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Research Article

INVESTIGATION ON THE CYTOTOXIC NATURE OF LEAF AND STEM BARK EXTRACTS OF *PROSOPIS CINERARIA*

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ABSTRACT

Objective: To investigate the cytotoxic activity of hydroalcoholic extracts of leaf and Stem bark of *Prosopis cineraria* using two human cancer cell lines such as HeLa and MCF-7.

Methods: The cytotoxic activity was evaluated by trypan blue exclusion and MTT assay. The apoptosis influencing activity of extracts in cancer cell lines were performed using Hoechst staining technique.

Results: Among the tested cell lines, Leaf extracts was more selective cytotoxic against HeLa and MCF-7 cell line than Stem bark extracts. In apoptotic study, morphological changes induced in HeLa and MCF-7 cells by both extracts were evidenced by using Hoechst staining and fluorescent microscopy. It was observed that there is marked increase in the number of MCF-7 and HeLa cells with the typical features of apoptosis, consisting in cells with condensed and fragmented nuclei.

Conclusion: The present study proves the traditional claim of the plant in the management of cancer.

Keywords: *Prosopis cineraria*, 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide, Apoptosis, Cytotoxic activity, Leaf, Stem bark.

INTRODUCTION

Cancer is one of the greatest killers worldwide and is spreading promptly. In medical science, the methods available to treat a cancer patient mainly include surgery, chemotherapy, and radiotherapy. As these known methods are very costly and have side effects with limitations of their use, there is need of effective and acceptable cancer therapeutics agents that should be non-toxic, highly efficacious against multiple cancers, palatable, cost effective and acceptable by human population [1]. *Prosopis cineraria* Linn [2] is a large tree, which belongs to the family Mimosaceae. It is called Kalpa plant in Ayurveda and Siddha literature. The whole plant is used in the Indigenous System of Medicine as folklore remedy for various ailments such as leprosy, dysentery, bronchitis, asthma, leukoderma, piles, muscular tremor and wandering of the mind. It is also known to possess anthelmintic, antibacterial, antifungal, antiviral, and anticancer activities [3]. This study is designed to evaluate the hydroalcoholic extracts of leaf and stem bark of *P. cineraria* (PCL and PCB) on HeLa and MCF-7 cell lines.

METHODS

Plant material

The plant specimens of *P. cineraria* were collected in the month of September from the Thiruvallur district, Tamil Nadu, India. The specimens were identified and authenticated by Prof. P. Jayaraman, Director of Plant Anatomy Research Centre, West Tambaram, Chennai. A voucher specimen (No: A-43/PARC) has been deposited in the same Institution.

Preparation of crude extract

The leaves and stem bark were extracted separately with 50% alcohol by cold percolation process to yield the respective extracts. The hydroalcoholic extracts of PCL and PCB were reduced to a molten mass by using rotary vacuum evaporator. The residue was then stored in a desiccator.

Cell lines

HeLa [4] and MCF-7 [5] cell lines were obtained from NCCS, Pune. The cells were cultured in Dulbecco's modified Eagle's medium in 10% FBS complete medium. The medium was supplemented with 10% heat inactivated fetal bovine serum, antibiotics. The cell lines were maintained at 37°C in a 5% CO2 incubator, and the media were frequently changed.

Cytotoxic activity

Trypan blue dye exclusion method

Principle

Trypan blue is a vital stain used to selectively color dead tissues or cells blue. It is a diazo dye. Trypan blue is recommended in dye exclusion procedures for viable cell counting based on the principle that live (viable) cells actively pump out the dye by efflux mechanism whereas dead (non-viable) cells do not. Hence in this assay, white transparent cells are viable cells and blue cells taking up the dye are dead cells.

Method

700 µl of a cell suspension was transferred to 24 well plates and incubated for 24 hrs in 5% CO_2. After incubation, 300 µl of varying concentrations of PCL and PCB (62.5-1000 µg/ml) was added and incubated for 24 hrs. 100 µl of cell suspension was taken in an Eppendorf tube and to that 100 µl of 0.4% trypan blue solution was added and mixed thoroughly. It was allowed to stand for 5-15 minutes. A small amount of trypan blue-cell suspension mixture was transferred to both chambers of a hemocytometer using a Pasteur pipette. All the chambers were filled by capillary action and not overfilled. From chamber 1 of the hemocytometer, the cells in the 1 mm center square and four 1 mm corner squares were counted. Non-viable cells stained with blue color. Viable and non-viable cells were counted separately [6].

$$\text{Percentage inhibition} = \frac{\text{Number of non-viable cells (stained)}}{\text{Total cells (stained and unstained)}} \times 100$$

3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide (MTT)

Principle

MTT measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a microtiterplate (MTP). It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and mitochondrial dehydrogenase produces water-insoluble formazan salt. This method involves by culturing the cells in a 96-well MTP and then incubating them with MTT solution for approximately 2 hrs. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and quantified with an enzyme-linked immuno assay (ELISA) plate reader. The absorbance directly correlates with the cell number. This is applicable for adherent cells cultured in MTP.

Method

About 0.1 ml of the cell suspension (containing 1×10^5 cells) and 0.1 ml of the compound (6.25-100 µg/ml in dimethyl sulfoxide [DMSO] such that the final concentration of DMSO in media is <1%) were added to the 96 well plates and kept in carbon dioxide incubator with 5% CO_2, at 37°C for 72 hrs. The blank contains only cell suspension and control wells contain 1% DMSO and cell suspension. After 72 hrs, 20 µl of MTT was added and kept in carbon dioxide incubator for 2 hrs followed by 80 µl of lysis buffer (15% SLS in 1:1 DMF and water). The plate was covered with aluminum foil to protect it from light. Then the 96 well plates were kept in a rotary shaker for 8 hrs. After 8 hrs, the 96 well plates were processed on ELISA reader for absorption at 562 nm. The percentage growth inhibition was calculated using the following formula and concentration of drug needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line [7].

% Growth inhibition = 100 – (Mean OD of test group/Mean OD of the control group) × 100

Apoptosis

To analyze the morphological apoptotic changes, 1×10^5 cells seeded in 96-well plates (37°C, 5% CO2), when logarithmic growth phase of cells was reached, PCL and PCB with final concentration of 500 µg/ml or 0.1% DMSO (negative control) were added, respectively. After 48 hrs, the cells were washed in phosphate-buffered saline (PBS) and stained for 10 minutes at room temperature in PBS containing 40% paraformaldehyde and 10 mg/ml Hoechst stain. HeLa cells for Hoechst staining were grown on sterilized cover slips and processed as described [8], with modifications. Briefly, after washing one time with PBS, cells were fixed with 3.7% formaldehyde in PBS for 10 minutes, washed one time with PBS, stained with 0.4 mg/ml Hoechst in PBS for 15 minutes, washed 2 times with PBS, and then one time with water. Cover slips were then air-dried and mounted with a slow fade (Molecular Probes) mounting media. Morphological evaluations of nuclear condensation and fragmentation were performed immediately after staining by means of the fluorescent microscope (Olympus, Japan) at 550 nm of emission.

RESULTS AND DISCUSSION

The cytotoxicity study by trypan blue exclusion method is a very simple method which can be carried out within a short time of 24 hrs. It is a precise method, which takes in to account the viable and also the dead cells in addition to estimation of inhibitory concentration 50% (IC_{50}) concentration. The IC_{50} of PCL and PCB was found to be 300, 310 and 320, 380 mg/ml against HeLa and MCF-7 cell lines respectively (Tables 1 and 2, Figs. 1 and 2). Among the tested cell lines, PCL was more selective cytotoxic against HeLa and MCF-7 cell line than PCB (Table 3 and Fig. 3).

The cytotoxic activity of PCL and PCB using two human cancer cell lines (i.e. HeLa and MCF-7) were evaluated with MTT assay. When the cells were treated for 72 hrs with various concentrations of PCL and PCB

Table 1: Cytotoxicity studies of PCL and PCB on MCF-7 by trypan blue dye exclusion technique

S. No	Concentrations (µg/ml)	Cell death in percentage	
		PCL	PCB
1.	1000	100	100
2.	500	100	58
3.	250	38.46	44
4.	125	35.84	39
5.	62.5	32.57	34.28
	IC_{50}	310	380

Average of three determinations, three replicates, IC_{50}, drug concentration inhibiting 50% cellular growth following the drug exposure. PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark

Table 2: Cytotoxicity studies of PCL and PCB on HeLa by trypan blue dye exclusion technique

S. No	Concentrations (µg/ml)	Cell death in percentage	
		PCL	PCB
1.	1000	100	100
2.	500	68	59
3.	250	46	46
4.	125	38	32
5.	62.5	29	20
	IC_{50}	300	320

Average of three determinations, three replicates IC_{50}, drug concentration inhibiting 50% cellular growth following the drug exposure, PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark

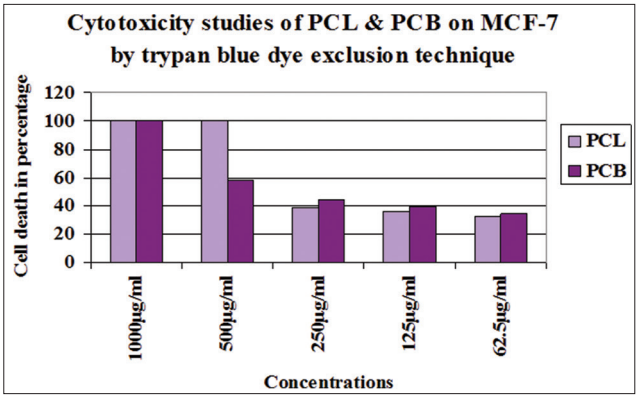

Fig. 1: Cytotoxicity studies of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on MCF-7 by trypan blue dye exclusion technique

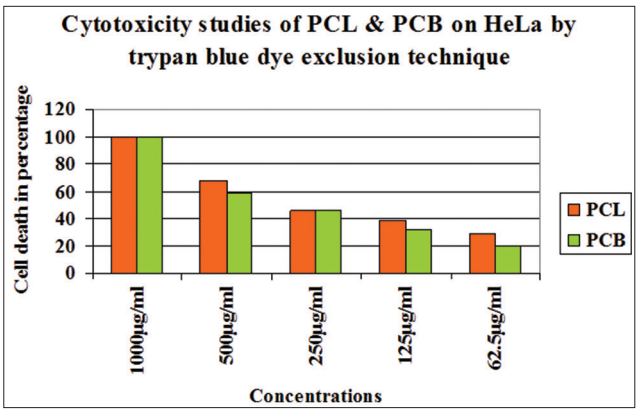

Fig. 2: Cytotoxicity studies of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on HeLa by trypan blue dye exclusion technique

Table 3: Cytotoxic effect of PCL and PCB on HeLa and MCF-7 cells by trypan blue assay

S. No	Treatment	Cell lines (µg/ml)	
		MCF-7 IC_{50}	HeLa IC_{50}
1.	PCL	310	300
2.	PCB	380	320

PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark, IC_{50}: Inhibitory concentration 50

Table 4: Cytotoxicity studies of PCL and PCB on MCF-7 by MTT assay

S. No	Concentrations (µg/ml)	Percentage inhibition	
		PCL	PCB
1.	500	71.74	67.49
2.	250	56.59	48.96
3.	125	50.7	41.46
4.	62.5	42.72	30.62
5.	31.25	29.83	19.39
	IC_{50}	115	260

Average of three determinations, three replicates, IC_{50}, Drug concentration inhibiting 50% cellular growth following 72 hrs of drug exposure, PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark, MTT: 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide

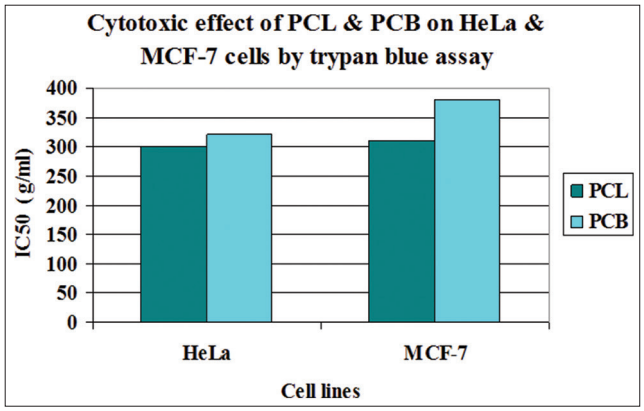

Fig. 3: Cytotoxic effect of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on HeLa and MCF-7 cells by trypan blue assay

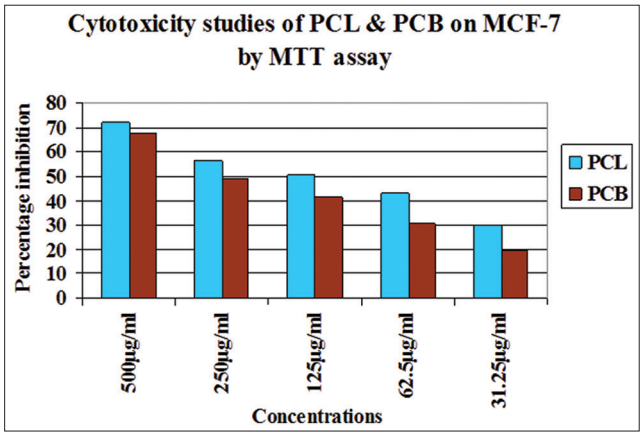

Fig. 4: Cytotoxicity studies of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on MCF-7 by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide assay

Table 5: Cytotoxicity studies of PCL and PCB on HeLa by MTT assay

S. No	Concentrations (µg/ml)	Percentage inhibition	
		PCL	PCB
1.	500	79.35	73.88
2.	250	68.36	56.84
3.	125	50.87	40.04
4.	62.5	33.86	21.48
5.	31.25	26.31	10.35
	IC_{50}	120	185

Average of three determinations, three replicatesm IC_{50}, Drug concentration inhibiting 50% cellular growth following 72 hrs of drug exposure, PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark, MTT: 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide

Table 6: Cytotoxicity studies of PCL and PCB on MCF-7 and HeLa by MTT assay

S. No	Compound	Cell lines (µg/ml)	
		HeLa IC_{50}	MCF-7 IC_{50}
1.	PCL	120	115
2.	PCB	185	260

PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark, IC_{50}: Inhibitory concentration 50%, MTT: 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide

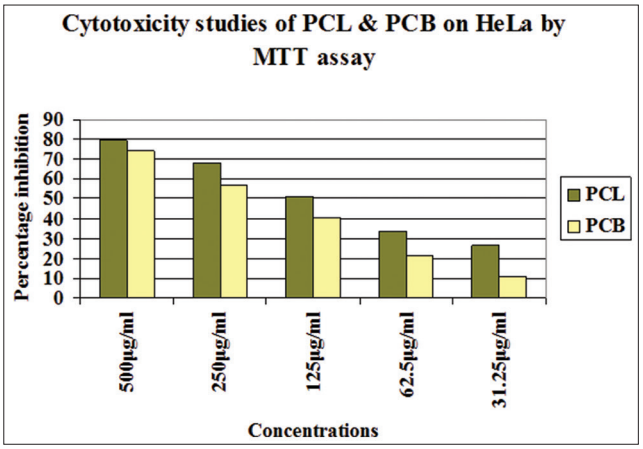

Fig. 5: Cytotoxicity studies of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on HeLa by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide assay

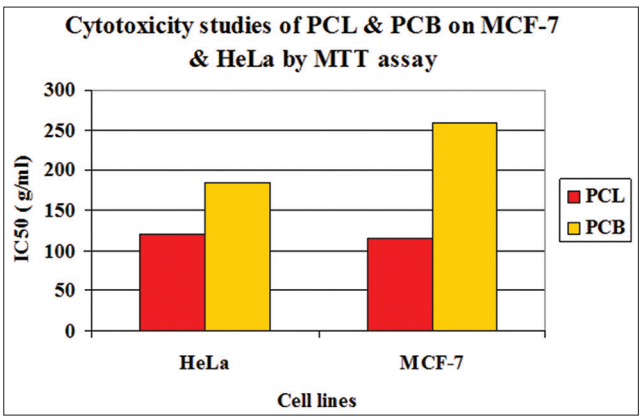

Fig. 6: Cytotoxicity studies of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on MCF-7 and HeLa by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide assay

(31.25-500 mg/ml), the relative cell survival progressively decreased in a dose-dependent manner. The growth inhibition 50% of PCL and PCB was found to be 120, 115 and 185, 260 mg/ml on HeLa and MCF-7 cell lines respectively (Tables 4 and 5, Figs. 4 and 5). Among the tested cell lines, PCL was more selective cytotoxic against MCF-7 and HeLa cell line than PCB (Table 6 and Fig. 6).

Table 7: Percentage of apoptosis on HeLa cells by treatment with PCL and PCB

S. No	Apoptotic cells in %	Control	PCL	PCB
1.	Percentage death	06	36	31
2.		11	43	40
3.		07	41	36
	Average	08	40.0	35.66
	SE	1.52	2.08	2.60

SE: Standard error, PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark

Table 8: Percentage of apoptosis on MCF-7 cells by treatment with PCL and PCB

S. No	Apoptotic cells in %	Control	PCL	PCB
1.	Percentage death	07	32	23
2.		10	36	32
3.		08	27	29
	Average	8.33	31.66	28
	SE	0.88	2.60	2.64

SE: Standard error, PCL: *Prosopis cineraria* leaf, PCB: *Prosopis cineraria* stem bark

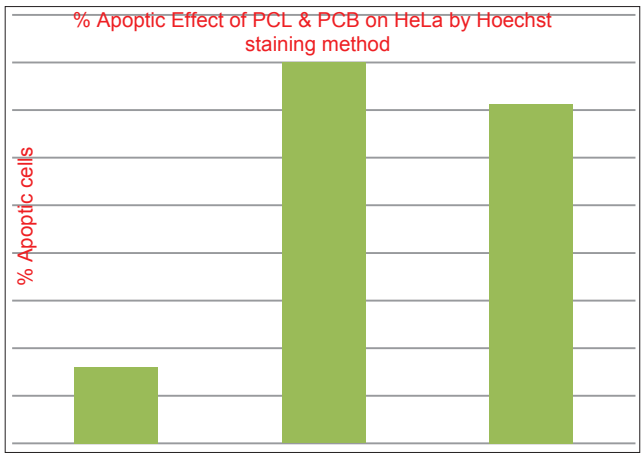

Fig. 7: Apoptotic effect of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on HeLa by Hoechst staining method

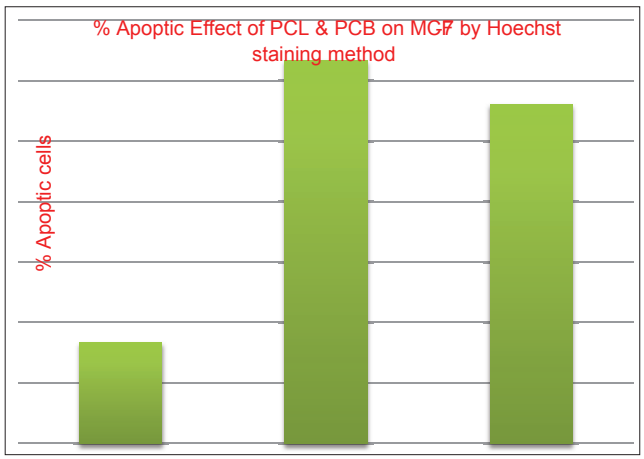

Fig. 8: Apoptotic effect of *Prosopis cineraria* leaf and *Prosopis cineraria* stem bark on MCF-7 by Hoechst staining method

Extensive apoptotic alterations were observed in PCL and PCB (500 µg/ml) that increased over time following the induction of apoptosis. PCL at 500 µg/ml concentration shows 31.66% and 40% apoptic cells against MCF-7 and HeLa respectively, whereas PCB at 500 µg/ml concentrations shows 28% and 35.66% apoptic cells against MCF-7 and HeLa respectively (Tables 7 and 8). From these data, the graphs were plotted showing the percentage of apoptic cells (Figs. 7 and 8). Upon a single dose of the PCL and PCB after 48 hrs, more than 30-40% of HeLa cells underwent apoptosis. The viable cells are uniformly blue whereas the apoptotic cells are blue (Figs. 9a and 10a) and contain bright blue dots in their nuclei representing the nuclear fragmentation and fragmented nuclei were detected by fluorescence. A marked increase in the number of MCF-7 and HeLa cells with the typical features of apoptosis, consisting in cells with condensed and fragmented nuclei was observed (Figs. 9b & c and 10b & c). Moreover, many cells were starting to detach from the substrate (not shown). This population appeared late after treatment and consisted of apoptotic bodies. Taken together, these data suggest that distinct stages of apoptosis can be identified by staining of cells with Hoechst. This assay should be useful for the detection and further characterization of cells

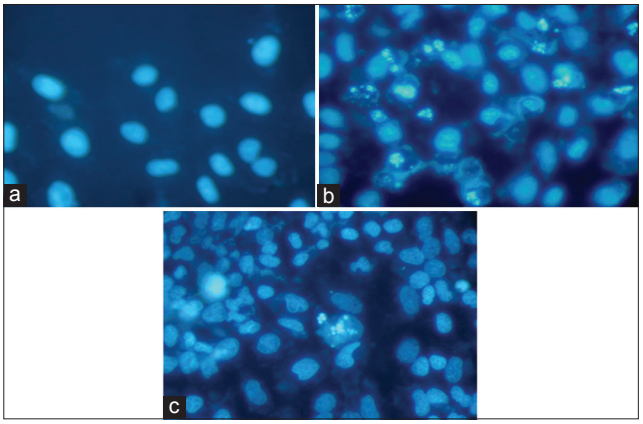

Fig. 9: (a) Hoechst stained HeLa cells that had not been exposed to *Prosopis cineraria* leaf (PCL) and *Prosopis cineraria* stem bark (PCB), (b) Hoechst-stained HeLa cells that had been exposed to PCL for 48 hrs (500 µg/ml), (c) Hoechst-stained HeLa cells that had been exposed to PCB for 48 hrs (500 µg/ml)

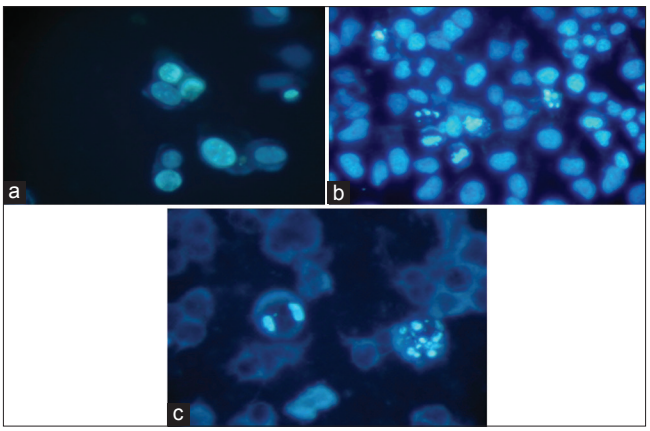

Fig. 10: (a) Hoechst stained MCF-7 cells that had not been exposed to *Prosopis cineraria* leaf (PCL) and *Prosopis cineraria* stem bark (PCB), (b) Hoechst-stained MCF-7 cells that had been exposed to PCL for 48 hrs (500 µg/ml), (c) Hoechst-stained MCF-7 cells that had been exposed to PCB for 48 hrs (500 µg/ml)

at different stages in the apoptotic process. The results suggest that PCL and PCB may exert their antitumor effects associated with two fundamental processes: Suppression of cell proliferation and induction of apoptosis on human MCF-7 and HeLa cell *in vitro*.

CONCLUSION

PCL and PCB, evaluated by trypan blue exclusion method and MTT assay were found to have a significant cytotoxicity effect against different tumor cell lines, such as HeLa and MCF-7. It was found that PCL and PCB reduced cell viability by inducing apoptosis on human MCF-7 and HeLa cancer cells. Therefore, it is concluded that PCL and PCB possess potent anticancer activity.

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