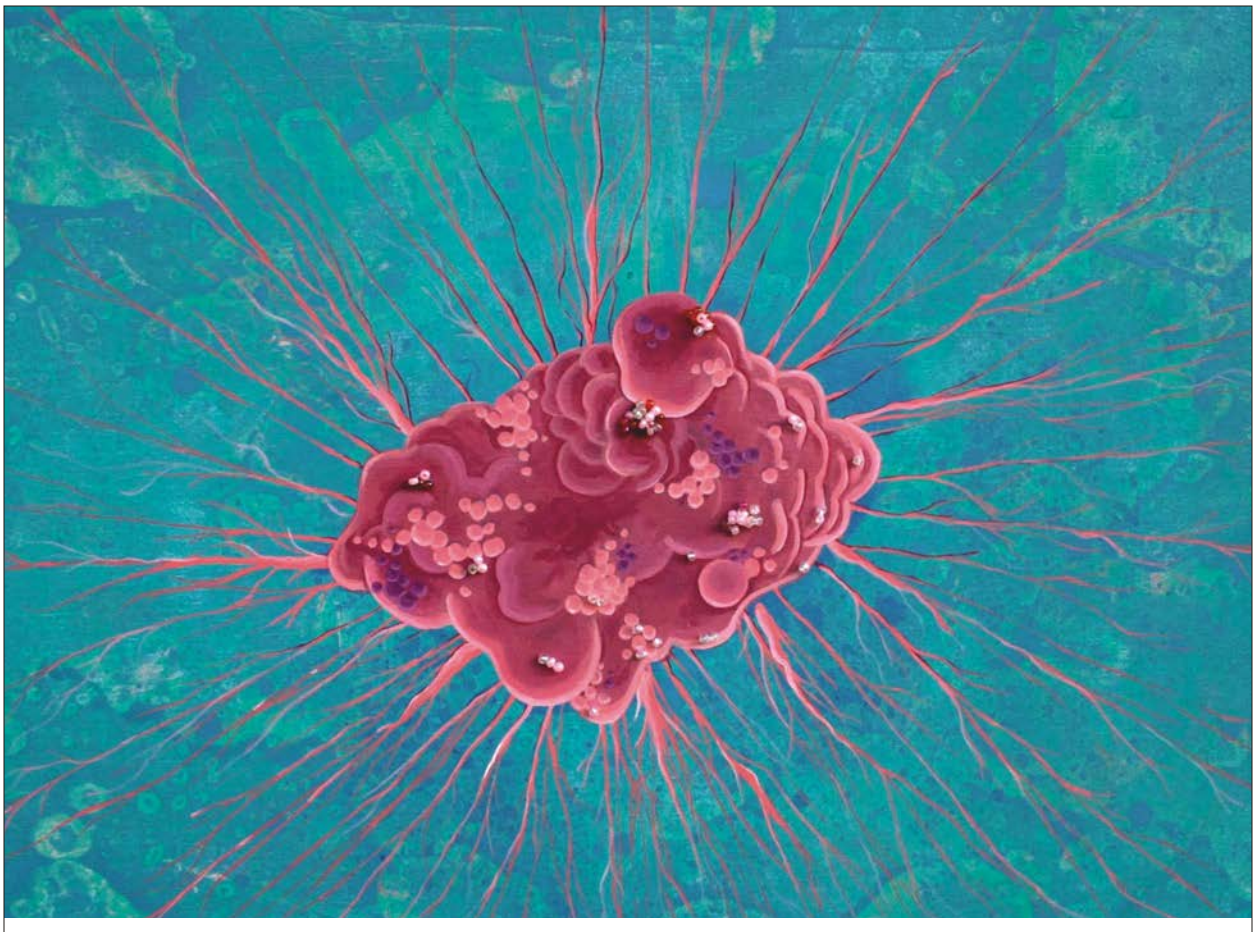

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Protective Effects of Rice Bran on Chemically Induced Colon Tumorigenesis may be Due to Synergistic/Additive Properties of Bioactive Components

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Abstract: In this study we examined the preventive properties of Rice Bran (RB) and germ on the incidence of azoxymethane induced colon tumorigenesis in Fisher 344 male rats. We also examined the cytotoxic and apoptotic properties of RB using an *in vivo* model. Tumor incidence (%) in C and RB 5% and RB 10% were 100, 55 and 64, respectively. Tumors/tumor Bearing Rats (TBR) were 3.8, 2 and 1.56 for C, RB 5% and RB 10%, respectively. Tumor size (mm) was larger in control (6.50) than in rats fed RB 5% and RB 10% (1.33 and 0.64). After 12, 24 and 48 h of incubation with RB extracts, LDH (%) release ranged from 2.25-46.79. Present results suggest that feeding RB at 5 and 10% levels significantly ($p<0.05$) reduced the incidence of AOM induced colon tumors in Fisher 344 male rats. We conclude that the protective effects of RB against colon tumorigenesis may possibly be attributed to the synergistic/additive actions of phytochemicals contained in RB.

Key words: Rice bran, antioxidant, azoxymethane, aberrant crypt foci, end-point tumor study

INTRODUCTION

Despite efforts focused on chemoprevention, colon cancer still remains the second leading cause of cancer deaths in the Unites States (American Cancer Society, 2008).

Diet plays a major role in both the prevention and onset of many chronic diseases such as cancer, cardiovascular diseases (CVD) and diabetes. Diets derived primarily from edible plant sources such as grains and cereals are beneficial for the prevention of diseases and maintenance of good health.

Dietary fiber is one of the most important sources of non-nutritive compounds that provide the potential of preventing chronic diseases. Numerous data have shown the importance of dietary fiber in improving levels of blood cholesterol, cardiovascular diseases (CVD), diabetes and cancers of the gastro-intestinal tract (Reddy, 1999; Levi *et al*., 2001; Peters *et al*., 2003; Mahadevamma *et al*., 2004). Although, there are a wide range of terms, dietary fiber originates mostly from plant sources such as fruits, vegetables, legumes and cereals; and is thus defined as the cell wall polysaccharides of plants that cannot be hydrolyzed by the human digestive system (Harris and Ferguson, 1993; Ferguson and Harris, 1996). Besides plant cell wall components such as cellulose, pectin and lignin, dietary fibers encompass resistant starches, oligosaccharides and non-starch polysaccharides such as seaweeds (e.g., gums and mucilages) (Coudray *et al*., 2002).

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Despite its health promoting capabilities, dietary fiber is classified as soluble or insoluble which according to Coudray *et al*. (2002) reflects the different physiochemical properties and their abilities to produce different biological effects. Among the numerous sources of dietary fibers, cereal brans are the most studied and their protective effects against diseases is noteworthy. One of the cereals of particular interest is rice. Rice is one of the most important cereal crops that is a staple for a majority of the world's population (Bird *et al*., 2000). When rice undergoes the milling process to remove the bran, almost all of its nutrients, i.e., minerals and vitamins are lost along with the bran.

Besides providing protein and minerals, rice bran is an excellent source of vitamin B and E, especially tocotrienols. It also contains polyphenolic compounds that have been shown to interfere with the proliferation or colony-forming ability of breast or colon cells (Hudson *et al*., 2000). Harris *et al*. (1998) stated that while different cereal bran species appears to have different protective effects when exposed to carcinogens, rice bran when compared to oat and barley brans has a superior protective effect. While many foods contain several disease fighting components, some such as rice bran posses' novel constituents such as tocotrienols, sitosterol ferulate, cycloartenol ferulate and gamma-oryzanol that have antioxidative and antigenotoxic activities (Yasukawa *et al*., 1998; Hudson *et al*., 2000; Nam *et al*., 2005). Other phenolic constituents such as phytic acid was shown to exhibit anticancer properties through the regulation of vital cellular functions such as signal transduction, cell proliferation and differentiation (Vucenik and Shamsuddin, 2006). The bio active components of rice bran, sitosterol ferulate, 24-methylcholesterol ferulate, cycloartenol ferulate and 24-methylenecycloartanol ferulate were shown to inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice (Yasukawa *et al*., 1998).

Despite the fact that rice bran is abundantly produced in the rice milling industry, it is still underutilized and is yet to gain recognition as an excellent source of phytonutrients with health promoting properties. As such, there is a need to explore it beneficial uses, especially as it relates to health.

Azoxymethane (AOM), a metabolite of 1, 2-Dimethylhydrazine (DMH) an organotropic colon carcinogen has been extensively used to induce colon carcinogenesis in susceptible laboratory animals (Papanikolaou *et al*., 1998; Dommels *et al*., 2003). The AOM is generally preferred to DMH because it is a more potent carcinogen than DMH based on molarity and has an enhanced chemical stability in solutions (Papanikolaou *et al*., 1998). Most studies have reported that two successive injections of AOM, 1 week apart, are adequate in inducing colon cancer in rats. Induction by AOM is the most popular experimental model often used to identify dietary modulations of colon cancer (Dommels *et al*., 2003).

Based on these observations, we examined the putative effects of Rice Bran (RB) with germ on azoxymethane (AOM) induced colon carcinogenesis in Fisher 344 male rats. We also determined the cytotoxic and antiproliferative effects of RB extracts in CaCo-2 colon cells.

MATERIALS AND METHODS

Animal Housing and Diets

After a one week period of acclimatization a total of 42 Fisher 344 male weanling rats (3-4 weeks old) (Harlan, IN) were assigned to 3 groups (Fig. 1). The Rats in the end point study were initially fed AIN-93G (Reeves *et al*., 1993a, b) as control (C) and AIN-93G +5% and 10% RB (The RiceX company, Phoenix, AZ) diets (Table 1) and switched to AIN-93M at 20 weeks of age until the end of the study. Relative humidity and temperature were

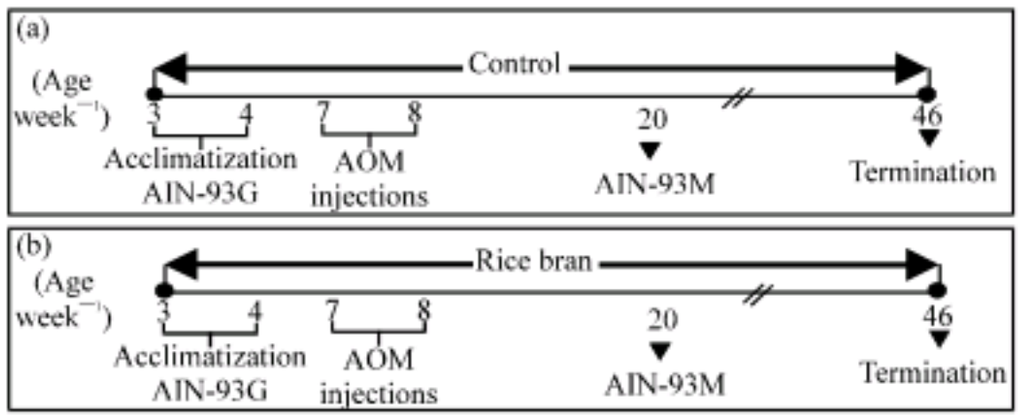

Fig. 1: Schematic representation of feeding (a) control and (b) RB diets in EPT study. Scale is not proportional, Control diet is based on AIN-93G/M (Reeves *et al.*, 1993a, b). Rice bran was fed at 5 and 10% levels, EPT: End point tumor

Table 1: Composition of diets[a] in experiment

Ingredients (g kg^{-1})	C (AIN-93G/M)	RB (5%)+(AIN-93G/M)	RB (10%)+(AIN-93G/M)
Cornstarch	397.5/465.7	397.5/465.7	347.5/415.7
Sucrose	100/100	100/100	100/100
Casein	200/140	200/140	200/140
Fiber	50/50	0/0	0/0
Soybean oil	70/40	70/40	70/40
Rice bran	0/0	50/50	100/100
Common ingredients[b]	182.5/204.3	182.5/204.3	182.5/204.3

Formulations of diets based on AIN-93G (American Institute of Nutrition, Reeves *et al*., 1993a, b); [a]Experimental diets: C-control; RB-rice bran. [b]Common ingredients (g): dextrose, 132; mineral mix (AIN-93G), 35; vitamin mix, 10; L-cystein, 3; choline bitatrate, 2.5

maintained at 50% and 21±1°C, respectively and dark and light cycles were held at 12 h intervals. Body weights were recorded every two weeks and daily feed intake was recorded. Diets were prepared biweekly and kept at 4°C for freshness. Ingredients for preparation AIN-93 diets were obtained from ICN (Costa Mesa, CA). The study was conducted at Alabama A and M University Food and Animal Sciences Department in July, 2007 and all protocols involving rats were approved by the Institutional Animal Care and Use committee of Alabama A and M University.

Aom Injection and Sample Collection

To induce ACF and colon tumors all animals except saline controls received 2 subcutaneous injections of azoxymethane (Sigma Chemicals, St. Louis, MO) at 7 and 8 weeks of age. The AOM was administered in saline at 16 mg kg^{-1} body. Rats were killed by CO_2 asphyxiation at 17 and 45 weeks of age and colons were removed and flushed with potassium phosphate buffer (0.1 mol L^{-1}, pH 7.2) and prepared for counting tumors. Colons were removed for ACF enumeration and liver samples and colonic mucosal scrapings were collected and stored at -80°C until analysis for Glutathione-S-Transferase (GST) activity. Cecum of rats was weighed and pH of cecal contents was noted.

Enumeration of Aberrant Crypt Foci (ACF)

Colons of rats from each group were flushed with PBS (0.1 M, pH 7.2) and ACF and crypts per focus were enumerated as described by Bird (2000).

Characterization of Colon Tumors

Colon tumors were collected and characterized as described by Shackelford *et al*. (1983), according to size, location, number and tumors per Tumor Bearing Rat (TBR).

Preparation of Liver and Colonic Tissues for Glutathione-s-Transferase (GST) Analysis

The GST activity in rat liver cytosol and colonic mucosal scrapings was assayed following the methods described by Habig *et al*. (1974). Briefly, 1 g of tissue sample was homogenized in 10 mL of potassium phosphate buffer (0.1 M, pH 7.2) and centrifuged at 10,000 rpm for 30 min. The clear supernatant was mixed with 1, chloro2, 4-dinitrobenzene (CDNB) (Fisher Scientific, Suwannee, GA), potassium phosphate buffer and glutathione reductase (Sigma chemicals, St. Louis, MO). One unit of GST activity is expressed as the amount of enzyme required to conjugate 1 µmole of CDNB with GSH per minute. The resulting product was analyzed using a UV/VIS dual beam spectrophotometer (Cary1/3, Varian) at 340 nm.

Preparation of Rice Bran Extracts

Defatted rice bran was extracted with 80% methanol (v/v) (at 1:10 ratio) for approximately 12 h at ambient temperature. The methanolic extracts were then filtered through Whatman No. 2 paper to remove residue. The filtrates were concentrated under restricted light using rotary evaporation (40°C) and the phenolic concentrate was made to a final volume of 10 mL with distilled water. The crude Rice Bran (RB) extracts were immediately stored at -80°C after flashing with nitrogen gas.

Cell Culture Experiment

Cell Culture

CaCo-2 cells were purchased from American Type Culture Collection (ATCC, Mannasas,VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1.0 mM sodium pyruvate. Cells were incubated at 37°C, 5% CO_2 until confluent (70-90%). For experiments, cells were seeded in 12 well tissue culture plates at a density of 3.28×10^5. Cells were incubated as previously specified until a monolayer developed. After the development of a monolayer (70-80% confluence), cells were rinsed twice with PBS and incubated for 24 and 48 h with 800 µL of fresh media (serum free) containing different concentrations (0-1000 µg mL^{-1}) of rice bran extracts. Serum free media containing no RB extracts served as control. After indicated incubation times, the culture supernatant was removed and used to assess lactate dehydrogenase (LDH) (EC 1.1.1.27).

LDH Assay

Cytotoxicity of RB was measured by the release of LDH from the CaCo-2 cells into the culture supernatant. The LDH release was quantified using a calorimetric cytotoxicity detection kit (LDH) (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Assay was performed in triplicate.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide) Assay

Cell viability was measured using an MTT assay kit (Roche Diagnostics, Indianapolis, IN). Briefly, Caco-2 cells were seeded at a density of 1.2×10^4 cells $well^{-1}$ into a 96-well plate in final volume of 100 µL of media. After 24 h post seeding, the cells were exposed to different concentrations of RB (0-1000 µg mL^{-1}) and maintained in culture for 24 and 48 h at 37°C in a humidified atmosphere containing 5% CO_2. After treatment, the cells were incubated for 3 h at 37°C with a solution of MTT following manufacturer's instructions. The absorbance of the reduced intracellular formazan product was read at 550/630 nm on a synergy HT micro plate reader. Each assay was performed in triplicate.

Statistical Analysis

Data from three experiments were combined (cell culture study) and analyzed using the SAS statistical program (2007). Results were performed by ANOVA and values are given as Means±SEM. Means were separated using Tukey's studentized range test. Differences between treatment groups were tested by student's t test and paired t test. Unless otherwise indicated the level of significance was considered significant at $p<0.05$.

RESULTS

Body Weight Gain, Feed Intake and Cecal Weight and pH

Although, daily feed intake was similar in the treatment groups in the ACF study, weight gain in rats fed Rice Bran (RB) was significantly ($p<0.05$) lower compared to rats fed the control (Table 2). We detected a 7 and 15% decrease in weight gain compared to the control. In the EPT study however, feed intake in the rats fed 10% RB was significantly ($p\leq0.05$) higher compared to the rats fed 5% RB and the control group. Even though daily feed intake and weight gain in rats fed RB was significantly ($p\leq0.05$) higher compared to the control group, among the rats fed RB, the group given 5% RB weighed significantly ($p\leq0.05$) more than the group fed 10% RB. Overall weight gain was 34 and 18% higher in the rats fed RB at 5 and 10%, respectively, compared to the control. In both the ACF and EPT studies, cecal weight in rats fed 10% RB was significantly ($p\leq0.05$) higher than in rats fed 5% RB and the control. The cecum was nearly twice as large in the 10% RB fed rats compared to the control and over 30% larger than their 5% counterparts. Since, cecal pH is inversely proportional to cecal weight, cecal pH was seen to be significantly ($p\leq0.05$) lower in 10% RB fed rats.

Aberrant Crypt Foci (ACF), Total Colonic Aberrant Crypts and Crypt Multiplicity

The ACF incidences in rats fed experimental diets are shown in Table 3. As detected, ACF in the distal colon were significantly ($p<0.05$) higher compare to distal colons in all the

Table 2: Weight gain, feed intake, cecal weight and cecal pH in Fisher 344 male rats

	*ACF study		
	Control	5% RB	10% RB
Weight gain (g 13 weeks^{-1})	212.70±9.0^{a}	197.20±6.50^{b}	180.00±5.9^{b}
Feed intake (g day^{-1})	12.60±0.2	13.83±0.49	13.70±0.4
Cecal weight (g)	2.25±0.2^{a}	1.92±0.08^{a}	1.15±0.2^{b}
Cecal pH	7.49±0.0^{a}	7.58±0.11^{a}	6.23±0.0^{b}
	EPT study		
	Control	5% RB	10% RB
Weight gain (g 41 weeks^{-1})	235.20±12.5^{b}	355.67±6.50^{a}	286.50±7.6^{b}
Feed intake (g day^{-1})	16.70±0.2^{b}	15.78±0.49^{b}	19.67±0.4^{a}
Cecal weight (g)	1.10±0.1^{b}	1.54±0.40^{b}	2.41±0.1^{a}
Cecal pH	7.72±0.0^{a}	7.54±0.40^{a}	6.62±0.0^{b}

Values are Means±SEM, *n = 4. ^{ab}Means in a row with the same superscript do not significantly differ ($p<0.05$) using Tukey's studentized test. RB: Rice bran

Table 3: Number of aberrant crypt foci in colon of azoxymethane-induced Fisher 344 male rats

Treatment	Proximal colon	Distal colon	Total ACF
Control (C)	40.0±0.94^{a}	107.60±1.70^{a}	147.60
5% RB	36.6±5.95^{a}	46.80±6.30^{b}	83.40
10% RB	22.0±0.75^{b}	36.25±2.30^{c}	58.25

Values are expressed as means±SEM; n = 4; ^{abc}Means in the same column with the same letter(s) are not significantly different by Tukey's studentized range test ($p\leq0.05$); RB: Rice bran

experimental groups. While there were no significant differences in the number of ACF in the proximal colons of rats fed the lower dose RB (i.e., 5%) and the control, ACFs were decreased by approximately 45% when compared to the group fed 10% RB. In the distal colon ACF were significantly ($p<0.05$) lower in the treatment groups compared to the control with reductions of 57 and 66%, respectively. Overall total number of ACF developed in the treatment groups were decreased by 44 and 61%, respectively. Similarly, total aberrant crypts/colon was significantly ($p<0.05$) lower in the treatment groups compared to the control with decreases of 55 and 71%, respectively (Table 4). Among the treatment groups we detected 30% and 35% reductions in total ACFs and total aberrant crypts/colon, respectively, when rats were fed 10% compared to 5% RB. Crypt multiplicity is crucial when using ACF as a colon cancer biomarker. ACF consisting of four or more crypts has been reported as putative premalignant lesions for colon cancer development (Seraj *et al*., 1997). In this study we noted that crypt multiplicity, especially aberrant crypts with 3, 4 and ≥5 foci was significantly ($p<0.05$) lower in the groups fed RB compared to the control (Fig. 2). The ACF with ≥3 crypts were predominantly seen in the distal colon.

Distal and Proximal Tumors

All the rats fed the control diet developed tumors compared to 54 and 64% in the groups fed RB at 5 and 10%, respectively (Table 5). While there were no tumors in the proximal colon in rats fed 10% RB, there was 55% incidence of proximal tumors in the 5% RB fed group. There was however, 100% tumor incidence in the distal colon of all rats. Earlier studies have shown that distal segments of the colon had significantly higher numbers of tumors than the proximal segments (Verghese *et al*., 2002).

Tumors/Tumor-Bearing Rat Ratios (TBR) and Tumor Size

The total number of tumors in the proximal and distal colon in the control group was significantly ($p<0.05$) higher compared to the rats fed RB. The RB fed rats had a 75% reduction in the total number of tumors/rat (Table 6). The number of tumors/rat in the distal colon was significantly higher than the proximal colon in all the experimental groups. Distal

Table 4: Aberrant Crypt Foci (ACF) and total crypts in Fisher 344 male rats

Groups	No. of rats in study	No. of total ACF/colon	No. of total aberrant crypt/colon	No. of total aberrant crypt/focus
Control	8	147.60	382.4[a]	2.60
5% RB	8	83.40	173.8[b]	2.10
10% RB	8	58.25	111.0[c]	1.89

Values are expressed as Means±SEM; n = 4; [abc]Means in a row similar superscripts are not significantly different by Tukey's studentized range test ($p\leq0.05$); RB: Rice bran

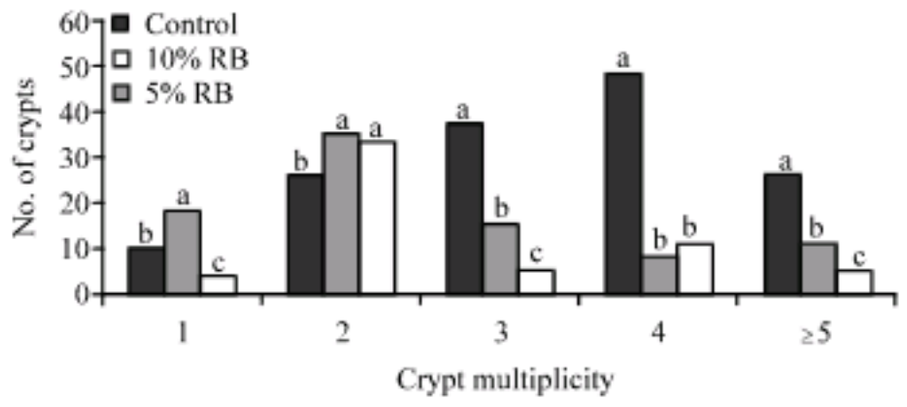

Fig. 2: Rice bran meal on crypt multiplicity in Fisher 344 male rat, [abc] Bars without a common letter significantly differ ($p<0.05$) using Tukey's studentized range test, RB: Rice bran

Table 5: Percent incidence (%) of colon tumors in Fisher 344 male rats

Treatment	N^1/N^2	Colon tumors (%)	Proximal tumors (%)	Distal tumors (%)
Control (C)	13/13	100	16	100
5% RB	6/11	54	55	100
10% RB	8/14	64	0	100

N^1 represents the number of rats with tumors; N^2 is total number of rats at the end of the experiment; RB: Rice bran

Table 6: Distribution and characterization of AOM induced colon tumors in Fisher 344 male rats

Treatment	N^1/N^2	Proximal tumors (n)	Distal tumors (n)	Total number of tumors (n)	Tumor size (n)	Tumors/tumor bearing ratio (TBR)
Control (C)	13/13	22.0^a	57^a	57	6.50^a	3.80^a
5% RB	6/11	3.0^b	12^b	12	1.10^b	2.00^b
10%RB	8/14	0.0^c	12^b	14	0.64^c	1.56^b

Values are Means±SEM, ^{ab}Means in a column with the same letter(s) do not significantly differ ($p<0.05$) using Tukey's studentized test. N^1 represents the number of rats with tumors; N is^2 total number of rats at the end of the experiment. RB: Rice bran

Table 7: Glutathione-S-Transferase (GST) activity in rats

	Glutathione S-Transferase activity (μmol mg^{-1} protein)		
Treatment	Hepatic (ACF study)	Hepatic (EPT study)	CMS (EPT study)
Control	20.38 ± 1.40^b	18.12 ± 2.3^b	3.14 ± 0.10^b
5% RB	31.34 ± 0.34^a	28.61 ± 1.1^a	6.20 ± 0.41^a
10% RB	38.32 ± 2.70^a	30.83 ± 1.1^a	5.84 ± 0.30^a

Values are expressed as Means±SEM. ^{ab}Means in the same column with the same letter are not significantly different by Tukey's studentized range test ($p\leq0.05$). CMS: Colonic mucosal scrapings, RB: Rice bran

tumors/rat was approximately 74 and 65% lower in rats fed 5% RB and 10% RB, respectively compared to the control. Tumor size (mm)/rat were over six and ten times larger in the control (6.50) compared to the rats fed 5% RB (1.1) and 10% RB (0.64), respectively. Tumors/tumor Bearing Rat Ratio (TBR) was significantly ($p\leq0.05$) lower in the rats fed RB compared to the control (Table 6). The 5% RB fed rats had a 47% reduction in TBR value, while the rats fed 10% RB exhibited the greatest reduction in TBR (65%) compared to the control.

Total Glutathione-S-Transferase (GST) Activity

The GST (μmol mg^{-1} protein) activity in the liver of rats fed RB in the ACF and EPT studies were significantly ($p\leq0.05$) higher compared to the control (Table 7). Residual GST activity in the Colonic Mucosal Scrapings (CMS) was also higher in the rats fed RB (6.2 and 5.84 for 5 and 10% RB) compared with those fed control (3.14). The GST activity in the hepatic tissue was higher compared to CMS, this is because the liver is the primary site for detoxification.

LDH Cell Viability Assay

The LDH release which represents cytotoxic effects on the cells corresponding to total cell lysis is shown in Fig. 3. Present study showed that LDH release as assessed in the cell culture medium after exposure to RB extracts following incubation for 12, 24 and 48 h was significantly ($p<0.05$) increased with increasing order of concentration. LDH release (%) ranged from a low of 2.25 (50 μg mL^{-1}) to a high of 46.79% (800 μg mL^{-1}). The highest release (46.79%) of LDH was seen after 48 h at all concentrations. The results also showed a dose dependant increase in LDH release when cells were exposed to RB extracts for 12 and 24 h.

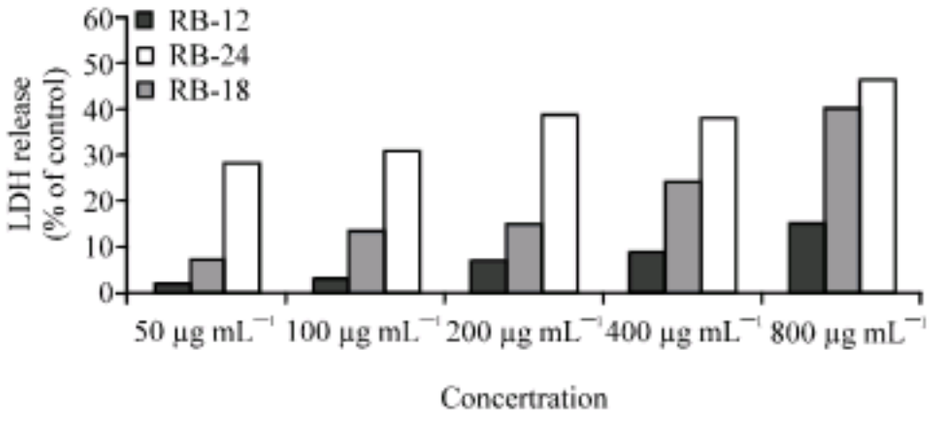

Fig. 3: LDH release in CaCo-2 cells after addition of different concentrations of RB extracts (50-800 µg mL^{-1}). Data are presented as Means±SEM of three replicates ($p<0.05$) using Tukey's studentized test

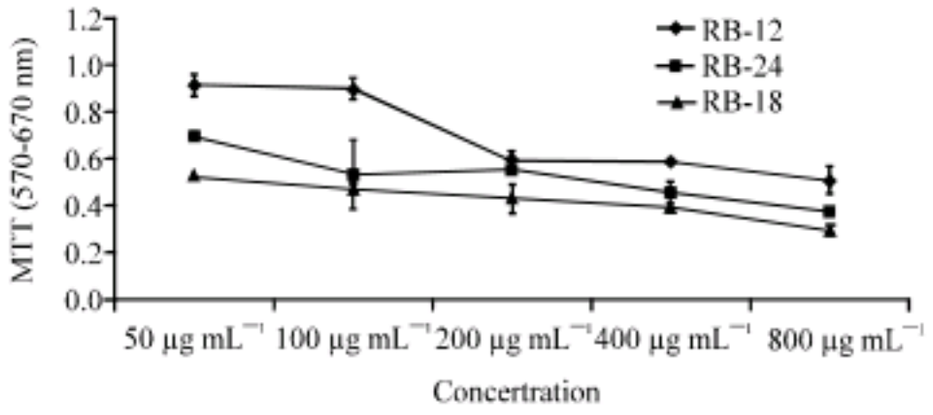

Fig. 4: The MTT in CaCo-2 cells after addition of different concentrations of RB extracts (50-800 µg mL^{-1}). Data are presented as Means±SEM of three replicates ($p<0.05$) using Tukey's studentized test

Cell Proliferation Using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Reduction Assay

To evaluate the effects of RB extracts on cell proliferation, CaCo-2 cells were cultured in RB-supplemented media (Fig. 4). The results indicated that RB extracts at all the concentrations tested inhibited CaCo-2 cell proliferation. Antiproliferative effects of RB extracts against CaCo-2 cells from decreasing order of concentration ranged from 0.51-0.92, 0.37-0.70 and 0.29-0.52 after 12, 24 and 48 h incubation.

DISCUSSION

The aim of this study was to determine the effects of Rice Bran (RB) with germ at 5 and 10% levels on AOM induced colon cancer in Fisher 344 male rats. Present results clearly indicate that rice bran reduced the number of ACF and colon tumors in Fisher 344 rats. One of the mechanisms associated with the observed results may be that the dietary fiber in rice bran which is slowly fermented may exert its protective effects through physical dilution of gut contents, by its dilution potential and fecal bulking capacity (Ferguson and Harris, 1996; Zoran *et al*., 1997). This property is thought to shorten transit time, thus leading to alterations in the mutagenicity of intestinal contents, mucosal cytokinetics and the subsequent effects on excretion of putative carcinogens (Ferguson and Harris, 1996).

Another aspect to consider is the production of butyrate. Butyrate regardless of the fiber source is linked with protection against the initial stages of colon carcinogenesis (Perrin *et al*., 2001). The primary function of butyrate is its ability to arrest the growth of

neoplastic colonocytes and inhibit the preneoplastic hyperproliferation induced by some tumor initiators and promoters (Valesquez *et al*., 1996; Dongowski and Proll, 2002).

Rice bran contains several components that may have contributed to the reduction in numbers of tumors seen in this study. Phytic Acid (PA), which is one of the constituents in rice bran, is a negatively charged molecule capable of binding proteins and starch (Rickard and Thompson, 1998). This leads to their reduced absorption and as a result increases fecal bulk (Rickard and Thompson, 1998).

The number of ACF in the distal colon has been found to be associated with markers of fermentation in the cecum, including SCFA and ATP concentration. Although, we observed a reduced incidence of ACF and tumors in the proximal compared to the distal colon in the RB fed groups compared to the control, rats fed rice bran also had significantly ($p \leq 0.05$) lower numbers of ACF and tumors in the distal colon compared to the control group. In some studies (Topping *et al*., 1997; Muir *et al*., 1998, 2004), fine fibers or some Resistant Starches (RS) were shown to ferment in the proximal region of the colon, while coarser fibers shifted the site of fermentation further down to the distal colon. On the other hand in humans or in rodents that have been experimentally induced with a chemical carcinogen, colon tumors appeared in the distal portions of the colon (Bufill, 1990; Holt *et al*., 1996; Henningsson *et al*., 2002). Magnuson *et al*. (2000) also reported that Fisher 344 male rats that received two successive AOM injections at 15 mg kg^{-1} $s.c.^{-1}$ beginning at 7 weeks of age developed tumors in distal portion of the colon.

In the EPT study, rats consuming 5% RB weighed significantly ($p \leq 0.05$) more and consumed similar amount of feed as the control. The control rats had a greater tumor burden compared to the RB fed groups and this may have caused malabsorption of nutrients leading to lower weight gain. Also, the increased energy available to this group could be as a result of the production of SCFA. In a study Zoran *et al*. (1997) postulated that luminal acetate and a large proportion of propionate, which is not used by the colonic epithelial cells, is transported to the liver and metabolized or utilized in lipid synthesis. We noted that weight gain in 10% RB fed rats was similar to the control. This may be due to the satiety effects associated with cereals rich in fiber. Furthermore, the intake of diets high in fiber decreases the intake of energy. Previous studies have shown a positive correlation between high-energy intakes and cancer risk (Andersson *et al*., 1996; Furberg and Thune, 2003; Slattery *et al*., 2005).

Present results showed a positive correlation between a lower cecal pH and reduction in ACF and tumor formation. Low pH, which results from fermentation of dietary fiber resulting in the production of Short Chain Fatty Acids (SCFA), is believed to prevent the overgrowth of pH-sensitive pathogenic bacteria. Earlier studies on other dietary fiber sources have indicated that the major genera of colonic bacteria, Bifidobacterium and Lactobacillus are generally not pathogenic. These organisms augment resistance to disease by reducing pathogenic and putrefactive bacteria by lowering pH (Keenan *et al*., 2006). Consequently, these organisms contribute to the stabilization of the microflora and ultimately to the health of the host. An acidic cecal pH is indicative of fecal pH reduction and fecal pH has been suggested to be a possible factor in suppression of colon tumorigenesis (Verghese *et al*., 2002a, b).

In the current study feeding RB at 5 and 10% levels resulted in 43 and 60% reductions in ACF study and in the EPT study by 47 and 65%, respectively. However, Kawakawa *et al*. (1999) reported 20% reduction in ACF in rats fed 2.5% rice germ. The authors also showed reductions of 28 and 46% when rats were fed 2.5% rice germ and gamma-aminobutyric acid (GABA)-enriched defatted rice-germ. It is possible that the increased reductions in ACF and

tumors observed in our study are a result of the increased dosage of RB that was fed to the rats. In addition, the RB used in this study contained equal mixtures of the bran and germ which may also have contributed to the tumor inhibitory effects observed.

To our knowledge, we show for the first time the effect of feeding rice bran on phase II enzyme (GST) activity. Present results indicated an increase in GST activity in rats fed RB based diets. The ability of butyrate, a byproduct from fermentation of dietary fiber which has been reported to increase phase II detoxifying enzymes such as glutathione S-transferase (GST) (Beyer-Sehlmeyer *et al*., 2003; Knoll *et al*., 2005) may have contributed to the detoxification of dietary carcinogens. Induction of GST is one of the chemopreventive effects of phytochemicals (Jung *et al*., 2006).

It is evident based on present results that the protective effects of grains such as rice bran on colon cancer might be attributed to the presence of dietary constituents other than fiber or antioxidants in these foods. Since, we cannot make the conjecture that the underlying reason for the reduced numbers of colon tumors in Fisher 344 rats may be attributed to fiber, it is possible to assume however, based on earlier studies that other agents besides fiber, may contribute to the antiproliferative, antioxidative and apoptotic activities of rice bran. Rice bran contains components such as fiber, polyphenols, phytic acid ferulic acid, oryzanols and other nutrients, the synergistic/additive effects of these compounds may have led to the enhanced increase in the chemopreventive effects seen in this study.

Lactate dehydrogenase (LDH) release assay and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay are the most commonly used indicators for quantitating cell viability and proliferation. The conversion of actively respiring cells, through a redox activity, converts water-soluble MTT into insoluble purple-colored MTT formazan. After solubilization a decrease in cellular MTT reduction could be an index of cell damage. On the other hand, LDH assay measures the release of the intracellular enzyme LDH upon damage of the plasma membrane and an increase in LDH release could be an index of cell damage.

Present study is one of a handful of studies to show the cytotoxic and antiproliferative properties of rice bran and germ extracts on colon cancer cell line CaCo-2. We detected cytotoxic and antiproliferative effects of RB extracts as measured by percentage LDH release and MTT against CaCo-2 cells after exposure periods of 12, 24 and 48 h in a dose-dependent and time-dependent manner. In a earlier experiment we showed the cytotoxic effect of Kidney Bean (KB) extracts against CaCo-2 cells (Boateng *et al*., 2008) and attributed the effect to constituents of KB such as flavonoids which have been reported to induce apoptosis in cancer cell lines (Paschka *et al*., 1998; Yang *et al*., 1998; Shen *et al*., 2003). Crude extracts from black Jamapa bean showed cytotoxic effect toward HeLa cells (Aparicio-Fernández *et al*., 2006). According to the authors, the cytotoxic effect of crude extract on HeLa cells may not be due to a single polyphenolic compound in the complex extract but to the sum of effects from different flavonoids in beans. In another study, Giron-Calle *et al*. (2004) found that different chickpea fractions had both cell growth-promoting and cell growth-inhibiting affects properties against CaCo-2 cell lines. Present data suggests that bioactive components present in RB extracts may be attributed for their possible anti-cancer effect. However, further studies are necessary before any definitive mechanisms can be recommended.

The present findings suggest that RB extracts exhibited antiproliferative properties in a dose dependant manner in CaCo-2 cells. Present results also showed significant antiproliferative activity with longer incubation periods. The RB extracts contain a mixture of polyphenolic compounds which display multiple effects (synergistic or antagonistic), thus

giving its bifunctional properties (cytotoxic and antiproliferative activities). In view of recent data it can be suggested that RB might have antitumor potential and that its possible role in the prevention of cancer warrants further investigations.

Since, rice bran is not widely consumed, the results of this study may provide the impetus for further research can enhance the utilization of rice bran in various nutritional formulas to promote gastro-intestinal health through alteration of gut microbiota. Rice bran can also be used in several baked and Ready to Eat (RTE) products and can also be used as a value added ingredient in meats, smoothies and infant weanling foods.

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