Original Articles
Toxicology International, *Sept-Dec 2015 / Vol-22 / Issue-3, 122-127*
ISSN (Print) : 0971-6580
ISSN (Online) : 0976-5131
DOI: 10.22506/ti/2015/v22/i3/137636

Assessment of Cytotoxicity of Aqueous Extract of *Euphorbia hirta* against Human Lung Carcinoma and Vero Cell Line

Prashant Y. Mali[1*] and Anil G. Jadhav[2]

[1]Department of Pharmacology, [2]Department of Pharmacognosy, Sandip Institute of Pharmaceutical Sciences, Sandip Foundation, Trimbak Road, Mahiravni, Nashik - 422213, Maharashtra, India; pymali2008@rediffmail.com

Abstract

Objective of the study was to assess the cytotoxicity of aqueous extract of *Euphorbia hirta* against human lung carcinoma (NCIH-522) and vero cell line. Fourier Transform Infrared spectroscopy (FTIR) analysis of an aqueous extract of *E. hirta* was studied. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was used to assess cytotoxicity of aqueous extract of *E. hirta*. Doxorubicin was considered as standard reference drug. Concentrations at range 1000–0.05 µg/ml of extract and doxorubicin were used. FTIR spectrum of aqueous extract of *E. hirta* was showed five peaks/centers such as 762.036, 1283.392, 1411.548, 2235.839 and 3749.054 at the wavelength region of 4000.00-650.00 cm−1. The 50% cell growth inhibition of aqueous extract of *E. hirta* and doxorubicin was 679.74 µg/ml and 531.14 µg/ml against human lung carcinoma cell line. It was 1363.65 µg/ml and 2392.71 µg/ml against normal vero cell line respectively. Aqueous extract of *E. hirta* was showed a good cytotoxic effect against human lung carcinoma cell line. It was due to the presence of functional group of phytocompounds detected in FTIR studies. The extract was found to be safe against normal vero cell line. Hence, *E. hirta* can be used as a good source of cytotoxic compounds. The isolation, identification, and elucidation of underlying mechanisms of these compounds will require and that could lead to develop alternative and complementary drugs useful in the treatments of tumors or cancers. Further, there is need to expand these studies using in vivo experiments, mechanistic-based assays such as tunnal, caspcase, and flow cytometry.

Keywords: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide Assay, *Euphorbia hirta* Linn., NCIH-522, Vero Cell Line

Received: 15-10-2015 **Revised:** 01-12-2015 **Accepted:** 21-12-2015

1. Introduction

Euphorbia hirta L. (Euphorbiaceae; Sp. Pl. [1753]) is erect or ascending annual with hairy stems, 6-20 inch or 15-50 cm high and opposite, elliptic-oblong or obovate-lanceolate leaves. Seeds are 0.8 mm long, ovoid-trigonous, slightly transversely rugose, light reddish brown. The plant is collected with or without roots when it is in flower and fruit and dried. It is common in the waste ground throughout the hotter parts of India and Ceylon-most tropical and subtropical countries. Traditionally, it is used in the treatment of tumors. The plant is chiefly used in the affections of childhood, in worms, bowel complaints, and cough. It is sometimes prescribed in gonorrhea. A decoction is used in asthma and chronic bronchial affections. Leaves are used in curing sores; their juice is sometimes squeezed into eyes to cure eye trouble. The root is given by the sandals to allay vomiting and the plant to nursing mothers when the supply of milk is deficient or fails. Juice of the plant is given in dysentery, colic, aphthae, and milk applied to destroy warts. In the form of tincture, it is considered useful in colic and dysentery, as vermifuge and in diseases of the genito-urinary tract. It is also used in gargles and as poultice. It has a depressant action on the heart and respiration and relaxes the bronchioles. The leaves are eaten as a vegetable

* *Author for correspondence*

that contain moisture (78.14%), protein (4.65%), ether extract (1.71%), ash (3.15%), Vitamin-C (44.32 mg/100 g). Various phytoconstituents have been identified in *E. hirta* that includes quercetin, triacontane, jambulol, euphosterol, gallic, melissic, palmitic, oleic and linoleic acids, l-inositol, xanthorhamnin (Kirtikar and Basu, 2006; Nadkarni and Nadkarni, 2007; Anonymous, 2003)), afzelin (I), quercitrin (II), myricitrin (III), (Liu *et al.,* 2007) rutin (IV), quercitin (V), euphorbin-A (VI), euphorbin-B (VII), euphorbin-C (VIII), euphorbin-D (IX), 2,4,6-tri-*O*-galloyl-β-D-glucose, 1,3,4,6-tetra-*O*-galloyl-β-D-glucose, kaempferol, gallic acid, and protocatechuic acid (Rastogi and Mehrotra, 2002; Rastogi and Mehrotra, 2002). *E. hirta* also contains β-amyrin, 24-methylene cycloartenol, β-sitosterol, heptacosane, nonacosane (Williamson, 2002), shikimic acid, tinyatoxin, choline, camphol, and quercitol derivatives containing rhamnose and chtolphenolic acid (Sood *et al.,* 2005). *E. hirta* has reported to have antiamoebic (Tona *et al.,* 2000), antibacterial (Ogbulie *et al.,* 2007; Suresh *et al.,* 2008), antidiarrheal (Galvez *et al.,* 1993), antimalarial (Liu *et al.,* 2007), antioxidant (Sharma *et al.,* 2007), analgesic, antipyretic and anti-inflammatory (Martinez et al., 1999; Lanhers *et al.,* 1991), antitumor (Loh *et al.,* 2009; Brindha *et al.,* 2010; Sandeep and Chandrakant, 2011), etc., activities.

Cancer has become a big threat to humans globally. As per the Indian population census data, the rate of mortality due to cancer in India was high and alarming with about 806,000 existing cases by the end of the last century. Cancer is the second most common disease in India responsible for maximum mortality with about 0.3 million deaths per year. This is owing to the poor availability of prevention, diagnosis, and treatment of the disease (Imran *et al.,* 2011). The limited success of clinical therapies including radiation, chemotherapy, immunomodulation, and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates that there is an imperative need of new cancer management. Chemoprevention involves the use of pharmacological, dietary biofactors, phytochemicals, and even whole plant extracts to prevent, arrest, or reverse the cellular and molecular processes of carcinogenesis due to its multiple intervention strategies (Mehta *et al.,* 2010). Based on the traditional uses of this medicinally important plant, the author has assessed cytotoxicity of aqueous extract of *E. hirta* against human lung carcinoma and vero cell line.

2. Materials and Methods

2.1 Chemicals and Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye powder (Serva Electrophoresis, New Delhi), dimethyl sulfoxide cell culture grade (Bioworld, New Delhi), Amphotericin-B (Himedia, Mumbai), Penicillin and Streptomycin Solution Stabilized (Sigma, Mumbai), EDTA (MP Biomedicals, New Delhi), DPBS/ modified 1X (Dulbecoo's Phosphate Buffer Saline without Ca^{+} and Mg^{+}) (Himedia, Mumbai), Fetal Bovine Serum (FBS), South American origin, 500 ml) (Sigma, Mumbai). All other chemicals used for this experimental work were of analytical grade.

2.2 Preparation of Extract

E. hirta Linn. plant was collected from the local region of Amalner Town, Jalgaon, Maharashtra, India and authenticated by taxonomist at the Centre for Postgraduate studies and Research in Botany, Pratap College, Amalner, Jalgaon, Maharashtra. The herbarium specimen was deposited in the same. The plant was dried, powdered, and defeated (1 kg) with petroleum ether (800 ml, 40–60°C) followed by methanol (900 ml, 55–60°C) and distilled water using percolation method of extraction for 07 days. The marc was filtered using a muslin cloth, extract gets concentrated and preserved in an airtight container under refrigeration for further use.

2.3 Fourier Transform Infrared Spectroscopy Analysis

Fourier Transform Infrared Spectroscopy (FTIR) (Agilent Cary 630, Diamond ATR) analysis of an aqueous extract of *E. hirta* was performed.

2.4 Maintenance of Cell Lines

Human lung carcinoma (NCIH-522) and Vero cell lines were procured from National Centre for Cell Science (NCCS), Pune. Stock cells of these cell lines were cultured in Dulbecco's Modified Eagles Media, supplemented with 10% FBS. Along with media, cells were also supplemented with 5% HBSS, penicillin, streptomycin, and amphotericin–B, in a humidified atmosphere of 5% CO_2 at 37°C until confluence reached. The cells were dissociated with 0.2% trypsin, 0.02% EDTA in phosphate

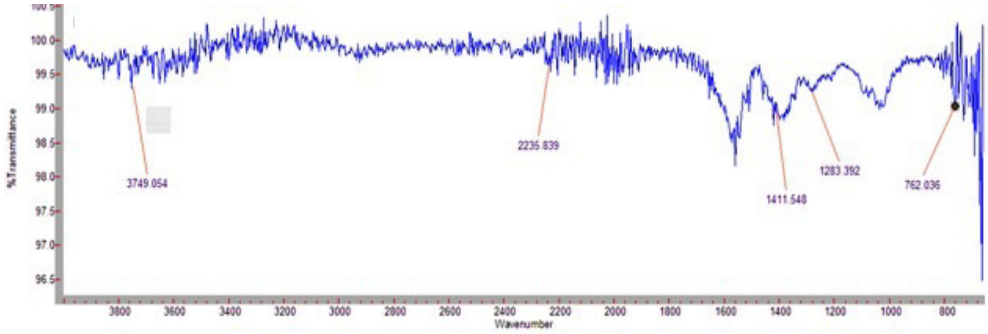


Figure 1. Fourier transform infrared spectroscopy spectrum showing peaks/centers in aqueous extract of *Euphorbia hirta.*

buffer saline solution. The stock cultures were grown initially in 25 cm^2 tissue culture flasks, then in 75 cm^2 and finally in 150 cm^2 and cytotoxicity experiment were carried out in 96 microtiter well plates.

2.5 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay

Assay protocol described by Mossmann with some modifications was adopted (Mosmann, 1983; Knick *et al.,* 1985; Skehan *et al.,* 1990; Skehan *et al.,* 1992; Mali, 2015; Mali and Gaikwad, 2015; Mali, 2016; Mali, 2015; Mali *et al.,* 2016; Mali *et al.,* 2016). Briefly, cell lines in exponential growth phase were washed, trypsinized and resuspended in complete culture media. Cells were seeded at a concentration of 2×10^4 cells/well in 96 well microtiter plate and incubated for 24 h during which a partial monolayer forms. The cells were then exposed to various concentrations of the test extracts and standard drug doxorubicin, (i.e., concentrations 1000–0.05 µg/ml). Control wells were received only maintenance medium. The plate was incubated at 37°C in humidified incubator with 5% CO_2 and 75% RH for a period of 24 h. At the end of 24 h, 10 µl MTT labeling mixture was added and incubates for 4 h. The absorbance was measured using microplate ELISA reader at wavelength 590 nm. The % cell growth inhibition or percentage cytotoxicity was calculated by the following formula,

$$\%\,\textbf{viability} = (A_T - A_B)/(A_C - A_B) \times 100$$

Where, A_T = Absorbance of treated cells (drug); A_B = Absorbance of blank (only media) and A_C = Absorbance of control (untreated).

% **cell growth inhibition** / % **cytotoxicity** = 100–% **cell survival**

2.6 Statistical Analysis

All the values of % cell growth inhibition were replicates of three independent observations (n = 3). The 50% cell growth inhibition (IC_{50}) was determined by interpolating concentrations (X-axis) versus %cell inhibition (Y-axis) by linear regression equation using Microsoft Excel, 2007, Microsoft Corporation, USA application.

3. Observations and Results

3.1 Fourier Transform Infrared Spectroscopy Analysis

Analysis of aqueous extract of *E. hirta* using FTIR spectrophotometer was performed at the wavelength region of 4000.00–650.00 cm^{-1} and resolution 4 cm^{-1} as shown in Figure 1 and Table 1. FTIR spectrum showed five peaks/centers that correspond to 900–650 (nonaromatics structures), 1300–1000 (C–O stretching) alcohol and phenols, 1300–1100 (aliphatic and anhydrides C–O stretching) esters, 1360–1250 (aromatic C–N stretching) amines, 1310–1250 (aromatic C–O stretching) esters, 1300–1270 (nitrate NO_2 symmetric stretching) various nitrogen-containing compounds, 1300–1000 (C–F stretching) organic halogen compounds, and 1390–1290 (SO_2 asymmetric stretching) sulfur group, 1600–1300 strong skeletal bands for aromatics and heteroaromatics, 2260–2100 weak (C≡C) stretching band of alkyne molecules, 3400–3500 (N–H) stretching band of primary amines and 3600–500 (N–H, C–H) stretching of ammonium (salts) compounds might be present in the said extract.

Table 1. Fourier transform infrared parameters and peak ranges of aqueous extract of *Euphorbia hirta*

Parameters/peak list			
Sensitivity	Resolution	Range	Peaks/centers
1	4 cm^{-1}	4000.00–650.00 cm^{-1}	762.036
			1283.392
			1411.548
			2235.839
			3749.054

3.2 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Assay

The IC_{50} of aqueous extract of *E. hirta* and doxorubicin was 679.74 µg/ml and 531.14 µg/ml against human lung carcinoma cell line. It was 1363.65 µg/ml and 2392.71 µg/ml against normal vero cell line respectively as shown in Figures 2-5 and Table 2.

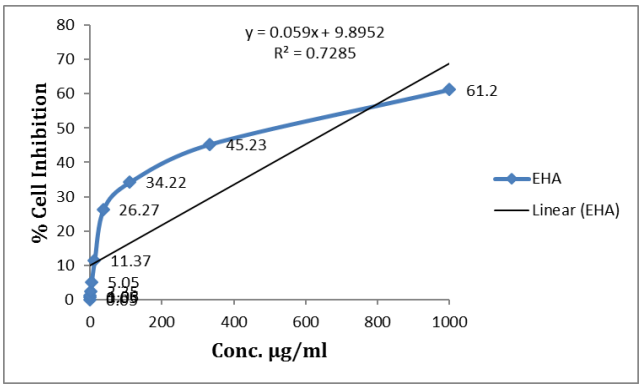


Figure 2. % cell inhibition of aqueous extract of Euphorbia hirta against NCIH-522cell line.

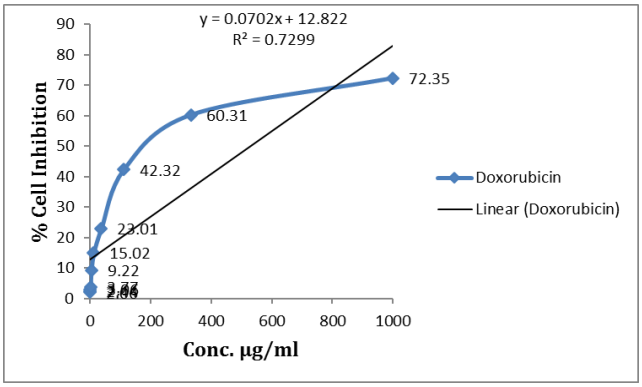


Figure 3. %cell inhibition of doxorubicin against NCIH-522 cell line.

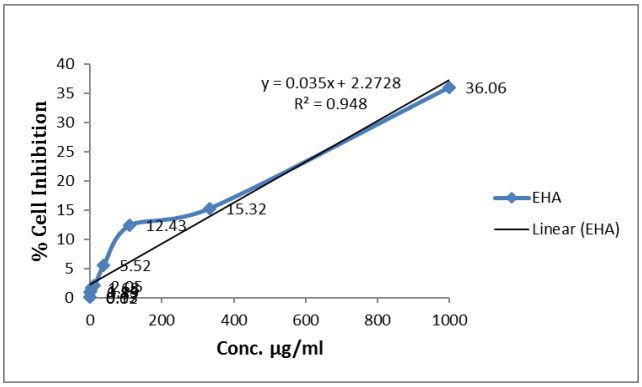


Figure 4. % cell inhibition of aqueous extract of Euphorbia hirta using vero cell line.

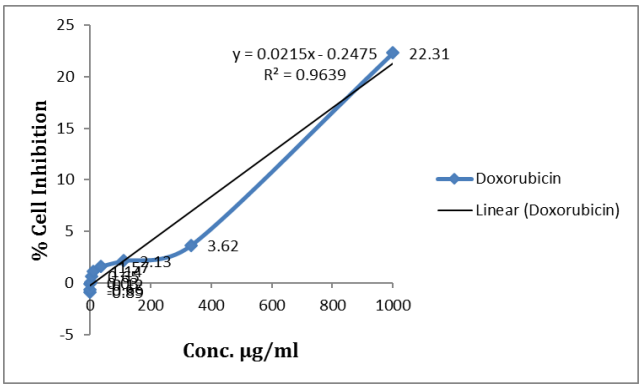


Figure 5. % cell inhibition of doxorubicin against vero cell line.

Table 2. Percentage of cell inhibition of aqueous extract of *Euphorbia hirta* against human lung carcinoma and vero cell line

Concentration (µg/mL)	NCIH-522 cell line		Vero cell line	
	EHA	Doxorubicin	EHA	Doxorubicin
0.05	0.05	2.06	0.02	−0.89
0.15	0.65	2.44	0.13	−0.65
0.46	1.06	3.06	0.89	−0.12
1.37	2.35	3.77	1.13	0.03
4.12	5.05	9.22	1.68	0.65
12.35	11.37	15.02	2.05	1.14
37.04	26.27	23.01	5.52	1.57
111.11	34.22	42.32	12.43	2.13
333.33	45.23	60.31	15.32	3.62
1000	61.2	72.35	36.06	22.31
IC_{50}	679.74	531.14	1363.65	2392.71

EHA=Aqueous extract of Euphorbia hirta, IC_{50}=50% cell growth inhibition

4. Discussion

Approximately, 60% of the anticancer drugs currently have been isolated from natural products from the plants and they have been proven to be a major source of novel agents with various pharmaceutical applications (Dai and Mumper, 2010; Mishra and Tiwari, 2011). To support these findings, the author has been studied FTIR analysis of an aqueous extract of *E. hirta* which could be a scientific base of the presence of cytotoxic phytocompounds in the extract. Hence, *E. hirta* can be used as a good source of cytotoxic compounds. The isolation, identification, and elucidation of underlying mechanisms of these compounds will require that could lead to develop alternative and complementary drugs useful in the treatments of tumors or cancers.

Cytotoxicity assays are performed to predict potential toxicity, using cultured cells which may be normal or transformed cells. These tests normally involved short-term exposure of cultured cells to test substances, to detect how basal or specialized cell functions may be affected by the substance, prior to performing safety studies in whole organisms. It can also provide insight towards the carcinogenic and genotoxic dispositions of herb-derived compounds and extracts. The ability of a plant extract to inhibit cellular growth and viability can also be ascertained as an indication of its toxicity. Assessment parameters for cytotoxic effects include inhibition of cell proliferation, cell viability markers (metabolic and membrane), morphologic and intracellular differentiation markers (O'Brien and Haskins, 2007). The last main consideration in cytotoxicity testing is the manner in which cells die. The mechanism of death can be quite important if cytotoxicity testing is initiated for ancillary safety concerns (e.g., off-target cytotoxicity from pharmaceuticals, cosmetics, and nutritional supplements), or specifically, as for identifying new chemical entities for cancer therapy. Simply stated, compounds that cause primary necrosis in cell culture may carry unacceptable cytotoxic liabilities whereas compounds that cause apoptosis can be preferable (Fink and Cookson, 2005; Majno and Joris, 1995; Wyllie *et al.,* 1980). Therefore, the aqueous extract of *E. hirta* was showed a good cytotoxic effect against human lung carcinoma cell line. It was due the presence of functional group of phytocompounds detected in FTIR studies. The extract was found to be safe against normal vero cell line. Further, there is a need to expand these studies by using *in vivo* experiments, mechanistic-based assays such as tunnal, caspcase, and flow cytometry.

5. Acknowledgments

The author would like to thank Dr. Vipul P. Patel, Department of Pharmaceutical Biotechnology, S. K. Patel College of Pharmaceutical Education and Research, Ganpat University, Mehsana, Gujarat, India, for providing the necessary facilities to performed MTT assay.

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