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Antioxidant and Cytotoxicity Effect of Rice Bran Phytic Acid as an Anticancer Agent on Ovarian, Breast and Liver Cancer Cell Lines

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ABSTRACT

Introduction: Phytic acid (PA) has been shown to have positive nutritional benefits. There are also claims that it is able to prevent cancer through its antioxidant capability. This study investigated antioxidant activity and cytotoxic effect of PA extracted from rice bran against selected cancer cell lines (i.e. ovarian, breast and liver cancer). **Methods:** Cytotoxicity activity of PA was investigated using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)]-2H-tetrazolium, inner salt] assay while the antioxidant activity of PA extract, commercial PA and butylated hydroxytoluene (BHT) was determined by using five different assays: ferric thiocyanate (FTC) and thiobarbituric acid (TBA) assay, β-carotene bleaching method, DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. **Results:** PA extracted from rice bran induced marked growth inhibition in ovary, breast and liver cancer cells with 50% growth inhibition concentration (IC_{50}) values of 3.45, 3.78 and 1.66 mM, respectively but exhibited no sensitivity towards a normal cell line (3T3). The PA extract was also found to exert antioxidant activity when tested using the FTC, TBA, FRAP and β-carotene bleaching methods but antioxidant activity could not be attributed to scavenging free radical species as measured by DPPH radical scavenging assay. **Conclusion:** The PA extract from rice bran displayed safe and promising anticancer properties in selected cancer cell lines and it is believed that its antioxidant capability is the likely contributor to the observed anticancer properties.

Keywords: Anti-cancer, antioxidant, phytic acid, rice bran

INTRODUCTION

Since the middle of the twentieth century, phytic acid (PA) has been recognised as an anti-nutrient for its ability to bind to, precipitate and decrease the bioavailability of di- and trivalent cationic minerals such as Fe^{3+}, Zn^{2+}, Mg^{2+} and Ca^{2+} (Zhou & Erdman, 1995; Minihane & Rimbach, 2002). However, in the early 1980s, more positive nutritional benefits attributed to PA have emerged. The possible effects of PA include a of lowering serum triglycerides and cholesterol, protection against cardio-vascular diseases and renal stone formation, and prevention against certain types of cancer. The proposed mechanisms of action include gene alteration, enhanced immunity

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and antioxidant properties (Matejuk & Shamsuddin, 2010).

Rice bran forms about 3-8% of rice grain. During milling, the germ and bran layers of rice are removed. Rice bran has only one principle use in Malaysia - as animal feed. Ironically, its utilisation in the food system is very limited despite it being a rich source of nutraceutical compounds, such as tocotrienols, tocopherols, oryzanol, minerals, lipid, protein, vitamins and phytic acid (Rohrer & Siebenmorgen, 2004). Both *in vivo* and *in vitro* studies demonstrate that PA reduces cell proliferation in different cell lines including colon cancer cells, erythroleukemia and human mammary cancer cells (Shamsuddin, 2002). Hence, PA may represent an alternative method for cancer treatment hence reducing dependency on drug treatment which is known for its many side effects. However, previous studies have been conducted on certain types of cancer only. To our knowledge, studies of PA on human breast cancer, ovary cancer and liver are limited.

METHODS

Chemicals and reagents

Linoleic acid, butylated hydroxytoluene (BHT), beta-carotene Type 1 (95%), DPPH (2,2-phenyl-1-picryl-hydroxyl) and thio-barbituric acid (TBA) were obtained from Sigma (St Louis, MO, USA), TPTZ (2, 4, 6-tripyridy-*s*-triazine) and ferric chloride from HmbG Chemicals (Germany), while ammonium thiocyate was from AJAX Chemical (Aubur, Australia), and ferrous chloride and ferrous sulphate ($FeSO_4$) were from BDH (England).

Dulbecco's Modified Eagle medium (DMEM), Earl Minimum Essential medium (EMEM), fetal bovine serum (FBS), Penicilin-streptomycin and Amphotericin B were from PAA Laboratory GmbH, (Austria). MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) was from Promega Co (USA) while commercial phytic acid (corn) was obtained from Sigma (USA). All other chemicals and reagents used were of the highest purity grade available.

Cell cultures

Human ovarian cancer (Caov-3), human breast cancer (MDA-MB-231), human liver cancer (HepG2) and mouse fibroblast (BALB/c 3T3) cell lines were purchased from American Type Culture Collection (ATCC) (USA).

Sample preparation

Malaysian local rice bran (*Oryza sativa* L., variety MR220) was obtained from BERNAS, the major rice miller in Malaysia. Rice bran was stabilised according to the method of Ramenzanzadeh *et al.* (1999). Stabilisation was undertaken to prevent oxidative rancidity during storage. After the stabilisation process, total lipid was extracted from rice bran samples by using hexane, using the modified method of Hu *et al.* (1996). Extraction of PA from rice bran was based on the procedures of Fruhbeck *et al.* (1995) with some modifications. The samples were added to hydrochloric acid (HCl) (1 g in 20 ml) in pH 1.0. The extraction was carried out at room temperature with constant shaking at medium speed in an orbital mixer. The resulting creamy mixture was centrifuged at 17,300 g for 30 min at 15°C and the supernatants were collected (Norazalina *et al.*, 2010). The modified method of Camire and Clydesdale (1982) was used to neutralise the phytate extract. The neutralised sample was then concentrated by freeze-drying and kept at –20°C.

Growth inhibition assay (MTS)

Caov-3, MDA-MB-231 and 3T3 were grown in DMEM while HepG2 was grown in EMEM supplemented with 10% (v/v) of FBS, 1% (v/v) Penicillin-streptomycin and 1% (v/v) amphotericin B at 37°C under 5% CO_2 and

95% air. To evaluate the effects of PA on the proliferation of the cells, a methyl-thiazol tetrazolium (MTS) assay (Promega, US) was used according to manufacturer's instructions. The cells were pre-incubated at a density of 1 x 10^5 cells / ml on 96-well microtitre plate for 24 h. The old medium was tapped out and PA extract (diluted in medium) ranging between 1.0 and 6.0 mM was added onto the plate. The plate was incubated at 37°C for a further 72 h. Then, 20 μl of MTS reagent was added into each well. This plate was incubated again for 2 h and finally the absorbance was read at 490 nm using the microplate reader (Tecan, Switzerland). In this study, the toxicity effect of phytic acid (extract and commercial) was determined by using a normal cell (3T3 cell line). The % recovery was graphed against the concentrations where 50% growth inhibition concentration (IC_{50}) values could be interpolated from the graph.

Ferric thiocyanate (FTC) assay

The FTC assay was carried out as described in the method of Osawa and Namiki (1981). Samples consisting of 4 mg of phytic acid extract from rice bran and commercial were weighed and dissolved in 3.9 ml of distilled water. Subsequently, 4.1 ml of 2.5% linoleic acid in 99.5% ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of distilled water were added and placed in an oven at 40°C in the dark. Butylated hydroxytoluene (BHT) was used in place of both samples in a similar solution as a comparison. A control was prepared using all solutions but without the sample. The absorbance was precisely measured 3 min after the addition of 0.1 ml, 0.02 M ferrous chloride in 3.5% HCl to the mixture by UV / VIS spectrophotometer at 500 nm. The optical density was taken every 24 h until one day after the maximum absorbance of the control was reached. Percentage of antioxidant activity was calculated using the following equation:

$$AA\ (\%) = \left[\frac{A_c - A_s}{A_c} \right] \times 100$$

where AA is antioxidant activity, A_c and A_s are the max absorbance values for control and samples, respectively.

Thiobarbituric acid (TBA) assay

The method of Ottolenghi (1959) was used to determine the TBA values of the samples. The formation of malonaldehyde is the basis for the well known TBA method used for evaluating the extent of lipid peroxidation. Two ml of 20% trichloroacetic acid aqueous solution and 2 ml 0.67% TBA aqueous solution were added to 1 ml of sample solution prepared from FTC method on day 1 and incubated in a similar manner. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and the absorbance of the supernatant was measured at 532 nm. Antioxidant activity was based on absorbance on the final day. Percentage of antioxidant activity was calculated using the equation that was used to calculate the antioxidant activity in the FTC method.

β-carotene-linoleate bleaching assay

The antioxidant activity of PA extract was evaluated using the β-carotene bleaching method following modification of the procedure described by Velioglu *et al.* (1998). One ml of 0.2 mg / ml β-carotene solution in chloroform was added to flasks containing 0.02 ml of linoleic acid and 0.2 ml of Tween-20. The chloroform was removed at 40°C using a rotary evaporator for 5-10 min. The resultant mixture was immediately diluted with 100 ml of distilled water and mixed for 1-2 min to form an emulsion. A mixture prepared similarly without β-carotene was used as a blank. A control, containing 0.2 ml of 80 % (v / v) methanol instead of extract, was also prepared. A 5 ml portion of the emulsion was added into a tube containing 0.2 ml of the sample extracts at 1 mg / ml.

The tubes were placed in a water bath at 40°C for 2 h. Absorbance was read at 470 nm at 15-min intervals using a UV-Visible spectrophotometer (UV-1601) (Shimadzu Corp., Kyoto, Japan). The antioxidant activity of each sample was calculated as percent inhibition relative to control using the following equation (Jayaprakasha, Sign & Sakariah, 2001).

$$AA\ (\%) = \left[1 - \frac{(A_0 - A_t)}{(A_0^{o} - A_t^{o})}\right] \times 100$$

where AA is antioxidant activity, A_0 and A_0^{o} are the absorbance values measured at zero time of incubation for sample extracts and control, respectively while A_t and A_t^{o} are the absorbance for sample extracts and control, respectively at t = 120 min.

DPPH radical scavenging activity

Free radical scavenging activity of PA extract was evaluated using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method of Shimada *et al.* (1992). One ml of freshly prepared 1 mM DPPH in methanol was added to test tubes containing 5 ml of the sample extracts. A control was prepared by adding 1 ml of DPPH solution to 5 ml of 80% methanol. Following storage in the dark for 30 min, the absorbance was read at 517 nm using a UV-Visible spectrophotometer. The percentage of free radical scavenging activity was calculated based on the following equation:

$$\text{Scavenging activity (\%)} = \left[1 - \frac{A_s}{A_c}\right] \times 100$$

where, A_s and A_c are the absorbance of the sample and control, respectively.

Ferric reducing antioxidant power (FRAP) Assay

The procedure described by Benzie and Strain (1996) was followed. The principle of this assay is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, coloured form in the presence of antioxidants. Briefly, the FRAP reagent contained 2.5 ml of a 10 mmol/l TPTZ (2, 4, 6-tripyridy-*s*-triazine) solution in 40 mmol/l HCl, 2.5 ml of 20 mmol/l $FeCl_3$ and 25 ml of acetate buffer; pH 3.6 was freshly prepared and warmed to 37°C. Aliquots of 0.1 ml samples were mixed with 0.3 ml distilled water and 3 ml of FRAP reagent. The absorbance of the mixture was measured at 593 nm after 4 min using a UV-Visible spectrophotometer. The 1 mmol/l $FeSO_4$ was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol/l $FeSO_4$. The change in absorbance between final reading selected and the blank reading was calculated for each sample and related to absorbance of a Fe standard solution.

$$\text{The FRAP value (mmol/l)} = \frac{\text{0-4 min } \Delta A_{593} \text{ of test sample}}{\text{0-4 min } \Delta A_{593} \text{ of standard}} \times [Fe^{2+}] \text{ standard (mmol/l)}$$

Statistical analysis

All experiments were carried out in three replicates and presented as mean ± standard deviation (SD). The data were statistically analysed by one-way ANOVA. The level of statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The study aimed at evaluating cytotoxicity of phytic acid extracts from rice bran and commercial (corn bran) on human liver cancer cells (HepG2), human breast cancer cells (MDA-MB-231) and human ovarian cancer cells (Caov-3). The viability of the cells was evaluated using the MTS method. Only viable cells have the ability to bio-reduce MTS tetrazolium compound into a coloured formazan product. The cytoxicity of phytic acid is expressed as IC_{50}, which is defined as the concentration at which the compounds cause a 50% decrease in MTS test. The IC_{50}

values of all the samples tested are given in Table 1. Results show that phytic acid from both samples induced cytotoxic activity against all cancer cell lines tested. Independent *t*-test showed that there was no significant difference between IC_{50} value of rice bran PA and corn PA on Coav-3. However, significant differences were found in IC_{50} value of rice bran PA and corn PA on MDA-MB-231 and HepG2 cell lines. Rice bran phytic extract was most cytotoxic (IC_{50} = 1.66 mM) against HepG2.

In order to identify the least cytotoxicity towards non-tumorigenic cells, the inhibitory effect of the rice bran PA was evaluated on 3T3 cells. This cell line is recommended by US National Institute of Environmental Health Sciences (NIEHS), Interagency Coordinating Committee in the Validation of Alternative Methods (ICCAM) to access basal cytotoxicity (NIEHS, 2001). It is important for an anticancer agent to exhibit cytotoxicity but such activities should be specific for cancer cells only (Nurul-Husna *et al.*, 2010). Our results showed that rice bran PA extract and commercial bran from corn did not cause any toxicity towards normal cells, 3T3 with <10% of cells being dead (results not shown). In addition, PA selectively inhibited cancer cells without affecting the normal cells and acted synergistically with standard therapeutics (Vucenik *et al*., 2005).

One of the proposed mechanisms on how PA can reduce the proliferation of cancer cells is through their antioxidant properties. Five methods were used to determine this activity, which were FTC and TBA assay, β-carotene bleaching method, DPPH radical scavenging activity and FRAP assay.

The FTC method is used to measure the amount of peroxide at the primary stage of linoleic acid peroxidation. In this method, peroxide produced during oxidation will react with ferrous chloride, decompose to a lower molecule compound and produce a reddish ferric chloride. As the antioxidant activity increases, the peroxide concentration decreases. Malonaldehyde (MDA) is a by-product of lipid peroxidation that is formed from the oxidation of linoleic acid. It can be measured by the TBA method. Therefore this method was used to determine the extent of lipid peroxidation. The antioxidant activity of the samples by FTC method showed all samples to have markedly inhibited the oxidation of linoleic acid when compared to the control. The values also were not significantly different between PA extracted from rice bran with commercial PA from corn but were significantly lower than BHT. For the TBA method, once again BHT had the highest antioxidant activity (p<0.05) followed by rice bran PA extract and corn PA. This result indicates that PA acts as antioxidant at an early stage of lipid peroxidation and after a certain duration, it become much less effective than BHT in stabilising linoleic acid.

Table 1. IC_{50} value of phytic acid (PA)

Samples	*IC_{50} (mM)*		
	Caov-3	*MDA-MB-231*	*HepG2*
Rice bran PA	3.45 ± 0.09a	3.78 ± 0.19a	1.66 ± 0.02a
Corn PA	3.76 ± 0.21a	5.13 ± 0.50b	4.96 ± 0.26b

Values are presented in mean ± standard deviation of three separate experiments (n = 3); different letters within the column are significantly different at p<0.05. IC_{50} value for 3T3 cannot be determined.

The mean antioxidant activity (AA) (%) of rice bran PA, corn PA and BHT as measured by β-carotene bleaching method were 93.36%, 92.559% and 109.61%, respectively (Table 2). One way ANOVA showed that there was no significant difference in the antioxidant activity of rice bran PA and corn PA. BHT, however, had significantly ($p<0.05$) higher antioxidant activity as compared to PA from rice bran and corn. This result indicates that the capability of PA to hinder the extent of β-carotene bleaching by neutralising the linoleate-free radical and other free radicals formed within the system is not as strong as BHT.

DPPH radical assays are based on the transfer of electrons from a donor molecule to the corresponding radical (Fugliano *et al.*, 1999). It is the simplest method to measure the ability of antioxidants to intercept free radicals. The scavenging effects of PA from both rice bran and corn as measured by DPPH assay were significantly lower (41.5%, 26.4%, respectively) when compared to BHT (95.2%). This finding is in accordance with those of Ahn *et al*. (2003) who also reported that non-irradiated PA did not show DPPH radical scavenging activity regardless of its concentration. However, the radical scavenging ability of PA increased after irradiation. So, it is suggested that PA might structurally change to have an electron donating effect after irradiation.

FRAP assay is a method for measuring the reducing power of antioxidants (Benzie & Strain, 1996). This assay measures the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above Fe^{3+}/Fe^{2+}. The FRAP values obtained for each sample are summarised in Table 3. Results showed that rice bran PA had higher FRAP capacity compared to BHT but lower than corn PA. The order of antioxidant effectiveness or reducing effect power was: corn PA> rice bran PA> BHT. Iron (Fe) can induce the production of free radicals which may cause DNA double strand to breaks and the activation of oncogene. Iron can also maintain the growth of malignant cells as well as the growth of the pathogen. On the other hand iron chelators can counteract cell damage. The same mechanism was proposed by Nelson *et al*. (1989) on how PA may lower the incidence of colonic cancer and protect against other inflammatory bowel diseases. Phytic acid reduced colon cancer via chelation of iron and suppression of iron-

Table 2. Antioxidant activity (AA) % of rice bran PA, corn PA and BHT using FTC and TBA assay, β-carotene system and DPPH assay

Sample	*Antioxidant activity (%)*			
	FTC	*TBA*	*β-carotene system*	*DPPH Assay*
Rice bran PA	74.76 ± 0.05a	40.05 ± 0.03a	93.36 ± 3.09a	41.5 ± 1.02a
Corn PA	74.89 ± 0.02a	34.42 ± 0.09b	92.55 ± 9.64a	26.4 ± 0.56b
BHT	86.02 ± 0.05b	64.30 ± 0.04c	109.61 ± 4.23b	95.2 ± 0.69c

Values are presented in mean ± standard deviation (n = 3); different letters within the column are significantly different at $p<0.05$.

Table 3. Antioxidant activity of rice bran PA, corn PA and BHT using FRAP assay

Sample	*FRAP value (mM)*
Rice bran PA	2.10
Corn PA	2.78
BHT	1.53

related initiation and promotion of carcinogenesis. Phytic acid, by binding free iron, will suppress a number of iron-driven oxidative reactions which also serve as a potent antioxidant function in the preservation of seeds.

Many studies have addressed the role of antioxidants such as vitamin A, C and E in the protection against cancers and cardiovascular diseases. These antioxidants, however, can also act as pro-oxidants in some circumstances. A previous study by Murata and Kawanishi (2000) reported that vitamin A and its derivatives cause oxidative damage to cellular and isolated DNA. Vitamin E and quercetin also cause oxidative DNA damage (Yamashita & Kawanishi (2000). Virtually, all putative chemopreventive antioxidants may have potential carcinogenicity. In contrast, oxidative DNA damage was not observed even when high concentrations of PA was used as shown by Midorikawa *et al.* (2001). They suggest that PA does not act as pro-oxidant, unlike other antioxidants such as vitamin A and E. Therefore, PA could potentially be a safe chemopreventive agent. The possible mechanism as to how PA could act as an antioxidant is by preventing the generation of highly reactive species by its metal-binding properties and not through scavenging free radical species that is also supported by our current finding. By the same mechanisms, dietary phytic acid could lower the incidence of cancer or inhibit oxidation during processing, preservation and storage of foods (Peterson, 2001).

CONCLUSION

Our findings further support that PA extracted from rice bran has potential for use in the prevention and therapy of selected cancer lines. PA antioxidant is the likely contributor to the observed anticancer properties. Further *in vivo* and human studies are needed to evaluate safety and clinical utility of this finding.

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