RESEARCH ARTICLE

Rice Bran Phytic Acid Induced Apoptosis Through Regulation of Bcl-2/Bax and p53 Genes in HepG2 Human Hepatocellular Carcinoma Cells

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Abstract

Phytic acid (PA) has been reported to have positive nutritional benefits and prevent cancer formation. This study investigated the anticancer activity of rice bran PA against hepatocellular carcinoma (HepG2) cells. Cytotoxicty of PA (0.5 to 4mM) was examined by MTT and LDH assays after 24 and 48h treatment. Apoptotic activity was evaluated by expression analysis of apoptosis-regulatory genes [i.e. p53, Bcl-2, Bax, Caspase-3 and -9] by reverse transcriptase-PCR and DNA fragmentation assay. The results showed antioxidant activity of PA in Fe^{3+} reducing power assay ($p \leq 0.03$). PA inhibited the growth of HepG2 cells in a concentration dependent manner ($p \leq 0.04$). After 48h treatment, cell viability was recorded 84.7, 74.4, 65.6, 49.6, 36.0 and 23.8% in MTT assay and 92.6, 77.0%, 66.8%, 51.2, 40.3 and 32.3% in LDH assay at concentrations of 1, 1.5, 2.0, 2.5, 3.0, and 3.5mM, respectively. Hence, treatment of PA for 24h, recorded viability of cells 93.5, 88.6, 55.5, 34.6 and 24.4% in MTT assay and 94.2, 86.1%, 59.7%, 42.3 and 31.6%, in LDH assay at concentrations of 1, 2.2, 3.0, 3.6 and 4.0mM, respectively. PA treated HepG2 cells showed up-regulation of p53, Bax, Caspase-3 and -9, and down-regulation of Bcl-2 gene ($p \leq 0.01$). At the IC_{50} (2.49mM) of PA, the p53, Bax, Caspase-3 and-9 genes were up-regulated by 6.03, 7.37, 19.7 and 14.5 fold respectively. Also, the fragmented genomic DNA in PA treated cells provided evidence of apoptosis. Our study confirmed the biological activity of PA and demonstrated growth inhibition and induction of apoptosis in HepG2 cells with modulation of the expression of apoptosis-regulatory genes.

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

Asian Pac J Cancer Prev, **15 (8)**, 3731-3736

Introduction

Phytic acid (PA) (inositol hexaphosphate, IP6) is a natural compound, present mostly in legumes including corn, soy beans, nuts, wheat bran and rice bran (Canan et al., 2011). In recent years, rice bran has been extensively examined for its biological activities and reported to have antioxidant , anti-inflamatory activity, lowering the risk of cancer formation, prevent coronary hearth diseases and decreased cholesterol level (Islam et al., 2011; Henderson et al., 2012). Rice bran have high content of PA ranging from 5.94 to 6.09g/100g (Liu et al., 2005). PA has been known for its beneficial effects on human health, particularly in the prevention of renal calculi, diabetes, cancer and parkinson's disease (Lee et al., 2005; Saw et al., 2007; Xu et al., 2008). In addition, PA has been reported for antioxidant, anticarcinogenic and hypolipidemic properties (Norazalina et al., 2010; Kang et al., 2012). PA is a strong chelator of multivalent metal ions, especially iron, zinc and calcium (Hurrel, 2004). In addition, dietary PA has ability to bind toxic trace elements and, thus, influence their solubility, absorption, and digestibility (Zhang et al., 2012). Researchers also reported that the iron-chelators inhibit the cancer cells growth reflects the importance of iron in a variety of crucial metabolic pathways including DNA synthesis and ATP production (Le and Richardson, 2004). It was also reported that, transferrin receptor expressed more abundantly in cancer cells than their normal counterpart (Shinohara et al., 2000). The primary function of transferrin is to transport iron through the blood (Shinohara et al., 2000).

Hepatocellular carcinoma is a most common cancer worldwide, about 500,000 new cases annually, representing the third largest cause of cancer-related death (Ferlay et al., 2010). Nutritional or dietary factors have attracting the great deal of interest due to their perceived ability to act as highly effective chemopreventive agents (Moshahid et al., 2009; Irfan et al., 2010). It is also considered a rational strategy for dietary approaches to prevent cancer. In fact increasing efforts are being made

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to isolate bioactive products from deity plants for their possible utility in cancer treatment.

PA (IP6) has been shown to inhibit growth of various types of cancer including prostate, liver, breast, colon and skin (Bode and Dong, 2000; Jenab and Thompson, 2000). The mechanisms of action against cancer cells growth have been reported the stimulation of genes toward against cell differentiation (Saied and Shamsuddin, 1998), alteration in signal transduction (Saad et al., 2013), anti-oxidant activity (Norhaizan et al., 2011) and increase in immunity (Fox and Eberl, 2002). However, molecular mechanism of cancer cell death after exposure of PA is still unclear. Therefore, the present study was undertaken to evaluate the effect of PA on growth and apoptosis regulatory genes like p53, Bax, Bcl-2, caspase-3 and -9 genes' of human liver cancer cells (HepG2). These genes regulates apoptotic mechanism, Bcl-2 gene protects cell death by inhibiting the apoptosis pathway (Song et al., 2014), whereas Bax gene is a regulators of apoptosis (Li et al., 2013). The p53 gene 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 (Song et al., 2014). 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 (Slee et al., 2001; Wang et al., 2013).

Materials and Methods

Reagent and chemicals

Phytic acid (PA) from rice bran (purity≥98% HPLC) 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).

Fe+3 reducing power assay

The Fe3+ reducing power of PA was determined by the standard method (Irshad et al., 2012). The extract (0.75mL) of various concentrations (0.2-4mM) was mixed with 0.75mL of phosphate buffer (0.2M, pH 6.6) and 0.75mL of potassium hexacyanoferrate (K3Fe(CN)6) (1%, w/v), followed by incubation at 50°C in a water bath for 20min. The reaction was stopped by adding 0.75mL of trichloroacetic acid (TCA) solution (10%) and mixture centrifuged at 800g for 10min. 1.5mL of the obtained supernatant was mixed with 1.5mL of distilled water and 0.1mL of ferric chloride (FeCl3) solution (0.1%, w/v) for 10min. The absorbance of reaction mixture was taken at 700nm. Ascorbic acid was used as positive reference. Higher value absorbance of the reaction mixture indicated greater reducing power.

DPPH radicals scavenging activity

DPPH radicals scavenging of PA was estimated according to the previously describe method (Irshad et al., 2014). DPPH radicals absorbed maximum at 517nm, which disappears with reduction by an antioxidant compound (s). Three milliliter (3mL) DPPH solution in methanol (0.1mM) was mixed with 100µL of PA (0.2-4mM). In control 100µL methanol (without PA) mixed with DPPH solution. The samples were incubated in a water bath for 20min at 37°C, and the decrease in absorbance at 517 nm was measured. Ascorbic acid was used as positive reference. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula: ***% Inhibiton=[(AC-AE)/AC]x100***, Where AC=absorbance of the control and AE=absorbance of tested samples.

Cell culture

Human hepatocellular carcinoma (HepG2) cell lines were 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 (100U/mL penicillin and 100mg/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 by Mehdi et al. (2011). The HepG2 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. HepG2 cells were treated with various concentrations of PA ranging between 0.1-4mM for 24 and 48h time points. Afterwards, the culture medium was replaced with fresh medium and 20µL of MTT (5mg/mL in PBS) was added to it and kept at 37°C for 4h. Formazan crystals formed in live cells by mitochondrial reduction of MTT were solubilized in DMSO (200µL/well) and the absorbance was measured at 570nm on iMark Microplate Reader (Bio-Rad, USA). All cytotoxicity assays were performed in triplicate and the percentage of cell survival was calculated using following formula: ***% Cell survival=[Experimental OD570/Control OD570]×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, HepG2 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 0.1 to 4mM were used to treat the cells lines for 24 and 48h 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 490nm on the iMark Microplate Reader (Bio-Rad, USA). The assay was performed in triplicate and the percent cytotoxicity was calculated as: ***% Cytotoxicity***

=[(Experimental OD490-Spontaneous OD490)/Maximum LDH released OD490]x100

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 (1-3mM) of PA for 48h. Treated and untreated HepG2 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. 1µg 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 25μL containing 2µL of cDNA, 1U taq DNA polymerase, 1.5mM MgCl2, 0.2mM 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).

DNA fragmentation assay

In order to perform DNA fragmentation assay, PA (at IC_{50} concentration for 48h) treated HepG2 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 $p<0.05$.

Table 1. Oligonucleotide Primer Sequences

P53	For- 5' CCAGCAGCTCCTACACCGGC 3'
	Rev- 5' GAAACCGTAGCTGCCCTG 3'
Bcl-2	For-5' GGTCGCCAGGACCTCGCCGC 3'
	Rev- 5' AGTCGTCGCCGGCCTGGCG 3'
Bax	For- 5' GAGCTGCAGAGGATGATTGC 3
	Rev-5'CCGGGAGCGGCTGTTGGGCT 3'
Caspase-3	For- 5' GTACAGATGTCGATGCAGC 3'
	Rev- 5' CACAATTTCTTCACGTGTA 3'
Caspase-9	For-5' CCTGCGGCGGTGCCGGCTGC 3'
	Rev-5' GTGTCCTCTAAGCAGGAGAT 3'
GAPDH	For- 5' GTGATGGGATTTCCATTGAT 3'
	Rev- 5' GGAGTCAACGGATTTGGT 3'

Results

Antioxidant activity

Antioxidant activity of PA was examined by two different method based on DPPH radicals scavenging and ferrous ions reducing activity. In ferric reducing power assay, antioxidant(s) reduces Fe3+ions into Fe2+, whereas in DPPH assays, antioxidant(s) donates H+to the DPPH radicals. The result showed that the PA significantly reduced Fe3+ ions in a dose dependent manner ($p\leq 0.04$) whereas DPPH assay did not show significant antioxidant activity (Figure 1). However, ascorbic acid significantly reduced DPPD radicals and Fe3+ions in both assays at same concentrations ($p\leq 0.03$).

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 HepG2 cells at 24 and 48h time points ($p<0.002$) (Figure 2). The IC_{50} value (evaluated after 48h) of PA against HepG2 cells were 2.49±0.61mM ($p\leq 0.04$). After 48h, PA decreased HepG2 cells viability by 84.7, 74.4, 65.6, 49.6, 36.0 and 23.8% at concentration of 1, 1.5, 2.0, 2.5, 3.0, and 3.5mM, respectively. Whereas, treatment of PA for 24h, decreased cells viability by 93.5, 88.6, 55.5, 34.6 and 24.4% at concentration of 1, 2.2, 3.0, 3.6 and 4.0 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 HepG2 cell viability by 92.6, 77.0%, 66.8%, 51.2, 40.3 and 32.3% at concentration of

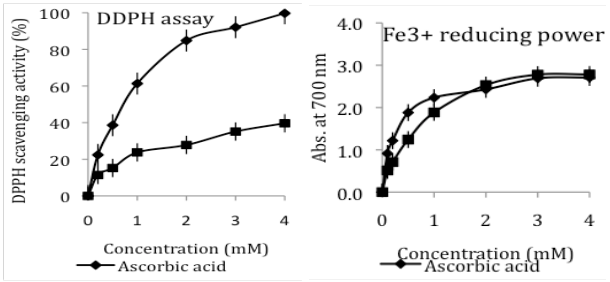

Figure 1. Antioxidant Activity of Phytic Acid. The Total Value Express as Mean±Standard Deviation (n=3)

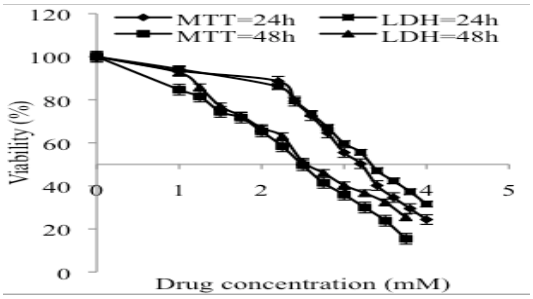

Figure 2. Cytotoxicity Trends of Phytic Acid Against HepG2 Cells, Determined by MTT and LDH Assays

1, 1.5, 2.0, 2.5, 3.0, and 3.5 mM after 48h, whereas after 24h, decreased of cells viability by 94.2, 86.1%, 59.7%, 42.3 and 31.6% at concentration of 1, 2.2, 3.0, 3.6 and 4.0mM, respectively. The cytotoxic activity profile of PA against HepG2 cells evaluated by MTT and LDH assays were significantly correlated at 24 and 48h time points ($r>0.969$). Interestingly, both bioactivity assays showed PA toxicity against HepG2 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 HepG2 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 3). At IC_{50} of PA, the p53 and Bax genes relative band intensity increased by 6.03 and 7.37 folds, respectively. However, PA treated HepG2 cells showed down-regulation of Bcl-2, and increased Bax to Bcl-2 ratio in a dose-dependent manner ($p\leq0.01$) (Figures 2-3). At IC_{50}, the Bax to Bcl-2 ratio was estimated to be 4.3.

Expression analysis of Caspase genes; the apoptosis activity in PA treated HepG2 cells were determined by expression analysis of Caspase genes. The results showed that PA treated HepG2 cells exhibited significant up-regulation of Caspase-3 and-9 genes in a concentration-dependent manner (Figures 4). Densitometry analysis revealed that, at IC_{50} of PA, Caspase-3 and-9 genes were

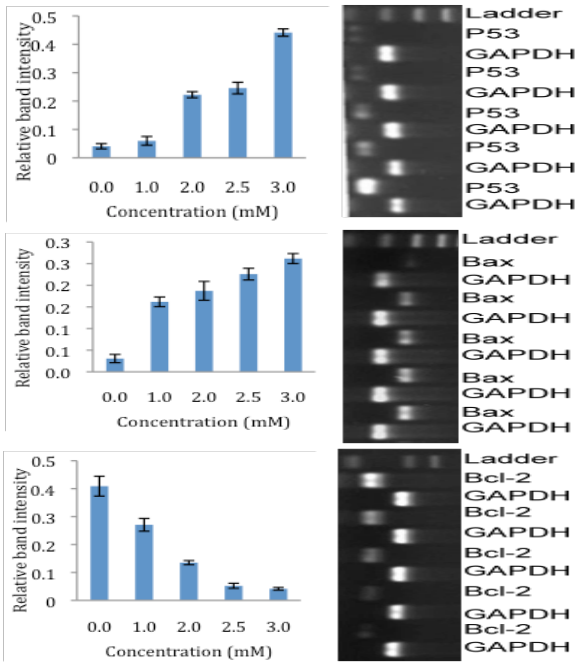

Figure 3. Expressional Analysis of p53, Bax and Bcl-2 Genes in HepG2 Cell Line Treated with Parthenolide for 48h

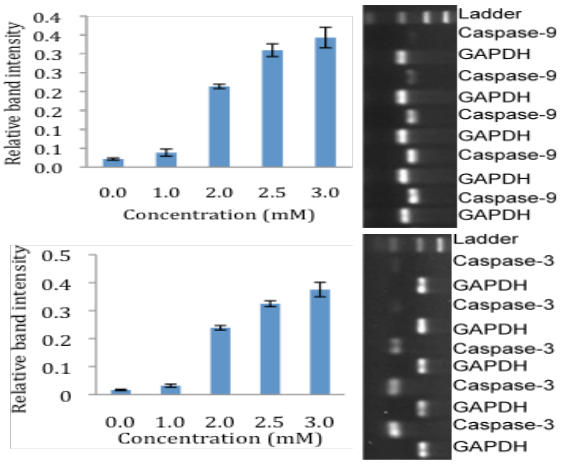

Figure 4. Up-regulation of Caspase-3, and -9 Genes in HepG2 Cell Line Treated with Phytic Acid for 48h Data Are Represented as Means±SD

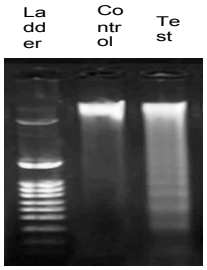

Figure 5. Genomic DNA Fragmentation of HepG2 Cell Treated with Parthenolide for 48 h

up-regulated by 19.71 and 14.49 folds, respectively ($p\leq0.03$).

DNA fragmentation analysis; genomic DNA fragmentation assay was performed in PA treated and untreated HepG2 cells in order to analyze the hallmark of apoptosis. DNA fragmentation assay revealed ladder like appearance in the gel (Figure 5). The 'laddering phenomenon' is a characteristic feature of apoptosis process in which the genomic DNA is cleaved into fragments by the endogenous endonucleases.

Discussion

Rice bran PA is a metal chelating natural compound, has been reported for anticancer activities in both *in vitro* and *in vivo* assays, however, the molecular mechanism of cancer cell death is still unclear. Present study examined the antioxidant activity by two different assays. PA showed antioxidant property in ferric reducing power assay, but did not significantly scavenge DPPH radical at same concentration. The result consistent with the previous findings where the high binding affinity of PA, especially to iron, has been recognized as a potent antioxidant and inhibitor of iron catalyzed hydroxyl radical formation (Graf and Eaton, 1990). The result also correlated with a finding in which PA acting as a potent inhibitor of iron-catalyzed radical formation by chelating free iron and blocking its coordination sites (Zajdel et al., 2013). Interestingly, iron-chelators has been used as therapeutics for the treatment of iron-overload disease and some of iron-chelator compounds act as potential role in cancer

therapy (Hatcher, et al., 2009). Iron-chelators expected to reduce iron availability, induce apoptosis in cancer cells through mechanisms that seem to involve mitochondrial activation (Richardson et al., 2009). Few 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 (Salettaet al., 2010).

In present study, rice bran PA induced HepG2 cell death concentration dependent manner with IC_{50} value of 2.49±0.61mM. 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 1.66mM, respectively, while no sensitivity against normal cell line (3T3) was reported (Norhaizan et al., 2011). However, anti-proliferative activity of IP6 was reported against HepG2 cells with IC_{50} value of 0.338mM (Vucenik et al., 1997). In addition, IP6 inhibited the proliferation of MCF-7 and HT-29 cells through arresting cells in the G0/G1-phase and inhibits DNA synthesis (El-Sherbiny et al., 2000; Nurul-Husna et al., 2010)

Apoptosis is regulated by the families of pro-and anti-apoptotic factors. 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 (Alabsi et al., 2013; Song et al., 2014). Semi-quantitative RT-PCR revealed down-regulation of Bcl-2 gene and up-regulation of p53 and Bax genes in HepG2 cells treated with PA (Figures 3-4). The PA treated HepG2 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 (Du et al., 2013). Pro-apoptotic gene, Bax is the most characteristic death promoting member of the Bcl-2 family (Fulda and Debatin, 2006). The Bax gene encodes a protein that is primarily localized to the cytosol where apoptotic stimulation is translocated to the mitochondria (Fulda and Debatin, 2006). 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 (Alabsi et al., 2013). However, Bcl-2 gene product acts as an anti-apoptotic agent by binding and antagonising with executioner molecules, such as Bax and Bak (Youle and Strasser, 2008). 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 (Youle and Strasser, 2008; Sun et al., 2013). On the similar lines, Hassan et al. (2013) reported that the p53 gene is a negative regulator of Bcl-2 gene and acts as a transcriptional activator of the Bax gene.

Caspase enzymes are mainly involved in the apoptotic cascade and lead to proteolysis of specific substrates associated with programmed cell death (Slee et al., 2001). Consistent with this possibility, PA treated HepG2 cells showed increased up-regulation of Caspase-9 gene and consequently activate Caspase-3 gene up-regulation (Figure 4). 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 (Errami et al., 2013). Our findings correlated with the previous findings in which iron-chelator compounds have been reported for multiple mechanisms in the antitumor activity (Richardson et al., 2009). 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 (Liang and Richardson, 2003). Also, reported that the iron depletion alters expression of many molecules that cause cell-cycle arrest (Fu and Richardson, 2007). Addition to previous findings, the present study first time reporting the consequences of apoptosis as a DNA fragmentation in the PA treated HepG2 cells (Figure 5). These findings provided information about the therapeutic function of PA against human liver cancer.

In conclusion, we conclude that rice bran PA inhibited the growth of hepatocellular carcinoma 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 N. Delhi, for providing the laboratory facility for this research study.

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