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Research Article

IN VITRO CYTOTOXIC AND APOPTOTIC ACTIVITY OF POLYSACCHARIDE RICH *MORINDA CITROFOLIA* FRUIT ON MCF-7 CELLS

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ABSTRACT

Morinda citrofolia fruit or noni is well-known traditional medicine that has both antioxidant and anticancer activity. This study was designed to investigate experimentally the anti-apoptotic effect of polysaccharide isolated from *M. citrofolia* fruit against MCF-7 breast cancer cells. Treatment of the MCF-7 cells with varied concentrations (1.0, 0.5, 0.25, 0.125, 0.062.5, and 0.03125 mg/ml) of polysaccharide isolated from *M. citrofolia* fruit resulted in dose-dependent cytotoxicity marked primarily by loss of cell viability in 3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide assay, and internucleosomal DNA fragmentation in DNA ladder assay. Furthermore, *M. citrofolia* fruit mediated cytotoxicity was found to be associated with transactivation of caspase 3 and p53, and down regulation of Bcl-2. From the result, it was proved that polysaccharide fractions of *M. citrofolia* fruit extract exhibited significant anti-proliferative and apoptotic activity thereby showing valuable application in developing anticancer drug products.

Keywords: MCF-7 cells, p53, Bcl-2, Caspase-3, *Morinda citrofolia* and polysaccharide.

INTRODUCTION

Cancer is a dreadful disease worldwide, and the treatment strategies for combating cancer severity have gained more importance to public health. Breast cancer remains main life-threatening cancer that affects women during their lifetime [1]. For the development of new anticancer drugs, drug combinations, and chemotherapy strategies by methodical and scientific exploration of the enormous pool of synthetic, biological, and natural products [2]. Cancer chemoprevention with strategies using foods and medicinal herbs has been considered as the main strategy in cancer control [3]; however, whether fruit, vegetable, or antioxidant micronutrient consumption is associated with a reduction in breast cancer incidence remains unresolved [4].

Morinda citrofolia Linn (Rubiaceae) also known as noni or Indian mulberry, is a small evergreen tree. The leaves are 8-10 inches long oval shaped, dark green and shiny, with deep veins. This is largely used in traditional medicine and has been heavily promoted for a wide range of uses; including arthritis, atherosclerosis, boils, burns, cancer, chronic fatigue syndrome, circulatory weakness, cold sores, congestion, constipation, diabetes, gastric ulcers, gingivitis, heart disease, hypertension and infections [5,6]. Fruit juice of *M. citrifolia* is a health promoting syrup and has various pharmacological properties including antioxidant and anti-inflammatory effects [7]. The fruit juice of *M. citrofolia* (noni) contains a polysaccharide-rich substance with antitumor activity as reported in the literature. However, there are no much reports available on the polysaccharide isolated from *M. citrofolia* fruit for its *in vitro* anticancer property, especially against MCF-7 cell lines. Hence, this necessitated to carry out the study for its effect on the induction of cytotoxicity in breast cancer cell line (MCF-7) which may prove its efficacy in the treatment of breast cancer. The *in vitro* cytotoxic effect of *M. citrofolia* fraction on the growth of human breast cancer cells and effect on expression of apoptotic genes were investigated in this experimental study.

METHODS

Chemicals

3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and trypsin were obtained from Sigma Aldrich Co., St. Louis, USA. Ethylenediaminetetraacetic acid (EDTA), glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl sulfoxide (DMSO) and propanol from E.Merck Ltd., Mumbai, India.

Cell lines and culture

MCF-7 (human breast adenocarcinoma cell line) cell line was cultured in DMEM supplemented with 10% inactivated FBS penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks, and all experiments were carried out in 96 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India).

Isolation of polysaccharide from *M. citrifolia* fruit extract

A total of 5 g of the extract was added to 10 ml of distilled water and heated at 85°C for 3 hrs. The filtrate was carefully collected and stored in the beaker. Na_2CO_3 was added and heated at 45°C for 3 hrs in the water bath. The filtrate was collected and stored. Both the filtrates were pooled together and the solvent was evaporated to obtain the final reside of carbohydrate. The collected residue was added to sodium acetate, and the precipitate formed thus was collected using ethanol [8].

Cell inhibition activity by MTT assay

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtiter plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 hrs interval. After 72 hrs, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hrs at 37°C in 5% CO_2 atmosphere. The supernatant was removed, and 100 µl of propanol was added and the plates were gently shaken

to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

DNA ladder assay

MCF-7 cells (3 × 10^6 /ml) were seeded into 60 mm petridishes and incubated at 37°C with 5% CO_2 atmosphere for 24 hrs. The cells were washed with medium and were treated with selected doses of test samples, the standard drug and incubated at 37°C, 5% CO_2 for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with G Bioscience apoptotic DNA ladder kit. The DNA samples were loaded onto 2% agarose gel electrophoresis and run 5 V/cm for 3 hrs. The gel was visualized under UVP gel doc and photographed.

Gene expression analysis (reverse transcriptase-polymerase chain reaction [RT-PCR])

The mRNA expression levels of p53, Bcl-2 and caspase 3 carried out using semi-quantitative RT-PCR. Briefly, the MCF-7 cells were cultured in 60 mm petridish and maintained in DMEM medium for 48 hrs. The DMEM medium was supplemented with FBS and amphotrecin. To the dish was added the required concentration of test sample (1000 µg/ml) and incubated for 48 hrs. Total cellular RNA was isolated from the untreated (control) and treated cells using tri reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by incubation for 1 hr at 42°C with M-MLV RT (Mercki, India) and oligo (dT) 18 primer (Mercki, India) according to the manufacturer's instruction. Then 3 µl of the reaction mixture was subjected to PCR for amplification of p53, Bcl-2 and caspase 3 cDNAs using specifically designed primers procured from Merck, India, as an internal control, the house keeping gene GAPDH was co-amplified in each reaction.

RESULT AND DISCUSSION

Cell inhibition activity by MTT assay

Medicinal plants are able to act through several mechanisms to provide protection against cancer. The percentage cytotoxicity of MCF-7 cells exposed to the polysaccharide to wide concentrations at 0.03125, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml was found to be 13.37±3.06, 15.80±2.00, 22.06±4.27, 24.68±2.51, 28.37±1.62, and 40.08±1.66% respectively. Dose-dependent anti-proliferative effect on the cell viability of MCF-7 was observed. The results have been summarized in Table 1 and Fig. 1. inhibitory concentration 50 (IC_{50}) (1.02 mg/ml) value indicated that the maximum cytotoxic effect of polysaccharide isolated fraction of *M. citrofolia* showed 50% reduction in cell viability upon treatment with highest dose (1 mg/ml).

The MCF-7 cell line type selected for the present investigation is an estrogen receptor positive type and estrogen responsive in nature [9]. Specific polysaccharide isolated from *M. citrofolia* exhibited a considerable cytotoxicity in MCF-7 cells and provide a scientific basis to develop anticancer drugs [10-12]. Previous reports reveal that anticancer and immune-modulatory potentials were found in the polysaccharide isolated from fruit extracts might have gained more attention in cancer medicine. Numerous anticancer polysaccharides already have been discovered in mushrooms, fruits, vegetables, algae, lichnens, yeast, and herbs. Lentinans, kresin, schizophyllan, β-D-glucans, and heteroglucans are some of the promising antitumor polysaccharide compounds [13]. These previous reports support the present study on anticancer property of *M. citrofolia* polysaccharide.

Table 1: Cytotoxic properties of test drug on MCF-7 cell line

Name of drug	Test concentration (mg/ml)	% Cytotoxicity	CTC_{50} (mg/ml)
Polysaccharide	1.0	50.08±1.66	1.02
	0.500	28.37±1.62	
	0.250	24.68±2.51	
	0.125	22.06±4.27	
	0.0625	15.80±2.00	
	0.03125	13.37±3.06	

Mean±SD of triplicates. SD: Standard devaiation

DNA laddering

DNA fragmentation was detected in MCF-7 cell line at 500 and 1000 µg/ml concentration after 72 hrs incubation while there no DNA fragmentation was observed in control (untreated) cells. However, the standard doxorubicin induced the fragmentation of the DNA at very low concentration (10 µg/ml) (Fig. 2). The results indicate that cytotoxic polysaccharide is present in *M. citrofolia* and exerted considerable DNA damage leading to apoptotic cell death in cancer cells. It also suggests that anticancer activity of *M. citrofolia* polysaccharide fraction was specific to estrogen receptor-positive breast cancer line MCF-7. Detection of DNA fragmentation remains the most acceptable hallmark of cytotoxicity and drug induced apoptosis in cancer cells [14]. This also serves as an essential biochemical marker for the measurement of drug mediated *in vivo* and *in vitro* cytotoxicity in the field of cancer research [15].

Expression analysis of p53, caspase-3 and Bcl-2 genes

The possible signaling pathways by which *M.citrifolia* polysaccharide induced apoptosis in MCF-7 cells was delineated by assessing the changes in the expression levels of apoptosis-regulating proteins. p53, caspase-3, and Bcl-2 were determined by the expression

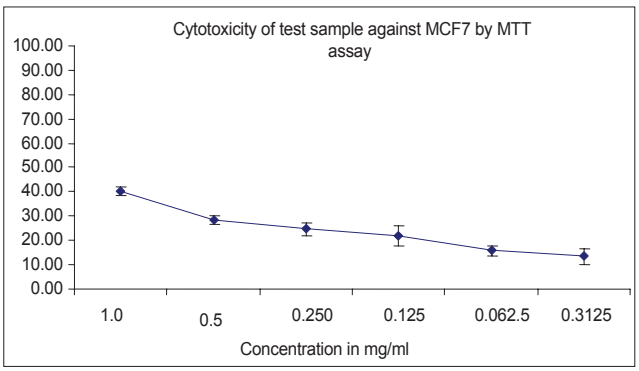

Fig. 1: Dose dependant cytotoxic effect of *Morinda citrifolia* on MCF-7 cells

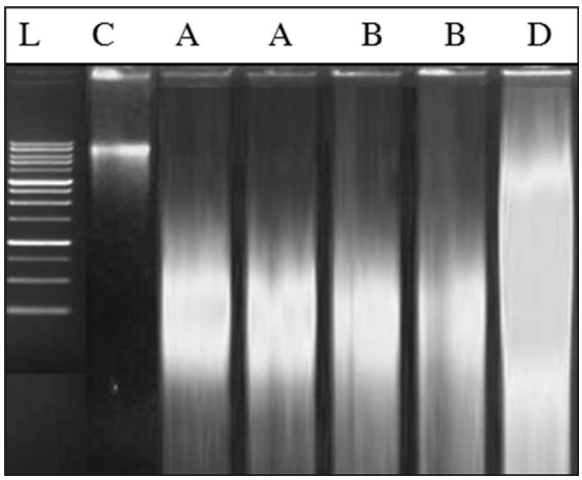

Fig. 2: DNA ladder assay, (Lane D: MCF-7 cell line treated with doxorubcin (10 µg/ml); B: MCF-7 cell line treated with test sample (1000 µg/ml); B: MCF-7 cell line treated with test sample (700 µg/ml); C: MCF-7 cell line (untreated); L: DNA marker 1000 bp)

analysis of these genes using RT-PCR. Results showed that *M.citrifolia* polysaccharide treatment induced DNA damage in MCF-7 cells and thereby leading to the activation of p53 when compared to control. It is evident that p53 activation tends to stimulate the cancer cells to undergo apoptosis (programmed cell death) (Fig. 3). The tumor suppressor protein p53 is mutant in over 50% of cancers and loss of p53 function is considered to be a key factor in the progression of any cancer. Cell viability of MCF-cells was reduced upon the treatment of *M. citrifolia* polysaccharide. The result suggests that activation of p53 is important and essential for cytotoxic activity of treatment. Doxorubicin treated cells served as a positive control and was found to have effectively up regulated the expression of p53 in the cancer cells [16]. Tumor suppressor protein p53 a key player in the cell death has been reported to involve in mitochondrial activated apoptosis pathway. The exposure of MCF-7 cells to *M.citrifolia* polysaccharide for 48 hrs was found to have resulted in up-regulation of caspase-3 (Fig. 4). Marked increase in the expression of procaspase-3 normally mediates proteolytic cleavage of poly(ADP-ribose) polymerase and activation of the apoptotic pathway. The results suggest that the cleavage/inactivation of caspases could lead to activation of both intrinsic and extrinsic apoptotic pathways [17]. Further the molecular events that occur upstream of caspase activation and the role of the intrinsic apoptotic pathway in *M. citrifolia* induced apoptosis was investigated, by examining the level of Bcl-2 expression, which are the crucial regulators of the intrinsic pathway of apoptosis. Following 48 hrs treatment, it was apparent that *M. citrifolia* treated MCF-7 cells exhibited considerable decrease in the levels of anti-apoptotic protein Bcl-2 with an increase in pro-apoptotic protein level of p53 (Fig. 5). This provides a strong indication that the intrinsic apoptotic pathway may play a role in *M. citrifolia* induced apoptosis in MCF-7 cells [18]. The present study is corroborating with the investigation of Alshatwi *et al.,* who investigated the antitumor activity of methanolic extract of lemon fruit (lemon extract) on the MCF-7 breast cancer cell line. This was investigated in MCF-7 cells via the mitochondrial pathway, as evidenced by the activation of caspase-3 and caspase-9 and the elevation of intracellular reactive oxygen species production. Caspase enzymes are mainly involved in the apoptotic cascade and lead to proteolysis of specific substrates associated with programmed cell death [19,20].

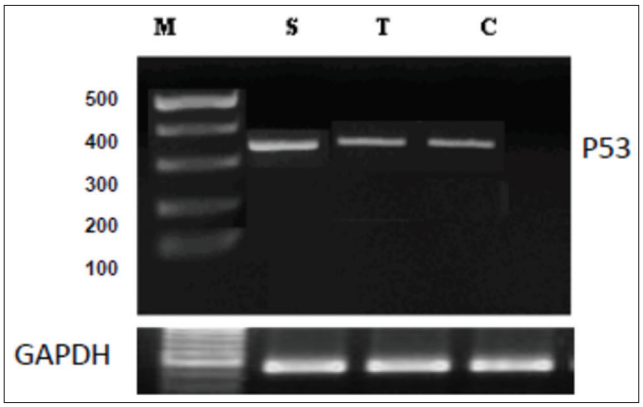

Fig. 3: Reverse transcriptase-polymerase chain reaction profile of p53 gene amplified from drug treated MCF-7 cells cells; (M - 100 bp ladder, S - cells treated with standard drug (doxorubicin), T - cells treated with test product; C - cell control)

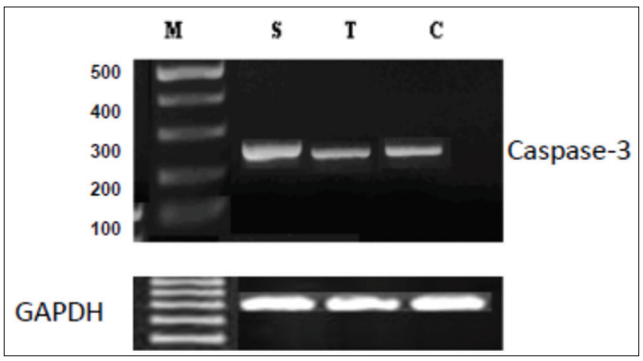

Fig. 4: Reverse transcriptase-polymerase chain reaction profile of caspase 3 gene amplified from drug treated MCF-7 cells. (M - 100 bp ladder, S - cells treated with standard drug (doxorubicin), T - cells treated with test product; C - cell control, product size: 262 bp)

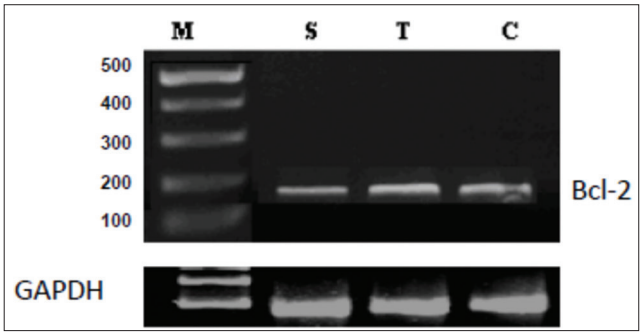

Fig. 5: Reverse transcriptase-polymerase chain reaction profile of Bcl-2 gene amplified from drug treated MCF-7 cells. (M - 100 bp ladder, S - cells treated with standard drug (doxorubicin), T - cells treated with test product; C - cell control, product size: 128 bp)

CONCLUSION

The study concludes that polysaccharide fraction isolated from the *M. citrofolia* fruit extract exerts anticancer activity against MCF-7 breast cancer cells. The *in vitro* cytotoxicity of this was associated with the up regulation of p53 and caspase-3 proteins expression, and down regulation of Bcl-2, which are involved in the apoptotic pathway. IC_{50} value of the polysaccharide fraction against MCF-7 cells is 1 mg/ml. DNA fragmentation was also found in the cells treated with polysaccharide. In conclusion polysaccharide fraction isolated from the *M. citrifolia* can be considered as good supplementary in the cancer prevention. The *in vitro* cytotoxicity assays offer quick, simple, and cost effective ways of testing the toxicity and forms an important tool for high through screening of fruit extract. Hence, the polysaccharide fraction isolated from extracts needs to be thoroughly studied using animal models. It can be concluded that noni fruit extract shows moderate toxicity against MCF-7 cells.

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