ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 7, Suppl 2, 2014

ISSN - 0974-2441

Research Article

ANTICARCINOGENIC ACTIVITY OF RICE BRAN PHYTIC ACID AGAINST HUMAN BREAST CANCER CELL LINE (MCF-7)

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Received: 25 March 2014, Revised and Accepted: 22 April 2014

ABSTRACT

Phytic acid (PA) has been reported for anti-inflammatory, antioxidant and anticancer activity. However, molecular mechanism of anticancer activity is not clear. This study investigated the anticancer activity of rice bran PA against breast cancer (MCF-7). Cytotoxicty of PA (0 to 7 mM) against MCF-7 cells was examined by MTT and LDH assays after 24 and 48 h treatment. Apoptotic activity was evaluated by expressional analysis of apoptosis-regulatory genes [i.e., p53, Bcl-2, Bax, caspase-3 and -9] by reverse transcriptase-PCR and DNA fragmentation assay. PA inhibited the growth of MCF-7 cells in a concentration dependent manner ($p \leq 0.04$). After 48 h treatment, cells viability was recorded 80.9, 71.1, 59.8, 36.6, 26.7 and 15.9% in MTT assay and 85.3, 72.6%, 62.3%, 42.1, 31.7 and 21.7% in LDH assay at concentration of 1.4, 2.2, 3.0, 3.8, 4.6, and 5.4 mM respectively. Hence, treatment of PA for 24 h, recorded viability of cells 84.6, 73.8, 61.0, 47.0, 28.8 and 17.3% in MTT assay and 87.8, 77.5%, 62.9%, 49.8, 35.7 and 23.3% in LDH assay at concentration of 2, 3, 4, 5, 6, and 7 mM, respectively. PA treated MCF-7 cells showed up-regulation of p53, Bax, caspase-3 and -9, and down-regulation of Bcl-2 gene ($p \leq 0.03$). At IC_{50} (3.4 mM) of PA, the p53, Bax, caspase 3 and -9 genes were up-regulated by 6.34, 4.90, 23.45 and 15.03 folds respectively. Also, the fragmented genomic DNA in PA treated cells showed the signs of apoptosis. Our study endorsed the biological activity of PA and demonstrated the PA induced growth inhibition and apoptosis in MCF-7 cells by modulating the expression of apoptosis-regulatory genes.

Keywords: Phytic acid, antioxidant, cytotoxicity, apoptosis, caspases, p53, Bax, Bcl-2, DNA fragmentation

INTRODUCTION

Phytic acid (PA) (inositol hexaphosphate, IP_6) is a highly phosphorylated molecule abundant in plants and legumes, including corn, soy beans, nuts, wheat bran and rice bran [1]. Nutritional or dietary factors are attracting the great deal of interest since long time due to their perceived ability to act as highly effective chemopreventive agents [2, 3]. Also, most of the natural products have been considered as rational strategy for cancer prevention due to its antioxidant properties without any harmful effect [4, 5]. Recently, PA extracted from rice bran has been extensively examined for its biological activities and reported to have hepatoprotective, antioxidant and anticancer properties [6, 7]. In addition, PA extracted from other plants, has been reported for beneficial effects on human health, particularly in the prevention of renal calculi, diabetes, cancer and parkinson's disease [1]. Extensive studies show that PA is a chemopreventive agent against cancers of the breast, colon, liver, leukemia, prostate, and skin [1]. The mechanisms of action against cancer cells growth have been reported the stimulation of genes toward against cell differentiation, alteration in signal transduction, anti-oxidant activity and increase in immunity [8, 9, 10, 11].

PA is a strong chelator of multivalent metal ions, and have ability to bind toxic trace elements and, thus, to influence their solubility, absorption, and digestibility [12]. In addition, iron-chelators inhibits the cancer cells growth reflects the importance of iron in a variety of crucial metabolic pathways including DNA synthesis and ATP production [13]. Cancer cells, as compared with their normal counterparts, generally have higher levels of the TfR13 [14] and take up iron from Tf [15].

Breast cancer is the second leading cause of death in woman, and estimated 5,22,000 death in 2012 (WHO, 2013). Since 2008, breast cancer incident has increased by more than 20%, while mortality has increased by 14% (WHO, 2013). Therefore, search of breast cancer prevention and therapeutic modality is a challenging area of research. In present study we intended to evaluate the effect of rice bran PA on growth and apoptosis regulatory genes (eg. p53, Bax, Bcl-2, caspase-3, and -9 genes) of human breast cancer cells (MCF-7). These genes regulate apoptotic mechanism in adverse conditions like stress, radiation and chemotherapy. The Bax gene is a pro-apoptotic regulator of apoptosis [16], whereas Bcl-2 gene protects cell death by inhibiting the apoptosis pathway [17]. p53 regulates the apoptosis by interacting with the Bcl-2 family and up-regulation of Bax gene expression through direct transcriptional activation of the Bax promoter with concomitant down-regulation of Bcl-2 gene [18, 19]. Caspase-9 initiates the cascade of apoptosis after release of mitochondrial cytochrom-c, whereas caspase-3 is a downstream caspase which play a pivotal role in the terminal phase of apoptosis [20, 21].

MATERIALS AND METHODS

Reagent and chemicals

Phytic acid (PA) from rice bran was purchased from Sigma-Aldrich (USA). Tissue culture media components were purchased from HiMedia (Mumbai, India). All chemicals and solvents were of analytical grade and purchased from Merck (Mumbai, India).

Cell culture

Human breast cancer cell line (MCF-7) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) [supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U / mL penicillin and 100 mg / L streptomycin)] at 37°C in a humidified atmosphere of 5% CO_2.

MTT assay

The cell survival was evaluated by MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide) assay as described [22]. The MCF-7 cells (~2x10^4 per well) were seeded overnight in a flat bottom 96 well plate (HiMedia, India) and incubated at 37°C in a humidified air atmosphere enriched with 5% (v/v) CO_2. MCF-7 cells were treated with various concentrations of PA ranging between 0-7 mM for 24 and 48 h time points. Afterwards, the culture medium was replaced with fresh medium and 20 µL of MTT (5 mg / mL in PBS) was added to it and kept at 37°C for 4 h. Formazan crystals formed in live cells by mitochondrial reduction of MTT were solubilized in DMSO (200 µL/ well) and the absorbance was measured at 570 nm on *i*Mark Microplate Reader (Bio-Rad, USA). All cytotoxicity assays were performed in triplicate and the percentage of cell survival was calculated using following formula:

$$\% \text{ Cell survival } = \frac{\text{Experimental } (OD_{570})}{\text{Control } (OD_{570})} \times 100$$

The mean percentage [± standard error of mean (SEM)] cell survival was plotted against the corresponding PA concentration and the 'best fit' was employed to derive the IC_{50} value.

Lactate dehydrogenase enzyme (LDH) leakage assay

For LDH leakage assay, MCF-7 cells (~2x10^4 per well) were seeded overnight in a flat bottom 96 well plate (HiMedia, India) and incubated at 37°C in a humidified air atmosphere enriched with 5% (v/ v) CO_2. Various concentrations of PA ranging from 0 to 7 mM were used to treat the cells lines for 24 and 48 h in triplicate. The treated cells were centrifuge at 3000 rpm for 5 min at 4°C. The cell free medium was used for the quantification of LDH enzyme following the commercially available Cytoscan™-LDH assay Kit (G-Biosciences, USA) protocol. The absorbance of the reaction mixture was measured at 490 nm on the *i*Mark Microplate Reader (Bio-Rad, USA). The assay was performed in triplicate and the percent cytotoxicity was calculated as:

$$\% \text{ Cytotoxicity } = \frac{\text{Experimental } (OD_{490}) - \text{Spontaneous}(OD_{490})}{\text{Maximum LDH released } (OD_{490})} \times 100$$

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression of apoptosis regulatory genes were examined after treating both the cell lines with different concentrations (0-4.2 mM) of PA for 48 h. Treated and untreated MCF-7 cells were harvested and washed with phosphate buffer saline (PBS) at 4°C. Total RNA was extracted using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instruction. RNA preparations were analyzed by agarose gel 1.8 (w/v) electrophoresis and found to be free of DNA contamination. 1 µg of total RNA was used for cDNA synthesis using RevertAidTM fisrt stranded cDNA synthesis Kit (Fermentas Life Science, USA) with random hexamers. cDNA was used for the detection of mRNA expressions of p53, Bcl-2, Bax, caspase-3, and -9 genes using specific oligonucleotide primers (Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The volume of PCR mixture was 25 µL containing 2 µL of cDNA, 1U taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 20 pmole of each gene specific oligonucleotide primer. The PCR reaction conditions were denaturation at 94°C for 30 sec, annealing at 52.7°C, 56.3°C, 65.4°C, 54.6°C, and 55.2°C for GAPDH, p35, Bcl-2, Bax, caspase-3 and -9, respectively, for 30 sec, and extension at 72°C for 30 sec (Eppendorf, Norwalk). The amplified products were checked on 2% agarose gel and documented on the Gel-doc system (Bio-Rad, USA).

Table 1: Oligonucleotide primers.

P53	F- 5' CCAGCAGCTCCTACACCGGC 3'
	R- 5' GAAACCGTAGCTGCCCTG 3'
Bcl-2	F- 5' GGTCGCCAGGACCTCGCCGC 3'
	R- 5' AGTCGTCGCCGGCCTGGCG 3'
Bax	F- 5' GAGCTGCAGAGGATGATTGC 3
	R- 5'CCGGGAGCGGCTGTTGGGCT 3'
Casp-3	F- 5' GTACAGATGTCGATGCAGC 3'
	R- 5' CACAATTTCTTCACGTGTA 3'
Casp-9	F- 5' CCTGCGGCGGTGCCGGCTGC 3'
	R-5' GTGTCCTCTAAGCAGGAGAT 3'
GAPDH	F- 5' GTGATGGGATTTCCATTGAT 3'
	R- 5' GGAGTCAACGGATTTGGT 3'

DNA fragmentation assay

In order to perform DNA fragmentation assay, PA (at IC_{50} concentration for 48 h) treated MCF-7 cells were harvested and washed with PBS at 4ºC. The cell pellets were used for genomic DNA fragmentation assay following the commercially available DNA Ladder Assay Kit (G-Biosciences, USA). The Fragmented DNA was analyzed on 1.8% (w/v) agarose gel and documented using the Gel Doc system (Bio-Rad, USA).

Statistical analysis

The mean value ± standard error of mean (SEM) was calculated from the samples (triplicate) for each experimental group. The statistical significance was determined with analysis of variance (ANOVA) test and statistical significance level was maintained at $p < 0.05$.

RESULTS

Cytotoxicity assays

MTT and LDH assay

MTT assays were performed for the screening of cell viability at various concentrations of PA. The assay results showed dose dependent decreased in viability of MCF-7 cells at 24 and 48 h time points ($p < 0.002$) (Figure 1 (A-B)). The IC_{50} value (evaluated after 48 h) of PA against MCF-7 cells were 3.40±0.65 mM ($p \leq 0.03$). After 48 h, PA decreased MCF-7 cells viability by 80.9, 71.1, 59.8, 36.6, 26.7 and 15.9% at concentration of 1.4, 2.2, 3.0, 3.8, 4.6, and 5.4 mM, respectively. Whereas, treatment of PA for 24 h, decreased cells viability by 84.6, 73.8, 61.0, 47.0, 28.8 and 17.1% at concentration of 2, 3, 4, 5, 6 and 7 mM, respectively.

Likewise, the LDH enzyme leakage assay had given positive response after treating with different concentrations of PA. Similar to MTT assay, LDH assay also revealed decrease of MCF-7 cell viability by 85.3, 72.6%, 62.3%, 42.1, 31.7 and 21.7% at concentration of 1.4, 2.2, 3.0, 3.8, 4.6, and 5.4 mM after 48 h, whereas after 24 h, decreased of cells viability by 87.8, 77.5%, 62.9%, 49.8, 35.7 and 23.3% at concentration of 2, 3, 4, 5, 6 and 7 mM, respectively. The cytotoxic activity profile of PA against MCF-7 cells evaluated by MTT and LDH assays were significantly correlated at 24 and 48 h time points ($r > 0.9712$). Interestingly, both bioactivity assays showed PA toxicity against MCF-7 cell line, despite their different working principles. As, in MTT assay, only metabolically active cells reduce MTT salt to purple formazan by mitochondrial succinate dehydrogenase enzyme, whereas, in LDH assay, the LDH enzyme is released into the culture medium after disruption of cell membrane integrity.

Expression analysis of Bcl-2, Bax, and p53 genes

Reverse Transcriptase-PCR was performed to study the expression analysis of Bcl-2, Bax and p53 genes in MCF-7 cells. The changes in mRNA expression levels were standardized by GAPDH expression. Densitometry analysis revealed the relative mRNA band intensity on the gel-doc system (Bio-Rad, USA). The treatments of PA showed up-regulation of p53 and Bax genes in a concentration-dependent manner (Figures 2). At IC_{50} of PA, the p53 and Bax genes relative band intensity increased by 6.34 and 4.90 folds, respectively. However, PA treated MCF-7 cells showed down-regulation of Bcl-2, and increased Bax to Bcl-2 ratio in a dose-dependent manner ($p \leq 0.02$). At IC_{50}, the Bax to Bcl-2 ratio was estimated to be 3.05.

Expression analysis of caspase genes

The apoptosis activity in PA treated MCF-7 cells were determined by expression analysis of caspase genes. The results showed that PA treated MCF-7 cells exhibited significant up-regulation of caspase-3 and -9 genes in a concentration-dependent manner (Figures 3). Densitometry analysis revealed that, at IC_{50} of PA, caspase-3 and -9 genes were up-regulated by 23.45 and 15.03 folds, respectively ($p \leq 0.04$).

DNA fragmentation analysis

Genomic DNA fragmentation assay was performed in PA treated and untreated MCF-7 cells in order to analyze the hallmark of apoptosis.

DNA fragmentation assay revealed ladder like appearance in the gel (Figure 4). The 'laddering phenomenon' is a characteristic feature of apoptosis process in which the genomic DNA is cleaved into fragments by the endogenous endonucleases.

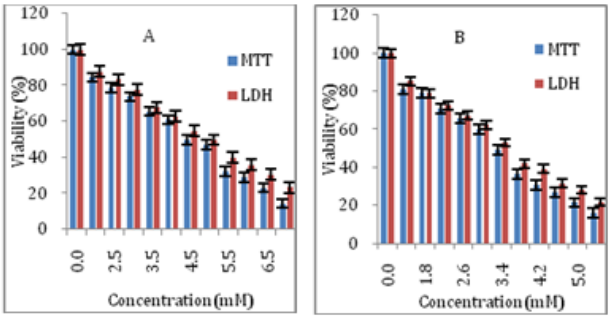

Fig.1: Cytotoxicity trends of phytic acid against MCF-7 cells, determined by MTT and LDH assays after 24 h(A) and 48 h (B).

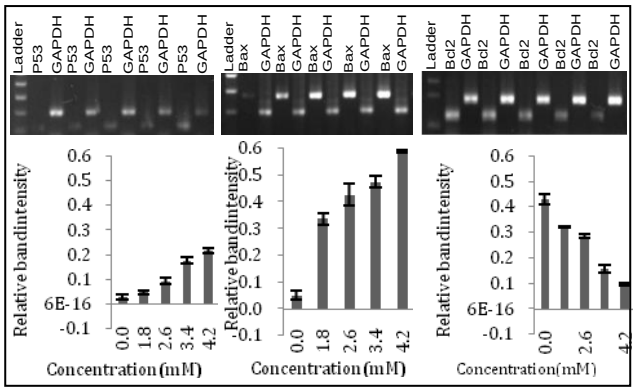

Fig.2: Expressional analysis of p53, Bax and Bcl-2 genes in MCF-7 cell line treated with parthenolide for 48 h. Data are represented as means ± SD.

DISCUSSION

Rice bran PA has been reported for anticancer activities in both *in vitro* and *in vivo* assays. Most of the iron-chelators have been used as therapeutics for the treatment of iron-overload disease and some of iron-chelator compounds act as potential role in cancer therapy [23, 24]. It was reported that iron-chelators reduced iron availability, and induced apoptosis in cancer cells through mechanisms that seem to involve mitochondrial activation [25, 26, 27]. Five iron-regulatory genes have been identified which are regulated differently in neoplastic cells and in normal cells and could play a role in the selective antitumor effects of iron-chelators [28].

In present study, rice bran PA induced MCF-7 cell death concentration dependent manner with IC_{50} value of 3.40±0.65 mM. In a similar finding, PA extracted from rice bran, inhibited the growth of ovary, breast and liver cancer cells with IC_{50} values of 3.45, 3.78 and 1.66 mM, respectively, while no sensitivity against normal cell line (3T3) was reported [11]. In addition, IP_6 inhibited the proliferation of MCF-7 and HT-29 cells through arresting cells in the G0/G1-phase and inhibits DNA synthesis [29, 30].

Pro- and anti-apoptotic factor regulates the apoptosis in most of the therapeutic intervention. The pro-apoptotic genes (e.g., p53 and Bax) and anti-apoptotic genes (e.g., Bcl-2) are generally involved in cellular proliferation and apoptosis [31]. Semi-quantitative RT-PCR revealed down-regulation of Bcl-2 gene and up-regulation of p53 and Bax genes in MCF-7 cells treated with PA (Figures 2). The PA treated MCF-7 cells also increased ratio of Bax to Bcl-2 gene regulation. The increased ratio of Bax to Bcl-2 gene translational product induced cell death process via apoptosis [32]. Pro-apoptotic gene, Bax is the most characteristic death promoting member of the Bcl-2 family [33]. The Bax gene encodes a protein that is primarily localized to the cytosol where apoptotic stimulation is translocated to the mitochondria [34]. In mitochondria it activates the release of cytochrome-c and forms a complex with other co-factors that triggers the activation of caspase-9 and initiates downstream caspase cascade leading to cell death [35, 21]. However, Bcl-2 gene product acts as an anti-apoptotic agent by binding and antagonising with executioner molecules, such as Bax and Bak [36]. The p53 expression leads to increase in p53 protein concentration which ultimately enhances the expression of Bax gene which is probably associated with further activation of pro-caspase genes [36, 37]. On the similar lines, Basu and Haldar (1998) reported that the p53 gene is a negative regulator of Bcl-2 gene and acts as a transcriptional activator of the Bax gene.

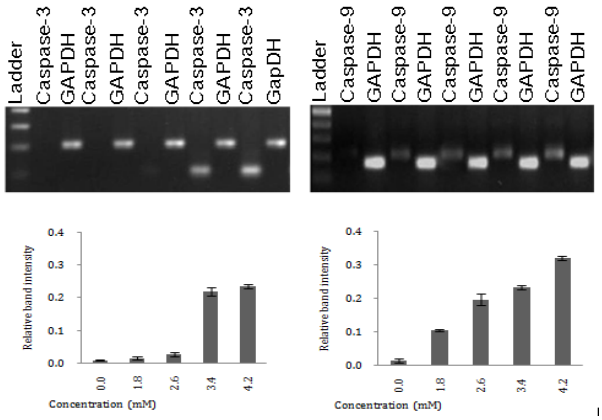

Fig.3: Up-regulation of caspase-3, and -9 genes in MCF-7 cell line treated with phytic acid for 48 h. Data are represented as means ± SD.

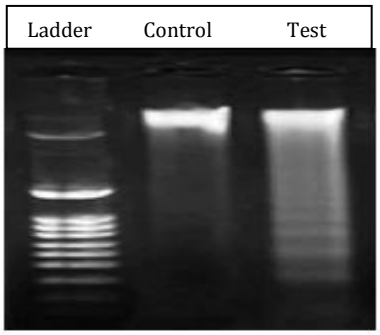

Fig.4: Genomic DNA fragmentation of MCF-7 cell treated with parthenolide for 48 h.

Caspase enzymes are mainly involved in the apoptotic cascade and lead to proteolysis of specific substrates associated with programmed cell death [20]. Consistent with this possibility, PA treated MCF-7 cells showed increased up-regulation of caspase-9 gene and consequently activate caspase-3 gene up-regulation (Figure 3). The caspase-3 is an important executioner caspases, which is activated by any of the initiator caspases. Active caspase-3 has variety of functions including activation of a latent cytosolic endonuclease, caspase activated deoxyribonuclease that cleaves genomic DNA into oligonucleosomal fragments [38, 39]. Our findings correlated with the previous findings in which iron-chelator compounds have been reported for multiple mechanisms in the antitumor activity [40]. Iron depletion results in inhibition of the Fe-containing enzyme, ribonucleotide reductase, which is critical for DNA synthesis [41]. Iron-chelators induced down regulation of Bcl-2, up-regulation of the pro-apoptotic protein Bax and p53 and increases caspase-3, -8, and -9 activities in cancerious cells [40]. Also, reported that the iron depletion alters expression of many molecules that cause cell-cycle arrest [42]. Addition to previous findings, the present study first time reporting the consequences of

apoptosis as a DNA fragmentation in the PA treated MCF-7 cells (Figure 4). These findings provided information about the therapeutic function of PA against human liver cancer.

CONCLUSION

We conclude that rice bran PA inhibited the growth of breast cancer cells. The cytotoxic activity of PA is possibly due to iron chelating properties that may induce apoptosis via p53 and caspase dependent pathways. Hence, we suggest that PA is a promising molecule and can be successfully exploited in cancer chemoprevention or chemotherapy. However, further studies are warranted to decipher the precise molecular mechanism of this bioactive compound to evaluate its anticancer properties.

ACKNOWLEDGEMENTS

Authors sincerely acknowledge the DST-FIST, Department of Biosciences, Jamia Millia Islamia New Delhi, for providing the laboratory facility for this research study. We also thank Dr. Md. Irshad, Anees Abbas and Md. Zafaryab, in the Department of Biosciences for reviewing and editing the manuscript.

DECLARATION OF INTEREST

Authors have no any declaration of interest

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