Effects of Bran Extracts from Thai Molecular Breeding Rices on Growth and Apoptosis in Human Promyelocytic Leukemia Cells

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ABSTRACT

Rice bran, the outer layer of brown rice, contains biologically active compounds which mediate chemo-preventive properties. Thus, in the present study, we evaluated the anti-carcinogenic effects of bran extracts from non-pigmented (Sinlek) and pigmented (Riceberry) rices on human promyelocytic leukemia cell line (HL-60 cells). The results showed that the treatment with rice bran extracts for 24 and 72 hours reduced cell viability in a dose- and time-dependent manner. Riceberry rice bran extract showed higher cytotoxic activity, corresponding with the lower IC_{50} compared to Sinlek variety. The low concentrations of both rice bran extracts which did not affect cell viability were able to inhibit DNA synthesis, whereas the higher selected concentrations that reduced viable cells could induced apoptosis. The apoptotic events were confirmed by chromatin condensation and nuclear fragmentation in cells after AO/EB nuclear staining as well as dose- and time- dependent increase of caspase activity. The findings suggested that bran extracts of new Thai rice varieties exhibited growth inhibitory effects on the human leukemia cells through cytostatic and cytotoxic mechanism. The results also indicated that killing leukemia cells by rice bran extracts partially involved activation of caspases and induction of apoptosis

Keywords: Rice bran; human leukemia cells; cytotoxicity; cytostatic; apoptosis

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INTRODUCTION

Cancer is one of the leading causes of death in the world, particularly in developing countries. In Thailand, cancer is one of the major public health problems and the number of new cancer cases of all sites increases from 81,000 in 1999 to approximately 125,000 by the year 2008.[1] Leukemia is the most common hematological malignancy and most common malignancies in Thai children.[2]

The risk factors of cancer are well established through the non-modifiable risk factors (age and heredity) and modifiable risk factor such as unhealthy diet, excessive energy intake, physical inactivity and tobacco use. Nutritional factor has been associated with the modifiable factor that presumed to account for approximately 35% of cancer deaths.[3] Higher consumption of fruits, vegetables as well as whole grains have been associated with reduces risks of numerous cancers.[4] These foods contain many biologically active chemicals, including nutrients and many nonnutritive constituents that have potential to reduce risk of developing of cancer.[5] Nowadays, many considerable researches are focused on the identification of the mechanism of action of compounds from dietary sources especially plant-derived substances (phytochemicals), which might prevent or postpone the onset of cancer. Phytochemicals are defined as bioactive nonnutrient plant compounds. It can be classified as carotenoid, phenolics, alkaloids, nitrogen-containing compounds and organosulfur compounds.[6] Phytochemicals have been reported to interfere with each stage of the carcinogenic process which include initiation, promotion and progression.[7] *In vitro* cell culture and animal cancer models have been used to evaluate the chemopreventive effects of phytochemicals. Phytochemicals have complementary and overlapping mechanisms of action in preventing initiation and suppressing promotion and progression of cancer cells.[8-11] Recently, considerable attention has focused on the sequence of events referred to apoptosis and the role which these processes may involve the pathogenesis and treatment of human diseases including cancer.[12] Apoptosis or programmed cell death plays a major role in the development of multicellular organisms and maintaining homeostasis of constant cell numbers in normal mammalian tissues.[13] Defects in apoptotic pathways are now thought to promote tumor formation, by allowing accumulation of neoplastic cells and by obstructing removal of genetic variants with enhanced malignant potential and promote tumor angiogenesis.[14] Therefore, induction of apoptosis has been recognized as a chemotherapeutic as well as a chemopreventive strategy for cancer control.

Rice, *Oryza sativa*, possesses special dietary importance in Asia. In Thailand, rice is not only the main staple crop but also a primary agricultural export. Rice bran is the outer layer of brown rice, obtained as a by-product of the rice milling industry. Nutrient analysis of rice bran indicates that it is a good source of protein, carbohydrate, unsaturated fatty acid, B-complex vitamins, vitamin E complex (tocopherols and tocotrienols), minerals, and dietary fiber. There are many evidences suggest that the bran layer rather than the rice kernel is the predominant location of biologically active constituents which are the chemopreventive and antitumor properties.[15-17] The bioactives present in rice bran include γ-oryzanol, phytosterol, polyphenols, flavones and proanthocyanidin, inositol and inositol hexaphosphate (IP6).[18] The phytochemical compounds of rice bran have been shown to have a wide range of biological activities, including anti-hyperlipidemia, anti-atherogenesis, anti-platelet aggregation and antioxidant property.[19] Furthermore, rice bran constituents possess antimutagenic,[15] antitumor promoting[17], and

cytotoxic properties.[20]

Rice is rich in genetic diversity, with a thousand of varieties throughout the world. Furthermore, the unmilled rice has many different colours, including brown, red, purple and black. These colorful rice varities are prized for their health properties. Nowadays, scientific technologies have made it possible to enhance the nutritional value of rice through modifying the genetic code. In this study, defatted rice bran samples (Sinlek and Riceburry), were obtained from Rice Science Center & Rice Gene Discovery Unit, Kasetsart University, Thailand. Sinlek (white brown rice), that was crossed breeding from Khaw kamdoichang X Chao Hom Nil 313-19-1-1, is the best source of iron and good iron bioavalability. Riceburry (purple brown rice), derived from Khaw kamdoichang X Chao Hom Nil 1000-11-2-26, has high level of antioxidant activity, polyphenol content, and β-carotene. However, preclinical evidence for anti-carcinogenic properties of these new Thai rice cultivars has been limited. Therefore, this study was conducted to assess growth inhibitory effects of bran extracts from pigmented (Riceburry) and nonpigmented (Sinlek) rices in human promyelocytic leukemia cell lines (HL-60). Cell viability, DNA synthesis and apoptosis induction were determined in cell treated with rice bran extracts. The results from this study would shed some light as the knowledge bases that demonstrate the health-promoting of these rice cultivars and provide scientific evidence for future research.

MATERIALS AND METHODS

Rice bran extract

The bran of pigmented (Riceberry) and nonpigmented rice (Sinlek) rice varieties were obtained from Rice Science Center & Rice Gene Discovery Unit, Thailand. Rice bran (100 gm) was extracted with 70% ethanol (once at 1,000 ml each). The active components were extracted by soaking the rice bran in solvent overnight at ambient temperature, and then, stirring was continued for 10 h. The extracts were filtered through Whatman No. 1 paper. The filtrates were concentrated using rotary evaporation and then lyophilized. The dried crude extracts were re-dissolved with water and were extracted with hexane to remove fat content. The aqueous portion of bran extracts were collected and re-lyophilized. The final dried extracts were stored at -30^{o}C until used.

Identification of active compounds in defatted rice bran

Bioactive compounds in defatted rice bran were quantified by Institute of Nutrition, Mahidol University. The extracts were analyzed for cyanidin, peonidin, vitamin E, β-carotene, and lutein by using HPLC (Water 515 HPLC Pump). Samples were diluted with mobile phase and then the peak area was identified base on RT values with standards of peonidin, vitamin E, β-carotene, and lutein. The cyanidin and peonidin concentration of sample extracts were quantitated from the peak areas relative to standard calibration curve.

Cells and cell culture

Human acute promyelocytic leukemia (APL) cell lines HL-60 were purchased from the Cell Lines Service (Eppelheim, Germany). Cells were maintained as suspension in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 ug/mL streptomycin (Gibco, USA) at 37°C in a humidified incubator containing 5% CO_2. Cells were grown in culture flasks and culture media was changed every two day. Trypsinization and subculture were performed when the cells propagated to the confluent condition.

Cytotoxicity assay

The cytotoxic effect of defatted rice bran extracts on HL-60 cells was determined by using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. The MTT bioassay is a cytotoxicity assay measuring the metabolic activity of the cells. The assay was carried out as previously described with some modifications.[21] Briefly, cells at the exponential phase were seeded at density of $1x10^4$ cells/ well in 96-well plates and incubated in media with or without test extracts. After a certain incubation period, culture media was removed and MTT (Chemicon, USA) was then added. The dye was cleaved by active mitochondria enzyme to produce purple formazan crystals. After 2 h incubation, iso-propanol containing 0.04 N HCl were added to each well to dissolve the formazan crystals, and the absorbance was read at 570 nm with an ELISA microplate reader (Bio-tek,USA). Survival of HL-60 cells after treatment with extracts was calculated using the following formula: viable cell number (%)=[OD_{570}(treatment well)/OD_{570}(control well)] x100. The median inhibitory concentration (IC_{50}) was assessed from the dose response curves.

Determination of DNA synthesis

HL-60 cells ($5x10^4$cells/ml) were seeded onto 96-well plates (200 µl/well) and treated with a range of concentrations of rice bran extract. Actinomycin-D was used as a positive control. After incubated with extract, DNA synthesis of HL-60 cells was assayed using ELISA for BrdU incorporation (Roche Diagnostics, Manheim, Germany). The assay was performed according to the manufacturer's instructions. Briefly, 2 h before the end of 24 and 72h treatment period with bran extract, cells were incubated for with 10 µM BrdU labeling reagent. Cells were then fixed and DNA was partial denatured in FixDenat-solution (30 min). After 30 min, FixDenat solution was removed, anti-BrdU-POD (monoclonal antibody from mouse conjugated with peroxidase) was added and incubation was continued for 90 min. The immune complexes were detected by using tetramethylbenzidine as substrate for 15 min, the reaction was stopped with H_2SO_4. The absorbance at 450 nm was measured by a micro-plate reader (Bio-tek). The result was present as a percent of BrdU incorporation compared to untreated cells. The cell viability was also determined in each BrdU incorporation assay.

Evaluation of apoptotic cell morphology

HL-60 cells were seeded in 24-well plates ($5x10^4$ cells/well) and incubated 2 days before being treatment with extract for 24, 48 h. Cells were harvested at different interval after being exposed to different concentrations of extract or without extract and were stained with a combination of the fluorescent DNA-binding dyes acridine orange and ethidium bromide (AO/EB 500 µg/ml) for 5 min. Afterwards, cells were observed under a fluorescence microscope (Nikon) according to the fluorescence emission and morphological features of chromatin condensation in AO/EB stained nuclei.[22] Minimum of 200 total cells were counted per sample, being classified as follows: (a) live cells with normal nuclei (LN: bright green); (b) live cells with apoptotic nuclei (LA: bright green, chromatin that was highly condensed or fragmented); (c) dead cells with normal nuclei (DN: bright orange, chromatin with organized structure); and (d) dead cells with apoptotic nuclei (DA; bright orange, chromatin that was highly condensed or fragmented). Apoptotic index and percentage of necrotic cells were calculated using the following formulae:

$$\% \text{ apoptotic cells (apoptotic index)} = \frac{LA+DA \times 100}{LN+LA+DN+DA}$$

$$\% \text{ necrotic cells} = \frac{DN \times 100}{LN+LA+DN+DA}$$

$$\% \text{ dead cells} = \frac{DN+DA \times 100}{LN+LA+DN+DA}$$

Caspase activity measurement

Caspase activation was measured using a fluorimetric caspase assay kit (Homogeneous caspases assay; Roche Diagnostics, Germany). The experiments were conducted according to the kit instructions. Cells ($2x10^4$cells/well) were grown in 96-well plates for 2 days prior to treatment. After treatment, the media was aspirated and added a substrate-containing lysis solution to the cells. After 2 h incubation at 37 ^{o}C, caspase-substrate cleavage was measured with a fluorescence microplate reader (Wallac 1420) with excitation wavelength of 485 nm and emission at 535 nm. The data were determined as fluorescence intensity. Higher fluorescence intensity indicated higher caspase activity.

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). Analysis of statistical significance between the control- and rice bran extract-treated groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test for pair wise comparison. The statistically significant difference was defined as $p < 0.05$.

RESULTS AND DISCUSSION

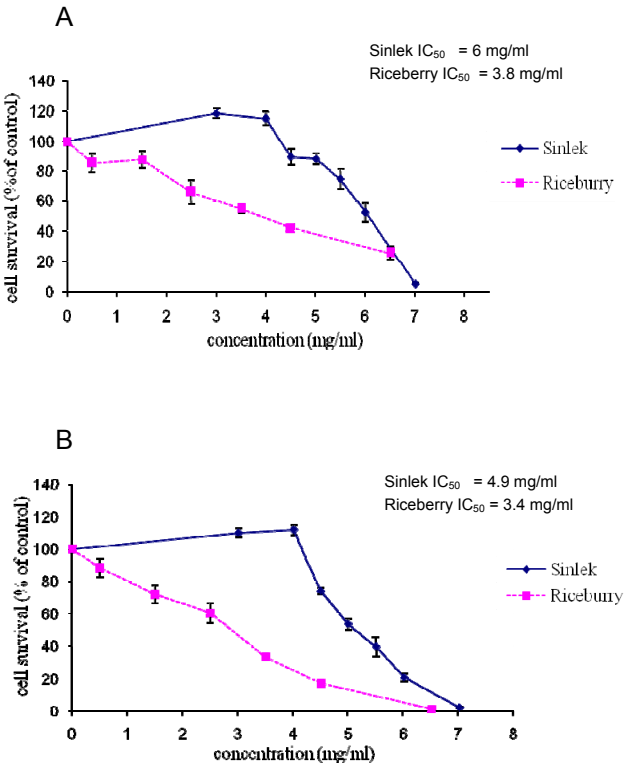
Cytotoxic effects of defatted rice bran extract on HL-60 cells

In the present study, the cytotoxic effect of defatted rice bran extracts (Riceberry and Sinlek) on HL-60 cells was characterized by conducting MTT assay. Cells were treated with various concentrations of extract for 24 and 72 h. The viability of cells was decreased significantly in a dose- and time-dependent manner (Figure 1). Riceberry bran extract showed a lower percentage of viability compared to Sinlek bran extract at all concentrations which corresponds with its lower IC_{50}. Since the lower the IC_{50} value indicated the higher cytotoxic activity of the extract from the pigmented rice. Parado *et al.* demonstrated that the rice bran enzymatic extract has the cytotoxic effect on HL-60 cells with the IC_{50} 2.3 mg/ml (at 72 h). Interestingly, rice bran extract did not exhibit cytotoxicity towards normal peripheral blood lymphocytes.[23]

Inhibition of DNA synthesis in HL-60 cells treated with rice bran extract

Cellular proliferation requires the replication of DNA; therefore, DNA synthesis indicated by BrdU incorporation is a parameter by which the rate of

Figure 1. Effect of bran extracts on HL-60 cell viability at 24 h (A) and 72 h (B). Data are reported as the means ± SEM of three separate experiments.



cell proliferation can be measured. After treatment for 24 or 72 h, both extracts from Sinlek and Riceberry rice bran caused a dose dependent reduction of DNA synthesis in HL-60 cells (Figure2). For the same dose range (2.5-3.5 mg/ml), more prominent effect resulted from Riceberry variety. To evaluate whether the reduced BrdU incorporation observed in treated cells was due to cytotoxic or a cytostatic effect, the cell viability was concomitantly assayed. At all concentration of both rice bran extracts, % DNA synthesis was less than % viability, particularly at 24 h. The extract of Sinlek inhibited DNA synthesis without loss of cell viability. These results suggested that growth inhibition in HL-60 cells might arise from the cytostatic and cytotoxic effect of rice bran extract. However, the DNA synthesis seemed to be more sensitive to rice bran extract when compared to the vital metabolic activity. This finding was in accordance with data from other cell culture studies.[24,25]

Apoptosis induction in HL-60 Cells by rice bran extract

In order to evaluate whether the cell death in rice bran extract-treated cells was in part due to the apoptosis or not, the role of this agent trigger cell death by apoptosis was investigated. The methodologies based on AO/EB staining and measurement of caspase activity, respectively for morphological, and biochemical analysis. The results showed that the cell treated with culture media containing 0.1 % DMSO had intact round shape nuclei containing euchromatin (Figure 3A), while cells treated with the test compound had smaller nuclei which exhibited condensed and fragmented chromatin as a hallmark of apoptotic cells (Figure 3B and 3C). After exposure to extracts of both Sinlek and Riceberry rice bran, the percentage of apoptosis was increased significantly in a dose- and time-dependent manner (Figure 4).

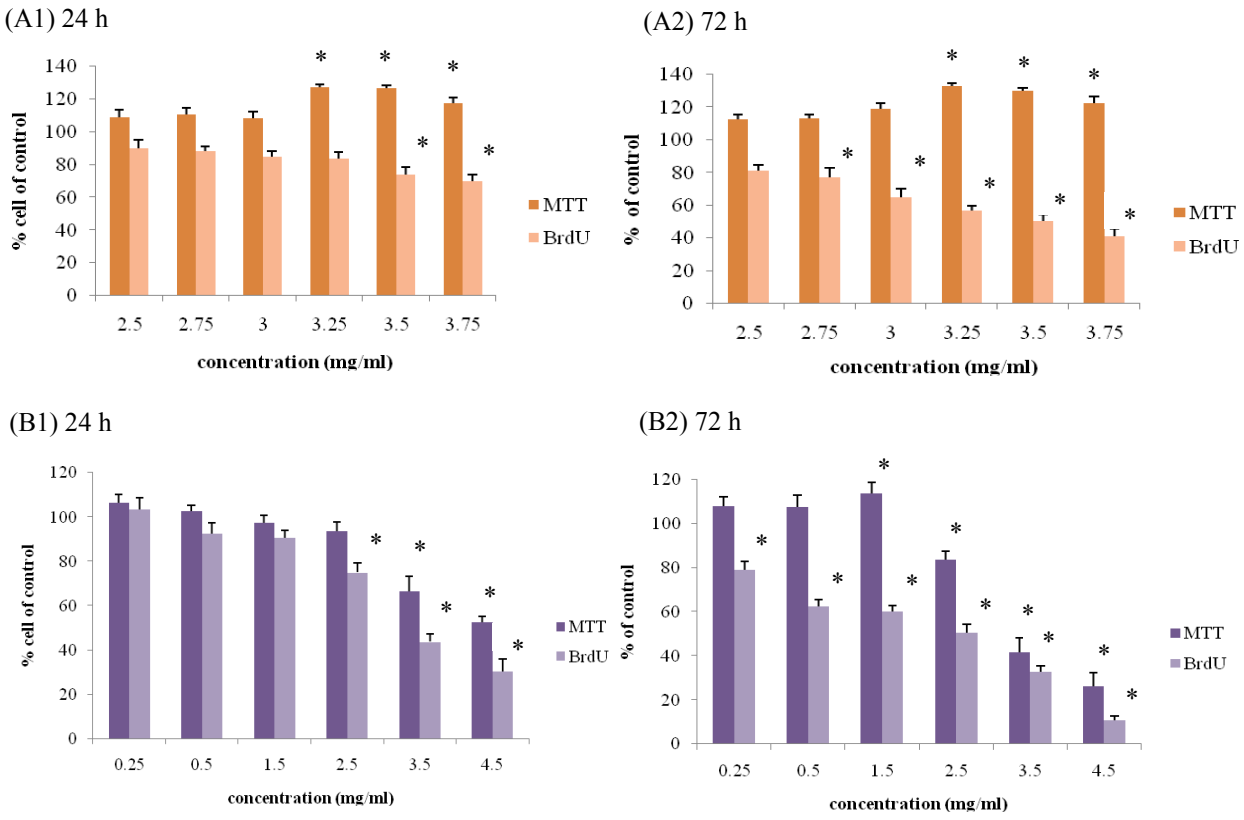


Figure 2. DNA synthesis in HL-60 cells treated for 24 and72 h with various concentrations of (A1-2) Sinlek (B1-2) Riceberry bran extract. Data are presented as mean values ± SEM from Three independent experiments. *P<0.05 mean value of treatment compared to control.

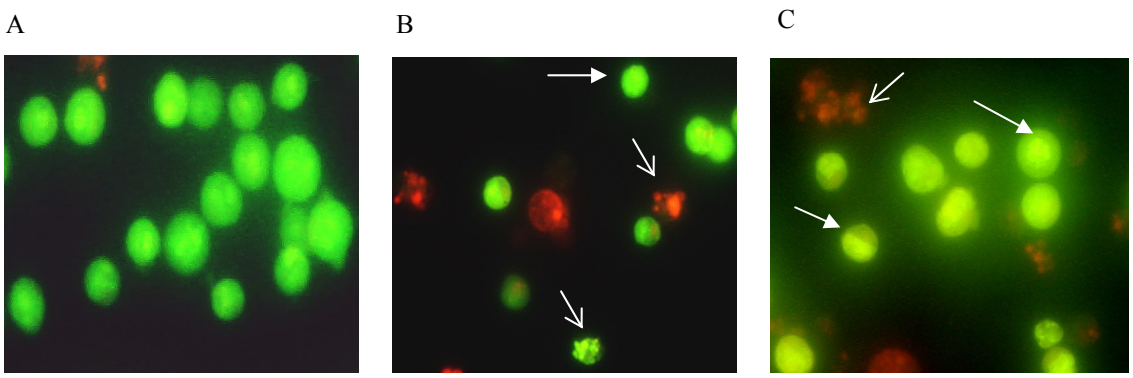


Figure 3. Apoptosis induction by extract of rice bran in HL-60 cells as monitored by AO/EB staining. Cell morphology was observed under fluorescence microscope. The cells with normal morphology were observe in the control group (A); The apoptotic nuclei were demonstrated by chromatin condensation (⟶) and nuclear fragmentation (⟶) were observed after 5.5 mg/ml Sinlek (B) and 3.5 mg/ml Riceberry treatment (C) for 48 h (x400)

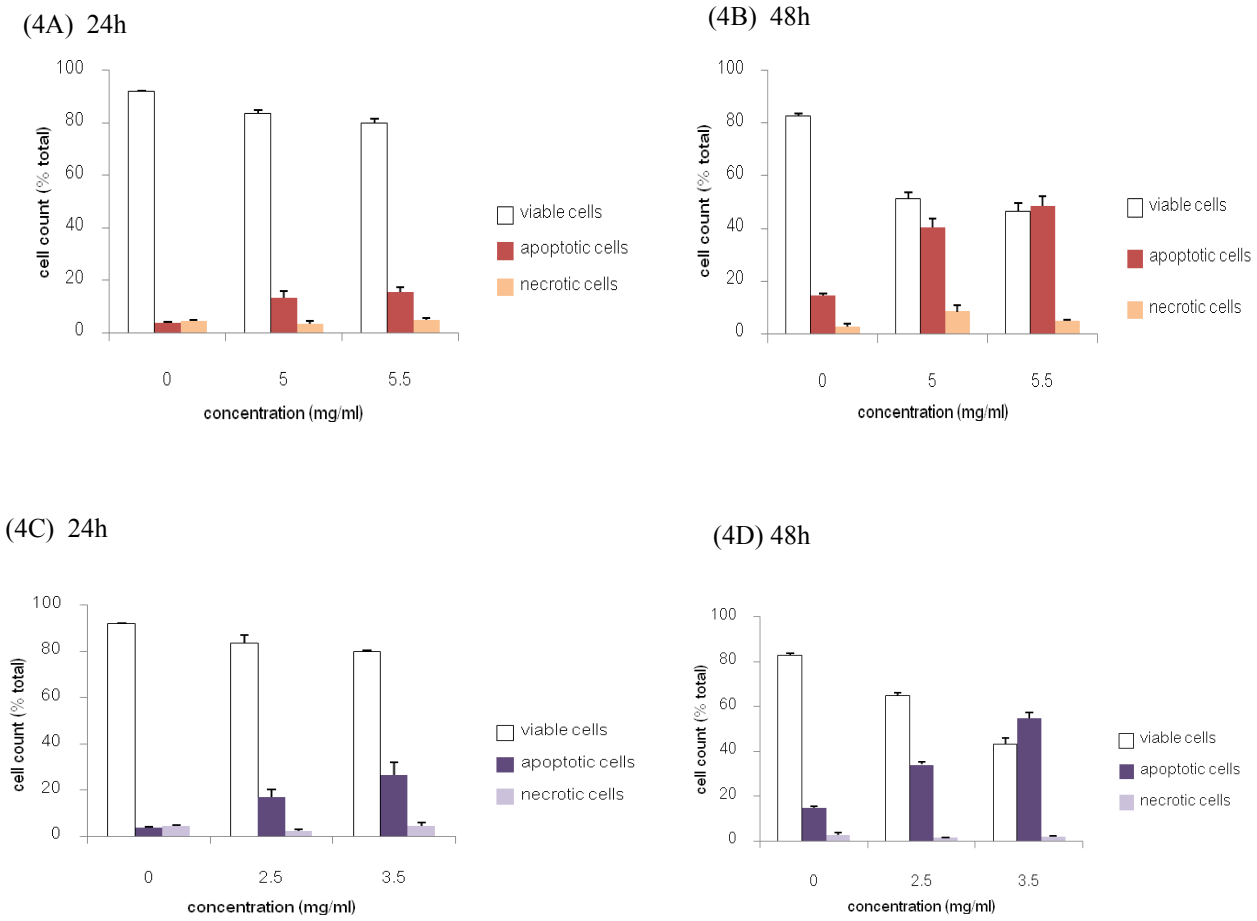


Figure 4. Fraction of viable, apoptotic and necrotic cells in HL-60 cells treated with various concentrations of Sinlek (A1-2) and Riceberry (B1-2) at the indicated times. Results are mean ± SEM from two independent experiments (n=6)

About 50% of cells became apoptosis within 48 h when bran extract of Sinlek and Riceberry were applied 5.5 and 3.5 mg/ml, respectively (Figure 4B and 4D). The results indicated that pigmented Riceberry bran had higher activity in induction apoptosis than did the extract from non-pigmented Sinlek bran. Furthermore, these experimental conditions did not increase the percentage of necrotic cells in the treated cells as compared to control.

Caspases play an important role in execution of apoptosis. Activation of caspases appears to be directly responsible for many of the morphological and biochemical features of apoptosis.[26] Fluorometric assay of caspase activity in HL-60 cells treated with different concentrations of extracts revealed that induction of apoptotic cell death by rice bran extract was through activation of caspases enzyme system. The activity of proteases tended to increase with increasing concentrations of rice bran extracts and duration treatment (Figure 5).

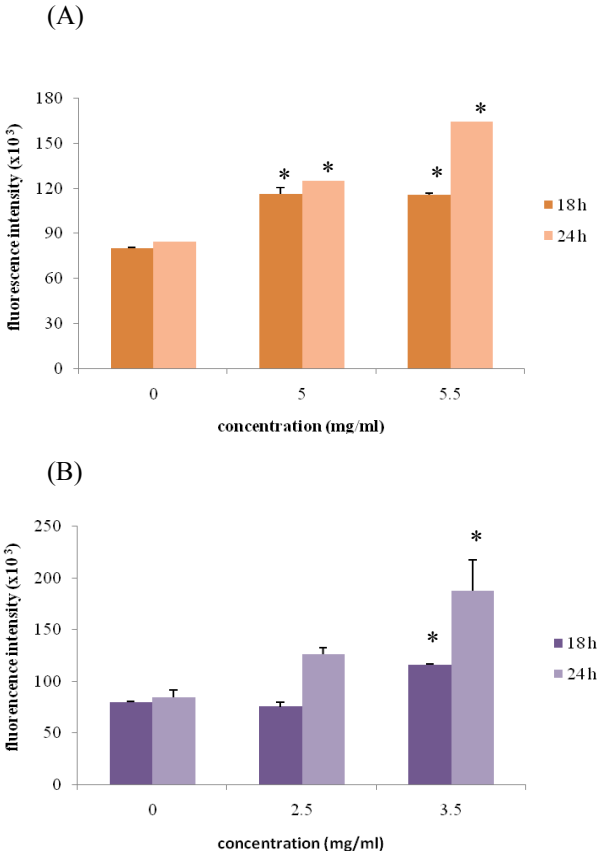


Figure 5. Effect of Sinlek (A) and Riceberry (B) rice bran on caspase activity after treatment with different concentrations of extracts for 18 and 24 hours. The values represented the means ± SEM (*P<0.05; statistical significance to control)

Carcinogenesis is a sequential multistep process, in which numerous molecular mechanisms play different crucial roles. The mechanism by which phytochemicals can modulate cancer risk including blocking initiation and by suppressing the later stages of promotion and progression.[27] From the results, it may be suggested that rice bran has a chemopreventive properties through suppressing activity. The one of possible mechanisms involving the *in vitro* anti-carcinogenic effects of rice bran may be due to their potent anti-oxidative properties. Cancer cells can exist under a condition of oxidative stress, as this increases their survival.[28] Oxidative stress activates redox signaling that may relate to oncogenic stimulation.[29] Mild oxidative has been shown to induce proliferation of human astrocytoma cells.[30] It was suggested that antioxidants could suppress cancer by reducing ROS stimulation cell growth, inducing apoptosis, maintaining normal cell cycle regulation, inducing phase II detoxification enzyme activity, suppressing tumor invasion and angiogenesis.[31] Identification of bioactive components showed that Riceberry rice bran extracts enriched with two anthocyanin compounds (cyanidin 150.81 and peonidin 66.76 mg/100g). Anthocyanins are water-soluble plant pigments and representatives of flavonoids which have strong free radical scavenging activities.[32,33] Anthocyanins also play a crucial role in preventing against mutagenesis and carcinogenesis by inhibit tumor cells proliferation, induce apoptosis in human colon carcinoma and leukemia cells.[34] From our results, Riceberry bran extracts exhibited strong antioxidant activity (data not present). The

scavenging free radical activity (DPPH) of Riceberry (pigmented) extract was about 5 times greater than that of Sinlek (non-pigmented) extract. This finding was consistent with a recent report by Nam *et al*[35] showing the bran of pigmented rice has greater antioxidant activities than those of non-pigmented cultivar. However, the greater vitamin E content (996.08 µg/100g) was found in Sinlek rice bran extract. Vitamin E shows promise as anticancer agents as it inhibits the expression of mutant tumor suppressor gene p53, cell cycle progression and cell proliferation by down-regulation of cyclin.[36,37] Several chemopreventive agents that are antioxidants at some concentrations become prooxidants at other concentrations.[38] Thus, the concentration and environment (redox status) in which they act may be important in promoting or inhibiting cancer cell growth.[39]

The principal anticancer effects of rice bran consist of cytotoxicity, anti-proliferation, anti-promotion, anti-invasion and induction of apoptosis.[40-45] In the present study, the rice bran extracts exhibited growth inhibition to HL-60 cells in a dose- and time-dependent manner. The growth inhibition potential assessed by measuring cell viability, DNA synthesis and apoptotic induction illustrated the greater inhibitory effects from bran of pigmented Riceberry rice compared to non-pigmented Sinlek. The superior effect of pigmented rice may result from two major anthocyanins: cyanidin and peonidin that contained in Riceberry bran. The growth inhibitory activity exerted by rice bran extracts appears to be due to both cytostatic and cytotoxic mechanism, depending on extract concentrations. At lower concentration, bran extracts from both rice varieties induced an appreciable inhibition of proliferation (BrdU incorporartion) without any significant loss of cell viability (MTT assay). These results suggested that at low concentration growth arrested via a cytostatic mechanism, whereas at higher concentrations, rice bran extracts induce significant cell death. Induction of apoptosis is an effective strategy for cancer chemoprevention. In the present study, the cells treated with rice bran extracts showed typical characteristics of apoptosis. The caspase activities in HL-60 cells exposed to bran extracts of both Riceberry and Sinlek rice for 24 h were significantly higher than those of the control. When exposure to rice bran extract was continued for another 24 h, the cells with apoptotic morphological features: DNA condense and fragment were predominantly presented. Consistent with cell cytotoxic and anti-cell proliferation assay, Riceberry bran had higher capability to induced apoptosis as compared to Sinlek variety.

Based on the results of this study, the potential anticarcinogenic activities of the extracts of Riceberry and Sinlek rice bran were demonstrated against human leukemia cells. Further study will be needed to clarify the molecular mechanism of rice constituents against cancer cells and *in vivo* studies may be provided to confirm the *in vitro* results.

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