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Research Article

In vitro growth inhibitory activity of *Prosopis cineraria* leaves in MCF-7 breast cancer cell line

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ABSTRACT

Phytotherapy which implies the use of plant derived products for the treatment of cancer has assumed higher value due to the reduction in the adverse side effects which results due to chemotherapy or radiation therapy. One such medicinal plant is *Prosopis cineraria,* which is known as Vanni. In the present study, the growth inhibitory potential of the extract was tested using a human breast cancer cell line MCF-7 (ER+, PR+) in the presence/absence of the standard chemotherapeutic drug tamoxifen by MTT, SRB, neutral red, LDH release, WST-1 and alamar blue assays. The safety of the extract was also evaluated using non-cancerous breast cell line HBL-100. The results showed that the extracts inhibited the growth of MCF-7 breast cancer cells.

Keywords: Anticancer activity; Cytotoxicity; Vanni; HBL 100; MCF-7; *Prosopis cineraria*

INTRODUCTION

Cancer is a crucial public health problem in both developed and developing countries and it represents the largest cause of mortality in the world (Newshan Behrangi *et al*., 2012). In India, breast cancer is the most common cancer among women in many regions and has overtaken cervical cancer, which was the most common type of cancer a decade ago. In females, breast malignancies may result due to hormonal abnormalities. Plants have been used for the treatment of many medical disorders for centuries and have been the source of several clinically useful cytotoxic and anti-inflammatory agents (Nataraj Jagannath *et al*., 2012). *Prosopis cineraria* is one such medicinal plant, which is used in traditional medicine. Despite its extensive use in traditional treatment, detailed studies focusing on anticancer properties have not been carried out. In view of this, in the present study an attempt was made to evaluate the cytotoxic activities of methanolic extracts of *Prosopis cineraria.* Previous studies in our laboratory showed that the methanolic extract of the leaves of the plant showed an ability to scavenge free radicals *in vitro* (Dharani *et al*., 2011). The extracts also improved the antioxidant status of oxidatively stressed goat liver slices. The cytotoxicity of the extract was also tested in *Saccharomyces cerevisiae* as a model for normal *in vitro* system. Hence the cytotoxicity of the extract was studied in breast cancer cells MCF-7 and non cancerous cells HBL 100.

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MATERIALS AND METHODS

Culturing of cells

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 number of cells (10^6 cells/ml) were seeded into 96-well plates respectively for carrying out various assays. The dose and time period of treatment were optimized in each cell line using MTT assay, which was used for the further experiments. The MCF-7 and HBL-100 cells were treated in the presence and/or the absence of leaf extracts (0.05mg) and with the standard chemotherapeutic drug tamoxifen-180µM and incubated for 24hrs in a 5% CO_2 and 95% humidity atmosphere.

Treatment groups

1. Cells alone

2. Cells+ Tamoxifen

3. Cells + methanolic extract of *Prosopis cineraria* leaves

4. Cells + *Prosopis cineraria* leaves + Tamoxifen

Cell viability assays

MTT assay

The extent of cytotoxicity in the treated cells was determined by the slight modification of the MTT dye reduction assay as described by Igarashi and Miyazawa

(2001). The medium was removed from the treated cells and incubated with 20µl of MTT (5mg/ml PBS) at 37ºC for 3 to 4 hours. After incubation, the liquid was removed and 150µl of acid-propanol was added and left overnight in the dark. The absorbance was read at 595nm in a microtiter plate reader (Anthos 2020, Austria). The optical densities of the control cells were fixed to be 100% viable and the percent viabilities of the cells in the other treatment groups were calculated.

SRB assay

The extent of survival in the cells induced by oxidative stress both in the presence, and in the absence of leaf extract was studied by sulphorhodamine B assay as proposed by Skehan *et al*. (1990). A portion of 50µl of ice cold 40% TCA was layered on top of the treated cells and incubated at 4^{o}C for one hour. The cells were washed with cold PBS. 100µl of SRB stain was added to each well for 30 minutes at room temperature. The unbound dye was removed with 1% acetic acid. Then 10mM tris (200µl) was added to solubilize the protein-bound dye and the plate was shaken gently for 20 minutes. The absorbance was read in a micro titre plate reader (Anthos 2020, Austria) at 492nm. The cell survival was calculated as the percent absorbance compared to the control (untreated) cells.

Neutral RED assay

The extent of neutral red uptake by the cells was done by the method of Borenfreund *et al*., 1990. After exposure of the cells to the test agent, the medium was removed. 0.2ml of neutral red containing medium was added per well and incubation was continued for 1 hour at 37ºC. Cells were then rapidly washed and fixed with a 0.2ml solution of 0.5% formalin-1% calcium chloride and the neutral red dye incorporated into the viable cells was released into the supernatant with 0.2ml of solution of acetic acid- 50%ethanol. Absorbance was recorded at 540nm with a micro titre plate spectrophotometer.

Cytotoxicity assays

Lactate dehydrogenase release

This assay was performed by following the instructions given in CytoscanTM LDH assay kit. Along with the treated cells, a spontaneous control (medium alone) and maximum LDH release control (cells in medium lysed using lysis buffer) were also taken. After incubation, the cells were centrifuged and 50µl of the supernatant was taken into a new ELISA reader plate; 50µl of the reconstituted substrate was added to each well and incubated in room temperature for 30 minutes. The reaction was stopped by adding 50µl of stop solution and the absorbance was recorded at 490nm, and the per cent cytotoxicity was calculated as follows:

$$\%\,Cytotoxicity = \frac{Experimental\ (OD490) - Spontaneous\ (OD490)}{Maximum\ LDH\ release\ (OD490)} \times 100$$

WST-1 Cytotoxicity assay

This assay was performed by following the instructions given in CytoscanTM WST-1 cell cytotoxicity assay kit. After treatment with plant extracts, 10µl WST-1/ CEC Assay dyes solution were added. After 4hours incubation in a cell culture incubator, the absorbance was measured at 420-480nm in a microplate reader with the reference wavelength of more than 600nm. The percent cytotoxicity was calculated using the formula.

$$\%\,Cytotoxicity = \frac{Cell\ Control - Experimental}{Cell\ Control} \times 100$$

Alamar blue metabolic assay

To the treated cells in 96-well plates 10µl of EZBlueTM reagent was added and wrapped in aluminium foil to avoid light exposure. The plates were then incubated in a cell culture incubator for 4h. The absorbance was read at 450nm (lower wavelength-λL) and 490nm (higher wavekength- λH) and percent reduction of EZ-Blue was calculated using the formula.

$$CF = \frac{A\ (\lambda H)}{A\ (\lambda L)}$$

A (λH) = Absorbance of EZBlueTM in medium at higher wavelength - Absorbance of only medium at higher wavelength

A (λL) = Absorbance of EZBlueTM in medium at lower wavelength - Absorbance of only medium at lower wavelength

Percentage reduction of EZBlueTM = {A(λL) - [A(λH) x CF] x 100}

RESULTS

Dose and time optimization

The cancer and non-cancerous cells were incubated with different concentrations of leaf extract ranging from 0.025mg to 0.4mg/5µl of DMSO for varying time intervals. The results showed that leaf extract at 0.05mg concentration and 24hrs incubation was very effective in cancer cells, and the same concentration was less cytotoxic to the non-cancerous cells. Hence, further experiments were carried out with the same concentration. The results are shown in Figure 1 & 2.

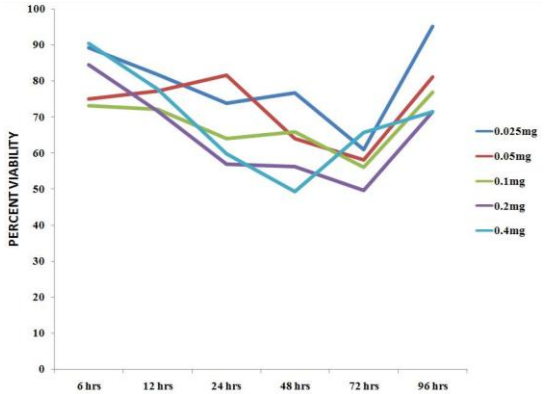

Figure 1: Dose and time optimization in HBL100

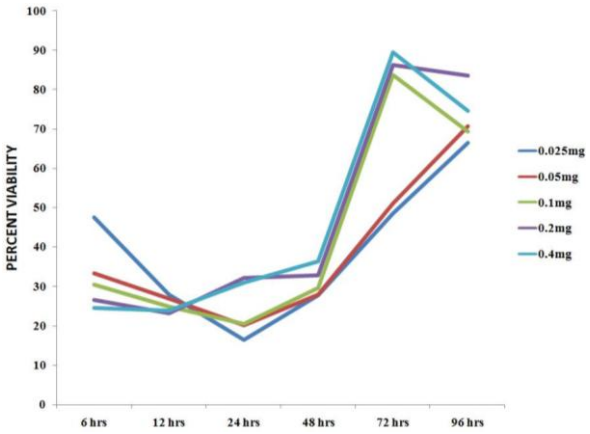

Figure 2: Dose and time optimization in MCF-7

Cell viability

The cell viability of both MCF-7 and HBL 100 was determined by MTT, SRB and neutral red assay. The standard drug tamoxifen reduced the viability of both HBL 100 and MCF-7 cells. On the other hand, the leaf extract reduced the percent viability of only MCF-7 cells to a large extent. There was slight cytotoxicity in HBL 100 cells. There was no difference in percent viability when extract was used in combination with tamoxifen in MCF-7 (Figure 3). Similar results were noticed in SRB and neutral red assay (Figure 4 &5) which were done to confirm the results of MTT.

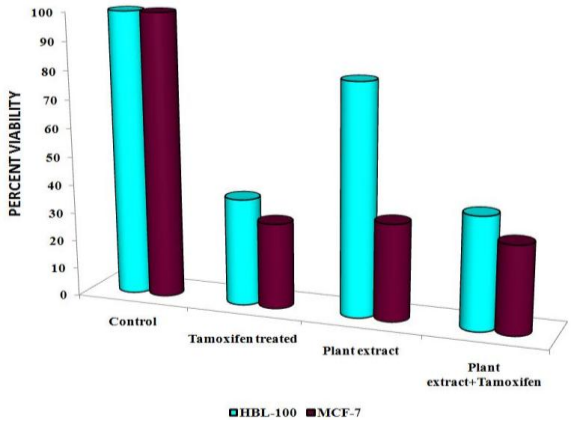

Figure 3: Effect of *Prosopis cineraria* leaf extract on the viability of cells as determined by MTT assay

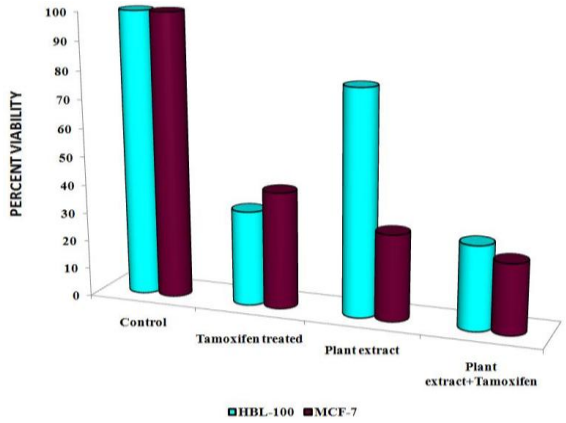

Figure 4: Effect of *Prosopis cineraria* leaf extract on the viability of cells as determined by SRB assay

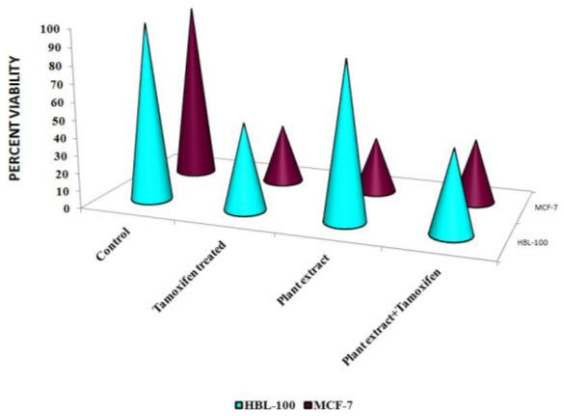

Figure 5: Effect of *Prosopis cineraria* leaf extract on the viability of cells as determined by neutral RED assay

Cytotoxicity assays

The results of WST-1 cytotoxicity (Figure 6) revealed that the treatment of cancer cells with extract caused the increase in percent cytotoxicity whereas in HBL 100 cells the toxicity was less compared to cancer cells. The tamoxifen caused a steep increase in cytotoxicity both in MCF-7 and HBL100 cells.

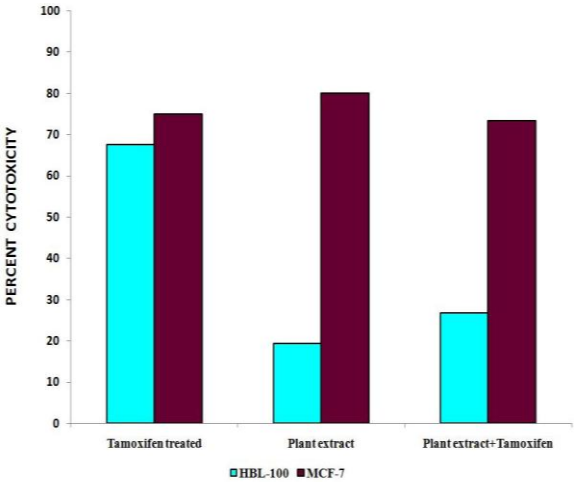

Figure 6: Effect of *Prosopis cineraria* leaf extract on the cytotoxicity of cells as determined by WST-1 cytotoxicity assay

The results of metabolic activity of the cells as revealed by alamar blue assay (Figure 7) indicated that the leaf extracts treated group showed less percent reduction of dye than the untreated control group. The combination of extract, and drug in cancer cells increased the reduction of dye than the drug alone treated group. This implies that the leaf extracts is cytotoxic to cancer cells.

The lactate dehydrogenase (LDH) assay also revealed that the cytotoxicity was increased in tamoxifen treated group of both MCF-7 and HBL 100 cells. The cytotoxicity was reduced in leaf extract treated group indicating the protective role of the extract in HBL 100 cells. On the other hand, increased toxicity was observed in combination treatment (Figure 8).

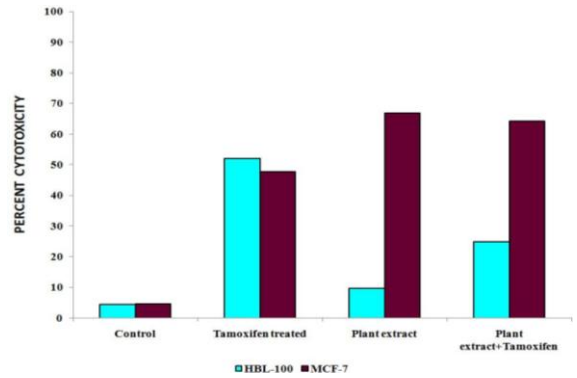

Figure 7: Effect of *Prosopis cineraria* leaf extract on the percent alamar blue reduction of cells as determined by LDH release assay

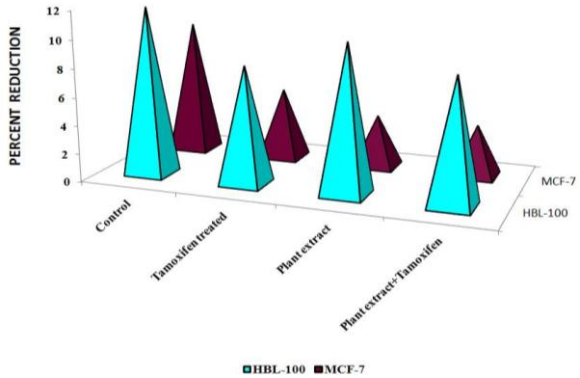

Figure 8: Effect of *Prosopis cineraria* leaf extract on the percent alamar blue reduction of cells as determined by alamar blue metabolic assay

DISCUSSION

The predominant aim of analyzing crude plant extracts are either to isolate bioactive agents for direct use as drugs or to identify bioactive compounds that can be used as lead substance in the preparation of semisynthetic drugs (Bernhard svejda *et al* 2010). In this study, we demonstrate the anticancer potential of *Prosopis cineraria* leaves in MCF-7 and HBL-100 cells. The extract at a concentration of 0.05mg showed cytotoxic to cancer cells at the same time it was less toxic to HBL100 cells. The plant extract treated group showed a decrease in viability of MCF-7. Our results are in agreement with the results obtained by Chuan Ding *et al* (2012) that casticin reduced the cell viability in a dose-dependent manner. At low concentration of 10 µM, statistically significant growth inhibition was observed after 24 h as compared to the control group. The maximum growth inhibition was observed at 80 µM, which was nearly 90%. Similarly, Thongchai Taechowisan *et al* (2007) have reported that 5, 7-dimethoxy-4-phenylcoumarin inhibited A427 cells by 42% and 5,7-dimethoxy-4-p-methylphenylcoumarin inhibited by 64%. The SRB assay showed that the extract itself could reduce the viability of MCF-7 cells and also the extract was not much toxic to HBL100 cells. In supportive of our results Aboul-Enein *et al* (2012) have reported that extracts from, *S. nigrum, Camellia sinensis* and *Glycyrrhiza glabra* showed anticancer activity more than 80% (89.7, 86.4 and 81%, respectively). Also five aqueous extracts possessed high anticancer activity more than 90% against HepG2. These results were in agreement with the results obtained by Nawab *et al*. (2011) who reported that exposure of aqueous extract of *S. nigrum* exerted an inhibitory effect on cell growth and colony formation of the prostate, breast and colorectal cells. Husein *et al*. (2011) reported highest cytotoxicity for ethanolic extracts of *A. palaestinum* against breast cancer.

However, another assay to confirm the cytotoxic activity of the extract was the WST1 cytotoxicity assay. The results revealed that the extract induced cytotoxicity in MCF-7 cells to a large extent. WST-1 assay was performed in KRJ-I and HF-SAR cells, both treated with *T. gracilis subfraction* TG-F28 (10 µg/ml) for 24, 48 or 72 h. Treated HF-SAR cells showed no reduction of cell viability compared to untreated control, whereas a decrease of cell viability was noted in the treated KRJ-I cells(Bernhard svejda *et al* 2010). Ce Zhang *et al* 2012 concluded that the cytotoxicity of S100A9 in SH-SY5Y neuroblastoma cells is greatly suppressed by the presence of sufficient amounts of Ab1–40 peptides.

LDH release assay was also performed to confirm cytotoxicity of the extract. This also confirmed the cytotoxicityof the extract by the release of LDH. Similar to other cytotoxic assays the LDH release was more in plant extract treated group than the control group. The toxicity was less in HBL 100 cells (9.64%) compared to tamoxifen treated group (52.14%). Wan Yong Ho *et al* 2012 reported that the basal amount of LDH release by the untreated monolayer culture was 10.90 ± 0.88% while by the untreated Multicellular Tumor Spheroid (MCTS), culture was 36.59±1.31% of the maximum LDH content. As the concentration of tamoxifen was elevated to 5.25, 10.5 and 21 mM, the release of LDH also increased in a concentration dependent manner in both types of cultures. *A. Montana* and *A. absinthium* protected fibroblast cells against hydrogen peroxide-induced oxidative damage, at the doses of 10 mg/L and 10–300 mg/L respectively, as determined by Neutral red and lactate dehydrogenase assays (Oana Craciunescu *et al*., 2012).

CONCLUSION

Thus, from the viability and cytotoxicity assays, It was concluded that *Prosopis cineraria* leaf extract possess anti cancer activity against MCF-7 breast cancer cells. The extract also proves to be non toxic to normal cells.

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