RESEARCH ARTICLE

Cytotoxic and Apoptotic-inducing Effects of Purple Rice Extracts and Chemotherapeutic Drugs on Human Cancer Cell Lines

Ratana Banjerdpongchai[1]*, Benjawan Wudtiwai[1], Korawan Sringarm[2]

Abstract

Pigmented rice is mainly black, red, and dark purple, and contains a variety of flavones, tannin, polyphenols, sterols, tocopherols, γ-oryzanols, amino acids, and essential oils. The present study evaluated the cytotoxic effects of purple rice extracts (PREs) combined with chemotherapeutic drugs on human cancer cells and mechanisms of cell death. Methanolic (MeOH) and dichloromethane (DCM) extracts of three cultivars of purple rice in Thailand: Doisaket (DSK), Nan and Payao (PYO), were tested and compared with white rice (KK6). Cytotoxicity was determined by 3-(4, 5-dimethyl)-2, 5-diphenyltetrazolium bromide (MTT) assay in human hepatocellular carcinoma HepG2, prostate cancer LNCaP and murine normal fibroblast NIH3T3 cells. MeOH-PYO-PRE was the most cytotoxic and inhibited HepG2 cell growth more than that of LNCaP cells but was not toxic to NIH3T3 cells. When PREs were combined with paclitaxel or vinblastine, they showed additive cytotoxic effects on HepG2 and LNCaP cells, except for MeOH-PYO-PRE which showed synergistic effects on HepG2 cells when combined with vinblastine. MeOH-PYO-PRE plus vinblastine induced HepG2 cell apoptosis with loss of mitochondrial transmembrane potential (MTP) but no ROS production. MeOH-PYO-PRE-treated HepG2 cells underwent apoptosis via caspase-9 and-3 activation. The level of γ-oryzanol was highest in DCM-PYO-PRE (44.17 mg/g) whereas anthocyanin content was high in MeOH-PYO-PRE (5.80 mg/g). In conclusion, methanolic Payao purple rice extract was mostly toxic to human HepG2 cells and synergistically enhanced the cytotoxicity of vinblastine. Human HepG2 cell apoptosis induced by MeOH-PYO-PRE and vinblastine was mediated through a mitochondrial pathway.

Keywords: Purple rice extracts - cytotoxicity - human cancer cells - apoptosis - chemotherapeutic drugs

Asian Pac J Cancer Prev, **14 (11)**, 6541-6548

Introduction

Purple rice is a range of rice types, some of which are glutinous rice. The pigmented rice has been consumed in Eastern and South-eastern Asia. It has been used for supporting kidney function, treatment of anemia and diabetes, promoting blood circulation and ameliorating sight in traditional Chinese medicine. The pigmented rice contains a variety of flavones, tannin, polyphenols, sterols, tocopherols, γ-oryzanols, amino acids, and essential oils. Several anthocyanins have been identified in the pigmented rice, including cyanidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-rutinoside, cyanidin 3, 5-diglucoside, malvidin 3-galactoside, peonidin 3-glucoside, and pelargonidin 3, 5-diglucoside (Deng et al., 2013). There are many bioactivities in anthocyanins, such as antioxidant and reactive oxygen species (ROS) scavenging, antitumor, anti-atherosclerosis, hypoglycemic, and anti-allergic activities (Deng et al., 2013).

Rice bran, a byproduct of the rice milling process, contains most of the phytochemicals. The phenolic, anthocyanin, and proanthocyanidin content of three brown, purple, and red rice brans isolated from different rice varieties using HPLC-PDA is high (Chen et al., 2012). Antioxidative capacities, which are determined by (2,2-diphenyl-1-picrylhydrazyl) DPPH and oxygen radical absorbance capacity (ORAC) and cell-inhibiting effects using an MTT assay, the light-brown bran has no effect, the purple bran exhibits a minor effect on leukemia and cervical cancer cells, and the red bran exhibits strong inhibitory effects on leukemia, cervical, and stomach cancer cells, based on the IC_{50} levels. High concentrations of protocatechuic acid and anthocyanins in purple bran and proanthocyanidins in red bran are identified (Chen et al., 2012).

The concentrations of lipophilic, solvent-extractable (free), and cell wall-bound (bound) phytochemicals and their antioxidant capacities from brans of white, light brown, brown, purple, and red colors, are compared by

*[1]Department of Biochemistry, Faculty of Medicine, [2]Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand *For correspondence: ratana.b@cmu.ac.th*

Min et al. (2011). Total proanthocyanidin concentration is highest in red rice bran, while total anthocyanin is highest in purple brans. The predominant anthocyanin is cyanidin-3-glucoside (Abdel-Aal et al., 2006). Red and purple brans contain high total phenolics and flavonoids determined by ORAC and DPPH (Min et al., 2011).

The distribution of chemical constituents is not even within a rice kernel. Nitrogen and the minerals are found to be more abundant in the outer than in the inner portion, but amylose is rich in the inner portion. The color of flour samples become red rice or purple rice because only the surface of hulled rice contains pigments (Itani et al., 2002).

There are several cultivars in Thai purple rice (*Oryza sativa* var. indica) such as Nan, Doi Saket and Payao. The aims of the study were to determine the cytotoxic effect of the methanolic (MeOH) and dichloromethane (DCM) extracts of these three purple rice cultivars compared to white rice (Kor Khor6) on human hepatocellular carcinoma HepG2 and prostate cancer LNCaP cells compared to murine normal fibroblast NIH3T3 cells. The combined effects with paclitaxel or vinblastine on growth inhibition of cancer cells were demonstrated. The mode and mechanisms of cell death were determined. Anthocyanin and γ-oryzanol amounts in PREs were measured.

Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO), paclitaxel, vinblastine, 3, 3-dihexyloxacarbocyanine iodide ($DiOC_6$), 2',7'–dichlorohydrofluorescein diacetate (DCFH-DA) and 3-(4, 5-dimethyl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma/Aldrich, St. Louis, MO, USA. RPMI-1640 medium, DMEM medium, Z-DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin), Z-LEHD-AFC (Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin), and Z-IETD-AFC (Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin) substrates were obtained from Invitrogen, USA. Annexin V-fluos staining kit was purchased from Roche, Indianapolis, IN, USA.

Rice materials and extraction

Purple glutinous rice (*Oryza sativa*), including 3 cultivars, viz., Nan, Doi Saket (DSK) and Payao (PYO), and white rice (Kor Khor6, KK6) were planted and collected in 2012 from Chiang Mai University, Chiang Mai, Thailand. The purple rice and white rice were separated into two portions and extracted with dichloromethane or methanol, which were then evaporated *in vacuo* at 45-50ºC and freeze-dried to obtain dry extracts. Percent yields of the dichloromethane (DCM) extracts of DSK, PYO, Nan, and KK6 were 2.00, 1.91, 1.64 and 3.05%, respectively, whereas percent yields of the methanolic (MeOH) extracts were 1.39, 1.15, 0.95, 0.88%, respectively. The dry extract was dissolved maximally in dimethylsulfoxide (DMSO) (20 mg/ml), which was then diluted in the medium and added to the cells at the indicated concentrations. The cells were treated with each extract at the concentrations of 0, 50, 100, 150, 200 μg/ml and incubated for 24 or 48 h.

Cell culture

Human prostate cancer LNCaP cells were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. Human hepatocellular carcinoma HepG2 cells and murine fibroblast NIH3T3 cells were gifts from Associate Professor Prachya Kongtawelert (Faculty of Medicine, Chiang Mai University). The prostate cancer LNCaP cells were cultured in RPMI-1640 medium with 25 mM $NaHCO_3$, 20 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin and supplemented with 10% fetal bovine serum. Human hepatocellular carcinoma HepG2 and normal mouse fibroblast NIH3T3 cells were cultured in DMEM medium. The dry powder extract was dissolved in dimethyl sulfoxide (DMSO) as a vehicle and the maximal volume used did not exceed 10 μl/ml of media. The cell lines were grown at 37°C in a 5% CO_2 atmosphere. The murine normal fibroblast cells and human cancer cells (1×10^6) were treated with methanolic and dichloromethane purple rice extracts at indicated concentrations and durations.

Cytotoxicity by MTT assay

HepG2 and LNCaP cells (3×10^5 cells/ml) and NIH3T3 (1×10^4 cells/ml) were cultured and incubated with methanolic and dichloromethane PREs (0, 50, 100, 150 and 200 μg/ml) at 37°C in 5%CO_2 atmosphere for 24 or 48 h. The cell viability was determined by using MTT assay (Wudtiwai et al., 2011). Briefly, MTT solution (sterile stock solution of 5 mg/ml) was added to cell media at the final concentration of 100 μg/ml and the solution incubated for 4 h at 37°C in a humidified 5%CO_2 atmosphere. The medium was then removed and cells were treated with DMSO for 30 min. The optical density of the cell lysate was measured at 540 nm with reference wavelength of 630 nm using microtiter plate reader (Biotek, USA) at 570 nm. The percentage of cell viability was calculated and 10, 20 and 50% inhibitory concentrations (IC_{10}, IC_{20} and IC_{50}) were determined and used for further experiments. The cytotoxic effect of the combined treatment was determined by MTT assay. The combined effects of the extracts (at IC_{20} level) with the chemotherapeutic drugs (paclitaxel or vinblastine, at IC_{20} level) were incubated for 48 h and determined by MTT assay in human HepG2 and LNCaP cells.

Determination of phosphatidylserine externalization in apoptotic cells

PRE-treated HepG2 cells were washed once in phosphate-buffered saline solution, centrifuged at 200× g and the cell pellet was suspended in 100 μl of binding buffer provided by the annexin V-fluos staining kit. Annexin V-FITC (20 μl) and propidium iodide (PI, 10 μl) were added and the cell suspension was left at room temperature for 15 min in the dark. Finally, 970 μl of binding buffer were added. Analysis was conducted using FACScan (Becton Dickinson, USA). Cells that were stained with annexin V-FITC, and annexin V-FITC together with PI, were designated as early and late apoptotic cells, respectively (Prommaban et al., 2012).

Determination of mitochondrial transmembrane potential and reactive oxygen species (ROS) production

Either 40 nM 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6$) for mitochondrial transmembrane potential (MTP) determination or 5 μM 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) for ROS detection were added for 15 min at 37°C before cells were subjected to flow cytometer. Cells were analyzed by a FACScan equipped with a 488 nm argon laser using CellQuest software (Becton-Dickinson, USA). Data were depicted as histograms and percentage of cells displaying loss of MTP or increase of ROS production.

Assay of caspase-3,-8 and-9 activities

Cleavage of the fluorogenic peptide substrates DEVD-AFC, IETD-AFC and LEHD-AFC indicative of caspase-3-, caspase-8- and caspase-9-like enzyme activity, was estimated. Cell lysates (1×10^6 cells) and substrate (50 μM) were combined in a standard reaction buffer and added to a 96-well plate. Enzyme-catalyzed release of AFC was measured by a fluorescence plate reader (Biotek, USA) using 355 nm excitation and 460 nm emission wavelengths.

Determination of anthocyanin and γ-oryzanol contents in purple rice extracts

Anthocyanin content was determined by Ryo method (Ryu et al., 1998). Briefly, each PRE (1 g) was dissolved in 0.5% trifluoroacetic acid (TFA) in 95% ethanol (20 ml), mixed and stirred for 9 h at room temperature, then filtered with filter paper No. 4, C18 cartridge and finally 0.45 μm filter before processing through HPLC-photodioarray (PDA) (Shimadzu, Japan). Detector system is photodiode array detector (520 nm), column 25 cm ×4.6 mm diameter column Allure C18 (Restek, USA), mobile phase A is 0.1% TFA in H_2O whereas mobile phase B is 0.1% TFA in methanol, flow rate 1.0 ml/min. The chromatogram was compared to the standard anthocyanins, viz., C3G and P3G.

The γ-oryzanol content was determined by HPLC method. Briefly, PRE (1 g) was dissolved in 0.5% TFA in 95% ethanol and stirred well at room temperature. HPLC profiles are as follows: UV-vis diode array detector (set 330 and 450 nm), column 25 cm ×4.6 cm of microsorb-MV C18, isocratic mobile phase with methanol: acetonitrile: dichloromethane: acetic acid (50:44:3:3) and flow rate of 1.4 ml/min. The chromatogram was obtained and compared to standard γ-oryzanol.

Statistical analysis

Results are expressed as mean±SD. Statistical difference between control and treated group was determined by one-way ANOVA (Kruskal Wallis analysis) at limit of $p<0.05$ from 3 independent experiments conducted in triplicate. For comparison between two groups, data were analyzed using Mann-Whitney U test.

Results

Cytotoxicity of PREs and/or chemotherapeutic drugs

Methanolic Payao purple rice extract (MeOH-PYO-PRE) was the most cytotoxic to the HepG2 cells compared to other extracts. Percent cell viability of cancer cells decreased dose- and time- dependently in both methanolic (MeOH) and dichloromethane (DCM) extracts of all purple rice cultivars. The sensitivity of the cells towards the extracts was as follows, viz., HepG2>LNCaP>NIH3T3 cells as shown in Figure 1 and 2. Murine normal fibroblast NIH3T3 cells were most resistant to the eight rice extracts (Figure 2). The 50% inhibitory growth concentration was detectable at 48 h to be 175.95±8.02 μg/ml MeOH-PYO-PRE affecting on HepG2 cells, whereas those of the other PREs were more than 200 μg/ml for both cancer cell lines. Paclitaxel or vinblastine was incubated with human HepG2 liver cancer cells and IC_{20} level was calculated to be 7.8±0.42 pM (picomolar) and 40.95±0.02 nM from inhibitory growth curve at 48 h treatment (Figure 3) whereas the IC_{50} levels of paclitaxel or vinblastine towards LNCaP cells were more than 2 μM (Table 1 and 2).

The cell viability of LNCaP and HepG2 cancer cells (when combined with MeOH- or DCM-PREs at IC_{20} levels

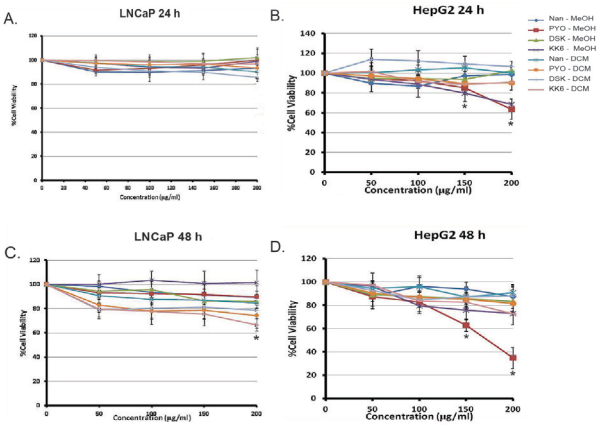

Figure 1. Cell Viability of Human Cancer Cells after Treatment with Purple Rice Extracts for 24 or 48 h by MTT Assay. The cytotoxicity of 24 h PRE treatment of **A)** LNCaP; **B)** HepG2; **C)** 48 h PRE treatment of LNCaP; and **D)** HepG2 cells. Shown as means±SD. The data were obtained from triplicate of 3 independent experiments. MeOH, methanol; DCM, dichloromethane; *$p<0.05$, compared to control

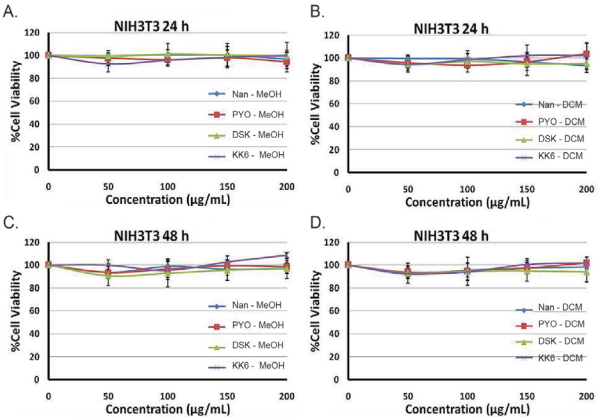

Figure 2. Cell Viability of Murine Normal Fibroblast NIH3T3 Cells after Treatment with Purple Rice Extracts for 24 or 48 h by MTT Assay. The cytotoxicity of 24 h **A)** MeOH-PRE; or **B)** DCM-PRE; or 48 h **C)** MeOH-PRE; or **D)** DCM-PRE. Treatment on NIH3T3 cells is shown as mean±SD. The data were obtained from triplicate of 3 independent experiments. MeOH, methanol; DCM, dichloromethane

Table 1. Cytotoxic Effect of Paclitaxel on Human Hepatocellular HepG2 and Prostate Cancer LNCaP Cells with IC_{10}, IC_{20} and IC_{50} Values at 24 and 48 h

Human cancer cell lines and incubation time	Paclitaxel IC_{10}	IC_{20}	IC_{50}
HepG2 24 h (μM)	0.0374±0.0305	0.105±0.09048	>2
HepG2 48 h (pM)	3.4±0.1	7.8±0.42	36.6±1.93
LNCaP 24 h (μM)	1.486±0.758	1.891±0.479	>2
LNCaP 48 h (μM)	0.215±0.143	0.747±0.448	>2

Table 2. Cytotoxic Effect of Vinblastine on Human Hepatocellular HepG2 and Prostate Cancer LNCaP Cells with IC_{10}, IC_{20} and IC_{50} Values at 24 and 48 h

Human cancer cell lines and incubation time	Vinblastine (nM) IC_{10}	IC_{20}	IC_{50}
HepG2 24 h	N.D.	80.18±0.09	>2000
HepG2 48 h	N.D.	40.95±0.02	561.92±0.02
LNCaP 24 h	288.65±0.01	717.39±0.01	>2000
LNCaP 48 h	N.D.	20.0±0.02	>2000

*N.D., not determinable

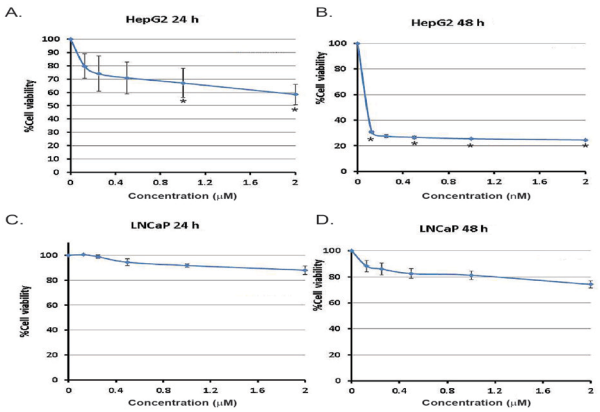

Figure 3. Cell Cytotoxicity of Human Cancer Cells after Treatment with Paclitaxel for 24 or 48 h by MTT Assay. Percent cell viability of HepG2 cells treated with paclitaxel for **A)** 24 h; **B)** 48 h and LNCaP cells treated with paclitaxel for **C)** 24 h; and **D)** 48 h. Shown as mean±SD. The data were obtained from triplicate of 3 independent experiments. *p<0.05 compared to control

plus paclitaxel or vinblastine at 20% inhibitory growth concentration) decreased as an additive effect (Figure 4A-4D and Figure 5A, 5B and 5D). However, 200 μg/ml MeOH-PYO-PRE and 41 nM vinblastine synergistically and significantly reduced cell viability of HepG2 cells as shown in Figure 5C.

Determination of phosphatidylserine externalization, reduction of mitochondrial transmembrane potential and ROS production

The growth inhibition might be caused by apoptosis, so the mode of cell death was determined by using annexin V-FITC/PI and flow cytometry. Annexin V specifically binds to phosphatidylserine, which will flip out to the outer layer of cell membrane in apoptotic cells. The MeOH-PYO-PRE combined with vinblastine-treated HepG2 cells underwent apoptosis 63% significantly compared to without treatment or treatment with vinblastine alone as shown in Figure 6A.

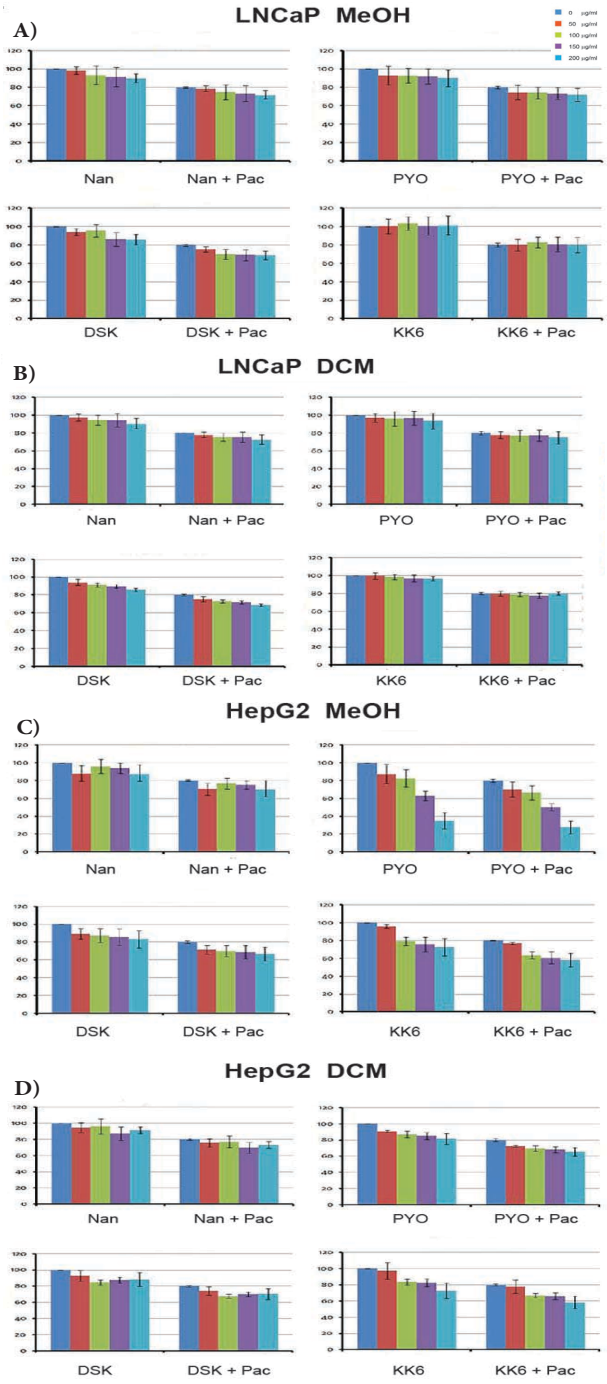

Figure 4. Cell Cytotoxicity of Human Cancer Cells after Combined Treatment with Purple Rice Extracts and Paclitaxel for 48 h by MTT Assay. Percent cell viability of LNCaP cells when treated with **A)** MeOH-PRE; **B)** DCM-PRE; HepG2 cells when treated with **C)** MeOH-PRE; and **D)** DCM-PRE, in the presence or absence of paclitaxel, is shown as mean±SD. The data were obtained from triplicate of 3 independent experiments. MeOH, methanol; DCM, dichloromethane; Pac, paclitaxel

Human hepatocellular carcinoma HepG2 cells treated with MeOH-PYO-PRE combined with vinblastine depolarized the mitochondrial transmembrane potential (MTP) with the reduction of $DiOC_6$ fluorescence determined by flow cytometer. Percent MeOH-PYO-PRE-treated HepG2 with loss of MTP increased significantly at the dose of 200 μg/ml. The combined MeOH-PYO-PRE plus vinblastine-treated cells also reduced MTP when

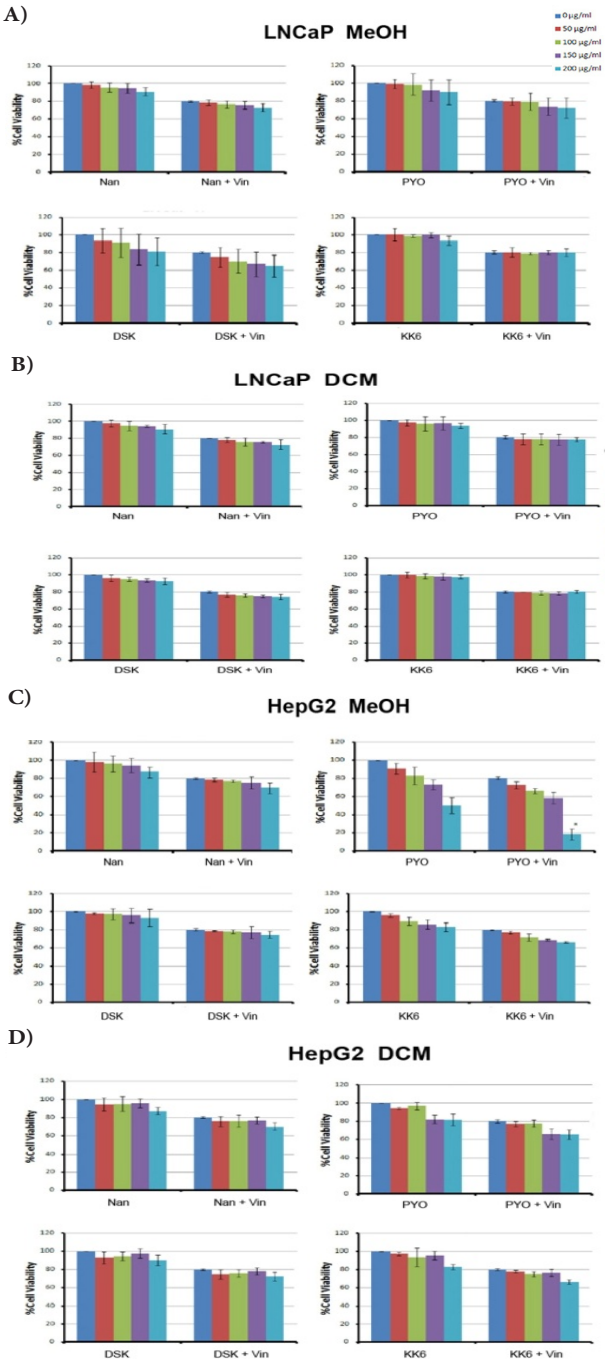

Figure 5. Cell Cytotoxicity of Human Cancer Cells after Combined Treatment with Purple Rice Extracts and Vinblastine for 48 h by MTT Assay. Percent cell viability of LNCaP cells when treated with **A)** MeOH-PRE; **B)** DCM-PRE; HepG2 cells when treated with **C)** MeOH-PRE; and **D)** DCM-PRE, in the presence or absence of vinblastine, is shown as mean±SD. The data were obtained from triplicate of 3 independent experiments.*p<0.05 compared to control, MeOH, methanol; DCM, dichloromethane; Vin, vinblastine

compared to control (without treatment) or vinblastine alone as shown in Figure 6B. However, there was no ROS production as demonstrated by DCFH-DA staining and FACscan (data not shown).

Induction of caspases-3, -8 and -9 activities

To confirm the pathway of apoptosis, caspase-3, -8 and -9 activities were measured by using fluorogenic substrates and the fluorescence microplate reader. The caspase-9 and -3 activities increased significantly in HepG2 cells treated with MeOH-PYO-PRE plus vinblastine at IC_{20} levels compared to control as shown in Figure 7A and 7C, whereas caspase-8 activity did not significantly alter (Figure 7B). This indicated the involvement of intrinsic or mitochondrial pathway of apoptosis which is consistent with the reduction of mitochondrial transmembrane potential.

Anthocyanin and γ-oryzanol contents

Purple rice contains the dark purple pigments in its bran (outer coat of the rice), which are mainly anthocyanins (Abdel-Aal et al., 2006). The content of anthocyanins was then measured to evaluate the amounts of pigments whether they were related to their cytotoxicity. Cyanin 3-glucoside (C3G) was the main component of anthocyanins in purple rice MeOH extracts. In MeOH-Nan-PRE, the total anthocyanins including C3G and peonidin-3-glucoside (P3G) were 7.75 mg/g, which were highest among the three cultivars tested. Total anthocyanins of DSK-PRE were 4.47 mg/g and those of PYO-PRE were 5.80 mg/g whereas there was no anthocyanin detected in KK6 rice extract as shown in Table 3. The anthocyanins could not be detected in dichloromethane extracts since anthocyanins are hydrophilic compared to dichloromethane, which are more hydrophobic.

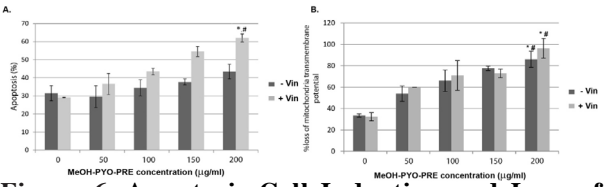

Figure 6. Apoptosis Cell Induction and Loss of Mitochondrial Transmembrane Potential of HepG2 Cells. A) MeOH-PYO-PRE-treated cells in the presence or absence of vinblastine at IC_{20} levels were examined by staining with annexin V-FITC/propidium iodide and using flow cytometry. Percentage of early apoptotic cells is shown. **B)** MTP was measured by staining the cells with $DiOC_6$ and proceeding through flow cytometer. Percentage of cells with loss of MTP is shown.*p<0.05 compared to control (without treatment) and #p<0.05 compared to vinblastine alone

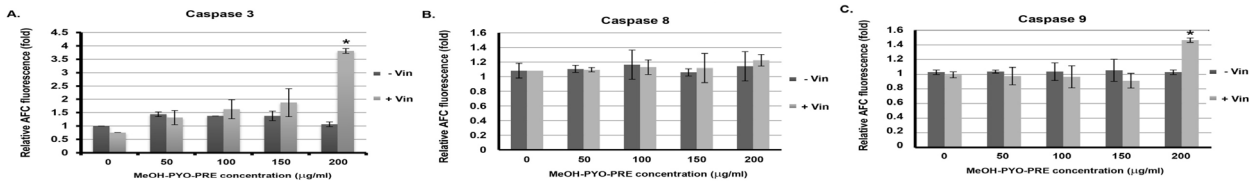

Figure 7. Effects of Methanolic Payao Purple Rice Extract and/or Vinblastine on Caspase-3,-8 and-9 Activities in HepG2 Cells. MeOH-PYO-PRE with or without vinblastine-treated HepG2 cells were determined for **A)** Caspase-3; **B)** Caspase-8; and **C)** Caspase-9 activities as described in the Materials and Methods.*p<0.05 compared to control (without treatment)

Table 3. Concentrations of Anthocyanins in the Methanolic Extracts of Three Cultivars of Purple Rice and White Rice

Cultivars	Concentrations (mg/g)		
	C3G	P3G	Total
Doi Saket (DSK)	2.5	1.97	4.47
Payao (PYO)	3.19	2.61	5.8
Nan	4.4	3.35	7.75
Kor Khor6 (KK6)	N.D.	N.D.	N.D.

*N.D., not detectable; C3G: cyanin 3-glucoside; P3G: peonidin 3-glucoside

Table 4. Concentrations of Gamma-oryzanol in the Dichloromethane and Methanolic Extracts of Three Cultivars of Purple Rice and White Rice

Cultivars	Concentrations (mg/g)	
	DCM	MeOH
Doi Saket (DSK)	36	2.18
Payao (PYO)	44.17	1.62
Nan	42.88	2.98
Kor Khor6 (KK6)	10.94	3.03

*DCM, dichloromethane; MeOH, methanol

The content of γ-oryzanol was detectable in both methanolic and dichloromethane extracts, but it was higher in dichloromethane extracts. The highest amount was found in DCM-PYO-PRE (44.17 mg/g) and the lowest level was MeOH-PYO-PRE (1.62 mg/g) as shown in Table 4.

Discussion

The grain anthocyanin content is easily influenced by the environment. The grain anthocyanin content of the P lines is much greater due to the reduced-grain weight, yield per plot and grain/brown rice thickness compared to the W lines. The small sink size is a key reason behind yield reduction of purple pericarp rice anthocyanin (Ji et al., 2012). C3G and P3G contents in PYO purple rice brans are more than those of DSK cultivar 1.2 folds (data not shown). But purple rice grains (DSK cultivar) contained C3G and P3G 1.8-fold more than Nan and PYO (unpublished data). The purple pigment in the grains of *Oryza sativa* is from the anthocyanin contents (Ryu et al., 1998).

The dietary polyphenol cyanidin, but not its glycosides, is a potent inhibitor of neurotensin- and epidermal growth factor-induced metabolic activity. It increases the free intracellular Ca^{2+} and cellular growth of cultured colon carcinoma cells *in vitro* (Briviba et al., 2001). The anthocyanins also inhibit tumor development *in vivo* (Kang et al., 2003). The aglycones of the most abundant anthocyanins in food, anthocyanidins and delphinidin are potent inhibitors of the EGFR, shutting off downstream MAPK and Elk-1 signaling cascades, contributing substantially to the growth-inhibitory effects on cancer cells (Meiers et al., 2001). Anthocyanidins/anthocyanins and anthocyanin-rich extracts induce TNF-alpha production and act as modulators of the immune response in activated macrophages (Wang and Mazza, 2002).

A standardized extract of black rice (*Oryza sativa* L. indica) pigmented fraction (BRE) containing known proportions of cyanidin 3-glucoside and peonidin 3-glucoside exhibits marked antioxidant activities and free radical scavenging capacities in an *in vitro* model system (Hu et al., 2003).

Oryza sativa cv. Heugjinjubyeo (Gramineae), anthocyanin-pigmented rice, having dark purple grains, is well known as enriched rice with an improved taste. From spectral analysis, the cytotoxic components from this rice trait are the anthocyanidins: cyanidin and malvidin. The 50% growth inhibitory concentrations (IC_{50}) of cyanidin and malvidin on U937, human monocytic leukemia cells, are 60 and 40 μg/mL, respectively. These compounds shows cytotoxicity through the arrest of the G(2)/M phase of cell cycle and induction of apoptosis (Hyun and Chung, 2004).

Two bioactive compounds, peonidin 3-glucoside and cyanidin 3-glucoside, from *Oryza sativa* L. indica are isolated and tested with many cancer cell lines. Human ductal breast carcinoma HS578T cells line is sensitive to peonidin 3-glucoside and cyanidin 3-glucoside. Peonidin 3-glucoside or cyanidin 3-glucoside treatment strongly inhibits cell growth via G2/M arrest. Regarding cell cycle-related proteins, peonidin 3-glucoside treatment results in down-regulation of protein levels of cyclin-dependent kinase (CDK)-1, CDK-2, cyclin B1, and cyclin E, whereas cyanidin 3-glucoside decreases the protein levels of CDK-1, CDK-2, cyclin B1, and cyclin D1. In addition, cyanidin 3-glucoside or peonidin 3-glucoside also induces caspase-3 activation, chromatin condensation, and cell death. Furthermore, anthocyanins from *O sativa* L. indica are evidenced by their inhibition on the growth of Lewis lung carcinoma cells *in vivo* (Chen et al., 2005).

Rice bran exerts beneficial effects towards several types of cancer, such as breast, lung, liver and colorectal cancer. The chemopreventive potential has been related to the bioactive phytochemicals present in the bran portion of the rice, viz., ferulic acid, tricin, β-sitosterol, γ-oryzanol, tocotrienols/tocopherols and phytic acid. These bioactive compounds contain scavenging activity of free radicals and block chronic inflammatory response. The anticancer effects of the rice bran-derived bioactive components are mediated through apoptosis induction, inhibition of cell proliferation and cell cycle progression alteration in malignant cells (Henderson et al., 2012).

The ethyl acetate extract of germinated brown rice (GBR) has higher total phenolic content and antioxidant capacity compared to brown rice. The GBR extract (up to 10 ppm) prevents H_2O_2-induced apoptosis in human SH-SY5Y neuronal cells. The protection of the cells by the GBR extract is linked to its ability to induce transcriptional changes in antioxidant (SOD 1, SOD 2 and catalase) and apoptotic (AKT, NF-Kβ, ERK1/2, JNK, p53 and p38 MAPK) genes that tends towards survival (Azmi et al., 2013). Black rice pericarp extract can inhibit proliferation, change the cell cycle distributions and induce apoptosis in human prostatic cancer cell PC-3. Its inhibitory effect is through promoting activation of the JNK and p38 signaling pathway (Jiang et al., 2013).

The water-soluble enzymatic extract from rice bran (EERB) induces MOLT-4 cell (human T cell acute

lymphoblastic leukemic) apoptosis in a dose-dependent way. Additionally, EERB exerts an immunoactivatory effect on N13 microglia cells, by inducing TNF-alpha (tumour necrosis factor-α) expression, which plays a key role in the innate immune response to infection (Revilla et al., 2013).

Brown rice fermented with *Aspergillus oryzae*, designated as FBRA, is a dietary fiber-rich food, and fully appreciated as one of the prebiotics, which are generally considered to be beneficial to the health of the body, because of stimulating the growth and/or the activity of bacteria in the digestive system. The exposure of human colorectal cancer HCT116 cells to FBRA extract reduces their viabilities in a concentration-dependent manner, and the cytotoxicity is attributed to the induction of apoptosis through the cellular oxidative stress. FBRA extract causes a significant elevation of Bax protein and a slight reduction of Bcl2 protein levels, and activates caspase-3 activity. Thus, FBRA extract can exert oxidative damage to the cells, resulting in apoptotic cell death by activating the mitochondrial pathway in human colorectal tumor HCT116 cells (Itoh et al., 2012).

The anthocyanin-rich extract from black rice (AEBR) reduces the viability of breast cancer cell lines MCF-7 (ER(+), HER2/neu(-)), MDA-MB-231 (ER(-), HER2/neu(-)), and MDA-MB-453 (ER(-), HER2/neu(+)) and induces apoptosis in MDA-MB-453 cells via the intrinsic pathway *in vitro* by activating caspase cascade, cleaving poly (ADP-ribose) polymerase (PARP), depolarizing mitochondrial membrane potential, and releasing cytochrome c. Oral administration of AEBR (100 mg/kg/day) to BALB/c nude mice bearing MDA-MB-453 cell xenografts significantly suppresses tumor growth and angiogenesis by inhibiting the expression of angiogenesis factors MMP-9, MMP-2, and uPA in tumor tissue (Hui et al., 2010).

The ethyl acetate extract of "Kurosu" (EK), Japanese traditional vinegar from unpolished rice, inhibits cell proliferation of human cancer cell lines, viz., colon adenocarcinoma (Caco-2), lung carcinoma (A549), breast adenocarcinoma (MCF-7), bladder carcinoma (5637), and prostate carcinoma (LNCaP) cells. Flow cytometry of EK-treated Caco-2 cells demonstrates a decrease of cell number in the G(2)/M phase and an increase in the sub-G1 phase (apoptotic). p21 mRNA expression is induced in EK-treated Caco-2 cells. Thus, EK causes G0/G1 arrest through p21 induction in Caco-2 cells (Nanda et al., 2004).

Paclitaxel enhances the polymerization of tubulins to stabilize microtubules. The drug binds specifically to the microtubule polymer and polymerizes tubulin even in the absence of cofactors such as guanosine triphosphate and microtubule-associated proteins. Paclitaxel blocks cell proliferation at the G(2)/M phase and such cells are unable to form a normal mitotic apparatus (Horwitz, 1994). The combined effect of all purple rice extracts on paclitaxel-induced cell cytotoxicity is not synergistic or antagonistic but additive (Figure 4A-D, 5A, 5B, 5D). Significant prevention of supercoiled DNA strand scission induced by reactive oxygen species (viz., peroxyl radicals and hydroxyl radicals) and suppression of the oxidative modification of human low-density lipoprotein are obtained when incubated with the rice extracts (Hu et al., 2003). Vinblastine rapidly induces Noxa and acutely sensitizes primary chronic lymphocytic leukemia cells to ABT-737 (a Bcl-2 inhibitor), which therefore enhances CLL cells to undergo apoptosis (Bates et al., 2013). The synergistic effect of MeOH-PYO-PRE on vinblastine-induced HepG2 cytotoxicity was found in HepG2 cells (Figure 5C) and such extract plus vinblastine induced apoptotic cell death. It has been reported that γ-oryzanol reduces plasma cholesterol in hypercholesterolemic hamsters (Wilson et al., 2007) and also suppresses the accumulation of cholesterol in arterial endothelium (atheroma) in hypercholesterolemic rabbits, which is its metabolic effect (Hiramatsu et al., 1990).

Human prostate cancer LNCaP cells are more resistant to PREs, paclitaxel and vinblastine than human hepatocellular carcinoma HepG2 cells with higher IC_{50} levels in LNCaP cells than those of HepG2 cells (Figure 1-3, Table 1 and Table 2). The mechanism of resistance in prostate cancer cells remains elusive and needs further investigation. The synergistic effect of MeOH-PYO-PRE may be of clinical use in reducing the dose of vinblastine in hepatocellular cancer treatment.

In conclusion, methanolic purple rice extract of Payao (MeOH-PYO-PRE) cultivar contained the highest inhibitory growth effect on human hepatocellular carcinoma HepG2 cells, which related to the high anthocyanin contents, i.e., cyanin 3-glucosides and peonidin-3-glucosides rather than to γ-oryzanol amounts. MeOH-PYO-PRE induced human HepG2 cell apoptosis via the mitochondrial pathway with the loss of MTP and activation of caspase-3 and -9.

Acknowledgements

The authors thank the National Research Council of Thailand (NRCT) and Agricultural Research Development Agency, grant No. 2555NRCT512321, for the financial support.

References

Abdel-Aal el-SM, Young JC, Rabalski I (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J Agric Food Chem*, **54**, 4696-704.

Azmi NH, Ismail N, Imam MU, Ismail M (2013). Ethyl acetate extract of germinated brown rice attenuates hydrogen peroxide-induced oxidative stress in human SH-SY5Y neuroblastoma cells: role of anti-apoptotic, pro-survival and antioxidant genes. *BMC Complement Altern Med*, **13**, 177-84.

Bates DJ, Danilov AV, Lowrey CH, Eastman A (2013). Vinblastine rapidly induces Noxa and acutely sensitizes primary chronic lymphocytic leukemia cells to ABT-737. Mol Cancer Ther, (in press).

Briviba K, Abrahamse SL, Pool-Zobel BL, Rechkemmer G (2001). Neurotensin-and EGF-induced metabolic activation of colon carcinoma cells is diminished by dietary flavonoid cyanidin but not by its glycosides. *Nutr Cancer*, **41**, 172-9.

Chen MH, Choi SH, Kozukue N, Kim HJ, Friedman M (2012). Growth-inhibitory effects of pigmented rice bran extracts and three red bran fractions against human cancer cells: relationships with composition and antioxidative activities.

J Agric Food Chem, **60**, 9151-61.
Chen PN, Chu SC, Chiou HL, et al (2005). Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis *in vitro* and suppress tumor growth *in vivo*. *Nutr Cancer*, **53**, 232-43.
Deng GF, Xu XR, Zhang Y, et al (2013). Phenolic compounds and bioactivities of pigmented rice. *Crit Rev Food Sci Nutr*, **53**, 296-306.
Henderson AJ, Ollila CA, Kumar A, et al (2012). Chemopreventive properties of dietary rice bran: current status and future prospects. *Adv Nutr*, **3**, 643-53.
Hiramatsu K, Tani T, Kimura Y, Izumi S, Nakane PK (1990). Effect of gamma-oryzanol on atheroma formation in hypercholesterolemic rabbits. *Tokai J Exp Clin Med*, **15**, 299-305.
Horwitz SB (1994). Taxol (paclitaxel): mechanisms of action. *Annal Oncol*, **5**, 3-6.
Hu C, Zawistowski J, Ling W, Kitts DD (2003). Black rice (*Oryza sativa* L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. *J Agric Food Chem*, **51**, 5271-7.
Hui C, Bin Y, Xiaoping Y, et al (2010). Anticancer activities of an anthocyanin-rich extract from black rice against breast cancer cells *in vitro* and *in vivo*. *Nutr Cancer*, **62**, 1128-36.
Hyun JW, Chung HS (2004). Cyanidin and Malvidin from *Oryza sativa* cv. Heugjinjubyeo mediate cytotoxicity against human monocytic leukemia cells by arrest of G(2)/M phase and induction of apoptosis. *J Agric Food Chem*, **52**, 2213-7.
Itani T, Tamaki M, Arai E, Horino T (2002). Distribution of amylose, nitrogen, and minerals in rice kernels with various characters. *J Agric Food Chem*, **50**, 5326-32.
Itoh M, Nishibori N, Sagara T, et al (2012). Extract of fermented brown rice induces apoptosis of human colorectal tumor cells by activating mitochondrial pathway. *Phytother Res*, **26**, 1661-6.
Ji ZJ, Wang XG, Zeng YX, et al (2012). Comparison of physiological and yield traits between purple- and white-pericarp rice using SLs. *Breed Sci*, **62**, 71-7.
Jiang W, Yu X, Ren G (2013). Inhibition effects of black rice pericarp extracts on cell proliferation of PC-3 cells. *Wei Sheng Yan Jiu*, **42**, 474-7.
Kang SY, Seeram NP, Nair MG, Bourquin LD (2003). Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. *Cancer Lett*, **194**, 13-9.
Meiers S, Kemény M, Weyand U, et al (2001). The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *J Agric Food Chem*, **49**, 958-62.
Min B, McClung AM, Chen MH (2011). Phytochemicals and antioxidant capacities in rice brans of different color. *J Food Sci*, **76**, 117-26.
Nanda K, Miyoshi N, Nakamura Y, et al (2004). Extract of vinegar "Kurosu" from unpolished rice inhibits the proliferation of human cancer cells. *J Exp Clin Cancer Res*, **23**, 69-75.
Prommaban A, Kodchakorn K, Kongtawelert P, Banjerdpongchai R (2012). Houttuynia cordata Thunb fraction induces human leukemic Molt-4 cell apoptosis through the endoplasmic reticulum stress pathway. *Asian Pac J Cancer Prev*, **13**, 1977-81.
Revilla E, Santa-María C, Miramontes E, et al (2013). Antiproliferative and immunoactivatory ability of an enzymatic extract from rice bran. *Food Chem*, **136**, 526-31.
Ryu SN, Park SZ, Ho CT (1998). High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. *J Food Drug Analysis*, **6**, 729-36.
Wang J, Mazza G (2002). Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW 264.7 macrophages. *J Agric Food Chem*, **50**, 4183-9.
Wilson TA, Nicolosi RJ, Woolfrey B, Kritchevsky D (2007). Rice bran oil and oryzanol reduce plasma lipid and lipoprotein cholesterol concentrations and aortic cholesterol ester accumulation to a greater extent than ferulic acid in hypercholesterolemic hamsters. *J Nutr Biochem*, **18**, 105-12.
Wudtiwai B, Sripanidkulchai B, Kongtawelert P, Banjerdpongchai R (2011). Methoxyflavone derivatives modulate the effect of TRAIL-induced apoptosis in human leukemic cell lines. *J Hematol Oncol*, **4**, 52-65.