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Cell death induced by methanolic extract of *Prosopis cineraria* leaves in MCF-7 breast cancer cell line

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ABSTRACT : *Apoptosis is a programmed cell death and is an active, defined process. It plays an important role in the development of multicellular organisms, regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. Cancer is one of the scenarios where too little apoptosis occurs, resulting in malignant cells that will not die and continue to proliferate. Apoptosis is one of the main therapeutic targets in cancer research. In the present study we did a comparative study on the apoptosis influencing activity of methanolic extract of Prosopis cineraria leaves in breast cancer cell line MCF-7 and non cancerous cell line HBL 100. Various staining techniques like Giemsa, ethidium bromide, Propidium iodide and Hoechst were performed both in cancerous cell line MCF-7 and noncancerous cell line HBL 100. The results revealed that plant extract caused a steep increase in apoptotic ratio in cancer cell line and not in HBL 100.*

Keywords —*Anticancer activity, apoptosis, MCF-7, Prosopis cineraria, vanni.*

I. INTRODUCTION

Over the past few decades a significant progress has been made in cancer prevention and treatment. But still the development of effective treatment regimens remains one of the greatest challenges in the area of cancer chemotherapy. Plant-derived natural products are becoming important as anti-cancer derivatives, including vincristine, vinblastine, paclitaxel and camptothecin, which are invaluable contributiors of nature to modern medicine [1-4]. Apoptosis, a form of programmed cell death, is highly organized and evolutionary conserved process characterized by membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and apoptotic bodies [5]. Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint of cell cycle or by induction of apoptosis or a combined effect of both cyclic block and apoptosis [6, 7]. Previous studies in our laboratory showed that the methanolic extract of the leaves of the plant showed ability to scavenge free radicals *in vitro* [8] and also possesses potent cytotoxic effect in breast cancer cell line MCF-7. The purpose of this study was to observe the effects of cell death by methanolic extract of *Prosopis cineraria* leaves in MCF-7 breast cancer cell line and noncancereous HBL100 cells. The results support that methanolic extract of *Prosopis cineraria* leaves possesses anticancer potential by induction of apoptosis.

II. MATERIALS AND METHODS

The two cell lines MCF-7 and HBL-100 were purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were incubated in a CO_2 incubator with 5% CO_2 and 95% humidity atmosphere. After attaining confluent growth, the cells were trypsinized using Trypsin-EDTA (PAA) and the required numbers of cells (10^6 cells/ml) were seeded into 6-well plates respectively for carrying out various staining. In each well of the 6-well plates, a clean, dry, sterile coverslip was placed before the cells were seeded. The MCF-7 and HBL-100 cells were treated in the presence and/or the absence of leaf extracts (0.05mg) and with the respective drug tamoxifen-180µM and incubated for 24hrs in a 5% CO_2 and 95% humidity atmosphere. For staining, after treatment, the cover slip was removed and placed on a glass slide, sealed with vaseline and used. The treatment groups were cells treated with extract of *Prosopis cineraria* leaves in presence and absence of standard anticancer drug.

Morphological Changes in the Cells

The morphological changes in the cells were followed in the presence and the absence of the leaf extract and/or the oxidant. The cells were fixed and stained with giemsa for 10 minutes and observed under the phase contrast microscope (Nikon, Japan) as explained by Chih *et al* 2001 [9]. The cells were then observed for morphological changes using a phase contrast microscope (Nikon, Japan) at 400X magnification. The apoptotic ratio was calculated using the formula,

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}}$$

Acridine Orange/Ethidium Bromide Staining

Apoptotic cells were identified by AO/EtBr staining described by Parks *et al* 1979 [10]. The combination of AO/EtBr staining technique is used to differentiate apoptotic and normal cells. To the treated cells, 10 µl of Ao/EtBr was added and spread by placing a cover slip over it. The stained slides were incubated at room temperature 5 minutes. The apoptotic cells with condensed chromatin and fragmented nuclei were identified by their red fluorescence and the normal cells were visualized by their green fluorescence which was counted by using an upright fluorescent microscope using B2A filter at 400X magnification.

Propidium Iodide (Pi) Staining

The nuclear changes in the apoptotic cells were observed by PI staining as described by Sarker *et al* 2000 [11]. The cells were permeabilized with 50µl of acetone: methanol (1:1) mixture at −20ºC for 10 minutes. Then 10µl of PI was added, spread by placing a coverslip over it and incubated at 37ºC for 30 minutes in the dark. The apoptotic cells with fragmented nuclei were detected using the green filter of a fluorescence microscope (Nikon, Japan) at 400X magnification. The apoptotic ratio was calculated by the formula mentioned earlier.

Ethidium Bromide (Etbr) Staining

The method proposed by Mercille and Massie 1994 [12] was followed to detect the nuclear changes in apoptotic cells, with minor modifications. EtBr (10µl) was added to the treated cells and spread by placing a coverslip over it. The slides were incubated for 5 minutes at room temperature. The apoptotic cells with condensed chromatin and fragmented nuclei were counted by using fluorescent microscope (Nikon, Japan) using G-2A filter at 400X magnification. The apoptotic ratio was calculated as before.

Hoechst 33342 Staining

The Hoechst 33342 staining assay was employed to observe morphology alterations of the cells and performed as reported by Yamakawa *et al* 2008 [13]. The treated cells were harvested and washed with PBS twice, and then incubated with Hoechst 33342 (1µg/ml) for 30 min at 37°C. The stained cells were visualized under an inverted fluorescence microscope (Moticam, Hong Kong) using Hoechst filter at 400X magnification.

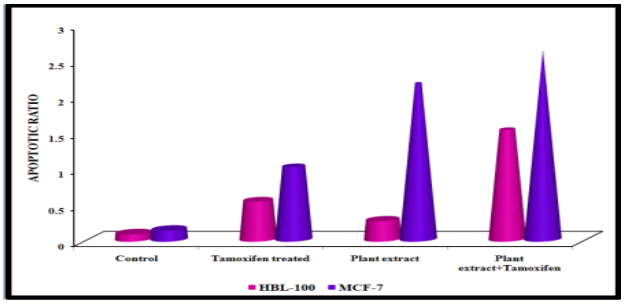
DAPI Staining

Apoptotic cells were detected with DAPI (4'-6'-diamidino-2-phenyl indole) staining technique as explained by Rashmi *et al* 2003 [14]. After the oxidant and/or plant extract treatment, the cells were fixed with 3% paraformaldehyde for 10 minutes and permeabilized with 0.2% Triton X-100. 10µl DAPI was added after placing a cover slip over the cells. The apoptotic ratio was determined by counting the cells with condensed chromatin and fragmented nuclei under inverted fluorescent microscope (Moticam, Hong Kong) using DAPI filter at 400X magnification.

III. RESULTS AND DISCUSSION

Morphological features of apoptotic cells include shrinkage, condensation of chromatin and cytoplasm, detachment of the cells from the neighbouring cells, fragmentation of the nucleus and membrane blebbing. These features were observed by Giemsa staining. The results showed that the tamoxifen caused an increase in apoptotic ratio both in MCF-7 and HBL 100. The plant extract increased the ratio only in MCF-7 and not in HBL 100 (Fig 1).

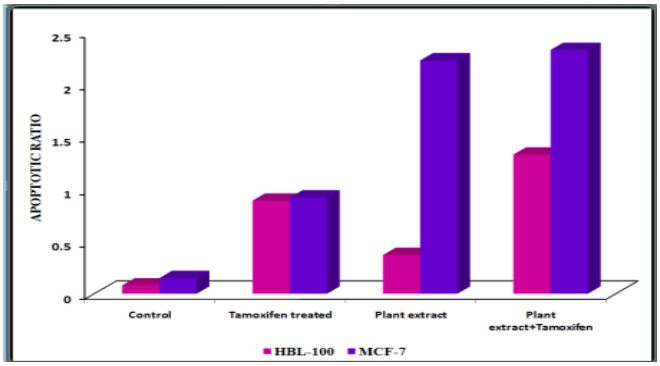
FIGURE 1



Effect of *Prosopis cineraria* leaf extract on the apoptotic ratio by Giemsa staining

The morphology of human leukemia HL-60 cells treated with Kuromoji (*Lindera umbellata)* essential oil (KEO) was observed using the Giemsa staining method. The study revealed that KEO as well as its major chemical linalool induced apoptosis and differentiation in human leukemia HL-60 cells [15]. Wright-Giemsa and Hoechst 33342 staining showed that curcumin induced morphological changes such as cell shrinkage and nuclear condensation, which are typical characteristics of apoptosis in KG1a, Kasumi-1 and U937 cells [16]. Allyl isothiocyanate (AITC) arrests cancer cells in M phase, rather than G_2 phase in human bladder cancer cells UM-UC-3 cells which were revealed by flow cytometry and Wright-Giemsa staining [17]. These literatures are in supportive of our findings that plant extract induced apoptosis in MCF -7 breast cancer cell line.

FIGURE 2



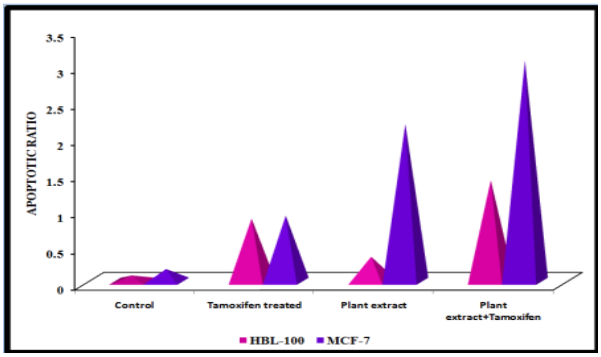
Effect of *Prosopis cineraria* leaf extract on the nuclear changes in cells (AO/EtBr staining)

The Acridine orange /ethidium bromide staining showed that the leaf extract induced apoptosis in MCF- 7 cancer cells (Fig 2). Our results are in agreement with the results of Kabeer *et al.,* 2012 [18] that the Acridine orange –Ethidium bromide staining showed Isodeoxyelephantopin could inhibit the proliferation of breast cancer cells and lung carcinoma cells and induce apoptosis in treated cells. Similarly AO/EB assay and FACS analysis clearly demonstrated that 5-Fluorouracil (5-FU) nanoparticles induce apoptosis in glioma (U87MG) and breast cancer (MCF7) cells compared with free drug [19].

Nuclear changes such as chromatin condensation around the nuclear membrane were noticed by ethidium bromide and propidium iodide staining (Fig 3 &4). The number of damaged cells was more in tamoxifen and plant extract treated groups in MCF-7. On the other hand the damage was less in HBL 100 cells. Various staining like ethidium bromide, propidium iodide and DAPI proved that leaf extract of *Rhinacanthus nasutus,* did not induce apoptosis in the chick embryo fibroblasts, but there was a marked increase in the number of apoptotic cells in the Hep2 cancer cells [20]. Sumathi *et al.,* 2011 [21] have reported that ethidium bromide staining proved that the *Euphorbia antiquorum* latex imparts complete protection to the primary cells which was exposed to etoposide and also latex does not induce apoptosis in normal cells but it modulates the apoptotic effects produced by etoposide. The results of PI/flow cytometer analysis revealed that a dose-dependent increase in the percentage of cells in sub-G1 phase was observed in cells treated with 0.5–4.0 μg/mL PEG-SeNPs. There was no significant changes in G0/G1, S, or G2/M phases were observed in treated cells, suggesting that cell death induced by PEG-SeNPs was caused mainly by cell apoptosis. PEG-SeNPs exhibited stronger growth inhibition on drug-resistant hepatocellular carcinoma (R-HepG2) cells than on normal HepG2 cells [22]. PI/flow cytometry analysis showed that bufalin induced the apoptosis of A549 cells in a dose and time dependent manner [23]. *S. litwinowii*-induced apoptosis in HeLa cell line, in which apoptotic cells were detected using PI staining of treated cells followed by flow cytometry [24].

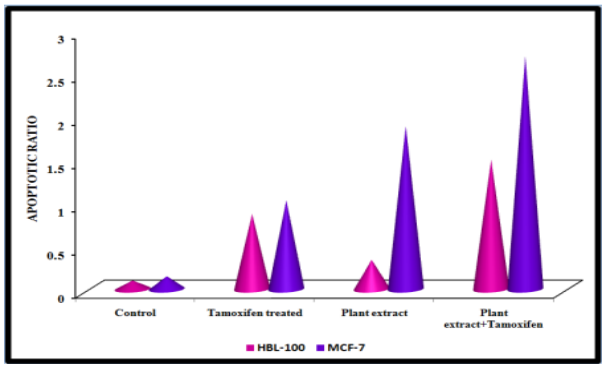
The results of the Hoechst 33258 staining showed that the nuclei of un- treated Mz-ChA-1 cells emitted low fluorescence intensity in a homogeneous manner, and the nuclear structure was intact whereas ESC-3-treated Mz- ChA-1 cells displayed typical morphological features of apoptosis: condensed chromatin, gradual disintegration of the nuclear membrane, and pyknotic (shrunken and dark) nuclei [25]. The nano encapusulated Ethanolic extract of *P. senega* (NEEPS) induced greater apoptosis of A549 cells than Ethanolic extract of *P. senega* (EEPS) [26]. Zerumbone-treated PANC-1 cells exhibited obvious apoptotic morphological changes in the nuclear chromatin, such as cell shrinkage, chromatin condensation, and cell nuclear fragmentation. In contrast, PANC-1 cells without zerumbone treatment presented the intact nuclear architecture [27]. The present study correlates with these supporting literatures that the methanolic extract of *Prosopis cineraria* leaves induce apoptotic cell death.

FIGURE 3



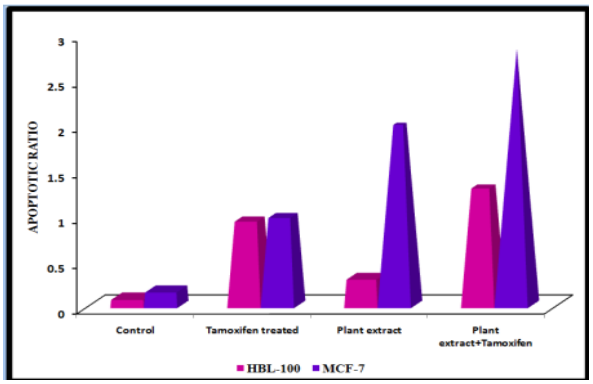
Effect of *Prosopis cineraria* leaf extract on the nuclear changes by PI staining

FIGURE 4



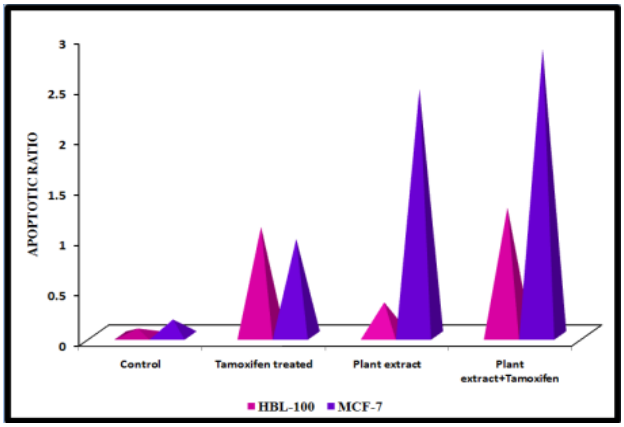
Effect of *Prosopis cineraria* leaf extract on the nuclear changes by EtBr staining

FIGURE 5



Effect of *Prosopis cineraria* leaf extract on the nuclear changes in cells (Hoechst 33342 staining)

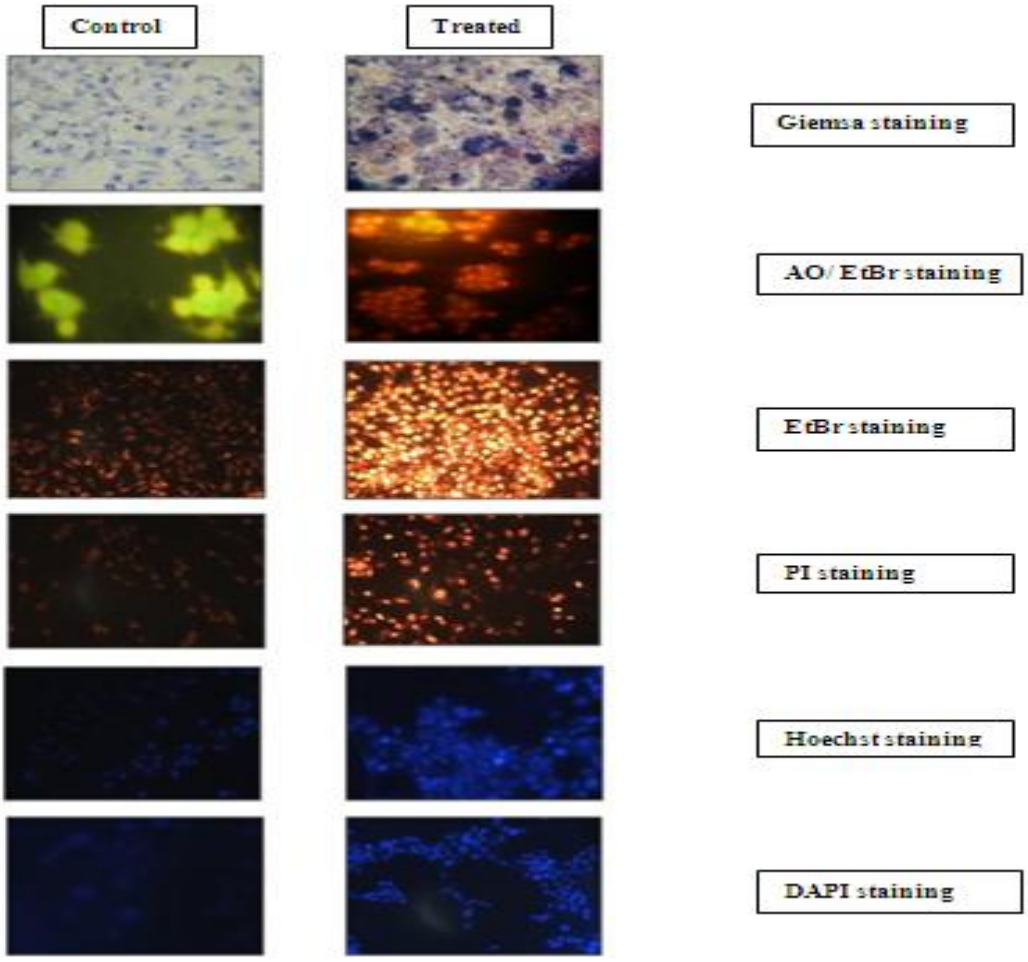
FIGURE 6



Effect of *Prosopis cineraria* leaf extract on the nuclear changes in cells (DAPI staining)

IV. CONCLUSION

This study showed that *Prosopis cineraria* leaves inhibit the proliferation of MCF-7 breast cancer cells with the involvement of apoptosis or programmed cell death. *Prosopis cineraria* could also be considered as a promising chemotherapeutic agent in cancer treatment. Further studies on the pathway of cell death would help to find a novel active natural compound for cancer.



Various Staining Techniques in Mcf-7

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