



AN IN VITRO STUDY OF CYTOTOXIC ACTIVITY OF *EUPHORBIA HIRTA* ON HEP-2 CELLS OF HUMAN EPITHELIOMA OF LARYNX

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ABSTRACT

The cytotoxic activity of the methanol extract of leaves of *Euphorbia hirta* on Hep-2 cells from human epithelioma of larynx was investigated in vitro using 3-(4, 5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT). The potency of each plant extract concentration was calculated in terms of percent decrease in viable Hep-2 cells as compared to the control value. The extract showed dose dependent antitumor activity. The MTT assay showed an anti proliferative activity (IC₅₀) at 625µg/ml of crude extract. Qualitative chemical screening tests were performed to detect the various phytochemicals present in the extract.

Keywords: *Euphorbia hirta*, Phytochemicals, HEp-2 cells, MTT assay, Cytotoxicity

INTRODUCTION

Cancer is a class of diseases in which the body cells become abnormal and divide indiscriminately. Cancer cells may become invasive and transform normal adjacent cells into malignant cells. They may also spread through the blood stream and lymphatic systems to other parts of the body to form metastatic tumors in distant organs. Cancer is caused by abnormalities in the genetic material of the transformed cells. Cancer may also be initiated by carcinogens, tobacco smoke, radiation, chemicals or infectious agents, especially some viruses. Death of the cells in any case is mediated by an intracellular activity either of two distinct mechanisms, necrosis or apoptosis. Cancers cause annually more than 13% of all human deaths. More than 70% of all cancer deaths occurred in low and middle income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (WHO estimate).

HEp-2 cells

The HEp-2 cell line was established in 1952 by A. E. Moore, L. Sabachewsky, and H. W. Toolan from tumours that had been produced in irradiated-cortisonized weanling rats after injection with epidermoid carcinoma tissue from the larynx of a 56-year-old male¹. A hardy cell line, HEp-2 resists temperature, nutritional, and environmental changes without loss of viability. It has supported growth of 10 of 14 arboviruses, measles virus, and it has been used for experimental studies of tumor production in rats, hamsters, mice, embryonated eggs and terminal cancer patient volunteers.

HEp-2 cell line has a high proliferation rate and a 23 hours cell cycle². In our research group, we recently identified the plant *Eclipta prostrata* with in vitro cytotoxic activity on HEp2 cells. The present study was carried out to verify the possible cytotoxic action of *Euphorbia hirta* on HEp2 cells, evaluating morphology and the number of viable cells after incubation with the plant extract in different concentrations and duration of exposure.

Euphorbia hirta

Euphorbia hirta Linn. of the family Euphorbiaceae is a medicinal, rhizomatous herb distributed in South Western Ghats of India and North East Coast of Tamil Nadu³. The plant is native to India but is a pan tropical weed. A small, erect or ascending annual herb reaching up to 50 cm, with hairy stem. The leaves are opposite, elliptical, oblong or oblong-lanceolate, with a faintly toothed margin and darker on the upper surface. The flowers are small, numerous and crowded together in dense cymes of about 1 cm in diameter. The fruits are yellow, three-celled, hairy, keeled capsules, 1-2 mm in diameter, containing three brown, four-sided, angular, wrinkled seeds⁴.

Leaves, stem and flowers are used for treating respiratory ailments especially cough, coryza, bronchitis and asthma. Worm infestations, dysentery, gonorrhoea, jaundice, pimples and digestive problems are also treated with *Euphorbia hirta*⁵.

Anticancer activity

Extracts of *Euphorbia hirta* have been found to show selective cytotoxicity against several cancer cell lines. The plant is useful in the effective treatment of cancers, particularly malignant melanoma and squamous cell carcinoma⁶.

MATERIALS AND METHODS

Plant material

Fresh plants of *Euphorbia hirta* was collected from Tiruchengode area of Tamil Nadu State in South India. The leaves of *Euphorbia hirta* washed, shade dried, pulverized in a blender and kept in an airtight container for experimental use.

Preparation of methanol extract

The dried and pulverised leaves of *Euphorbia hirta* was subjected to successive soxhlet extraction with methanol. The extract was evaporated to dryness by leaving the extract in hot air oven at 50°C for 72 – 96 hrs. The condensed form of extract collected. The yield of prepared extract was 7.2%.100mg of the extract was dissolved in 1 ml of Dimethyl sulphoxide (DMSO) to prepare the stock solution (100 mg/ml).

Phytochemical screening

Qualitative chemical tests were carried out using extracts from plant to identify the phytochemicals⁷⁻¹³. A 10mg/ml leaf extract was used for the tests. The phytochemicals detected in methanolic extract of *Euphorbia hirta* are tannins, flavonoids, saponins, proteins, steroids, alkaloids and anthraquinones (Table-1).

Table 1: Phytochemical constituents of *Euphorbia hirta*

S. No.	Phytochemicals	Methanol extract of leaves of <i>Euphorbia hirta</i>
1.	Tannins	(++) moderate
2.	Flavonoids	(+) low
3.	Saponins	(-) very low
4.	Proteins	(-)very low
5.	Steroids	(+) low
6.	Alkaloids	(+) low
7.	Anthraquinones	(++) moderate

Evaluation of cytotoxic activity

Preparation of HEP-2 cell suspension

A subculture of HEP-2 cells in Eagle's Minimum Essential Medium (EMEM) was trypsinized, after discarding the culture medium. To the disaggregated cells in the flask 25ml of EMEM with 10 % FCS was added. The cells suspended in the medium by gentle passage with the pipette and the cells homogenized.

Seeding of cells

1ml of the homogenized cell suspension was added to each well of a 24 well culture plate and incubated at 37°C in a humidified CO₂ incubator with 5% CO₂. After 48 hrs incubation the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells cytotoxic assay was carried out.

Cytotoxicity assay

The assay is carried out using (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT). MTT is cleaved by mitochondrial Succinate dehydrogenase and reductase of viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity¹⁴⁻¹⁵.

Preparation of working herbal extracts

0.5ml of stock (100 mg/ml) herbal extract was dissolved in 4.5 ml of DMSO giving a concentration of 10mg/ml. The fresh working suspension filtered through 0.45 µm syringe filter prior to the assay.

Using the 10mg/ml concentration herbal extract nine serial doubling dilutions of the extract of 500µl each was prepared in DMSO to get the concentration of the extract as indicated in Table-2 and the diluted extracts transferred to 10 wells of a 12 well culture plate. 500 µl of 48hr culture of Hep-2 cells at a concentration of

10⁵cells/ml was added to each well. Two wells receive only cell suspension without extract and they serve as control. The plate incubated in a humidified CO₂ incubator at 37° C for 4 - 6 hrs. The plate was microscopically examined for confluent monolayer of cells, turbidity and toxicity.

MTT assay¹⁶⁻¹⁹

After incubation, the medium from the wells aspirated carefully and discarded. Each well washed with EMEM without FCS. 200µl of MTT solution (5mg/ml of PBS, pH 7.2) added to each well. The plate incubated for 6-7 hrs at 37°C in an incubator with 5% CO₂. After incubation 1ml of DMSO added to each well and mixed with pipette and left for 45seconds at room temperature. Purple color formazan formed in the wells. The suspension transferred to a spectrophotometer cuvette and absorbance values read at 570nm using DMSO as blank. The % cell viability and % cell death were calculated with the following formulae:

Cell viability % =

Mean OD of wells receiving each plant extract dilution/ Mean OD of control wells x 100

Cell death % =

1 - (OD of sample/OD of control) x 100

A chart was plotted using the % cell viability in Y-axis and concentration of the plant extract in X-axis. Another chart was prepared using the % cell death in Y-axis and concentration of plant extract in X-axis. Cell control and solvent control were included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments. The OD readings are shown in Table-2. The MTT assay showed an anti proliferative activity (IC₅₀) of HEP-2 cells at 625µg/ml of crude leaf extract of *Euphorbia hirta*.

Table 2: Cytotoxicity on Hep2 cells- by methanol extract of *Euphorbia hirta* leaves (MTT Assay)

S. No.	Concentration (mg/ml)	Dilution	Absorbance at 570 nm	%Cell viability	%Cell inhibition
1.	10.0	Neat	0.14	19.44	80.56
2.	5.0	1:1	0.19	26.38	73.62
3.	2.5	1:2	0.22	30.55	69.45
4.	1.25	1:4	0.30	41.66	58.34
5.	0.625	1:8	0.38	52.77	47.23
6.	0.3125	1:16	0.44	61.11	38.89
7.	0.156	1:32	0.52	72.22	27.78
8.	0.078	1:64	0.66	91.66	8.34
9.	0.039	1:128	0.70	97.22	2.78
10.	0.0195	1:256	0.71	98.61	1.39
11.	Cell control	--	0.72	100.00	0.00
12.	Cytotoxic changes observed		Cell condensation, Cell shrinkage & Cell death		

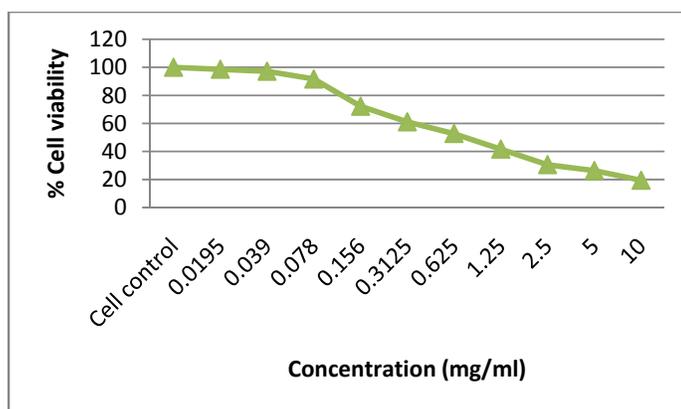


Fig. 1: Effect of methanol extract of *Euphorbia hirta* leaves on Hep-2 cell viability

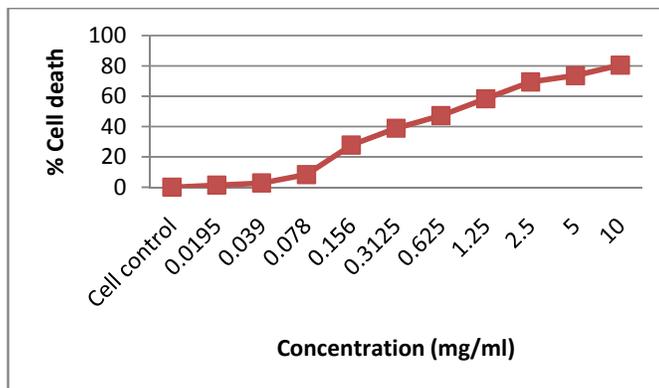


Fig. 2: Effect of methanol extract of *Euphorbia hirta* leaves on Hep-2 cell death

RESULTS AND DISCUSSION

Traditionally many medicinal plants, which possess the ability to prevent and even to stall the progress of cancer, were in use. Plants possess certain chemicals, which have the ability to modify the physiological function of cells and hence act as anti-cancer drugs to arrest the proliferation of cancer cells. The mode of action of the drugs is unknown but successfully integrating our documented knowledge of plant properties and modern technological tools, effective anti-cancer drugs can be derived from plant sources and their mechanism can be elucidated²⁰⁻²².

The present need is to develop drugs that can potentially target cancer cells by means of their inherent difference to normal cells. The development of such drugs with differential action will be very valuable in cancer chemotherapy without the observed side effects. The methodology involves use of cancer cell lines to test the efficacy of the plant extracts in vitro.

In the present study only the methanol extract of leaves of *Euphorbia hirta* was tested for the antitumor activity and it showed most effective inhibition of HEP-2 cell proliferation. The best potential was obtained at certain optimal concentrations of the extract. Accumulating evidence clearly indicates that apoptosis is a critical molecular target by dietary bioactive agents for the prevention of cancer.

The potential use of *Euphorbia hirta* as therapeutic agent holds great promise as the isolation of one or more cytotoxic chemicals from crude extract and the judicious use of such chemicals can control the progression of cancer and also can prevent the formation of tumour in individuals who are highly susceptible to developing a tumour.

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