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CYTOTOXICITY OF METHANOLIC EXTRACT OF *PISONIA ACULEATA* L. LEAF

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ABSTRACT

Cytotoxicity of Methanolic extracts of *Pisonia aculeata* (MPA) was evaluated using three human cancer cell lines [Human Laryngeal Epithelial Carcinoma cell (Hep2), Human Colon Cancer cell (HT-29) and Human Breast cancer cells (HBL-100)]. The *in vitro* cytotoxicity was done by Tryphan blue dye exclusion method, MTT assay and Apoptosis. Cells were treated for 72 hrs with various concentration of MPA (31.25-500 µg/ml), the relative cell survival progressively decreased in a dose dependant manner. The IC_{50} of the MPA was found to be 280, 200 & 340 µg/ml by Tryphan blue dye exclusion method & 130,100 &170 µg/ml by MTT assay. Among the tested cell lines, MPA was more selective cytotoxic against HT-29 cell line than Hep2 & HBL-100 cells. Extensive apoptotic alterations were observed in MPA (500 µg/ml) by Hoechst staining & more than 30% of cells underwent apoptosis upon a single dose (500 µg/ml) of the MPA after 48 hrs.

KEYWORDS: **Cytotoxicity, Pisonia *aculeata* leaf, Hep2, HT-29 , HBL-100, Human cancer cell lines, MTT, Hoechst staining, Apoptosis.**

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INTRODUCTION

Tissue culture has been used to screen many anticancer drugs, as there is clear correlation between the *in vitro* and *in vivo* activities of potential chemotherapeutic agents. There is scientific justification for cytotoxicity testing in tissue culture, since animal models are in many ways inadequate for predicting the effects of chemicals on humans since there are many metabolic differences between species. Cytotoxicity studies involve the analysis of morphological damage or inhibition of zone of outgrowth induced by the chemicals tested[1]. The methods used for the evaluation of cytotoxicity i.e. blue dye exclusion assay in that the Tryphan blue was a vital stain used to selectively color dead tissues or cells blue based on the principle that live (viable) cells actively pump out the dye by efflux mechanism where as dead (non-viable)cells do not and MTT[(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide] which measures the metabolic activity of the viable cells. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt which is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number [2]. *Pisonia aculeata* Linn. (Nyctaginaceae) is a large scandent shrub, which holds an important place in folklore medicine. It is extensively used by native medical practitioners and tribes for treating swelling, rheumatic pains, jaundice and tumors. Preliminary phytochemical screening of the extract showed the presence of alkaloids, triterpenes, Phenolic compounds, flavonids and glycosides. The present study was focused on the analysis of morphological damage or inhibition of zone of outgrowth induced by the chemicals tested[3-4].

MATERIALS AND METHODS

Plant materials

Fresh leaves of *Pisonia aculeata* were collected from the surrounding of Tirupati district (517502), Andhra Pradesh, India, in the month of April and May 2010 and authenticated by Dr. K. Madhava Chetty, Asst. Professor, Department of Botany, Sri Venkateswara University, Tirupati AndhraPradesh, India.

Preparation of extracts

The leaves of *Pisonia aculeata* were dried under shade, then powdered with a mechanical grinder, passed through sieve No 40 and stored in an airtight container for further use. The coarse powder was extracted with 1-1.5L of petroleum ether (60-80°C) by continuous hot percolation using Soxhlet apparatus, after completion of extraction, it was filtered and the solvent was removed by evaporation. The marc left after petroleum ether extraction was dried and extracted with 1–1.5L of methanol (70-80°C) by the same procedure as above. The extract was stored in desiccator. [5-6]

Cell lines and culture medium

Hep-2 (Human Laryngeal Epithelial Carcinoma cell), HT-29(Human Colon Cancer cell), HBL-100(Human Breast Cancer cell) were used in this study. These were obtained from National Center for Cell Sciences, Pune.

Assay for proliferation studies

(A) Tryphan Blue dye exclusion assay:[7-10]

Cell suspensions were prepared by incubating for 24hr in 5.0% CO_2. 300μl of MPA (62.5-1000 μg/ml) were added. After incubating for 48hr, 100 μl of cell suspension and 100 μl of 0.4% Trypan Blue solution were taken in an Eppendorf tube, mixed thoroughly and allowed to stand for 15 min. Cover slip was placed and Pasteur pipette was used to transfer a small amount of Trypan Blue-cell suspension mixture to both chambers of a hemocytometer. The edge of the cover-slip was carefully touched with the pipette tip and allowed each chamber to fill by capillary action. Starting with chamber 1 of the hemocytometer all the cells in the 1 mm center square and four 1 mm corner squares were counted and % inhibition was calculated by following formula.

$$\% \text{ Inhibition} = \frac{\text{Number of non viable cells (stained)}}{\text{Total number of cells (stained and unstained)}} \times 100$$

(B) MTT(3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) method:[11-14]

Accurately measured 0.1ml of the cell suspension (containing 1x10^5cells) and 0.1ml of the MPA (31.25-500 μg/ml) in Di methyl sulphoxide (such that the final concentration of DMSO in media is less than 1%) were added to the 96 well plates and kept in carbon dioxide incubator with 5.0% CO_2, at 37°C for 72 hr. After 72hr, 20μl of MTT was added and kept in carbon dioxide incubator for 2 hr followed by 80μl of lysis buffer (15% SLSin 1:1 DMF and water). The plate was covered with aluminum foil to protect it from light. Then 96 well plates were kept in rotary shaker for 8 hr and were processed on ELISA reader for absorption at 562 nm. Blank contains only cell suspension and control wells contain 1% DMSO and cell suspension were also similarly treated. The readings were averaged and viability of the test samples was compared with DMSO control. The percentage growth inhibition was calculated using the following formula

$$\% \text{ Growth Inhibition} = \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \times 100$$

Hoechst staining and photo microscopy: [15-18]

To analyze the morphological apoptotic changes, 1 X 10^5 cells were seeded in 96-well plates (37°C, 5.0% CO_2). When logarithmic growth phase of cells was reached, the MPA with final concentration of 500 µg/ml or 0.1% DMSO (negative control) was added, respectively. After 48 hr the cells were washed in phosphate-buffered saline (PBS) and stained for 10 min at room temperature in PBS containing 40% w/v formaldehyde and 10 mg/ml Hoechst 33258. Hep2, HT-29 & HBL-100 cells for Hoechst staining were grown on sterilized cover slips and processed after washing one time with PBS, cells were fixed with 3.7%w/v formaldehyde in PBS for 10 min, washed one time with PBS, stained with 0.4 mg/ml Hoechst (Molecular Probes, Eugene) in PBS for 15 min, washed two times with PBS, and then one time with water. Cover slips were then air-dried and mounted with Slow Fade (Molecular Probes) mounting media. Morphological evaluations of nuclear condensation and fragmentation were performed immediately after staining by means of fluorescent microscope (Olympus, Japan) at 550 nm of emission.

RESULTS

Table 1
Cytotoxicity studies of MPA on Hep2, HT-29 & HBL-100 cells by Typhan Blue Dye Exclusion Technique

Concentrations (µg/ml)	Hep 2 % cell death	HT-29 % cell death	HBL-100 % cell death
1000	82±1.54	89.65±2.0	76±1.2
500	59±2.71	69±1.10	59±1. 07
250	48±0.73	57±1.4	41±1.3
125	27±0.84	35± 2.5	23±0.9
62.5	12.5±0.50	17.5±3.0	8.9±0.4
IC_{50} (μg/ml)	**280**	**200**	**340**

Readings are expressed as mean ± sem. Average of 3 determinations, 3 replicates

IC_{50} , drug concentration inhibiting 50% cellular growth following the drug exposure. By trypan blue exclusion method,the IC_{50} of MPA was found to be 280, 200 & 340 μg/ml against Hep2, HT-29 & HBL-100 cell lines respectively (Table 1). Among the tested cell lines, extract of *P. aculeata* was more selective cytotoxic against HT-29 cell line than Hep2 & HBL-100 cells.

Table 2
Cytotoxic effect of MPA on Human cancer cell lines by MTT assay

Concentrations (μg/ml)	Hep 2 % inhibition	HT-29 % inhibition	HBL-100 % inhibition
500	78±1.52	85±2.08	73±2.51
250	69±4.58	72±2.08	58±2.00
125	49±2.64	57±2.00	43±1.52
62.5	32±1.52	33.6±1.76	27±1.52
31.25	16±1.52	19.45±1.25	14±1.52
IC_{50} (μg/ml)	**130**	**100**	**170**

Readings are expressed as mean ± sem, Average of 3 determinations, 3 replicates

$IC_{50,}$ Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure. By MTT, the IC_{50} of the MPA was found to be 130, 100 & 170 μg/ml on Hep2, HT-29 and HBL-100 cell lines respectively (Table 2). Among the tested cell lines, MPA was more selective cytotoxic against HT-29 cell line than other cells.

Apoptosis

Apoptosis or programmed cell death is a highly conserved, tightly controlled cell suicide process that is regulated by many different intracellular and extracellular events to ablate neoplastic cells in normal physiological functions. Apoptosis as an intrinsic suicide serves to remove excess, damaged or infected cells in metazoans.

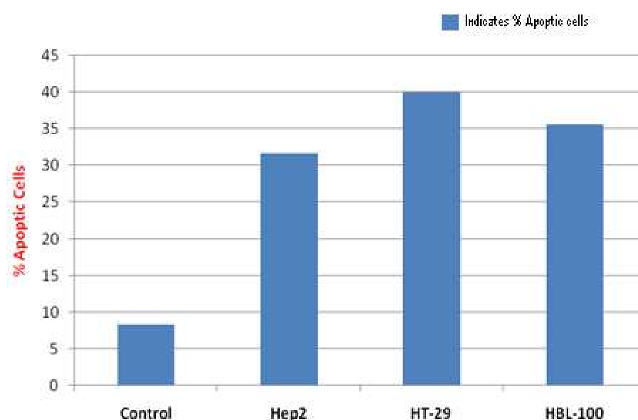

Figure 1
Graph– Percentage of apoptic cells after staining with Hoechst.

The nuclear morphological changes by Hoechst staining were assessed and found that, more than 30% of Hep2, HT-29 & HBL-100 cells underwent apoptosis upon a single dose (500µg/ml) of the MPA after 48 h and 50%−60% of the cells fragmented nuclei were detected by fluorescence. Cells treated with (500 µg/ml) dose of the MPA were prone to apoptosis after 48 h (Fig. No.1). The viable cells were uniformly blue, whereas the apoptotic cells were blue and contain bright blue dots in their nuclei, representing the nuclear fragmentation.

DISCUSSION

This study was undertaken to scientifically prove the traditional claim of extract of *P. aculeata* possessing anticancer activity. *P. aculeata* is known and reported for its antitumor[5,6], hepatoprotective and antioxidant activity[19]. However no studies have been reported for the cytotoxic effect of this plant. An attempt was made to determine the cytotoxicity of MPA by Trypan Blue exclusion, MTT, Hoechst staining methods. Trypan Blue exclusion method was very simple & precise. The IC_{50} of MPA was found to be 280, 200 & 340 µg/ml against Hep2, HT-29 and HBL-100 cell lines respectively. The finding from the study reveals that MPA is more cytotoxic against HT-29 cell line than Hep2 & HBL-100 cells. The IC_{50} of MPA by MTT was found to be 130,100 &170 µg/ml on Hep2, HT-29 and HBL-100 cell lines respectively. Among the tested cell lines, MPA was more selective cytotoxic against HT-29 cell line than other cells. The nuclear morphological changes by Hoechst staining method were assessed and found that more than 30% of Hep2, HT-29 & HBL-100 cells underwent apoptosis upon a single dose (500 µg/ml) of the MPA after 48 hrs and 50%−60% of the cells fragmented nuclei were detected by fluorescence. Cells treated with (500 µg/ml) dose of the MPA were prone to apoptosis after 48 hrs. Therefore, it was proved that the MPA possess potent antitumor on human Hep2, HT-29 & HBL-100 cancer cells.

CONCLUSION

Therefore, it can be concluded that the MPA possess potent antitumor activity on human Hep2, HT-29 & HBL-100 cancer cells. Further studies can be undertaken for the isolation and characterization of active chemical constituent from *P. aculeata* which may act as a lead compound for the development of potential anticancer drugs.

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