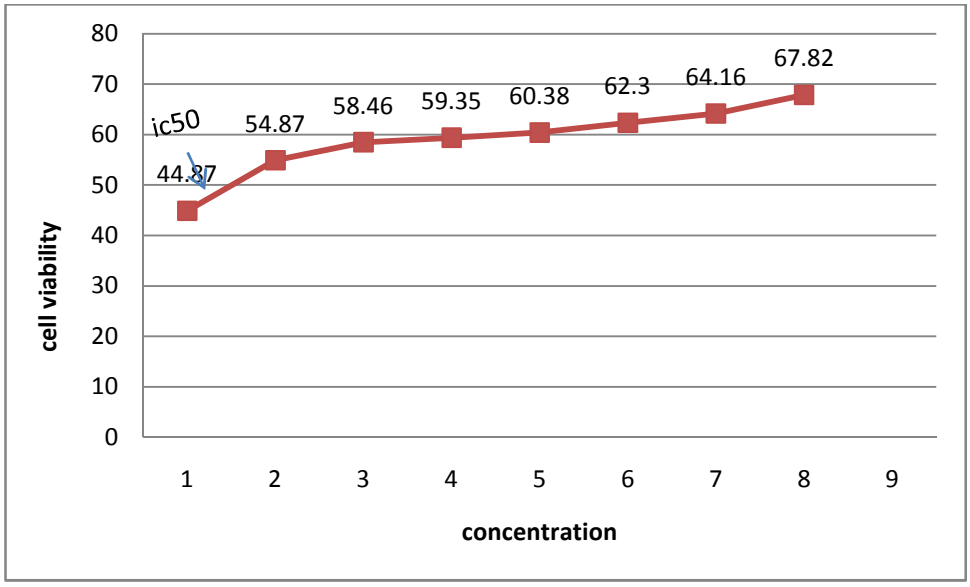


Fig 2-cytotoxicity and IC50 value of aqueous sample of Curcuma pseudomontana on BHK-21 cell line
1, 2,3,4,5,6,7,8 shows the dilution concentration of plant extract by DMSO (20mg-20ngm)

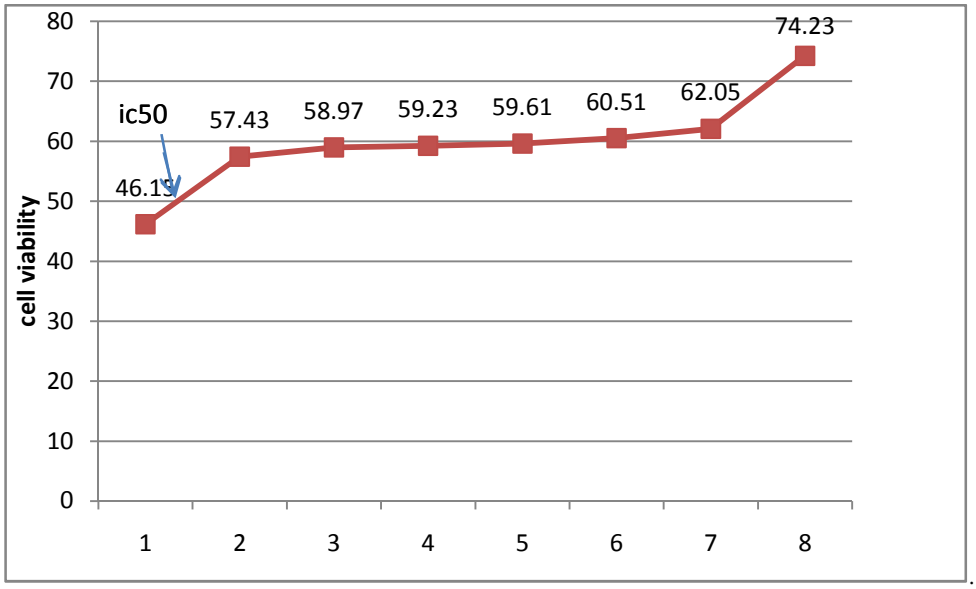


Fig 3- cytotoxicity and IC50 value of ethanolic sample of Curcuma pseudomontana on BHK-21 cell line
1, 2,3,4,5,6,7,8 show the dilution concentration of plant extract by DMSO (20mg-20ngm)

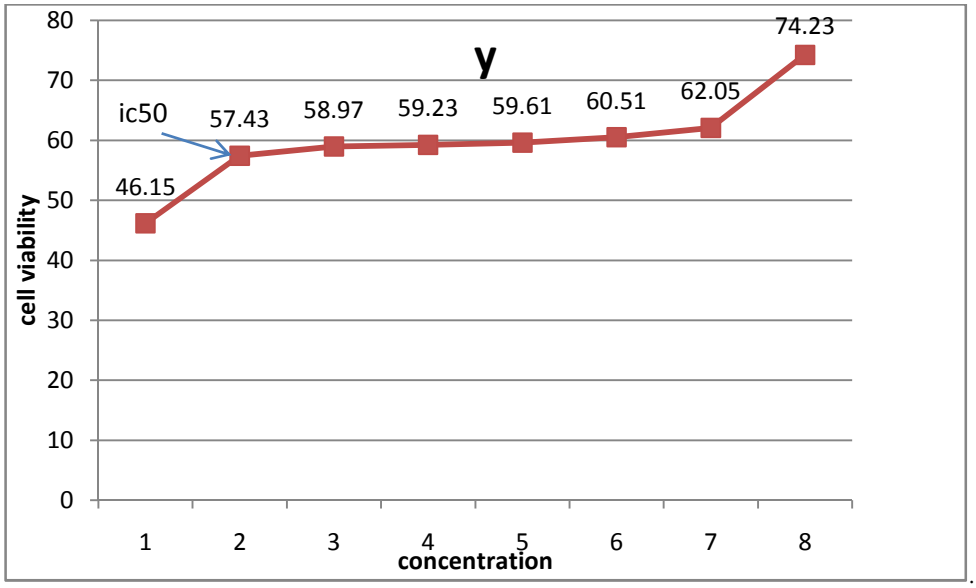


Fig 4- cytotoxicity and IC50 value of methanolic sample of Curcuma pseudo Montana on BHK-21 cell line
1, 2,3,4,5,6,7,8 shows the dilution concentration of plant extract by DMSO (20mg-20ngm)

CONCLUSION

This is the first report of anti cancer and antiproliferative/cytotoxic of *Curcuma pseudomontana*. Other many different species of curcuma show anti cancer activity. Different plant extract of*Curcuma pseudomontana* contain certain types of active compounds, these active compounds show many types of activity (eg.antimicrobial, anticancerous etc.).These active compounds are extracted with appropriate solvent (organic/inorganic).Selection of solvent depend upon the type of active compound. Curcumin is main active compound of turmeric plant and many researchers prove this. Further research needed in this direction to use this safely for cancer patients.

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