Introduction

In Thailand, colorectal cancer is the third most prevalent form of cancer in males and the fifth in females (Khuhaprema and Srivatanakul, 2008). Aside from genetic predisposition, age and behavioral risk factors include high intake of fats and low fiber. Therefore, improving diet and lifestyle or receiving food supplements with natural chemopreventive agents may help prevent colon cancer (Hemminki, 1993). Some natural phytochemicals have been shown to have preventative effects on colon cancer, such as curcumin, which could induce apoptosis in HCT-116 human colon cancer cells (Watson et al., 2008). Cranberry extracts and cranberry juice demonstrated antiproliferative activity against two colon cancer cell lines; HT-29 and LS-513 (Vu et al., 2012). Moreover, red wine inhibited colon adenocarcinoma cell proliferation by G2/M cell cycle block and reduction in cyclin D1 expression (Gomez-Alonso et al., 2012).

The most common forms of rice consumed in Thailand are white and brown rice. Rice genotypes with red, purple or black bran layers have been traditionally cultivated in southeast Asia as well (Sutharut and Sudarat, 2012). Purple rice (Oryza sativa L. indica) is cultivated widely in the lower northern area of Thailand. This variety of rice has been used as a traditional Thai medicine as well. Kum Doi Saket, a variety of northern purple rice contains several bioactive compounds, including anthocyanins, gamma-oryzanol and vitamin E derivatives (Boonsit et al., 2010). Punyatong (2008) reported that anthocyanin from purple rice extract inhibited the growth and induced apoptosis on myeloma cells (x63). Purple rice extract (Oryza sativa L.) and the anthocyanin aglycones in PRE inhibited VEGF-induced angiogenesis of HUVECs mediated by inhibition of both cell proliferation and migration (Tanaka et al., 2012). In a previous study, we had found that purple rice extract could inhibit the progression of DMH-induced ACF and the growth of colon cancer cell lines by inducing aberrant cells to undergo apoptosis (Unpublished data). Therefore, in the present study we investigated the effect of a purple rice extract supplemented rat diet on the initiation stage of DMH-induced colon carcinogenesis, focusing on DMH metabolism.

Dimethyhydrazine (DMH) is a carcinogen that can induce colon cancer (Povey et al., 2002). Humans may receive hydrazine compounds via environmental pollution...
and contaminated food. In mammals, DMH is oxidized by liver xenobiotic metabolism phase I enzymes (cytochrome P450 2E1; CYP2E1). The product, methylazoxymethanol (MAM) is then conjugated with UDP-glucuronic acid by the phase II enzyme (Uridine diphospho(UDP)-glucuronyl transferase; UDPGT) and is transported in glucuronide conjugated form to the colon via the bile. Bacterial β-glucuronidase has been suggested to play a role in human colon carcinogenesis (Manoj et al., 2001), as it is a key enzyme for the final activation of DMH metabolites to free reactive MAM in the colonic lumen. MAM could generate methylating adducts at guanine bases to form O6-methylguanine, which is believed to be of primary importance as a precarcinogenic lesion for colon carcinogenesis (Godschalk et al., 2003).

An Aberrant Crypt Focus (ACF) is a preneoplastic lesion of the rat or human colon. They are used as relevant biomarkers to investigate the initiation step of colon carcinogenesis and are thus used in chemoprevention studies. (Alrawi et al., 2006; Bird, 1995) Therefore, this study investigated effect of purple rice extract mixed diet on DMH-induced aberrant crypt foci formation in regard to the molecular level mechanism.

Materials and Methods

Chemicals

The carcinogen 1, 2-dimethylhydrazine (DMH) was obtained from Tokyo Chemical Industry. P-nitrophenyl-β-D-glucuronide, E coli β-glucuronidase, glutathione, uridine diphosphate glucuronic acid (UDPGA), 1-chloro-2,4-dinitro benzene (CDNB), glycogen, guanine and O6-MeG were purchased from Sigma-Aldrich Chemical Co. Tris-HCl and sucrose were obtained from Thermo Fisher Scientific Inc. Phenol-chloroform-isoamylalcohol was obtained from Research Organics Inc. Other reagent grade chemicals were obtained from Merck Millipore Bioscience (Thailand).

Rice samples extract and mixed diet preparation

Sticky purple rice variety Kum Doisaket used in this study was supplied by the purple rice research unit, Chiangmai University. Purple rice was ground and stirred in 80% ethanol. An ethanolic extract solution was then inactivated at -80°C to expand colonic mucosa, and stored on ice for 15 min. The colon samples were fixed in 10% formaldehyde for at least 24 hr. The flattened colons were stained with 2% methylene blue and the size and numbers of ACF were then scored under a light microscope at 40x magnification and recorded according to the criteria of Bird (Bird, 1987).

Bacterial β-glucuronidase activity

This assay was according to the modified method of Deeptha et al (Deeptha et al., 2006). Weighed rat feces from each group were suspended in 0.1M cold PBS (1:5 w/v) and ground. Fecal suspensions were centrifuged at 2,000×g for 5 min and then the enzyme supernatant was collected to determine β-glucuronidase activity by p-nitrophenyl-β-D-glucuronic acid hydrolysis. The feces supernatant was added to a reaction mixture of 0.01 M PBS (pH 7.0), 0.1 mM EDTA, 3.0 mM p-nitrophenyl-β-D-glucuronide and distilled water. The reaction was started by adding 100 μl of fecal supernatant, then run at 37 °C for 15 min and stopped with 4.0 ml of 0.2 M glycine buffer (pH 10.4) in 0.2 M NaCl. The absorbance was measured at 450 nm and the enzyme activity was calculated.

Phase II enzyme activity determination

The preparation of cytosolic and microsomal fractions: The liver tissues were excised and rinsed with cold 0.154 M KCl. Two grams of tissue were minced and homogenated using Ultra-Turrax disperser (IKA® T10 basic) in an ice bath. The homogenate was centrifuged at 15,000×g for 20 min, and the supernatant was continuously centrifuged at 100,000×g for one hour. The cytosolic supernatant was carefully transferred into small vials and was then used to measure glutathione+ transferase (GST) activity. The microsomal pellet was gently suspended in ice cold 0.154 M KCl for measuring uridinediphosphoglucuronyl transferase (UDPGT) activity.

Glutathione+ transferase activity: GST activity was
measured by monitoring the conjugation of 1-chloro-2,4-dinitro benzene (CDNB) with glutathione according to methods of Habig et al (Habig et al., 1974). The 100 mM Phosphate buffer and 0.5 mM CDNB mixture were pre-incubated for 10 min at 37°C and the reaction was started by adding 5.0 mM GSH, followed immediately by 0.1 ml of the supernatant fraction. The absorbance at 340 nm was measured at 5 minutes and the value for the reaction mixture without GSH subtracted, and the enzyme activity was then calculated from the absorbance.

Uridine diphosphoglucuronyl transferase activity: This assay was done using p-nitrophenyl phosphate (PNP) as the substrate according to the modified method of Woodcock and Wood (Woodcock and Wood, 1971). The reaction mixture had 0.4 ml total volume containing 0.1 M Tris Hcl (pH 8.5), 4 mM magnesium chloride, 0.5 mM PNP, 2 mM UDPGA and 50 µl of the microsomal fraction. The assay was carried out at 37°C in a shaking water bath for 30 min. The reaction was stopped by adding an equal volume of 0.2 N trichloroacetic acid (TCA). After centrifugation at 10,000 g for 5 min, 0.5 ml of the supernatant was added to 1.0 ml of 0.5 N NaOH. The rate of PNP disappearance was measured at 405 nm, and enzyme activity was then calculated from the absorbance.

Measurement of O6-Methylguanine DNA adducts by HPLC

The procedure for detection of DNA adducts was based on the method described by Iwitzki et al. (Iwitzki et al., 1998). Frozen liver and colon were defrosted on ice. Total DNA was isolated by phenol-chloroform and DNA in aqueous phase was precipitated with cold ethanol. One milligram of DNA was hydrolyzed in 1M HCl at 95°C for 1 hour (acid hydrolysis). An aliquot of the mild acid hydrolysate was filtered and analyzed for guanine and O6-MeG content by high-pressure liquid chromatography (HPLC) using an Agilent PL-SCX1000A cation exchange column (250mm×4.6 mm). After 3 min, an equal volume of 0.2 N trichloroacetic acid (TCA). After centrifugation at 10,000 g for 5 min, 0.5 ml of the supernatant fraction. The assay was carried out at 37°C in a shaking water bath for 30 min. The reaction was stopped by adding an equal volume of 0.2 N trichloroacetic acid (TCA). After centrifugation at 10,000 g for 5 min, 0.5 ml of the supernatant to 1.0 ml of 0.5 N NaOH. The rate of PNP disappearance was measured at 405 nm, and enzyme activity was then calculated from the absorbance.

Results

Effect of purple rice extract on DMH-induced ACF formation in the initiation stage

To determine the effects of PRE on the DMH generated colon carcinogenesis in the initiation stage, the total numbers of ACF/rat and number of aberrant crypts were scored. During the experiment period, the body weights of rats were not significantly different between control and experimental groups. The mean number of ACF and crypt/focus in rat colon are shown in Table 1. All rats treated with DMH exhibited ACF distributed throughout the colon, (Groups 3-5) while no ACF were observed in the NSS-treated group (Group 1-2). Interestingly, rats treated with purple rice extract in the diet with DMH administration showed a significantly lower total number of ACF compared to DMH-treated alone (p<0.05), but there were no significant differences between the two dosage groups. Moreover the multiplicity of ACF (number of AC/focus) was not significantly different among groups. These results suggest that PRE in the diet modulated DMH metabolism causing ACF formation in the initiation stage.

Effect of purple rice extract on DNA adducts in DMH-treated rats

To determine the effect of PRE on the DMH generated DNA adducts in colonic epithelial cells, the level of O6-MeG was determined. O6-MeG levels in the colon over 12

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<th>Table 1. The Effect of Purple Rice Extract on DMH-induced Aberrant Crypt Focus Formation in Wistar rats in the Pre-initiation Stage</th>
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<tr>
<td>Treatment, n, ACF/Rat, % inhibition</td>
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<tr>
<td>Negative (NSS) 6, 0±0.0</td>
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<tr>
<td>NSS+PRE 2%, 6</td>
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<tr>
<td>Positive (DMH) 6</td>
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<td>DMH+0.2% PRE 6</td>
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<td>DMH+2% PRE 6</td>
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a Means SD of 6 rats in each group, b Significantly different from positive, p<0.05.

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<th>Table 2. Effect of Purple Rice Extract on Bacterial β-Glucuronidase Activity in rat Feces</th>
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<tr>
<td>Groups, Fecal beta-glucuronidase activity (nmol min⁻¹mg⁻¹), Day 1, Before inject DMH (1), After inject DMH (1), Before inject DMH (2), After inject DMH (2)</td>
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<tr>
<td>Negative (NSS) 14.7±0.8</td>
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<tr>
<td>Positive (DMH) 13.7±0.7</td>
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<tr>
<td>Diet control (2% PRE) 15.4±2.5</td>
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<tr>
<td>0.2% PRE + DMH 14.9±2.1</td>
</tr>
<tr>
<td>2% PRE + DMH 9.8±2.7</td>
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a MeansSD of 3 rats in each group, b Significantly different from negative control group in the same collecting time, p<0.05, c Significantly different from positive DMH group in the same collecting time, p<0.05.

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h after the DMH injection are shown in Figure 2. In the colon, the DMH treated groups had significantly increased levels of O\(^6\)-MeG (p<0.05) compared to saline-treated group. In purple rice diet treated groups both 0.2 and 2% significantly reduced the level of O\(^6\)-MeG in the colon (p<0.05) when compared to the DMH treatment alone. However there was no difference in the level of O\(^6\)-MeG between high and low doses. Moreover, the levels of DNA adducts in the livers of DMH treated rats were similar in both PRE and normal diet groups. These results suggested that PRE in the diet could inhibit DMH-induced O\(^6\)-methyl DNA adduct formation in the colonic mucosa related to ACF formation.

**Effect of purple rice extract on phase II xenobiotic metabolism enzyme**

Enzymatic activities of GST and UDPGT are shown in Figures 3 respectively. In the DMH treated groups, GST and UDPGT activity were significantly elevated compared to non-treated (p<0.05). However in the purple rice extract treated together with DMH administration, the GST and UDPGT activities were not significantly different compared to DMH treatment alone. The results suggest that the stimulation of liver GST and UDPGT enzymatic activity respond to eliminate reactive MAM via bile secretion. The purple rice extract had no effect on phase II enzymatic activity, was similar to the DNA methylation levels in the parallel livers of treated rats.

**Effect of purple rice extract on bacterial \(\beta\)-glucuronidase activity in rat feces**

Bacterial \(\beta\)-glucuronidase enzyme has been considered as a key enzyme for the final activation of DMH metabolites to carcinogens in the colonic lumen. The effect of PRE on bacterial \(\beta\)-glucuronidase activity is shown in Table 2. The activities of fecal \(\beta\)-glucuronidase in each group were comparable on the first day and pre-injected DMH in all groups. After DMH administration, rat feces exhibited significantly higher \(\beta\)-glucuronidase activity in both weeks 1 and 2 (p<0.05) compared to the non-treated group. On the other hand, purple rice extract-treated rats, both 0.2% and 2% doses of DMH showed lessened fecal \(\beta\)-glucuronidase activity compared to the DMH-treated alone group, both after 1\(^{st}\) and 2\(^{nd}\) DMH injections (Figure 4). The results indicated that purple rice extract exhibited a preventive effect on DMH-induced ACF formation via the inhibition of \(\beta\)-glucuronidase activity, which might alter the metabolism of the carcinogens in the colon.

The inhibitory effect of PRE on \(E\) coli \(\beta\)-glucuronidase in vitro was also investigated. The enzymatic activities of \(\beta\)-glucuronidase treated with 50, 100 and 200 µg/ml PRE were decreased at all doses (Figure 5a). Figure

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![Figure 3](image3.png)

**Figure 3. Effect of Purple Rice Extract on Phase II Xenobiotic Metabolism Enzyme.** Rat liver homogenate was determined for a) Glutathione-S-transferase; b) Uridine-diphosphoglucuronyl transferase. Each bar represent the mean±SD of duplicated value of 5 rats. Significantly different from negative control; *p<0.05

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![Figure 4](image4.png)

**Figure 4. Effect of Purple Rice Extract on Bacterial \(\beta\)-Glucuronidase Activity in Rat Feces.** Each bar represent the mean±SD. *Significantly different from negative control; p<0.05, ** Significantly different from DMH treatment group; p<0.05
Purple Rice Extract Reduction of DMH-Induced Aberrant Crypt Foci by Inhibition of Bacterial β-Glucuronidase

Discussion

This study has demonstrated the preventive effect of purple rice extract (PRE) supplemented diet on the initiation stage of colon carcinogenesis in rats that received colon carcinogens. The phytochemicals in purple rice include high amounts of hydrophilic phenolic compounds; anthocyanins and proanthocyanidins (Sompong et al., 2011). There are several research reports on the biological activity of these phenolic compounds. The bran extracts of riceberry contain bioactive constituents that have putative cancer chemopreventive properties. Both the dichloromethane and the methanolic extracts that have putative cancer chemopreventive properties. The inhibition of ACF formation by alteration (x-axis) without Km alteration (x-axis). This result confirmed that PRE could inhibit bacterial β-glucuronidase activity in the rat colon.

The inhibition of ACF formation by alteration of DNA methylation might be one effect of PRE on xenobiotic metabolism of DMH in the colon and decreased both DNA adducts and ACF in the rat colon (Suaeyun el. al. also reported that the reduction of O-MeG in colonic mucosa was more correlated to ACF in colonic mucosa than in the liver. The inhibition of ACF formation was more correlated to ACF in colonic mucosa. However, O-MeG in the liver had no significant difference. Pfohl-Leszkowicz et al. (1995) than in the liver. Purple rice extract reduced the total number of ACF when compared to DMH-treated rats but the effects of the dosage were not significant. These results suggest that 0.2% PRE was a sufficient dose to inhibit ACF formation.

In this protocol rats were treated with PRE mixed diet for 1 week before induction of ACF by DMH in order to study the modulating effect of xenobiotic metabolism in rat liver and the final outcome marker, ACF at 5 weeks of the experiment (Raju, 2008). Purple rice extract reduced the total number of ACF when compared to DMH-treated rats but the effects of the dosage were not significant. These results suggest that 0.2% PRE was a sufficient dose to inhibit ACF formation.

There are a number of possible mechanisms for preventing DMH induced ACF formation. To clarify the mechanism of PRE on DMH-induced ACF formation, the modulating effect of PRE on DMH metabolism was investigated. Methylazoxymethanol (MAM), which is an electrophilic methylating agent (Samanta et al., 2008), can methylate at a number of guanine bases of DNA in colonic mucosa cells. O-Methylguanamine is believed to be proportional to the number of ACF. Our results showed that the PRE had an inhibitory effect on the formation of O-MeG in rat colonic epithelial cells (p<0.05), which suggests that the PRE may decrease DNA adducts, resulting in the reduction of ACF formation in the colonic mucosa.

There are several reports showing that chemopreventive agents have stimulating effects on phase II xenobiotic metabolism enzymes. Caffeic acid, garlic oil, sinigrin and propyl gallate all showed an ability to induce phase II enzymes (Manson et al., 1997). As expected, after DMH administration, both phase II enzymes (GST and UDPGT) were elevated due to the xenobiotic conjugation process. Nirmala et al. reported that the enzyme activity levels of both GST and UDPGT were significantly higher in all the carcinogen treated animals compared to controls (Nirmala et al., 2010). Bock et al. explained that UDP-GT activity increase together with the decrease of cytochrome P-450-dependent monoxygenase may contribute to the resistance of preneoplastic hepatocytes to the cytotoxic actions of chemical carcinogens (Bock et al., 1982). Unfortunately, PRE did not stimulate hepatic...
GST and UDPGT activity over the range of DMH used. Therefore, phase II enzyme activities in the DMH treatment with or without PRE were similar in regard to MAM-glucuronide conjugation and excretion. This could indicate that reactive MAM was eliminated from the rat liver regularly, which resulted in an unchanged level of O6-MeG in the liver. Suaceyun et al. also reported that orally administered lemon grass extract inhibited O6-MeG formation in the colonic mucosa and significantly inhibited ACF formation, and that the magnitude of the decrease in DNA-binding paralleled the decrease in ACF formation, but it did not inhibit O6-MeG in the liver (Suaceyun et al., 1997). Therefore, hepatic O6-MeG has not been shown related with colonic ACF formation.

Intestinal bacteria elevated the deconjugating enzyme when presented with xenobiotic conjugates in the colon for carbohydrate utilization by the cleaving process. Beta-glucuronidase enzyme has been considered as a key enzyme for deconjugation of MAM-glucuronide to free MAM in the colonic lumen, resulting in an increase in DNA adduct level and ACF (Gadelle et al., 1985). Some dietary substances had shown inhibitory effects on bacterial β-glucuronidase activity in vitro. Manoj et al. reported that dietary fibers isolated from coconut or black gram can reduce the formation of colon tumors induced by the carcinogen DMH by reducing the activity of the intestinal as well as fecal β-glucuronidase (Manoj et al., 2001). Moreover, dietary caraway markedly suppressed ACF development and bacterial enzyme β-glucuronidase activities during the initial stages of colon carcinogenesis induced by DMH (Deeptha et al., 2006). Interestingly, our results showed that fecal β-glucuronidase activity of purple rice extract-treated rats were decreased, caused the reduction of free MAM in the colonic lumen. This result was parallel to the inhibitory effect of PRE on ACF and DNA adducts in the colonic mucosa. PRE also inhibited E. coli β-glucuronidase in vitro. The enzyme kinetics showed that PRE could inhibit β-glucuronidase in a noncompetitive manner (Figure 5b). Various phytochemical substances have inhibitory effects on β-glucuronidase activity. Extracts of Chondria crassicaulis showed an inhibitory effect against beta glucuronidase as a mixed competitive (Sekikawa et al., 2002) while Serres et al. also reported that proanthocyanidin-A2 from cranberry could inhibit β-glucuronidase directly in a non-competitive manner (Serres et al., 1997). However, the inhibition mechanisms of phytochemicals on β-glucuronidase were dependent on the active compounds mixed in those extracts.

In conclusion, purple rice extract inhibited β-glucuronidase activity in the colonic lumen, resulting in a decrease of MAM-glucuronide deconjugation. The reduction of free MAM might reduce colonic mucosa DNA adducts and ACF in this DMH-induced colon carcinogenesis model. Therefore, purple rice could be further developed as a nutraceutical product for colon cancer prevention in Thailand.

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References

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