

Full Length Research Paper

Antiproliferation and apoptosis induction of phytic acid in hepatocellular carcinoma (HEPG₂) cell lines

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Phytic acid is a naturally occurring polyphosphorylated carbohydrate, present ubiquitous in plants and animals. It is not only a natural antioxidant, but may also be the precursor/storage of intracellular inositol phosphates, important for various cellular functions and potential as anticancer compound. A prominent anticancer action of phytic acid has been demonstrated both *in vivo* and *in vitro* in a variety of tumor types, possibly through inhibition of tumor cell growth and differentiation. In this study, the growth inhibitory effect of phytic acid extracted from rice bran on hepatocellular cell lines (HEPG₂), cell cycle modulation and apoptosis induction were undertaken. Phytic acid prove to induce growth inhibition and differentiation in HepG₂ in a dose and time-dependent manner with IC₅₀ value of 17.0 µg/ml. Analysis of flow cytometry was performed for the analysis of cell cycle and apoptosis. Treatment of phytic acid against HepG₂ also resulted in cell cycle arrest in HEPG₂ cell at G₂/M phase cell cycle arrest. Besides, Annexin V-assay and propidium iodide confirmed the apoptosis occurred early and late in the cell line. In conclusion, with the results taken from our findings, phytic acid extracted from rice bran was revealed as a potent candidate for adjuvant chemotherapy and prevention of cancer.

Key words: Phytic acid, rice bran, apoptosis, anticancer, cell cycle.

INTRODUCTION

In cancer prevention, a better strategy is an important ways for controlling cancer other than finding newer and better therapeutics. Cancer prevention can be approached through prophylactic supplements and vitamins. A series of epidemiological studies confirmed by clinical interventions, and animal model studies have pointed that major components of fiber including phytic acid present in wheat bran, grains and legumes may protect against cancer (Matejuk and Shamsuddin, 2010). Besides, phytic acid is also shown as one of the biologically active components of fiber that is responsible in anticancer effect. Phytic acid inhibited tumor growth by induction

of apoptotic cell death, cell cycle arrest and differentiation of several human cancer cell lines including colon (Sakamoto et al., 1993), liver (Vucenic et al., 1998a), prostate (Singh et al., 2003; Zi et al., 2000) and breast (Shamsuddin et al., 1996; Vucenic et al., 2005). Since phytic acid is obtained in our everyday diet and ability to quickly absorb from gastrointestinal tract and safe, the continuing investigation has been conducted against this novel biological agent against HepG₂ cell lines.

Herein, we revealed that *in vitro* administration of phytic acid on HepG₂ cells significantly induced growth inhibition and differentiation. Moreover, the anti-proliferative effects and the mechanism intriguing selectivity of phytic acid for normal and malignant cells deserve further investigations. Phytic acid is one of the bioactive compounds that are being intensively studied to evaluate their effects on

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health. Phytic acid is a naturally occurring compound that is abundantly found in grains, cereals, nuts, and foods that are high in fiber content (Somasundar et al., 2005). In rice bran, it occurs at 9.5 to 14.5% by weight (Jariwalla, 2001). The effect of phytic acid extracted from rice bran has not been evaluated in hepatocellular carcinoma (HepG₂) cell lines; therefore, we hypothesized that phytic acid would significantly inhibit the cell growth rate of HepG₂ cell lines.

MATERIALS AND METHODS

Cells and culture conditions

Human hepatocellular carcinoma cell line (HepG₂) was purchased from American Type Culture Collection (ATCC) (USA). HepG₂ cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Sample preparation

In a stabilization process of rice bran, method of Ramezanzadeh et al. (1999) was referred. The purpose of stabilization process was to prevent oxidative rancidity during storage. Total lipid was extracted from rice bran by using hexane regarding method of Hu et al. (1996) with slight modification. Extraction of phytic acid from rice bran was conducted regarding to the Fruhbeck et al. (1995). The extraction was centrifuged at 17300 g for min at 15°C and the supernatant were collected (Norazalina et al., 2010). The modified method of Camire and Clysdale (1982) was used to neutralize to the phytate extract. The sample was neutralized and concentrated by freeze-drying and kept at -20°C.

Growth inhibition assay

HepG₂ cell and 3T3 cells were cultured in DMEM supplemented with 10% FBS and 1% of penicillin-streptomycin. Cells were incubated at 37°C under 5% CO₂ in a humidified atmosphere. Microculture tetrazolium salt (MTT) assay were performed to determine the proliferation of cell lines as described by Pillai et al. (2004) with slight modification. This assay based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells were seeded at a density of 1 × 10⁵ cells/well microtitre plates for overnight. The next day, the medium was removed and phytic acid (diluted in FBS-free medium) containing increasing concentrations (from 0 to 50 µg/ml) were added into each well. After 72 h of phytic acid exposure, 20 µl MTT reagent (5.0 mg/ml) was added to each well for another 4 h at 37°C. The formazan product was solubilized in 100 µl dimethyl sulfoxide (DMSO) and the results were measured on a Microplate reader (Tecan, Switzerland) using a wavelength of 570 nm. The median inhibitory concentration (IC₅₀) is defined as the phytic acid concentration at which cell growth was inhibited by 50% from the dose-response curves. The percentage of cytotoxicity was calculated as follows: Cytotoxicity (%) = (1 - A₅₇₀ of experimental well) / A₅₇₀ of control well).

Cell cycle analysis

HepG₂ cells were plated at a density of 1 × 10⁵ cells in 25 mm² culture flasks. After 24 h, cells were exposed to phytic acid at three

different concentrations (17, 15 and 19 µg/ml). After 24, 48 and 72 h exposure cells were collected in the original medium. Cells were pelleted by centrifugation at 500 g at 4°C and washed with phosphate-buffered saline (PBS). Cells were resuspended in 70% ice-cold ethanol and further incubated at -20°C for 2 h. Cells were washed with PBS and resuspended in 50 µl of RNase solution (10 mg/ml) and stained with propidium iodide (1 mg/1 ml). After incubation for 30 min, cells were analyzed using flow cytometry (Beckman Coulter, USA).

Detection of apoptotic cell death by flow cytometry

Annexin V-FITC Apoptosis Detection Kit 1 (BD Bioscience) was used to detect early and late apoptotic activity HepG₂ cells after 24, 48 and 72 h of incubation. After treatment of the different forms of phytic acid at concentrations of 15, 17 and 19 µg/ml, cells at a density of 1 × 10⁵ cells were trypsinized, washed twice with ice-cold PBS, and resuspended in 100 µl of 1 × binding buffer (0.1 M Hepes/NaOH, pH 7.4 and 1.4 M NaCl₂, 25 mM CaCl₂). Then, 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added to each sample and gently vortexed before incubated in the dark for 15 min. Subsequently, 400 µl of 1 × binding buffer was added and the fluorescence of the cells was immediately analyzed by flowcytometry (Beckman Coulter, USA).

Statistical analysis

Biostatistical analyses were done using the SPSS 18.0 software package. All experiments were repeated at least three times. All data are reported as mean ± SD and statistical significant difference was considered to be present at p < 0.05. *Post hoc* comparison of individual concentration means with the control was completed using the Turkey's test.

RESULTS

Effect of phytic acid on growth inhibition of HepG₂ cells

Firstly, we determined the effect of phytic acid extracted from rice bran on the proliferation of HepG₂ cells. Phytic acid induced a dose-dependent decrease in vital formazan dye accumulating cells after 72 h of treatments, ranging from 0 to 100 µg/ml (Figure 1). The mean of IC₅₀ value of rice bran phytic acid was 17 µg/ml. For cell cytotoxicity analysis, phytic acid extracted from rice bran did not show any toxicity towards normal cells, 3T3 with no IC₅₀ was determined (result not shown).

Cell cycle analysis of phytic acid on HepG₂ cell

Based on the growth of inhibitory response of phytic acid extracted from rice bran on HepG₂ cell lines, we then assessed its effect on cell cycle progression using flow cytometry. The changes in the cell cycle distribution of HepG₂ cells after being treated for 24, 48 and 72 h with extracted phytic acid at their respective IC₅₀ ± 2.0. Analysis of the cycle as shown in Figure 2a showed that phytic acid increased the G2/M phase cells due to the

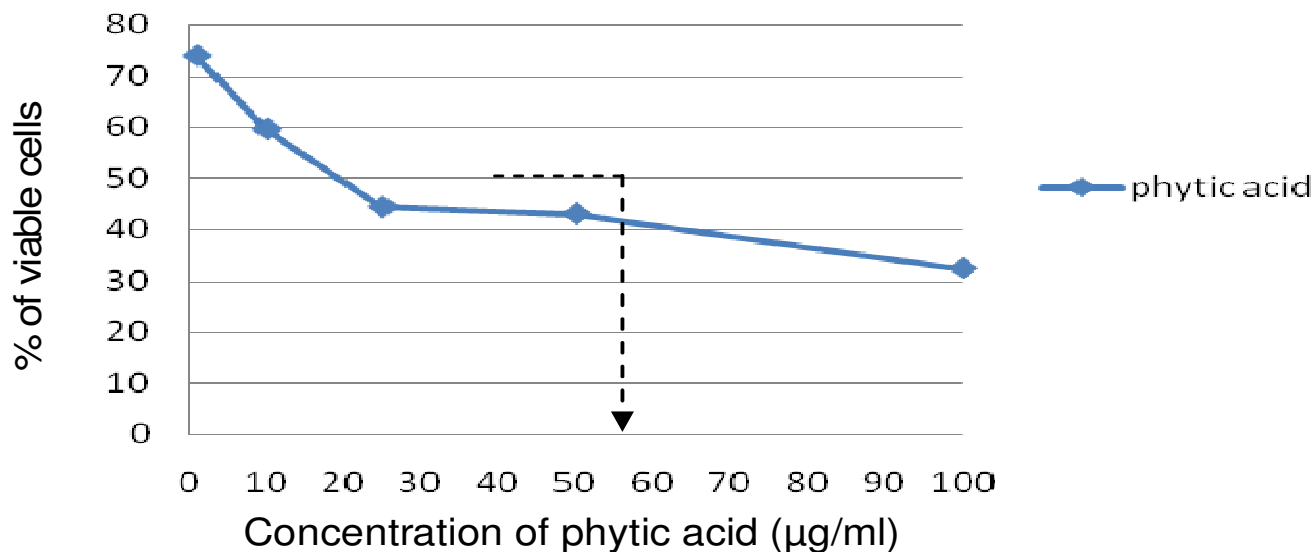


Figure 1. Cellular growth inhibition of extracted phytic acid from rice bran in hepatocellular carcinoma (HepG₂). The cell viability was measured by MTT assay after 72 hours culture. Data shown are means \pm standard deviations.

increasing of phytic acid dosage (15, 17 and 19 $\mu\text{g/ml}$) after 24 h. In addition, longer exposure time lead to an additional increase of accumulation of 43 to 49% \pm 1.1 of G2/M phases compared to control showing 39 \pm 2.47% (Figure 2b).

Measurement of apoptosis analysis

We determined any possible involvement of apoptosis induction by phytic acid extracted from rice bran that may have contributed in its strong growth inhibitory effect at increasing doses and longer treatment times. The annexin V/PI assay proved phytic acid extracted from rice bran significantly increased total apoptosis of HepG₂ cells. Phytic acid also increased the early and late apoptotic HepG₂ in a dose and time dependent manner. The total apoptotic cell death significantly increased after 24 h treatment with phytic acid (15 $\mu\text{g/ml}$) as compared to the control ($p < 0.05$). Phytic acid significantly increased the number of early (93 \pm 2.1%) and late apoptotic (58.73 \pm 2.3%) HepG₂ cells in dose dependent manner (Figures 3a and 4) compared to control only <3% of cell death ($p < 0.05$). In addition, phytic acid also significantly increased the number of early apoptotic (94 \pm 2.2%) and late apoptotic (79 \pm 2.3%) HepG₂ cells in a time dependent manner compared to control <2% of cell death ($p < 0.05$) (Figures 3b and 5).

DISCUSSION

The anticancer effect of phytic acid has been revealed in several studies both *in vivo* and *in vitro* (Sakamoto et al.,

1993; Vucenic et al., 2004; Somasundar et al., 2005; Matejuk and Shamsuddin, 2010). However, most study did not compare the different sources of phytic acid. Therefore, in this study, effect of phytic acid extracted from rice bran could be observed. Study from Reddy et al. (2000) proved that wheat bran is more effective than corn bran or oat bran, suggesting that the modifying effect of dietary fiber on the production of putative tumor promoters depends on the type of fiber consumed. Phytic acid which is among the components of dietary fiber has been suggested to play a significant role and contributes to inhibit colon cancer (Shamsuddin, 1995).

Various theories have been proposed for the anticancer activity of phytic acid. The most prominent effect of phytic acid has been documented in cancer prevention by controlling tumor growth, progression and metastasis. The anticancer action of phytic acid involved are boosting immunity, antioxidant properties, reducing cell proliferation and inducing differentiation of malignant cells (Matejuk and Shamsuddin, 2010). The major finding of the present study is that phytic acid extracted from rice bran induced growth inhibition, disruption of cell cycle progression and increased apoptosis on HepG₂ cells.

Phytic acid extracted from rice bran strongly induced growth of inhibition on HepG₂ cell lines in a dose dependent manner, with the IC₅₀ value 17.0 $\mu\text{g/ml}$ after 72 h (Figure 1a). The identifying of least cytotoxic effect of extracted phytic from rice bran acid towards non-tumorigenic cells was conducted on normal cell line, 3T3 (IC₅₀ cannot be determined). The analysis was performed as recommended by US National Institute of Experimental Health Sciences (NIEHS), Interagency Coordinating Committee in the Validation of Alternative Methods (ICCAM) in order to access basal cytotoxicity

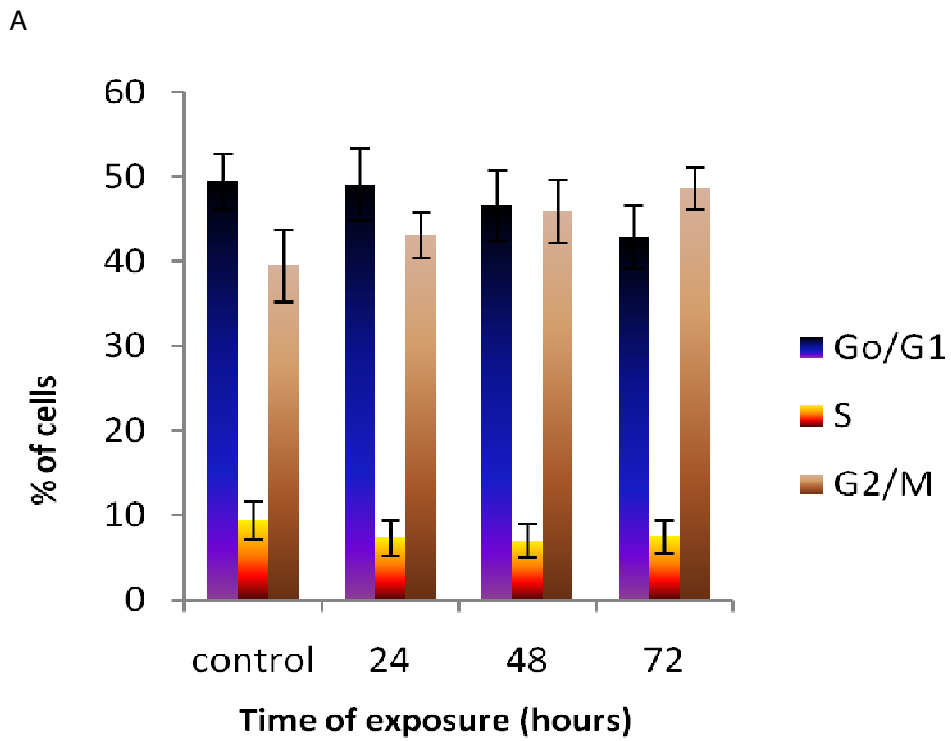
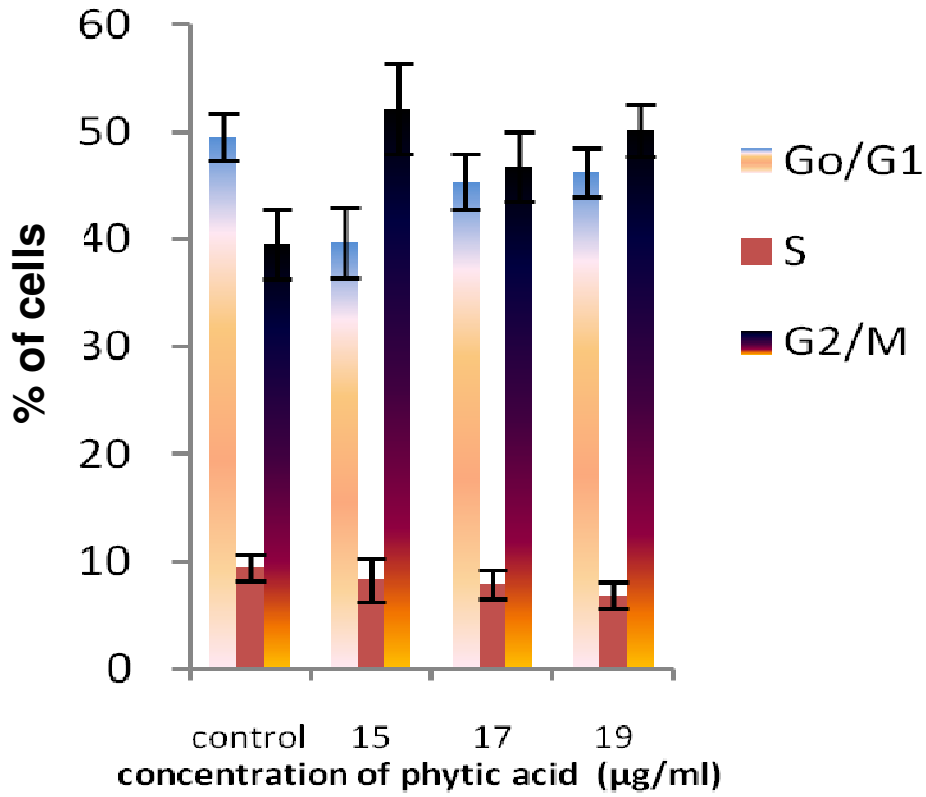
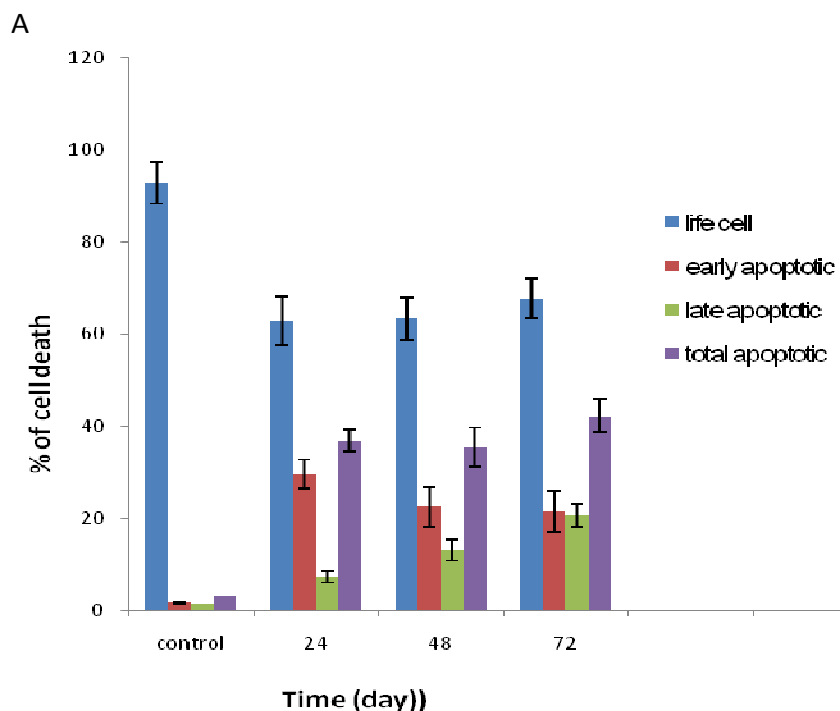
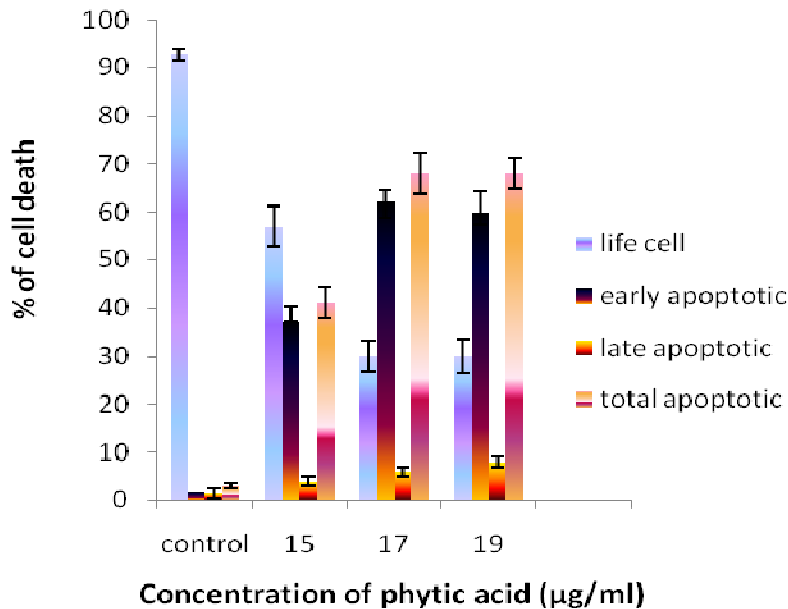


Figure 2. Effect of phytic acid on cell cycle in HepG₂ cells. The distribution of cells in the sub-G₁, G₀/G₁, S and G₂/M phases of the cell cycle in different dosage (a) and different exposure times (b). Each value is presented as mean ± standard error of mean of triplicate determinations.



B

Figure 3. Apoptotic activity, determined by the Annexin V assay, induced early and late apoptosis in HepG₂ cell lines when treated with phytic acid in different dosage (A) and different exposure time (B). Each value is presented as mean ± standard error of mean of triplicate determinations.

(NIEHS, 2001). It can be hypothesized that phytic acid normalized the rate of cell growth. Although, the exact mechanism of the cytotoxicity of phytic acid against HepG₂ cells is not entirely clear, many possible

mechanisms have been proposed for the growth inhibition of phytic acid in cultured cells and animal models. These mechanisms include induction of apoptosis and immunoregulation such as promoted

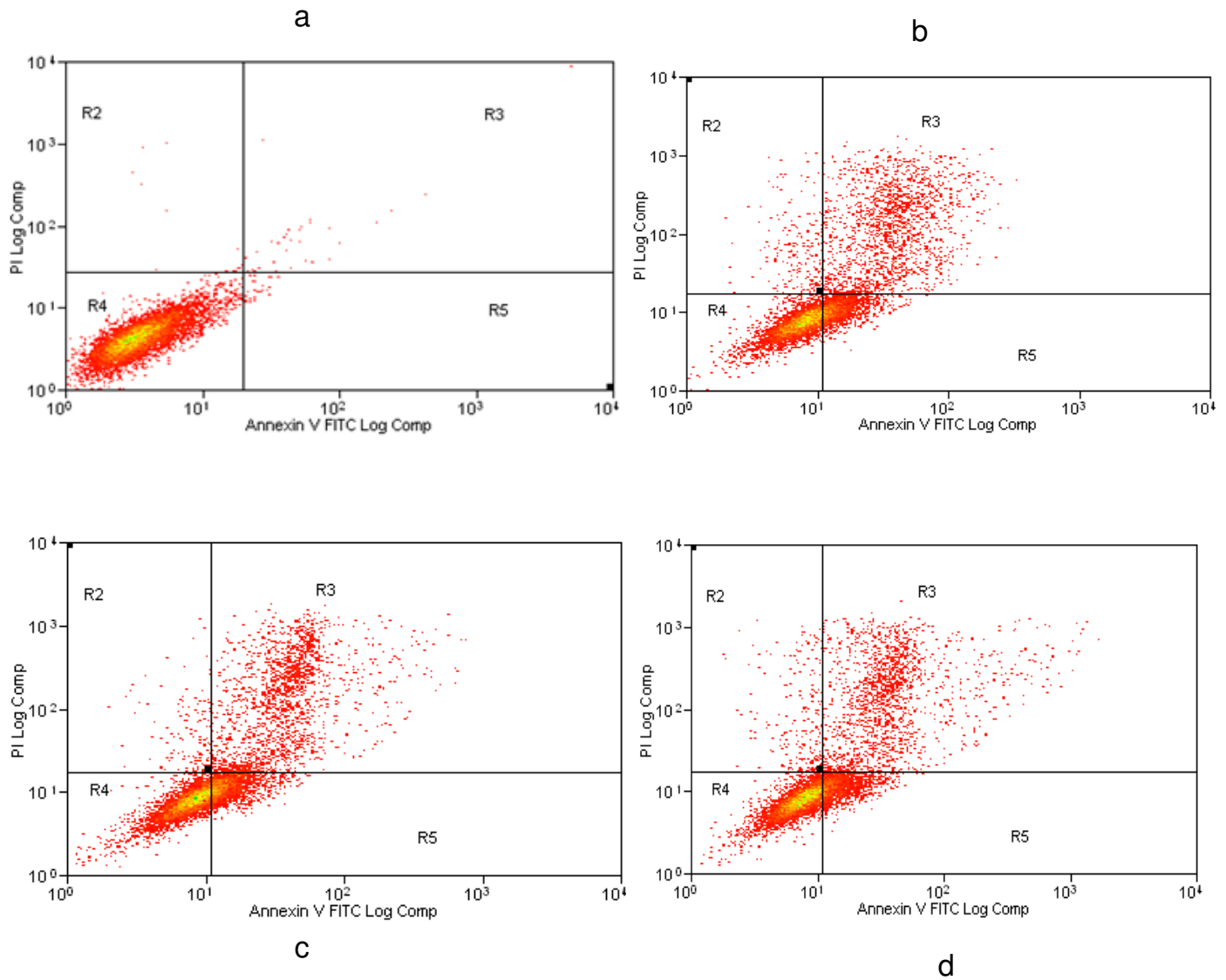


Figure 4. Apoptosis detection by flow cytometry and fluorescent markers Annexin-V FITC/PI. (a) Control (b) Treatment (15 $\mu\text{g/ml}$) (c) Treatment (17 $\mu\text{g/ml}$) (d) Treatment (19 $\mu\text{g/ml}$). Cell populations in bottom left, bottom right, top right and top left quadrants represented the proportion of viable cells, necrotic cells, late apoptotic cells and early apoptotic respectively. These results were representative of three experiments and at least 10,000 cells were analyzed per experiment.

lymphocytes and IL-2 production by splenocytes (Xu et al., 2007).

Since cell cycle progression is an essential event for cellular growth, we also examined whether the cell growth inhibitory effect by phytic acid and/or mediated through an alteration in a specific phase of the cell cycle progression. In this experiment, flow cytometric analysis of cells (both attached and floating cells) was conducted after staining cells with propidium iodide. The results obtained in the present study provide convincing evidence that phytic acid exerts its effects on cell cycle progression. As shown by the cell cycle analysis by PI staining (Figure 2), there was an increase in cell population in G2/M phase after HepG₂ cells were treated

with different concentration of phytic acid extracted from rice bran indicated that phytic acid induced a cell cycle arrest at G2/M phase. Cell cycle arrest in G1 phase of G2/M phase has been demonstrated as one of anticancer mechanism. The previous study reported by Sherbiny et al. (2001) found that treatment with phytic acid cause G₁ cell cycle arrest in mammary cancer cell lines MCF-7 and MDA-MB 231, and in HT-29, a human colon cancer cell line. However, the G1 arrest was not observed in our study when cell were treated with phytic acid, which could be due to the use of different types of cancer cell lines.

Apoptosis is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. A tumor occurs when the balance of cell

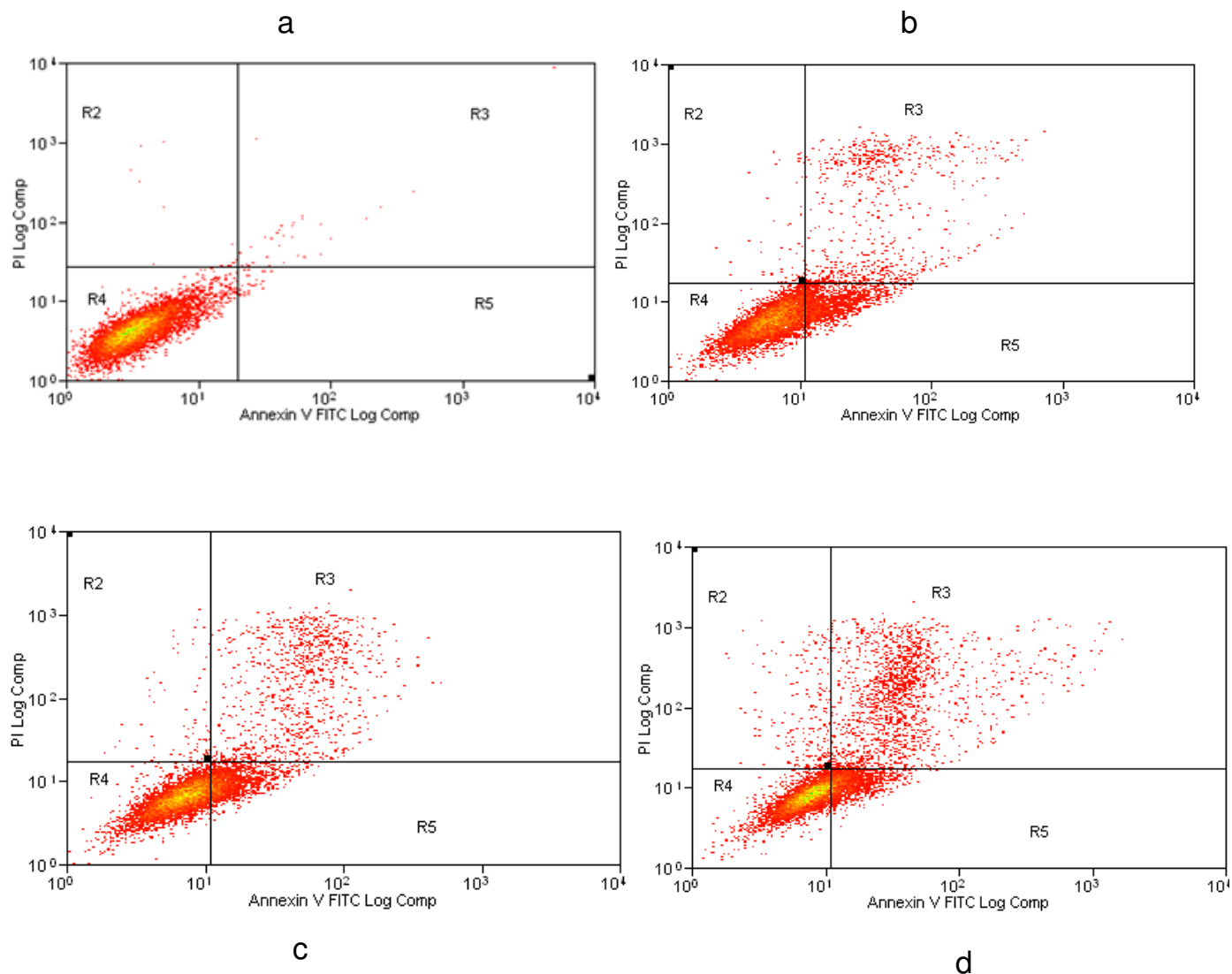


Figure 5. Apoptosis detection by flow cytometry and fluorescent markers Annexin-V FITC/PI. The experiments were performed after (a) Control (b) 24 h incubation time (c) 48 h incubation time (d) 72 h incubation time. Cell populations in bottom left, bottom right, top right and top left quadrants represented the proportion of viable cells, necrotic cells, late apoptotic cells and early apoptotic respectively. These results were representative of three experiments and at least 10,000 cells were analyzed per experiment.

proliferation and cell death are broken (Xu et al., 2007). Induction of apoptosis is an effective strategy for cancer therapy (Sun et al., 2004). Induction of apoptosis and cycle arrest are the most common mechanisms studied on the chemotherapeutic drugs in recent years (Zhou et al., 2008). Previous study reported that phytic acid induces apoptosis *in vivo* and *in vitro* in prostate, breast, cervical cancer, pancreas, melanoma, Barrett's adenocarcinoma and KS (Kaposi's sarcoma) cell lines, involving cleavage of caspase 3, caspase 9 and poly ADP-ribose polymerase (PARP), an apoptotic substrate, in a time and dose-dependent manner (Matejuk and Shamsuddin, 2010).

In this study, the cell treated with extracted phytic acid from rice bran demonstrated typical characteristics of

apoptosis. We could observe increases in both early and late apoptotic activity in HepG₂ cell lines. The observed increases in early apoptotic activity may indicate that the cells were in static, non-proliferative state, while the cells were in late apoptotic cycle and the cell death was imminent (McFadden et al., 2008). We also incorporated the induction of HepG₂ apoptotic cell death by phytic acid extracted from rice bran in a dose and time dependent manner.

Several molecular mechanisms can be suggested for the observed apoptosis effect of phytic acid in HepG₂ cell. Agarwal et al. (2003) proved that phytic acid inhibits nuclear kappa B (NFκB) in different models of cancer such as prostate. Nuclear factor kappa B (NFκB) is an early-response gene involved in cell growth and

apoptosis, and has been implicated in various cancers. Whereby, Karmakar et al. (2007) demonstrated that phytic acid down regulates cell survival factors BIRC-2 (baculovirus inhibitor-of-apoptosis repeat containing-2) and telomerase and up-regulates calpain and caspase-3 activities in malignant glioblastoma cells in promoting apoptosis. Further important role in apoptosis is suggested by finding that inositol hexaphosphate kinase-2 is a physiologic mediator of cell death (Nagata et al., 2005). In addition, phytic acid has been shown to regulate pro-apoptotic BCL-2 family of genes (Diallo et al., 2008).

In summary, extracted phytic acid from rice bran significantly decreased growth of hepatocellular carcinoma, HepG₂ *in vitro* without any toxicity effects. Phytic acid proved the antiproliferative effect through altering cell growth and apoptotic activity. These findings suggest that phytic acid has the potential to become an effective adjunct for hepatic cancer. Besides, since the occurrence of phytic acid compound in safe, naturally occurring foods make it an attractive natural therapy for dietary and conventional treatment and possible prevention of hepatocellular carcinoma. Further *in vivo* and human studies are needed to evaluate safety and clinical utility of this agent in patients with hepatic cancer treatment.

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