

IN VITRO ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITY OF CURCUMA AERUGINOSA SUPER CRITICAL CO₂ EXTRACT IN HUMAN HELA CELL LINE.

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ABSTRACT

The antiproliferative and antioxidant potential of Curcuma aeruginosa extracts were investigated. The extracts were isolated by Super Critical fluid extraction method. SCF extracts were subjected to column chromatography was resulted in six fractions. These fractions were subjected to Total antioxidant assay. One of the fractions showed comparatively high antioxidant activity. And this fraction was subjected to antiproliferative activity test on HeLa Cell line was initially procured from National Centre for Cell Sciences (NCCS) Pune. The cell viability was determined using MTT assay. The percentage of viability is found to be lowered (43.16±1.16) with increased concentration (100µg/ml) of the sample. In contrast percentage of viability was more (82.67±1.15) when the concentration of the sample less (6.25µg/ml).

Keywords: Antioxidant activity, HeLa cell, SCF extracts, MTT Assay

INTRODUCTION

Cancer is a disease in which cells in the body grow out of control. Cancer is always named for the part of the body where it starts, even if it spreads to other body parts later. When cancer starts in the cervix, it is called cervical cancer (Lutgens L., et al. 2010). Cervical cancer is the fourth most common forms of cancer in women worldwide. The disproportionately high burden of cervical cancer in developing countries and elsewhere in medically underserved populations is largely due to a lack of screening that allows the detection of precancerous and early stage cervical cancer (Parkin DM., et al. 2008). Risk factors for cervical cancer include human papillomavirus (HPV) infection (90% of cases), smoking, a weak immune system, and birth control pills, starting sex at a young age and having many sex partners (Mitchell RS., et al. 2007). Several approaches manage the treatment of cervical cancer, including chemotherapy and radiotherapy. However, other strategies with fewer side effects are necessary for treatment and prevention. Relevant approaches particularly food-based entities stay essential in reducing the risk of cancer (Follen M., et al 2003). There are several cell lines for human cervical cancer such as HeLa, C4-1 and SW756. The HeLa cell line is a cervical cancer cell line that is commonly used in experimental research and is aggressive in culture.

Zingiberaceae family also known as ginger family comprises a number of approximately 52 genera and over 1300 species of aromatic plants (Kress WJ., et al. 2002). The rhizomes of Curcuma species (*C. aeruginosa*, *C. amada*, *C. aromatica*, *C. brog*, *C. caesia*, *C. malabarica*, *C. rakthakanta*, *C. sylvatica* and *C. zedoaria*) are also pharmacologically important and are possess antioxidant, antimicrobial, anti-inflammatory and cytotoxic properties. Literature has reported Curcuma caesia has anti-inflammatory, hepatoprotective, antioxidant, antiasthmatic, antitumour, stomachic and carminative properties (Sahu B., et al. 2013). The anti-proliferative activity of *C. caesia* was reported against three human cancer cell lines- (MCF-7) human breast cancer, (HCT-116) human colon cancer and (PA-1) ovarian cancer using the SRB (sulforhodamine B) assay (Shaikh A M., et al. 2016). Additionally, this natural polyphenol has been described as an anticancer agent, both in vitro and in vivo on a wide range of cancer types, such as colon, pancreatic, liver, cervical, pulmonary, thymic, brain, breast and bone cancer (Bar-Sela G., et al. 2010). The aim of this study was to determine the antioxidant activity of SCF extracts obtained from the rhizome of *C. aeruginosa*, and to perform preliminary in vitro tests regarding a possible antiproliferative HeLa Cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium Himedia).

Material and Methods

Collection of Plant material and extraction: Plant material was collected in February 2015 from Waynad district of Kerala, India. Taxonomic identification of the plants was carried out by Dr. S John Britto, Director at the Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, Tamilnadu, India. Voucher specimens (RHT 68570) is submitted at the Rapinat Herbarium. Fresh rhizomes of Black turmeric (*Curcuma aeruginosa*) was

collected sliced into small pieces, shade dried and powdered extracted through Super Critical Fluid Extraction (SCF) method.

Purification of extract by column chromatography

35g silica was suspended in hexane and was transferred in to the column without any breakage or channels. 1g of crude dissolved in 5ml ethanol was loaded on the column and was allowed to get adsorbed. The adsorbed components were then eluted by solvents based on its polarity. Hexane, Ethyl acetate and Methanol were the solvents used for elution. (Table:1) 20 ml was used for each elution. The eluted solvent was collected in different fractions. It was observed that with the increase in polarity the colour of the fraction changed from the colourless to yellow. Then the fractions allowed to condensation for volume reduce.

Total anti-oxidant activity by phosphomolybdenum method

The total antioxidant capacity of the extract was evaluated by the phospho-molybdenum method, according to the procedure described by Prieto et al.(23). 10 μ L of each fraction were combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm using a UV-VIS spectrophotometer against blank after cooling to room temperature. Methanol (10 μ L) in the place of extract was used as the blank. The total antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic with methanol

Invitro antiproliferative determination by MTT assay

Hela Cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Himedia). The cell line was cultured in 25 cm² tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100 μ g/ml), and Amphotericin B (2.5 μ g/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO₂ incubator (Galaxy[®] 170 Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100 μ l cell suspension (5x10⁴ cells/well) was seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO₂ incubator.

Preparation of plant extracts and compound stock:

1 mg of each plant extract or compound was added to 1ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 μ m Millipore syringe filter to ensure the sterility.

Antiproliferative effect Evaluation:

After 24 hours the growth medium was removed, freshly prepared samples in 5% DMEM were five times serially diluted by two fold dilution (200 μ g, 100 μ g, 50 μ g, 25 μ g, 12.5 μ g in 100 μ l of 5% MEM) and each concentration of 100 μ l were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO₂ incubator.

Antiproliferative effect by MTT Method:

Fifteen mg of MTT (Himedia, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30 μ l of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO₂ incubator for 4 hours. After the incubation period, the supernatant was removed and 100 μ l of MTT Solubilization Solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

Results and Discussion

Total antioxidant capacity by Phosphomolybdenum Method:

The total antioxidant capacity of 6 fractions obtained through column chromatography was compared with standard ascorbic acid. An increase in absorbance at 695 nm indicated the total antioxidant capacity of the extracts. The highest activity obtained from the fraction of Hexane: Ethyl acetate (1:1) and the Concentration of antioxidants in $\mu\text{g}/10\mu\text{L}$ of sample is 588.125 (Table 1: Fig. 1).Further analysis had done based on this fraction.

Antiproliferative effect by Direct Microscopic observation:

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICAPS™ HD camera) and microscopic observation were recorded as images.(Fig: 2,3) Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Curcuma aeruginosa SCF Extract Effectively Decreased Cell Proliferation

The results of MTT assay indicated that the active fraction showed an inhibitory effect on proliferation in all doses on HeLa cell line compared to the controls after 24 and 72 hours. It was found that with an increase in the dose 6.25, 12.5,25,50,100 $\mu\text{g}/\text{ml}$ of extract reduced the survival rate of HeLa cells (Fig 4). A relationship was detected between the reduction of cell viability and increasing concentrations of extract. The lowest cell survival rate occurred after administration of 100 $\mu\text{g}/\text{mL}$.

Conclusion

In conclusion, our finding indicated that active fraction of SCF extract has antitumor activity against HeLa cell line in a dose and time dependent manner after 24 and 48 hours. We suggested that this extract might be considered as a therapeutic candidate in cancer therapy. Nevertheless, more examination should be done in vitro and preclinical settings before conducting clinical trials; and its efficiency on normal cells should be clarified in future studies.

Fraction NO:	Column fraction	OD at 695nm	Conc of antioxidants in $\mu\text{g}/10\mu\text{L}$ of sample
1	100% Hexane	0.651	81.375
2	Hexane : Ethyl acetate (7:3)	3.081	385.125
3	Hexane: Ethyl acetate (1:1)	4.705	588.125
4	Hexane: ethyl acetate (3:7)	1.995	249.375
5	100% Ethyl acetate	0.226	28.25
6	Ethyl acetate:Methanol (99:1)	0.027	3.375

Table: 1- Purification of SCF extract by column chromatography

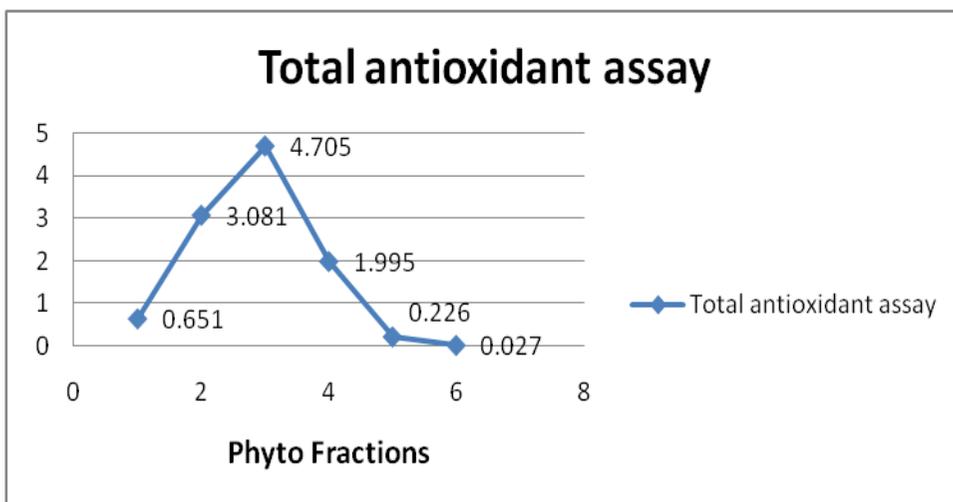


Figure:1- Total Antioxidant assay

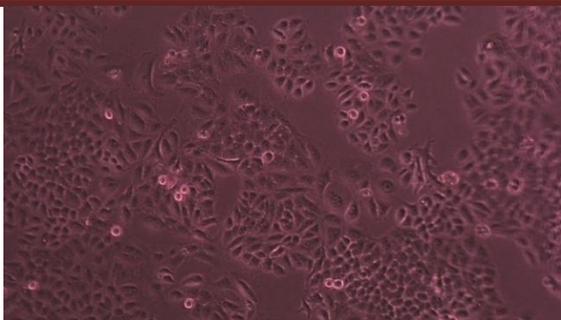


Fig: 2 Control

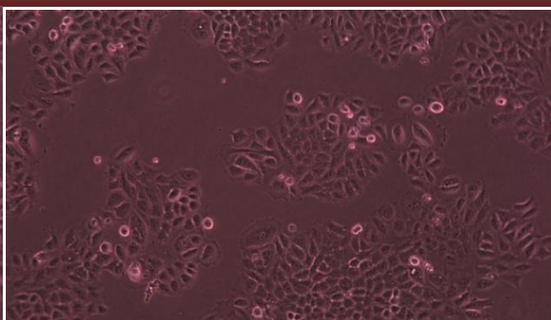
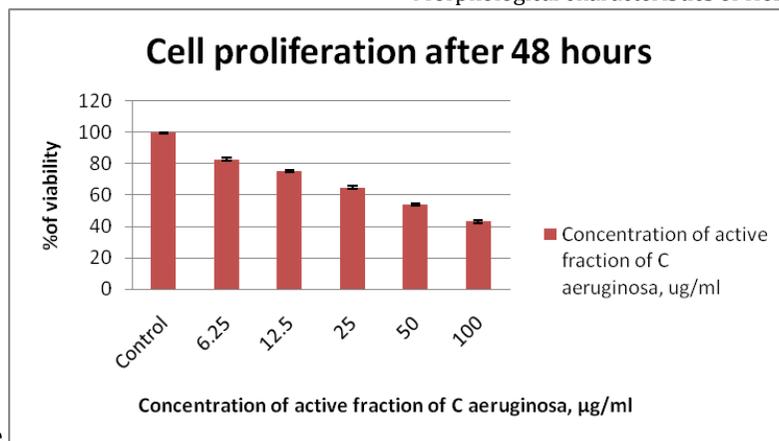
Fig:3 Effects of Active fraction of *C.aeruginosa* on Morphological characteristics of HeLa cells

Fig:4- HeLa Cell Viability after Treatment with Different Concentrations of active fraction of SCF Extract after 48 Hours

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