RESEARCH ARTICLE

Purple Rice Bran Extract Attenuates the Aflatoxin B1-Induced Initiation Stage of Hepatocarcinogenesis by Alteration of Xenobiotic Metabolizing Enzymes

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Abstract

Pigmented rice bran has been suggested to be a valuable source of beneficial phytochemicals. We investigated genotoxic and anti-genotoxic effects of purple rice bran extract (PRBE) in rats using a liver micronucleus assay. Purple rice bran was extracted with methanol, obtaining large amounts of phenolic compounds, including anthocyanins and small amounts of gamma-oryzanol. The experimental protocols were divided into two sets. Male rats were divided into three groups. Group 1 was a negative control, while Groups 2 and 3 were fed with 100 and 500 mg/kg bw of PRBE, respectively, for 28 days. PRBE had no effect on micronucleus formation or xenobiotic metabolizing enzymes in rat liver. Experiments concerning the effect of PRBE on AFB_1 showed that PRBE significantly lessened the amount of micronucleated hepatocytes in AFB_1 treated rats. Furthermore, it modulated metabolic activation of AFB_1 metabolism in the liver by suppressing activity and protein expression of CYP1A2, CYP3A and CYP 450 reductase, and enhancing phase II enzymes including GST and UGT. Overall, purple rice bran extract was not genotoxic in rats. It exhibited anti-genotoxicity by modulation some xenobiotic enzymes active in AFB_1 metabolism.

Keywords: Purple rice bran - aflatoxin B1 - hepatocarcinogenesis - xenobiotic metabolizing enzymes

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Introduction

The rice bran or outer layer of the rice grain contains a number of micronutrients and valuable phytochemicals. Several studies have shown that pigmented rice bran contains higher amounts of beneficial phytochemicals such as phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, phytic acid, free fatty acids and methyl ester derivatives (Leardkamolkarn et al., 2011; Jun et al., 2012; Goufo and Trindade, 2014) than colorless rice bran. Colored rice bran also has numerous biological and pharmacological properties (Choi et al., 2010; Yang et al., 2011; Jang et al., 2012) including anti-cancer activity. Brown rice bran prevented dimethylhydrazine-induced colon cancer in rats (Li et al., 2011), and red and purple rice brans inhibited human cancer cell growth through the action of phenolic, anthocyanin, and proanthocyanidin constituents (Chen et al., 2012; Pintha et al., 2014).

Hepatocellular carcinoma is considered to be a top rank cancer in developing countries. Although viral hepatitis B and C infections seem to be a pivotal cause of human liver cancer in Asian countries, this could be controlled by vaccination. Aflatoxin B1 (AFB_1) produced by *Aspergillus* species fungi is classified as a carcinogen 1A and is known to be the most potent hepatocarcinogen in humans and animals. AFB_1 contamination in daily food is one cause of human liver cancer, although it is difficult to completely avoid AFB_1 exposure (Fan et al., 2013; Hamid et al., 2013).

Chemopreventive agents from plants are now considered to be a strategy for reduction of cancer incidence. Many previous *in vitro* and *in vivo* studies have demonstrated that natural products are able to reduce aflatoxicosis, for example Korean red ginseng and neem flower (Tepsuwan et al., 2002; Kim et al., 2011). Our group found that the alcoholic extract of purple rice (*Oryza sativa* L. var. *indica*) grain cultivar Kum Doisaket exhibited antimutagenic effects against AFB_1 in the Ames test (Punvittatyagul et al., 2014). Furthermore, the cultivar Kum Phayao was highly cytotoxic to human hepatocellular carcinoma HepG2 cells when compared to other northern Thai purple rice varieties (Banjerdpongchai et al., 2013). Nevertheless, there are no data concerning the effects of purple rice bran on the AFB_1 induced initiation stage of hepatocarcinogenesis in an animal model. Therefore, this study was designed to evaluate the genotoxicity and antigenotoxicity of the methanol extract of the bran of Kum Phayao using a liver mironucleus assay, and to

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investigate its inhibitory mechanism through xenobiotic metabolizing enzyme systems.

Materials and Methods

Chemicals and reagents

Aflatoxin B1 (AFB_1), standard gallic acid, catechin, γ-oryzanol and resorufin sodium, cytochrome c, uridine 5'-diphospho- glucuronic acid (UDP-GA), and ethoxyresorufin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methoxyresorufin was purchased from Wako Pure Chemicals (Osaka, Japan). 1-chloro-2, 4-dinitrobenzene (CDNB) was purchased from Fluka (Buchs, Switzerland). Collagenase type IV prepared from *Clostridium histolyticum* and 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Gibco/ Invitrogen Corp. (Carlsbad, CA, USA). β-Nicotinamide adenine dinucleotide phosphate (β-NADPH) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Extraction and chemical determination of purple rice bran

Purple rice (*Oryza sativa*), cv. Kum Phayao, was cultivated during August-November, 2013 in Chiang Mai University's agricultural fields, Chiang Mai, Thailand. After dehulling, rice bran was ground using a rice milling machine and soaked in dichloromethane for 72 hours at room temperature. The resulting filtrate was filtered using Whatman No. 1 sheet with a vacuum pump, concentrated by a rotary evaporator at 40°C and freeze-dried with a vacuum lyophilizer. Then the residues were soaked in methanol and concentrated via the same process. The dried extract was stored at -20°C until use. The hydrophilic parts, including total phenolic compounds and flavonoids of rice bran extract, were determined by colorimetric methods described elsewhere (Inboot et al., 2012). Various phenolic acids, flavonoids and anthocyanins were confirmed by a reverse phase HPLC technique with a C18 column (4.6 x 250 mm, 5 μm). The mobile phase for phenolic acids and flavonoids was composed of methanol and 3% acetic acid at flow rate of 1 ml/min and detected at wavelengths of 260, 280, 320 and 360 nm (Punvittatyagul et al., 2014). For anthocyanins, a mobile phase containing 0.1% trifluoroacetic acid (TFA)-H2O and 0.1% TFA-methanol with a flow rate of 1 ml/min was used, and evaluated at a wavelength of 520 nm (Ryu et al., 1998). The γ-oryzanol was analyzed at flow rate 2 ml/min of mobile phase consisting of methanol, acetronitrile, dichloromethane and acetic acid and was measured at a wavelength of 330 nm (Sankam et al., 2013).

Animals

Three weeks old male Wistar rats (approximately 70 g body weight) were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The rats were maintained in controlled environments at a temperature of 25±1°C under a 12hr dark-light cycle. Water and standard pellet diet were provided *ad libitum*. The rat protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University.

Genotoxicity and antigenotoxicity of purple rice bran extract

To study the genotoxicity of purple rice bran extract (PRBE), male Wistar rats were divided into 3 groups, 6 rats per group. Group 1, a negative control group, received 5% Tween-80, while Groups 2 and 3 were fed with 100 and 500 mg/kg body weight of methanol extract, respectively, for 28 days. On day 29, a partial hepatectomy was done in order to accelerate mutated hepatocyte proliferation. Four days after the operation, all rats were anesthetized by thiopental injection. The hepatocytes were isolated by two-step collagenase perfusion (Sankam et al., 2013). The hepatocytes were stained with DAPI and counted under a fluorescent microscope (×400), at least 2000 hepatocytes. The micronucleated hepatocytes were identified following the criteria of Igarashi et al. (2010).

To study antigenotoxicity of PRBE, Groups 1 and 2 were administered 5% Tween-80 orally, whereas Groups 3-4 were given low and high doses of PRBE. On days 21 and 25, Groups 2-4 were intraperitoneally injected with 200 μg/kg bw of AFB_1, while Group 1 was injected with a saline solution (Figure 1). On day 29, a partial hepatectomy was performed. All rats were sacrificed 4 days after the operation to isolate single hepatocytes for micronuclei determination.

Preparation of liver cytosolic and microsomal fractions

The liver was pulverized with homogenizing buffer using a tissue homogenizer. The homogenate was cold-centrifuged at 14,000 rpm for 20 min. The supernatant was further centrifuged at 30,000 rpm for 1 hour. The resulting supernatant was a cytosolic fraction and the remaining pellet was a microsomal fraction. The protein concentration of each fraction was quantified by the Lowry method using bovine serum albumin as a standard.

Determination of the activities of phase I metabolizing enzymes

The activities of cytochrome P450 (CYP) 1A1, 1A2 and 3A were determined by methoxyresorufin-O-demethylation (MROD), ethoxyresorufin-O-deethylation (EROD) and erythromycin N-demethylation (ENDM) methods, respectively (Chatuphonprasert and Jarukamjorn, 2012). Briefly, the reaction mixture containing 0.01 M Tris and 0.05 mM methoxyresorufin or ethoxyresorufin was added to 1 μM standard resorufin sodium or 10 mg/

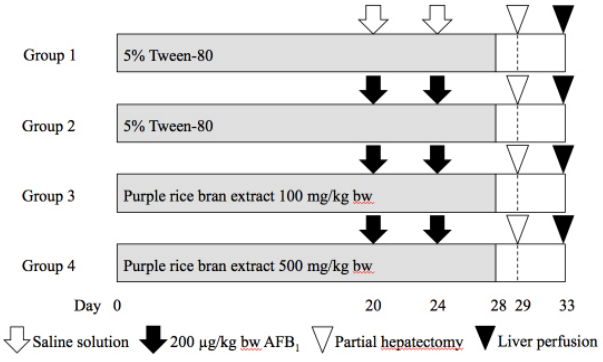

Figure 1. The Protocol for the Study of Anticlastogenicity of the PRBE

ml microsomal fraction and 0.5 mM NADPH. The activity was evaluated with a spectrofluorometer at excitation and emission wavelengths of 520 and 590 nm, respectively. The determination of CYP 3A activity was done by adding 5 mM NADPH into a reaction mixture containing phosphate buffer saline, 10 mM erythromycin and 150 mM MgCl2 with a standard formaldehyde or 10 mg/ml of liver microsome. After incubation at 37°C for 20 min in a shaking water bath, the reaction was terminated by adding 12.5% trichloroacetic acid. The supernatant obtained from centrifugation for 15 min was mixed with Nash reagent and incubated at 50°C for 15 min. The enzyme activity was recorded using a spectrophotometer at a wavelength 405 nm.

The activity of NADPH-cytochrome P450 reductase was evaluated according to the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Yim et al., 2005). The reaction mixture containing 0.3 M potassium hydrogen phosphate buffer (pH 8.7), 1 mM cytochrome c Type IV and 50 mM potassium cyanide was pre-incubated with liver microsome at 37°C for 2 min followed by addition of 10 mM NADPH. The activity was measured by spectrophotometry at OD 550 nm and calculated using a molar coefficient of 21 mM^{-1} cm^{-1}.

Determination of the activities of phase II metabolizing enzymes

The glutathione-S transferase (GST) activity was measured by colorimetry (Sankam et al., 2013). The liver cytosol was added into a reaction mixture containing 0.2 M phosphate buffer and 10 mM GSH. The reaction occurred when CDNB substrate was added to produce a CDNB-GSH conjugate. After incubation at 37°C for 20 sec, the enzyme activity was measured in a spectrophotometer at OD 340 nm and was calculated by using a molar coefficient of 9.6 $M^{-1}cm^{-1}$.

The activity of UDP-glucuronyltransferase (UGT) was measured (Summart and Chewonarin, 2014). Five mg/ml liver microsomes were pre-incubated with reaction mixture comprised of 200 mM Tris, 40 mM magnesium chloride and 5 mM *p*-nitrophenol in the dark at 37°C for 5 min. Then 20 mM UDP-GA as a co-enzyme was added to the reaction and incubated in the dark at 37°C for 20 min. The reaction was stopped with 10% TCA and centrifugation at 10,000 rpm for 5 min. The supernatant was removed and mixed with 1 M NaOH. The activity of UGT was measured by spectrophotometry at a wavelength of 405 nm.

Determination of protein expressions of phase I and II metabolizing enzymes

The hepatic xenobiotic metabolizing enzymes were separated using sodium dodecyl sulfate-polyacrylamide denaturing gel electrophoresis. The proteins were transferred to a nitrocellulose membrane for 1 hour and washed with phosphate buffer saline-0.05% Tween-20 (TPBS). After blocking with non-fat dry milk in TPBS for 2 hours, the membrane was incubated with primary specific antibody for 1 hour. The membrane was further incubated with secondary antibody containing peroxidase-conjugated anti-rabbit IgG for 30 min. Protein expression was detected by using Horseradish Peroxidase as a chemiluminescent substrate and exposed to x-ray film to evaluate band intensity.

Statistical analysis

The experimental data were reported as means±SD. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests. The differences were considered as significant when $p<0.05$.

Results

Major compounds of purple rice bran extract

The percentage yield of methanol extract of purple rice bran was 7.91±0.35%. The amounts of major constituents in the extract are shown in Table 1. Total phenolic compounds, flavonoids and gamma-oryzanol in the purple rice bran extract contained 9.131, 5.310 and 0.587 %, respectively. Protocatechuic acid and vanillic acid were identified by our detection system (Figures 2A-B). The major anthocyanin found in purple rice bran variety Kum Phayao was cyanidin-3-glucoside (Figures 2C-D). We could not detect gallic acid, catechin, epicatechin, p-coumaric acid, ferulic acid, rutin, quercetin, cyanidin-3-rutinoside, or malvidin-3-glucoside in our purple rice bran extract.

Effect of purple rice bran extract on micronucleus formation and xenobiotic metabolizing enzymes in rat liver

The administration of PRBE for 28 days did not

Table 1. Phytochemical Determination of Purple Rice Bran Extract

Compounds	Content (mg/g extract)
Total phenolic compounds	91.31 ± 5.16
Protocatechuic acid	1.00 ± 0.66
Vanillic acid	0.73 ± 0.39
Total flavonoids	53.10 ± 2.66
Cyanidin-3-glucoside	1.24 ± 0.27
Peonidin-3-glucoside	0.76 ± 0.12
Gamma-oryzanol	5.87 ± 0.01

*Values established as mean±SD

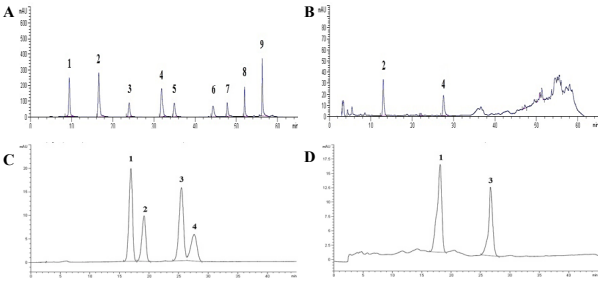

Figure 2. Chromatograms of Standard Mixture and PRBE. (A) Standard of phenolic acids and flavonoids, **(B)** phenolic acids and flavonoids in PRBE; peaks 1: gallic acid, 2: protocatechuic acid, 3: catechin, 4: vanillic acid, 5: epicatechin, 6: p-coumaric acid, 7: ferulic acid, 8: rutin, and 9: quercetin, **(C)** Standard of anthocyanins, **(D)** anthocyanins in PRBE; peaks 1: cyanidin-3-O-glucoside, 2: cyanidin-3-rutinoside , 3: peonidin-3-O-glucoside, 4: malvidin-3-glucoside

impact on body weight and did not alter the number of micronucleated hepatocytes, binucleated hepatocytes or mitotic cells compared with the control group (Table 2). Furthermore, 100 and 500 mg/kg bw of PRBE did not modulate the activities of xenobiotic-metabolizing enzymes in rat livers (Table 2).

Antigenotoxic effect of purple rice bran extract on AFB_1-induced micronucleated hepatocytes formation in rats and the possible inhibitory mechanism

Rats treated with AFB_1 in this protocol were examined for had a significant increases in the number of micronucleated hepatocytes, binucleated hepatocytes and mitotic cells compared to the negative control. The

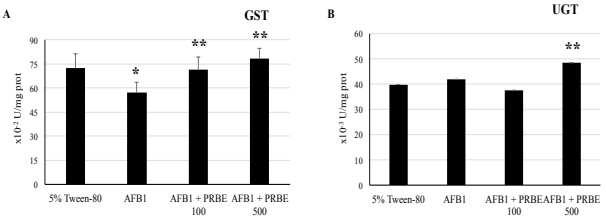

Figure 4. Effect of PRBEon the Activities of Phase II Hepatic Xenobiotic Metabolizing Enzymes of AFB_1-Induced Rats; (A) Glutathione-S transferase (GST) and **(B)** UDP-glucuronyltransferase (UGT). The data represent the mean±SD with *p<0.05 when compared with 5% Tween-80 group and **p<0.05 when compared with AFB_1 group

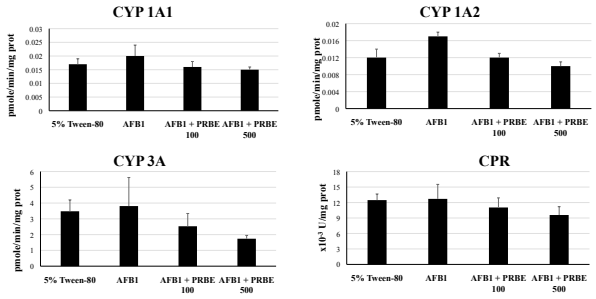

Figure 3. Effect of PRBE on the Activities of Phase I Hepatic Xenobiotic Metabolizing Enzymes of AFB_1-Induced Rats; (A) Cytochrome P450 (CYP) 1A1, **(B)** CYP1A2, **(C)** CYP3A and (4) NADPH-cytochrome P450 reductase (CPR). The data represent the mean±SD with *p<0.05 when compared with 5% Tween-80 group and **p<0.05 when compared with AFB_1 group

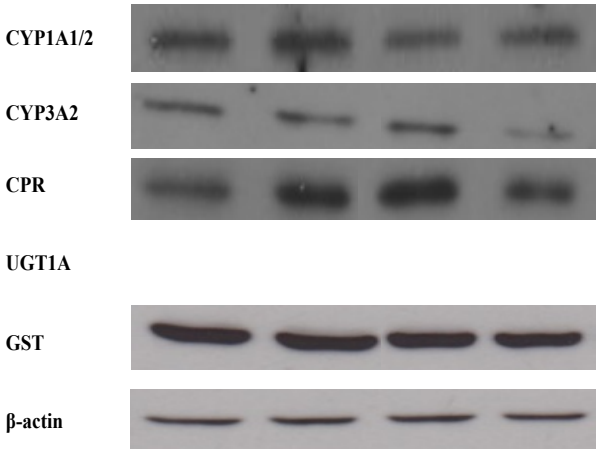

Figure 5. Effect of PRBE on the Expression of Some Xenobiotic Metabolizing Enzymes

Table 2. Effect of Purple Rice Bran Extract on Rat Liver Micronucleus Formation and Activities of Xenobiotic Metabolizing Enzymes

Parameter	5% Tween-80	Purple rice bran extract (mg/kg bw)	
		100	500
Rat body weight			
Initial (g)	110.0 ± 19.8	100.0 ± 0.0	98.3 ± 4.1
Final (g)	279.2 ± 22.9	291.7 ± 20.2	283.3 ± 11.3
MNH (per 1,000 Hep)	2.95 ± 0.76	3.46 ± 1.03	3.41 ± 0.81
BNH (per 1,000 Hep)	1.18 ± 0.30	1.19 ± 0.22	1.25 ± 0.24
MI (%)	1.19 ± 0.41	1.36 ± 0.23	1.34 ± 0.27
CYP 1A1 (pmole/min/mg prot)	0.18 ± 0.03	0.19 ± 0.03	0.18 ± 0.03
CYP 1A2 (pmole/min/mg prot)	0.25 ± 0.03	0.25 ± 0.02	0.25 ± 0.04
CYP 3A2 (pmole/min/mg prot)	12.45 ± 1.64	11.42 ± 0.80	11.15 ± 0.59
CPR (x10-3 U/mg prot)	9.90 ± 0.96	9.90 ± 0.74	10.14 ± 1.47
UGT (x10-3 U/mg prot)	36.07 ± 4.26	33.92 ± 8.00	40.21 ± 9.79
GST (x10-2 U/mg prot)	70.44 ± 5.00	69.82 ± 8.24	73.41 ± 14.84

*Values established as mean ± SD; MNH: micronucleated hepatocytes, BNH: binucleated hepatocytes, MI: mitotic index; Hep: hepatocyte

Table 3. Anticlastogenicity of Purple Rice Bran Extract by Liver Micronucleus Assay in Rats

Test group	Rat body weight (g)		MNH	BNH	MI
	Initial	Final	(per 1,000 Hep)	(%)	(%)
5% Tween-80	99.0 ± 6.5	284.0 ± 26.3	1.80 ± 0.67	0.82 ± 0.21	0.85 ± 0.21
AFB_1	97.0 ± 5.7	280.0 ± 27.6	8.68 ± 1.67*	1.82 ± 0.19*	1.93 ± 0.38*
AFB_1 + PRBE 100	98.6 ± 5.6	278.0 ± 18.9	3.99 ± 0.41**	1.36 ± 0.37**	1.31 ± 0.25**
AFB_1 + PRBE 500	95.8 ± 3.8	274.2 ± 9.7	4.16 ± 0.60**	1.30 ± 0.16**	1.34 ± 0.26**

*Values established as mean±SD; MNH: micronucleated hepatocytes, BNH: binucleated hepatocytes, MI: mitotic index, PRBE: purple rice bran extract; (*) indicates statistical significance at p<0.05 compared with 5% Tween-80 group; (**) indicates statistical significance at p<0.05 compared with AFB_1 group; Hep: hepatocyte

purple rice bran extract significantly reduced micronucleus formation and mitotic index in the liver of AFB_1-initiaed rats (Table 3). We also found that AFB_1 treatment enhanced hepatic CYP1A1 and CYP1A2 activities and suppressed detoxifying enzyme activity in rats. However, purple rice bran extract diminished the activities and the expression of some phase I enzymes, including CYP1A1, CYP1A2, CYP3A2 and NADPH-cytochrome P450 reductase and improved the activities of detoxifying enzymes, GST and UGT (Figures 3-5).

Discussion

The methanol extract of purple rice bran presented no mutagenicity in rat liver but it inhibited AFB_1 -induced mutagenicity in rats by decreasing the number of micronucleated hepatocytes. AFB_1 is metabolized by CYP1A and 3A families. The ultimately produced AFB_1, AFB_1-8, 9-epoxide, can covalently bind with DNA in the liver forming AFB_1-N^7-guanine DNA adduct in hepatocytes resulting in DNA mutation (Hamid et al., 2013). The initiating consequence persists when DNA damage is neither correctly nor completely repaired prior to DNA synthesis. This initiation step of AFB_1-induced hepatocarcinogenesis occurs rapidly and is irreversible. Our study was designed to detect the micronucleus, a lesion caused by chromosomal fragmentation in the liver, within several days after AFB_1 treatment and induction of cell division by partial hepatectomy. Thus micronucleated hepatocytes are recognized as one biomarker of hepatocarcinogenesis.

Purple rice (*Oryza sativa* var. indica) is cultivated in Northern and Northeastern Thailand. This present study found that the purple rice bran extract did not increase the number of micronucleated or binucleated hepatocytes, nor did it alter the mitotic index of hepatocytes, indicating a lack of mutagenic and mitogenic activities. Furthermore, the purple rice bran extract could prevent the first stage of AFB_1-initiated hepatocarcinogenesis in rats. It inhibited some metabolic activating enzymes involved in AFB_1 metabolism including CYP1A1, CYP1A2, CYP3A2 and NADPH-cytochrome P450 reductase. These phase I enzymes are commonly induced by AFB_1 to generate a more polar structure, which is highly reactive (Bbosa et al., 2013).

The detoxifying enzyme system aims to remove xenobiotic substances from the body. The metabolites produced by metabolic action of phase I enzymes are mostly eliminated by conjugated reactions with some large polar molecules such as glucuronic acid, sulfate and glutathione by UDP- glucuronyltransferases (UGT), sulfotransferases and glutathione S-transferase (GST), respectively (Shimada, 2006). The purple rice bran extract modulated the activities of some hepatic detoxifying enzymes including GST and UGT in AFB_1-initiated rats. These data suggest that purple rice bran extract might inhibit micronucleus formation in rat liver by attenuation of some xenobiotic metabolizing enzymes in AFB_1 metabolism.

Cancer chemoprevention is now considered to be a target for cancer treatment. Cancer chemopreventive agents in natural products can be classified into 2 types depending on their actions (Surh, 2003). Blocking agents involve the early stage of carcinogenesis by regulation of procarcinogen transformation and either improvement of the efficiency of the detoxifying system or via the DNA repair process. The agents that are able to suppress cancerous cell proliferation of initiated cells in promotion and progression stages are called suppressing agents. Our results suggest that the active components in purple rice bran extract that influence AFB_1 bioactivation may act as blocking agents.

Phenolic compounds have been well recognized as chemopreventive phytochemicals. Purple rice bran extracted by methanol contained high amounts of phenolic compounds including phenolic acids, flavonoids and anthocyanins. Numerous studies have shown that flavonoids can either inhibit several isozymes of cytochrome P450 or enhance phase II metabolizing enzymes (Moon et al., 2006). Furthermore, protocatechuic acid, by either itself or as a metabolite of cyanidin, was found to be an inhibitor of CYP1A1 and CYP1A2 as well as an inducer of GST in rat liver (Krajka-Kuzniak et al., 2005; Kay et al., 2006). γ-Oryzanol is one beneficial constituent of rice bran; however, it has a slightly inhibitory effect on cytochrome P450 function (Umehara et al., 2004). These data indicate that some flavonoids and protocatechuic acid might be the important xenobiotic metabolizing enzyme modulators in purple rice bran extract.

In summary, the methanol extract of purple rice bran contained high amounts of phenolic compounds and exhibited cancer chemopreventive effects on the AFB_1-induced initiation stage of hepatocarcinogenesis in rats. The inhibitory mechanism might be partly due to the modulation of some xenobiotic metabolizing enzymes involved in AFB_1 metabolism.

Acknowledgements

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