Cytoprotective and Cytotoxic Effects of Rice Bran Extracts on H_2O_2- Induced Oxidative Damage in Human Intestinal Caco-2 Cells

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ABSTRACT

Oxidative stress is considered to play a prominent role in the causation of chronic diseases. At present, Rice Science Center, Thailand used plant biotechnology technique to develop new rice strains with increasing nutritive values. Some rice varieties such as Sinlek brown rice and Riceberry pigmented rice showed high levels of antioxidant contents and activities, especially in bran portion. Therefore, the aim of this study was to determine the effects of these bran extracts on H_2O_2-induced oxidative stress in Caco-2 cells. Human intestinal Caco-2 cells were co-incubated with the bran extracts and H_2O_2 for 3 h. Cytotoxic effects of both bran extracts were also evaluated and cell viability measured by MTT assay. Cell growth was inhibited in dose- and time-dependent manner by both bran extracts. The doses at which non-toxic and approximate to IC_{50} were used to determined effects of the rice bran extracts on H_2O_2 induced oxidative stress. Non-toxic concentrations of both bran extracts protected cells against H_2O_2-induced oxidative stress as antioxidant property. On the other hand, high concentrations enhanced oxidative stress mediated reduction of cell viability. This may result from the pro-oxidant activity that express at high concentrations of the extracts. Sinlek bran extract exhibited antioxidant and pro-oxidant activity higher than Riceberry bran. In addition, the high concentrations of both bran extracts inhibited cell growth in dose and time-dependent manner. Further studies are needed to clarify the potential activities and properties of these bran extract before using as supplement in food or medicine.

Keywords: Oxidative stress, cytoprotective, H_2O_2, rice bran, antioxidant, pro-oxidant

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INTRODUCTION

Reactive oxygen species (ROS) or oxygen-derived free radicals have been implicated in various pathological conditions involving chronic diseases such as cardiovascular disease, stroke and cancer.[1-3] It is an important mediator of damage to key cellular structures; lipids, proteins and DNA. The epidemiological findings indicate that consumption of fruits, vegetables and whole grains is strongly associated with reducing risk of cancer and other chronic diseases. These plant foods are the major sources of phytochemicals which have antioxidant and anticancer properties.[4-6] Rice (*Oryza sativa* L.) is a basic staple crop for a large part of the world including Thailand which is one of the *world's largest rice* exporters. Rice bran is a relatively abundant by-product of rice milling process and contains various antioxidant factors showing beneficial effects on human health. Rice bran is not only a source of *high quality proteins* but also rich sources of *fiber*, antioxidant *vitamins* and b*ioactive compounds*. Several studies have demonstrated the abundant bioactives with corresponding high antioxidant, anticarcinogenic and antimutagenic activities in rice bran extracts.[7-10] In the rice consuming world, nutritious rice grains can become a main ingredient in designing food for specific neutraceutical and functional food formulations. Recently, Rice Science Center, Thailand has developed some new Thai rice strains with increasing nutritive values by using plant biotechnology technique. Some rice varieties such as Sinlek brown rice and Riceberry pigmented rice showed high contents and activities of various antioxidants especially in bran portions.[11] Therefore, in this present study, we determined the chemical antioxidant activities of bran extracts from Sinlek and Riceberry rices. In addition, the bran extract of each rice variety was evaluated for its effects on H_2O_2 mediated oxidative stress in human colon cells.

MATERIALS AND METHODS

Samples and reagents

Bran of brown rice, Sinlek and pigmented rice, Riceberry were obtained from Rice Science Center & Rice Gene Discovery Unit, Thailand. Rice bran samples from experimental rice field were stored at -20 ºC until use. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin and streptomycin were obtained from Gibco (Invitrogen, USA). Colorimetric (MTT) kit for cell survival and proliferation were obtained from Chemicon international, Inc. H_2O_2 was purchased from Carlo Erba Reagents Company.

Extraction of rice bran

The extraction of rice bran was performed according to previous method.[12] Briefly, 100g of rice bran was extracted with ethanol - water (70:30 v/v) by stirring for 10 h at room temperature. The filtrate of extract was passed through Whatman filter paper No.1. Solvent was removed by rotary evaporator. Then the sample was lyophilized, re-dissolved and extracted with hexane to remove residual fat. Finally, the aqueous phase of extract was lyophilized and aliquoted into a test tube for storage at -20 ºC until used.

Identification of active compounds in defatted rice bran

Bioactive compounds in defatted rice bran were quantified at Institute of Nutrition, Mahidol University. The extracts were analyzed for cyanidin, peonidin and vitamin E. The analysis was performed using a high performance liquid chromatography (HPLC). Samples were diluted with mobile phase and then the peak area was identified base on RT values with standards of cyanidin, peonidin and vitamin E. The concentration of active com-

pounds in sample extracts was quantitated from the peak areas relative to standard calibration curve.

Measurement of antioxidant capacity in defatted rice bran

The antioxidant activity of the defatted rice bran extracts was determined at Institute of Nutrition, Mahidol University. A number of assays were performed for antioxidative activities; oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), and 2, 2-Diphenyl-l-picrylhydrazyl (DPPH) free radical scavenging activity. These assays were carried out according to the previously described procedures.[13-15]

Cell culture

Caco-2 human colon cancer cells were kindly provided by Asst. Prof. Dr. Ratchanee Kongkachuichai at Institute of Nutrition, Mahidol University. Cell line (passage 32-36) were routinely grown in DMEM media supplemented with 10% FBS and 1 % penicillin/streptomycin in humidified incubator maintained 37 ºC and supplied with 95% air and 5% CO_2. Cells were seeded at a density of 10^4 cells/well onto 96 well plates and maintained for 48 h before experiments.

Cytotoxicity assays

The MTT assay was used to evaluate the cytotoxicity of the rice bran extract. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells. An increase in number of living cells results in an increase in total metabolic activity, which leads to a stronger color formation. In the experiment, bran extracts were dissolved in completed media with 0.1% v/v DMSO at different concentration (0-10 or 0-20 mg/ml for Sinlek and Riceberry respectively). Cells were exposed to 200 µl of each treatment solution except, control cells that were treated with 0.1% DMSO presented in media without bran extracts. After 24 and 72 h of incubation, treatment medium was replaced with 100 µl of completed media and 10 µl of MTT solution was added to each well. After 2 h of incubation, the media was removed and 100 µl of isopropanol/ 0.04N HCL was added to each well to solubilize the formazan. The plate was read using a micropate reader (Bio-tek, USA) at a wavelength of 570 nm with a reference wavelength of 630 nm. The results were expressed as the percentage of viable cells compare with the control (% cell viability).

H_2O_2-induced oxidative stress in Caco-2 cells

Caco-2 cells were cultured in 96-well plates for 48 h before oxidative stress induction. Culture media was removed, cells were washed once with PBS and then, H_2O_2 was added at the concentration ranging from 0.3 to 10 mM. After 3 h of incubation, cell viability was measured by the MTT assay as described above.

Effect of rice bran extracts on H_2O_2-induced inhibition of cell growth

The effects of rice bran extracts on H_2O_2 induced oxidative stress and suppressing cell growth was done by co-incubation method. The cells were incubated in 200 µl of culture medium containing 1 mM H_2O_2 with or without various concentrations of the extracts (0 to 7.5 mg/ml of Sinlek and 0 to 20 mg/ml of Riceberry rice bran). After 3 h of incubation, the cells were washed once with phosphate buffered saline (PBS) and 100 µl of fresh medium was added. Then, cell viability was measured by the MTT assay and the treated cells were observed for morphology under inverted microscope.

Statistical Analysis

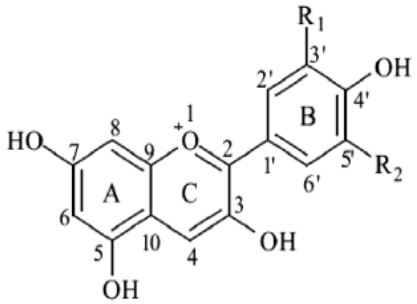
All results were expressed as mean ± SEM. The

statistical significance of differences between groups in cytotoxicity tests was determined by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison tests. Student's t-test was used for analysis the effects of rice bran extracts on H_2O_2 treated cells. $P<0.05$ was accepted as the statistically significant level.

RESULTS

Analyses of bioactive components and antioxidant activities of rice bran extracts

Table 1 shows the antioxidant properties and bioactive constituents of the experimental rice bran extracts. The major bioactive components in pigmented Riceberry bran were two phenolics; cyanidin and peonidin (Fig. 1) whereas non-pigmented Sinlek variety contained vitamin E. The results of antioxidant activities were in accordance with those observed in a previous study that found pigmented rice bran had significant higher antioxidant activities than white rice bran. It may be partially due to the anthocyanins those found only in the bran of Riceberry rice. Major and minor proanthocyanidin in rice as refer to cyanidin and peonidin which generally found in pigmented of fruits and vegetables have important roles in reducing risk of cancer and other chronic diseases because of their free radicals scavenging capacities.[16-19]



Name	R1	R2
Cyanidin	OH	H
Peonidin	OCH_3	H

Figure 1. Chemical structure of anthocyanidins

Cytotoxicity assay

As illustrated in Fig.2, the cytotoxicity of rice bran extracts on Caco-2 cells was observed after incubation at 24 and 72 h. Bran extracts significantly decreased viability of Caco-2 cells in a dose- and time- dependent manner. After 24 h incubation, the IC_{50} was 7.5 mg/ml for Sinlek whereas 20 mg/ml of Riceberry rice bran extract reduced cell growth to approximately 60% of control. At 72 h, extracts from both varieties had IC_{50} lower than those at 24 h. With the lower IC_{50} values, Sinlek showed a greater cytotoxic activity compared to Riceberry. For subsequent experiments the doses equal or close to IC_{50} were used to examine the effect of the bran extracts.

H_2O_2 -induced oxidative stress and reduction of viability in Caco-2 cells

Caco-2 cells were treated with various concentrations of H_2O_2 (0.3, 0.5, 1, 5 or 10 mM). Following 3 h of incubation after H_2O_2 treated (Fig.3), cell viability was determined by MTT assay. The results showed that H_2O_2 significantly reduced cell viability dependently on doses. At a concentration of 1 mM, the cell viability was about 60%. There-

Table 1 The antioxidant activity and active compounds of defatted rice bran extracts.

	Sinlek	Riceberry
FRAP (µmole TE/ml)	146.03	407.85
ORAC (µmole TE/ml)	705.89	1,735.88
DPPH (µmole TE/ml)	1,078.16	5,198.48
Cyanidin (mg/100g)	n.d.*	150.81
Peonidin (mg/100g)	n.d.	66.76
Vitamin E (µg/100g)	996.08	n.d.

* n.d., not detected

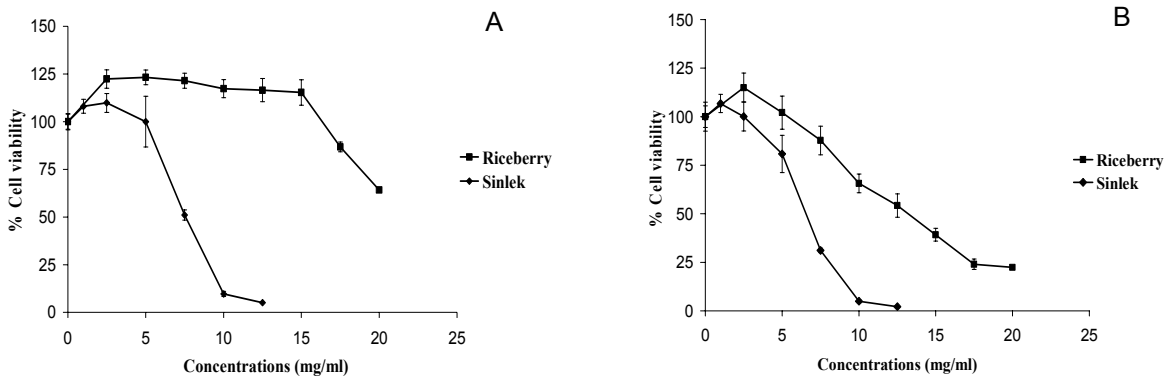


Figure 2. Cytotoxic effect of rice bran extracts on Caco-2 Cells. Caco-2 cells were treated with various concentrations of rice bran extracts for 24 h (A) and 72 h (B). After incubation, cell viability was measured by MTT assay. Data were means ± SEM (n≥6).

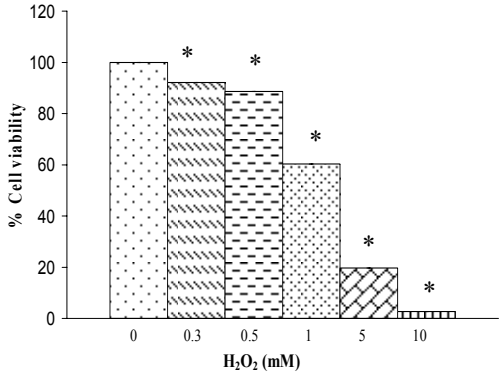


Figure 3. H_2O_2-induced reduction of viability of Caco-2 cells. Caco-2 cells were treated with various concentrations of H_2O_2 (0.3, 0.5, 1, 5, 10 mM). After 3 h of incubation, cell viability was measured by MTT assay. Data were means ± SEM (n=3). * p<0.05 compared to control group.

fore, for investigation the effects of rice bran extracts on oxidative induced cell damage, we used 1 mM as the concentration of H_2O_2 to induced oxidative damage.

Effect of the rice bran extracts on H_2O_2-induced oxidative stress in Caco-2 cells

As shown in Fig.4A and 4B, Sinlek and Riceberry bran extracts in the highest concentration used in this experiment did not affected cell viable. When the rice bran extracts with 1 mM H_2O_2 were added simultaneously on Caco-2 cells, low concentrations (0.005-0.5 mg/ml) of Sinlek extract

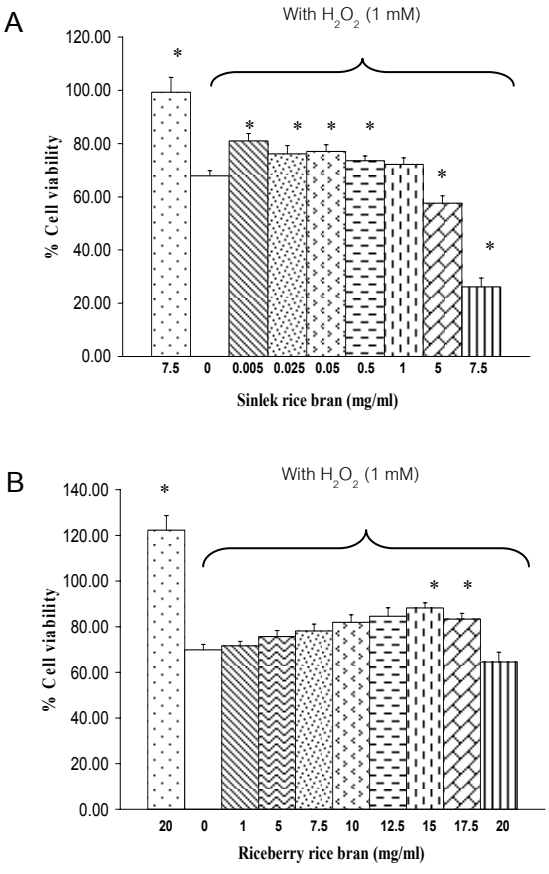


Figure 4. Effect of rice bran extracts on H_2O_2-induced cell damage in Caco-2 cells (co-incubation) Caco-2 cells were treated with 1 mM of H_2O_2 with various concentrations of rice bran extracts: (A) Sinlek or (B) Riceberry. After 3 h of incubation, cell viability was measured by MTT assay. Data were means±SEM (n≥6). * p<0.05 compared to control group.

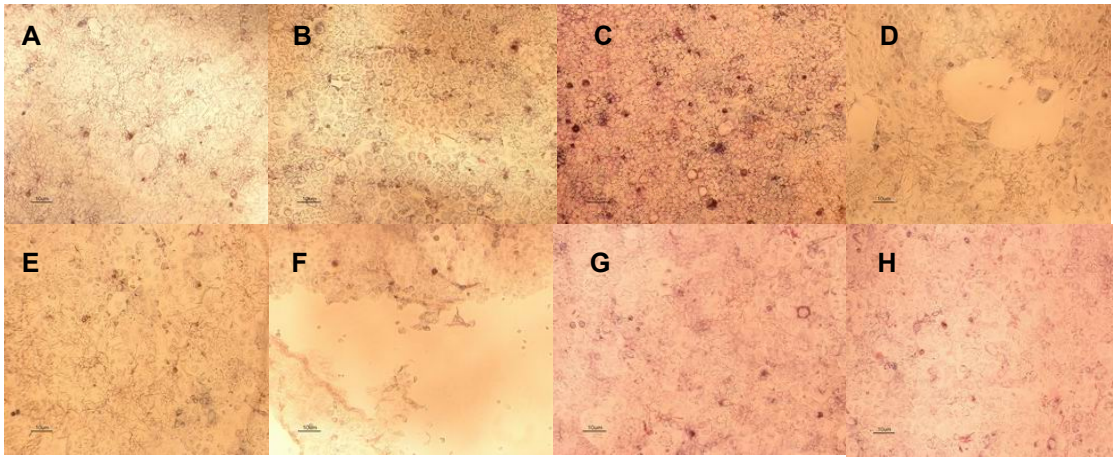


Figure 5. Effect of rice bran extracts on H_2O_2-induced cell damage in Caco-2 cells. Rice bran extracts were added together with H_2O_2. At 2 h after the end of 3 h incubation period, cells were incubated with MTT and then observed under phase contrast microscopy. (A) non -H_2O_2 treated groups (B) 7.5 mg/ml Sinlek (C) 20mg/ml Riceburry (D) 1 mM H_2O_2 (E) 1 mM H_2O_2 /0.005 mg/ml Sinlek (F) 1 mM H_2O_2 / 7.5 mg/ml Sinlek (G) 1 mM H_2O_2/ 17.5 mg/ml Riceburry (H) 1 mM H_2O_2 / 20 mg/ml Riceburry

could protect a number of cells by significant increased cell viability compared to H_2O_2 treated control group. However, the protective effect was not seen in higher concentrations (5, 7.5 mg/ml), on the contrary, it caused a significant dose dependently decrease in cell viability after H_2O_2 exposure.

Similar results were found from the cells exposed to Riceberry bran extract with H_2O_2. The cell viability had a trend of increase according to concentration of bran extracts (Fig.4B). At concentration of 15 and 17.5 mg/ml it caused significant protective effect against H_2O_2. However, at concentration of 17.5 mg/ml, cell viability started to decline and the protective effect was lost at the highest concentration of 20 mg/ml.

Morphological observations

The morphologies of the Caco-2 cells co-incubated in rice bran extracts with or without H_2O_2 were examined after 3 h of incubation by optical microscopy (Fig. 5). Without treatment, cells were intacted and well attached to the culture plate (A). Cells incubated in the highest concentration of Sinlek (B) and Riceberry (C) bran extracts appeared normal without any signs of impairment. When the oxidative stress was induced by 1 mM H_2O_2, the prominent morphological changes were observed. Cell membrane aberration and a detachment of monolayer were clearly observed. In contrast, the membrane damage and monolayer detachment induced by H_2O_2 treatment were completely prevented by co-incubation with low concentration of both rice bran extracts (E) and (G). However, co-incubation with some higher concentrations of bran extracts, cell loss and detachment (F) and membrane aberration (H) were noticed again. The results of cell morphologies were consistent with those of cell viability determination.

DISCUSSION

By using the chemical assays, we found that Riceberry rice bran had a higher antioxidant activity than Sinlek rice bran. Regarding method of analysis, ORAC assay is based on the measurement of antioxidant inhibition of reactive oxygen species (ROS) induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer. DPPH is based on

the measurement of the reducing ability of antioxidants in test compounds toward DPPH· which after reaction, DPPH color is lost. Ferric reducing antioxidant power (FRAP) is based on the measurement of reduction of ferric 2 , 4 , 6 - tripyridyl-s-triazine (TPTZ) to ferrous-TPTZ and give blue color by test compound. Our finding agree with a previous study,[11] it showed that the bran extract of pigmented Riceberry rice had higher values of ORAC, DPPH and FRAP than the bran extract of non-pigmented Sinlek. It may be related with bioactive compounds identified in this rice bran. Especially, cyanidin and peonidin that expressed higher levels in Riceberry rice bran. Both cyanidin and peonidin is naturally occurring polyphenolic compound that give the intense color to many fruits and vegetables. They are a group of flavonoids that represent the aglycones of most anthocyanins in plants. In current studies, they indicated that these flavonoids have a potential antioxidant, anti-inflammatory and antitumor in many cell lines.[20] The structures which support these activities were illustrated in Fig. 1 . The flavonoids are recognized for both their ability to donate electrons and stop chain reactions. These activities attribute to the phenolic hydroxyls, particularly in the 3´, 4´OH of the (B) ring and the activities increase with the number of OH groups in ring (A) and (B). However, they lack the ketone structure in ring (C) for contribute to metal chelating activity.[21-23] Therefore, the higher contents of cyanidin and peonidin including their suitable structures may promote the higher ORAC and DPPH activities of Riceberry as compared to Sinlek cultivar.

The MTT cytotoxicity assay showed that Sinlek rice bran had a higher cytotoxic potential on Caco-2 cells than Riceberry rice bran. To investigate the health benefits of these rice brans, the effects of bran extracts from Sinlek and Riceberry rice on H_2O_2–induced oxidative stress in Caco-2 cells were examined. H_2O_2 was used to induce ROS formation in this experiment because of its ability to pass freely across cell membranes. It is precursor of highly oxidizing, tissue-damaging radicals such hydroxyl radical as known to be highly toxic to many systems and generate from nearly all sources of oxidative stress.

When 1 mM H_2O_2 and bran extracts were added concomitantly, Sinlek rice bran extract had higher ability to protect cells against H_2O_2-induced oxidative stress than Riceberry rice bran extract. Sinlek showed preventive effect on cells at lower concentration than Riceberry rice bran. Rice bran counteract against the H_2O_2-induced decrease in cell viability may at least partly result from its antioxidant and free radical scavenging properties. Nonetheless at the high dose range, Sinlek bran extract also exerted a promoting effect on H_2O_2-induce oxidative stress in Caco-2 cells at the lower concentration than Riceberry rice bran extract. Some flavonoids such as quercetin reduced intracellular production of ROS and produced peroxide in the culture medium at the same time. It was found that low concentrations of quercetin enhanced total antioxidant capacity while high concentrations decreased total antioxidant capacity of the cells.[24] Some phenolic substances can exert pro-oxidant activities under certain conditions, such as in the presence of transition metal ions or alkalis.[25,26] Metal-mediated autoxidation of some phenolic phytochemicals generates semiquinone radicals, resulting in the enhancement of redox activity to produce reactive oxygen species including H_2O_2. Thus, some phenolic phytochemicals may exert antimutagenic and antitumor-promoting activities at relatively low doses, whereas at high doses, some excess antioxidant may instead exhibit acute toxicity or carcinogenicity. It is possible that a pro-oxidant activity of extracts could be linked to their beneficial effects in cancer therapy while dietary plant phenolics have been reported

to protect cells from oxidative-induced cell toxicity.[27,28] The present study finding indicated that bioactive compounds in rice bran may act as both antioxidant or pro-oxidant depend on the redox status of the cells and concentration of the extracts.

In summary, Sinlek and Riceberry rice bran extracts behaved differently in cell free and cell based tests. Our present study showed that Sinlek and Riceberry rice bran possessed a high antioxidant activity in cell free system. Exposed Caco-2 cells to rice bran extracts and H_2O_2 simultaneously revealed that at the low concentration, rice bran extract exhibited antioxidant activities whereas at the high concentration, they seemed to be pro-oxidant enhanced oxidative stress decrease in cell viability. Sinlek and Riceberry rice bran extract by themselves, at the high dose range leaded to cell death in dose and time-dependent manner. Therefore, further studies in these rice bran extracts are needed to clarify their potential activities and properties before using as supplement in food or medicine.

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