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Determination of Cytotoxicity of Latex and Methanol Extract of *Euphorbia helioscopia* Leaves on Vero Cell Line with MTT Assay

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Abstract.- The objective of this study was to evaluate the cytotoxic potential of latex and leaves methanol extract of *Euphorbia helioscopia* on Vero cell line. Aliquots (100 µl) of eight different concentrations (1000-0.0128 µg/ml) of each sample were incubated (with 5%CO_2) with 100 µl of 0.5% MTT solution for 48 h at 37°C. Active mitochondria of viable cells cleaved tetrazolium ring of MTT and consequently purple colored formazan was formed. Then multi-well plate was read in ELIZA reader at 570nm. Latex showed 57% cell survival percentage at highest (1000 µg/ml) while leaves methanol had 34% cell survival percentage at same concentration. IC_{50} of leaves methanol extract, calculated from linear regression equation, was 5.27 µg/ml. It was concluded that latex was devoid of cytotoxicity within concentrations ranged from 1000 to 0.0128 µg/ml, whereas leaves methanol extract showed cytotoxicity at 5.27 µg/ml concentration.

Keywords: Cell viability, latex, methanol extract of *Euphorbia helioscopia* leaves.

INTRODUCTION

Several methods such as trypan blue assay, water-soluble tetrazolium (WST) assay, sulforhodamine assay, clonogenic assay, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,diphenyltetrazolium bromide] assay can be employed for measuring cytotoxicity of plant extracts (Hussain *et al.*, 2009). Among many traditional techniques, MTT is most multifaceted and quantitative method. This is rapid assay because it is devoid of any washing steps like other staining techniques as washing increases the processing time and sample variations. MTT assay measures the number of viable cells as living cells break tetrazolium ring of MTT inside active mitochondria only (Mosmann, 1983).

Euphorbia helioscopia belongs to family *Euphorbiaceae* which is rich in medicinally active plants (Kinghorn and Evans, 1975; Webster, 1994). It is used traditionally in different ailments (Wu *et al.*, 1991) and has been extensively studied for number of pharmacological activities like antibacterial, antifungal, phytotoxicity, antiviral, anticancer etc. (Uzair *et al.*, 2009; Farhat *et al.*, 2011; Ramezani *et al.,* 2008; Wang *et al.,* 2012).

Chemicals or compounds possessing anti-angiogenic property should not be cytotoxic or their anti-angiogenic property should be at doses that are remarkably less than their cytotoxic dose (Hussain *et al.*, 2009). Cytotoxic anticancer drugs show more toxic effects on normal rapidly growing cells in the body as compared to anti-angiogenic compounds so anti-angiogenic compounds can arrest the uncontrolled proliferation of neoplastic cells at subcytotoxic doses. Our earlier work has confirmed anti-angiogenic potential of *Euphorbia helioscopia* (Uzma *et al.*, 2014b). This plant was selected for investigation on the basis of its folklore and medicinal value in Pakistan and other countries. Cytotoxicity assay is imperative to unveil the antitumor and other unwanted effects of compounds.

The objective of the current study was to investigate the cytotoxic potential of this plant quantitatively, on Vero epithelial cell line obtained from kidney of an African green monkey (Yasumura and Kawakita, 1963), by using MTT colorimetric assay.

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MATERIALS AND METHODS

Plant material

Euphorbia helioscopia was collected from suburbs of Lahore, identified and authenticated by a taxonomist of Botany Department, Government College University, Lahore. A voucher specimen (1501) was deposited in their herbarium. Latex was collected from stem in dried bottles. Plant was shade dried; stem and leaves were separated and ground. Fine powder was used for extraction.

Preparation of extract and its dilutions

Extract was prepared by two methods 1) hot sequential extraction with soxhlet using solvents in increasing order of polarity (petroleum ether, chloroform, and methanol) and 2) cold extraction (maceration) using water and methanol as solvents. Excessive solvent was evaporated on rotary evaporator and semisolid extract was collected in the pre-weighed beakers and then dried in oven at 40^{O}C. Latex and leaves methanol extract were selected, based on their *in-vitro* antioxidant activity in our earlier work, in this study (Uzma *et al.,* 2014).

Phosphate buffer saline (PBS) was used for dilution of extract and latex. For both samples, 1 mg/mL stock solution was prepared then their five fold serial dilutions (*viz.,* 1000, 200, 40, 8, 1.6, 0.32, 0.064, 0.0128 µg/ml) were used for the assay.

Chemicals

Dulbecco's modification of Eagle medium [DMEM] (Sigma-Aldrich, Germany), Fetal bovine serum (PAA Laboratories, Austria), MTT (MP Biomedicals, Germany), Trypsin versen (Biowest, France), Trypan blue dye (Sigma-Aldrich, Germany), Phosphate buffer saline [PBS] (Sigma-Aldrich, Germany) were purchased from local market.

Vero cell line and its sub culturing

Vero cell line was obtained from cell culture laboratory of Microbiology Department, University of Animal and Veterinary Sciences, Lahore, Pakistan. Trypan blue (dye) exclusion method was adopted to measure the cell viability. Dead cells appeared blue (Freshney, 2010). Formula used for determination of % cell viability is given as under:

$$\% \text{ Viable cells} = [\text{Number of viable cells per mL} / \text{Total number of cells per mL}] \times 100$$

Vero cells were grown in the 75 cm^2 cell culture flasks (Karrel Flasks, Corning, USA) that contained sterilized DMEM with 10% fetal bovine serum (FBS). These flasks were incubated at 37^{o}C for 24 h with 5% CO_2 in incubator. Flasks were observed under inverse microscope (Olympus CK40, Japan) to see the confluent monolayer of cells unless there was 80-90% confluency (Freshney, 2010).

Sub culturing of Vero cells

Growth medium was removed from confluent monolayer of Vero cells, and then cells were washed with 10 mL of PBS to remove trypsin inhibitors that were present in serum. Confluent monolayer of cells was fixed inside the cultural flask. Five milliliter of 1X trypsin-EDTA was added to flask and incubated for 2-3 minutes in order to detach the cells. Gentle shaking and taping helped in detaching cells. DMEM (5 mL) with 10 % FBS was added to it to inactivate the trypsin- EDTA. Cells were washed down in the media, pipetting was done many times to break the clumps of cells. This cell suspension was collected in sterile tray and 10 mL DMEM with 10 % FBS was added to it. All steps were done under laminar flow hood (Freshney, 2010).

Cytotoxicity assay

Ninety six well plates (Corning, USA) were opened under laminar flow hood and 100 µl of cell suspension containing 10^5 cell/mL was added to each well except column 1 and 12, row A and H of well plate with multi-pippettor and incubated at 37°C for 24 h with 5% CO_2. These empty wells served as positive control. Plates were observed under inverse microscope to check the confluent monolayer of cells. Medium was removed and fresh medium of same volume was added and 100 µl of tested samples were poured with micropipette into wells containing confluent monolayer of cells except column 2 and 11 of the well plate, these two column's wells served as negative control, and

incubated at 37°C for 48 h with 5% CO_2. All the wells were emptied and 100 µl of 0.5% (5mg/mL) MTT solution was added to each well and incubated at 37°C for 48 h with 5% CO_2. A purple colored formazan appeared in the wells when viable cells reacted with MTT. Then MTT solution was removed and 100 µl of DMSO (5%) was added and mixed with pipette to dissolve formazan and left for few minutes at room temperature and optical density was measured at 570 nm (Twentyman and Luscombe, 1987) using ELISA (enzyme linked immunosorbent assay) reader (Type- 355, Model 2005–05, Thermo, China). Percentage of cell survival was calculated according to Mosmann (1983).

$$\text{Cell Survival \%} = \frac{[\text{Tested sample (mean OD)} - \text{Negative control (mean OD)}]}{\text{Positive control (mean OD)}} \text{ X } 100$$

Statistical analysis

Results were presented as mean ± S.E.M. Pearson correlation was applied to find out the relationship between latex and leaves methanol treated groups. Correlation was considered significant at 0.01 levels (2-tailed).

RESULTS AND DISCUSSION

Leaves methanol extract and latex showed concentration dependent cytotoxic effect in proliferating Vero epithelial cell line as determined by calculating the cell survival percentage. Cells were exposed to eight different concentrations, from 1000 to 0.0128 µg/ml, for both the samples. Latex showed no cytotoxicity as cell survival percentage at highest concentration (1000 µg/ml) was 57% (Fig. 1A). Comparatively leaves methanol extract had IC_{50} value 5.27 µg/ml calculated with linear regression equation Y= -6.8095X + 85.893, R^2= 0.9819 (Fig. 1B). The highest correlation (r = 0.973) was found between latex and methanol extract of leaves results. No growth in negative control wells (column 1 and 12, row A and H of well plate) and 90% cell viability in positive control wells (column 2 and 11 of the well plate) were observed.

The endpoint of MTT colorimetric assay is purple colored formazon crystals produced by viable cells activity. Mitochondria in the living cells convert MTT metabolically into formazon. The formation of formazon has direct relation with viable cells; as dead cells cannot generate formazon (Kappler *et al.,* 1981).

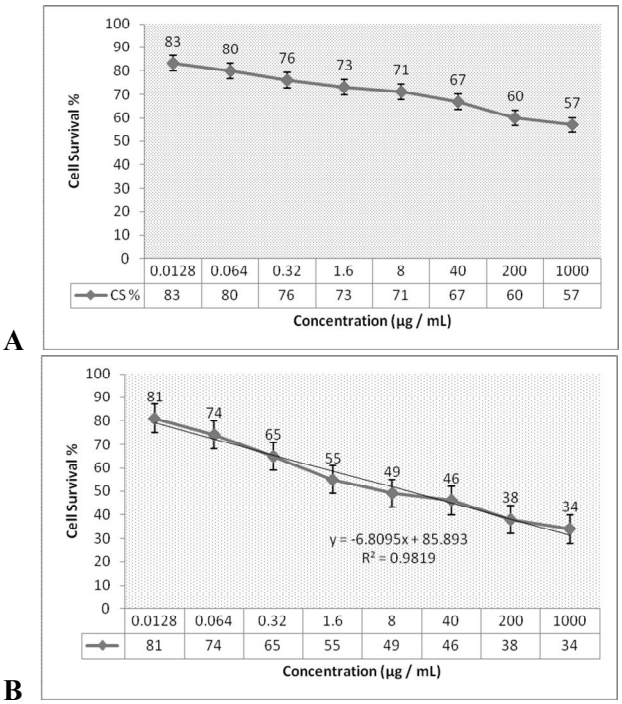

Fig. 1. Cytotoxic effect of different concentrations of latex (A) and methanol extract of leaves (B) on Vero cell line.

The latex and methanol extract of leaves showed promising anti-angiogenic activity in our previous study (Uzma *et al.*, 2014b). MTT assay was performed to find out the potential mechanism behind anti-angiogenesis activity of *Euphorbia helioscopia*. Angiogenesis is the formation of new blood vessels from pre-existing vessels, it plays pivotal role in tumor growth (Pepper, 1997) and oxygen supply to ischemic tissues (Tsurumi *et al.,* 1996). Vascular endothelial growth factor (VEGF) is a main mediator in vessels neogenesis, Permeability factor is another term used for VEGF. VEGF enhances the expression of endothelial nitric oxide synthase (eNOS), which may paly important role in VEGF-induced angiogenesis (Bouloumie *et al.*, 1999). VEGF binds with specific receptors present on endothelium and promotes cellular events such as endothelial proliferation, migration and

degradation of extracellular matrix components (Ferrara and Davis-Smyth, 1997). A good anti-angiogenic substance should be able to inhibit angiogenesis without killing vascular endothelial cells. The vascular endothelial cells should be controlled for the inhibition of vascularization without killing them. Cytotoxicity studies help to rule out the possibility of the involvement of cell death in anti-angiogenesis. Therefore, it is important to evaluate anti-angiogenic compounds for cytotoxicity.

In this study latex showed 83 % cell survival at lowest concentration *i.e.* 0.0128 µg/ml and 57 % cell survival at 1000 µg/ml (highest concentration). IC_{50} cannot be calculated as minimum cell survival percentage is greater than 50%. So the use of latex is non-toxic on normal endothelial cell line (Vero cell line). The leaves methanol extract had IC_{50} value 5.27 µg/ml. At highest and lowest concentrations, the cell survival values were 34% and 81 % respectively. The crude extract with IC_{50} < 20 µg/ml is considered as highly toxic (Mahavorasirikul *et al.,* 2010). According to this, the leaves methanol extract is highly toxic with IC_{50} value 5.27 µg/ml.

CONCLUSIONS

Latex did not show cytotoxicity within experimentally used concentration range *i.e.* 1000-0.0128 µg/ml as its IC_{50} was found beyond this limit. Whereas IC_{50} value of leaves methanol extract was 5.27 µg/ml.

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