

Black Rice Bran Extracts and Fractions Containing Cyanidin 3-glucoside and Peonidin 3-glucoside Induce Apoptosis in Human Cervical Cancer Cells

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Abstract

Anthocyanin of pigmented rice inhibits the growth of cancer cells. The cytotoxicity and apoptosis-inducing properties of local black rice (cv Cempo Ireng) extracts and fractions, which contain anthocyanin including cyanidin 3-glucoside and peonidin 3-glucoside, on human cervical cancer cell line (HeLa cells) has been evaluated. The pigmented rice bran was extracted and fractionated using methanol-HCl. The MTT test was performed on HeLa cell cultures to observe the IC_{50} value. Preparative TLC was performed to obtain the fractions of black rice bran. Cyanidin 3-glucoside and peonidin 3-glucoside were identified in the pigmented rice bran extract and fractions using UHPLC. Flowcytometry analysis was performed to measure the percentage of apoptotic cells. Our results suggest that the fractions are more toxic than the methanolic crude extract with IC_{50} values of $85.95 \pm 5.56 \mu\text{g/mL}$ (the lowest one) and $408.13 \pm 51.9 \mu\text{g/mL}$, respectively. The concentration of cyanidin 3-glucoside and peonidin 3-glucoside in the methanolic extract were 1.89 and 0.84 $\mu\text{g/mg}$, respectively. The apoptosis induction by fractions F2 and F4 (52 and 55%) were significantly higher compared to fraction F3 and F5 (30 and 33%) and doxorubicin (21%). Cyanidin 3-glucoside was detected in F4 (0.14 $\mu\text{g/ml}$) while peonidin 3-glucoside in F2 (0.012 $\mu\text{g/ml}$), however both were not detected in F3 and F5.

Keywords: Cempo Ireng, cyanidine 3-glucoside, peonidin 3-glucoside, apoptosis, HeLa cells

Introduction

Indonesia is rich in terms of biodiversity, containing many local black rice cultivars. One of them is cultivated in the Special Region of Yogyakarta Province (DIY) named as 'Cempo Ireng' (Kristamtini *et al.*, 2013). Previous studies reported that Cempo Ireng contains high level of anthocyanin and antioxidant activity, thus normalize serum lipid profile in hyperlipidemia rats (Kristamtini *et al.*, 2012; Pratiwi *et al.*, 2014).

Deng *et al.*, (2013) and Banjerdpongchai *et al.*, (2013) reported that black rice or pigmented rice contains variety of flavones; including anthocyanins, tannin, polyphenols, sterols, tocopherols, γ -oryzanols, amino acids, and essential oils which are mostly concentrated in the rice bran. Varieties of the pigmented rice are distributed in many countries such as India (Mohanlal *et al.*, 2011), China (Xia *et al.*, 2006), Thailand (Banjerdpongchai *et al.*, 2013), Japan (Tanaka *et al.*, 2008; Matsuba *et al.*, 2010.), Korea (Kim *et al.*, 2008; Lee, 2010) and Indonesia (Suliartini *et al.*, 2011; Kristamtini *et al.*, 2012). The bio-activity of phytochemical compounds in the pigmented rice has been shown as potential cure to metabolic diseases and trigger apoptosis in cancer cells (Deng *et al.*, 2013).

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The aleurone layer of black rice contains 43.2% of total anthocyanin, mostly consists of cyanidin 3-glucoside and peonidin 3-glucoside which possess antioxidative activity in chemical and biological model system (Hu *et al.*, 2003; Xia *et al.*, 2006). Cyanidin 3-glucoside and peonidin 3-glucoside also inhibit tumor cell growth both in vitro and in vivo. These compounds may exert cell growth inhibition through an arrest of the G2/M phase of the cell cycle, inhibition of cell proliferation, or induction of apoptosis. So far, these two compounds of anthocyanins have been observed can enhance the inhibitory effect of doxorubicin on cell growth of HS578T cells (human breast carcinoma) and promoted to be a potential candidate for cancer chemoprevention (Chen *et al.*, 2005). Hyun and Chung (2004) also reported that anthocyanins isolated from *O. sativa* cv Heugjinjubyeo showed cytotoxicity through the arrest of G2/M phase of the cell cycle and induction of apoptosis in monocytic leukemia cells. Furthermore, the inhibitory effect of cell invasion on various cancer cells, including HeLa cells, by these compounds was also reported previously (Chen *et al.*, 2006).

This study aims to evaluate the apoptotic activity of local black rice bran (*Oryza sativa* L. Indica var cv Cempo Ireng) extract and fractions on human cervical cancer cells (HeLa cells).

Materials and Methods

Chemicals and cell lines

Methanol, HCl, n-heksan, n-butanol, glacial acetic acid, silica gel powder 60 F₂₅₄ were obtained from Merck. Tripsin, MTT [3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide], penicillin-streptomycin, fetal bovine serum (FBS), fungizone and RPMI medium were obtained from Gibco. Phosphate Buffer Saline (PBS) was obtained from Sigma, whereas dimethylsulfoxide (DMSO) from Merck and doxorubicin from Ebewe. Human cervical cancer cells (HeLa) were obtained from

a culture collection of the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM).

Rice materials and extraction

Black rice (*Oryza sativa* L. Indica) cv Cempo Ireng was planted and collected from rice farmer Mr. Jamasto from Sayegan, Sleman DIY in 2014. Black rice bran extraction was performed by modifying Kim *et al.* (2008) which has been modified by Abdel-Aal *et al.* (2006). The rice bran (100g) was macerated using 1 L of methanol, whereas BRB was also extracted with water as a solvent using a procedure that modified from Tan *et al.* (2013): HCL 1 N (85:15) for 48 hours, while being stirred occasionally to prevent saturation. After being rested for 2 nights, the bran mass was filtered using Whatman No. 1 filter. The maceration process was repeated twice, with 24 hours of resting period between each process. The methanolic extract was evaporated using waterbath at 60°C to obtain the dry extract.

The dry extract was dissolved in 5% of the media (2.5% DMSO and 97.5% of RPMI) as a stock solution. Cells were treated with extract at the concentrations of 2000, 1000, 500, 250, and 125 µg/mL and the maximal DMSO concentration in each well was 0.2%, followed by 48h incubation.

Cell culture

Propagation of HeLa cells was conducted by the following procedures established by the Laboratory of Parasitology, Faculty of Medicine UGM. Human cervical cancer HeLa cells were cultured in RPMI medium containing 2% penicillin-streptomycin, 0.5% of fungizone and 10% FBS. The methanolic extract, in the form of dry powder, was dissolved in dimethyl sulfoxide (DMSO) as a vehicle with a maximal volume of 10 µl/mL media. The cell were grown at 37°C in a 5% CO₂ atmosphere. The cells (5x10³) were treated with methanolic extracts of rice bran at indicated concentrations and durations.

Cytotoxic test by MTT assay

Cytotoxic test was conducted by modifying Nurhayati *et al.* (2015). One hundred μL of human cervical cancer (HeLa cells) suspension with a density of $5 \times 10^3/100 \mu\text{L}$ media was distributed into 96-well plate and incubated for 24 hours. After incubation, 100 μL of rice bran methanolic extract in different concentrations was added into the well. As a control, 100 μL of doxorubicin in different concentration was used, and 100 μL of culture medium was left without treatment. The cells were incubated for 48 hours in the incubator with 5% CO_2 and 95% O_2 flows. At the end of incubation, culture media were removed and 110 μL of MTT solution (5 mg/mL PBS) was added, followed by 3-4 hours incubation. MTT reaction was stopped by adding stopper reagent SDS (100 μL).

Cell-containing micro-plate was sealed for ± 5 minutes, wrapped with aluminum foil and incubated for overnight at room temperature. The assay results were analyzed using an ELISA reader at 595 nm wavelength. The cell death percentage was calculated based on the formula $[(A-D)-(B-C)]/(A-D) \times 100 \%$, in which A = control absorbance, b=extract absorbance, C=extract control absorbance, and D=media control absorbance. The value of Inhibitor Concentration 50 (IC_{50}) was determined statistically using probit analysis with SPSS 13 statistic application.

Fractionation of black rice bran methanolic extract

The methanolic extract of black rice bran (1g crude extract homogenized in 3 mL methanol) was fractionated and isolated using preparative-thin layer chromatography with silica-gel as stationery-phase and buthanol as mobile phase. The solvents are buthanol : acetic acid: water (4:1:5 v/v) after being concentrated for 17 hours. The extract was dotted on the silica plate using capillary pipe at about 1.5 cm from the bottom. The silica plate was put in the solvent jar and incubated for 3 hours (when the eluen reached about 1.5 cm from the upper edge). Bands on the plate were exposed to UV

254 and UV 366 nm. The bands were marked on the Rf side and scraped carefully. The fractions were macerated using methanol:HCl 1% to eliminate the silica powder. Each fraction was filtered and dried up using waterbath at 60°C.

Cytotoxicity test of fractions

Each fraction was tested for the cytotoxic activity on HeLa cells using MTT method with dosage $> \text{IC}_{50}$ of black rice bran methanolic extract.

Apoptosis Analysis Using Flow cytometry

Apoptosis analysis was performed using flowcytometry using annex V-FLUOS staining kit. HeLa cells were treated using active fractions of the black rice methanolic extract and doxorubicin for 48 hours. The cells were harvested using 0.25% trypsin and washed with PBS. The cells were treated using 100 μL of Annexin-VFLUOUS staining kit, and incubated in dark condition for 10 minutes at 25° - 27°C. The obtained data of apoptotic and necrotic cells were analyzed using flowcytometer FACSCalybur (Becton-Dickinson).

Quantification of cyanidine 3-glucoside and peonidin 3-glucoside

The concentration of cyanidin 3-glucoside and peonidine 3-glucoside in the fractions were detected and measured by using Ultra High Performance Liquid Chromatography (UHPLC-Perkin Elmer, PhotoDiode Array or PDA detector and column C-18). The separation process was performed using linear proportion starting from 13% B to 24% B for 8 minutes and increased by 100% in 14 minutes with flow velocity of 0.3 ml/minute. The mobile phase consists of formic acid of 3% in the Mili-Q water (A) and Me-OH UHPLC grade (B). The chromatogram was recorded at an absorbance of 520 nm. Injection volume was 20 μL of cyanidin 3-O-glucoside and peonidin 3-O-glucoside. Quantification was performed by calibration of the standard curve and repeated for 3 times.

Statistical analysis

Results are expressed as mean \pm SD. Statistical differences between control and samples were determined by one-way ANOVA (Kruskal Wallis analysis) at the 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 and Discussion

Methanol is a polar solvent suitable to extract phenolic compound such as flavonoid from the black rice bran, especially anthocyanines including cyanidin 3-glucoside and peonidin 3-glicoside. Water, which is also a polar solvent, possesses different polarity with methanol, thus comparing both solvents to extract the active compounds of black rice bran is of interest. The rendement productions from methanol and water

extraction process and colour of black rice bran extracts is shown in Table 1.

Methanolic and water extract of black rice bran showed different colours, indicating that each extract contains different portions of pigmented compounds. Anthocyanin is soluble in water, while some pigmented-lipid compounds such as vitamin E are not soluble.

TLC-Chromatogram of methanolic and water extract of black rice bran

Chromatogram-TLC profiles of methanolic and water extracts of black rice bran showed 6 and 5 spots of compounds respectively (Table 2). Each extract showed different patterns, except for Rf 0.14 which is similar. Methanolic extract at Rf 0.75 or spot no 5, showed a clear spot which is similar to rutin (flavanoids standards), suggesting that the anthocyanin compounds, such as

Table 1. Rendement and colour of black rice 'Cempo Ireng' bran extract

Solvent	Rendement (%)	Colour of extract
Methanol	20.60	Dark red
Water	28.47	Dark brown

Table 2. Spots and Retardation factor (Rf) on TLC of black rice bran extracts

Extract	No. of spots	Rf
Methanol	6	0.14, 0.28, 0.46, 0.62, 0.75, 0.88
Water	5	0.14, 0.20, 0.36, 0.56, 0.73

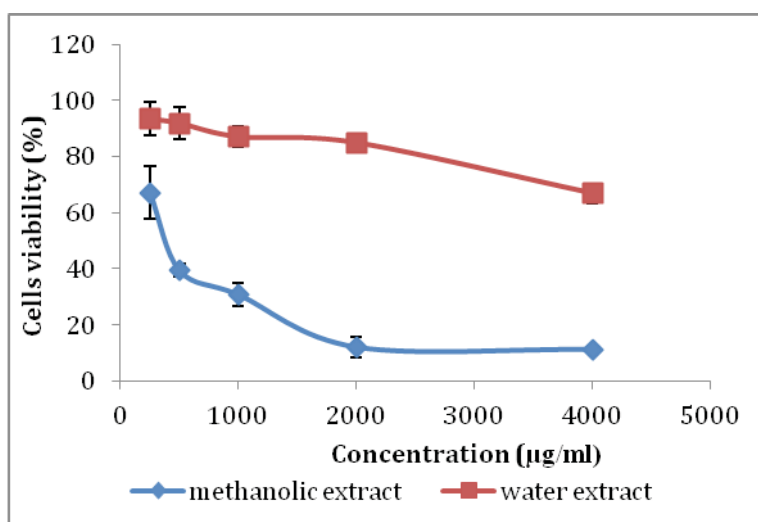


Figure 1. HeLa cells treated with fractions of methanolic and water extract of black rice bran. The cells viability shows decreasing in HeLa cells cultures after treated with methanolic extract compare to water extract treatment.

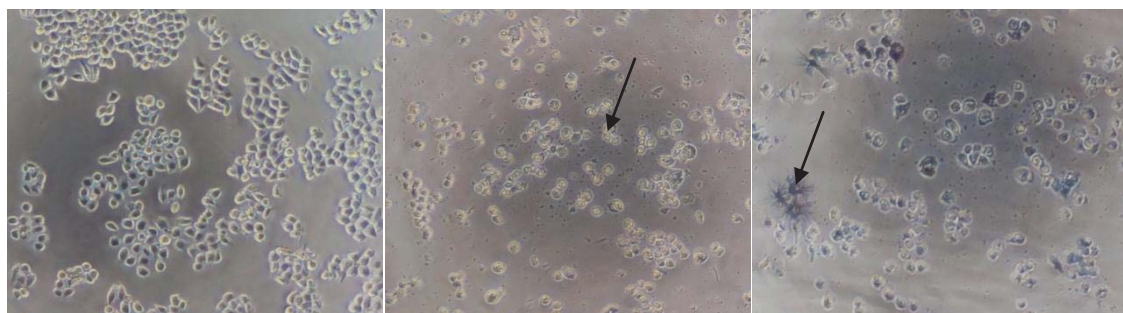


Figure 2. HeLa cells morphology in control media (a), methanolic extract treatment media (b) and 4 h incubated in MTT media. The death cells are pointed by the arrow.

cyanidin and peonidin, of black rice extract most likely are contained in this spot.

Cytotoxic activity of methanol extract of black rice bran

Methanolic and water extracts of black rice bran were evaluated for cytotoxicity activities on HeLa cells. Methanolic and water extracts of black rice bran showed different responses on HeLa cells. The Table 3 and Figure 1 showed that the methanolic extract has IC_{50} of 408.13 $\mu\text{g}/\text{mL}$, which is much lower than the water extract. This result might be due to the difference in compounds contained in both extracts. The water extract of black rice bran was not toxic, suggesting that the concentration of cytotoxic compound (IC_{50} value) on cells should be less than 1000 $\mu\text{g}/\text{mL}$ (Meyer *et al.*, 1982).

Table 3. The IC_{50} values of black rice bran methanolic extract on HeLa cells (48 h incubation)

Treatment	IC_{50} ($\mu\text{g}/\text{ml}$)
Methanolic extract of black rice bran	408.13 \pm 51.9 0
Water extract of bran	22,735.26 \pm 17,872.15
<i>Doxorubicin</i>	0.46 \pm 0.06

The cytotoxicity tests results of the methanolic and water extracts correlates with the abnormality of HeLa cells morphology after treatment (Figure 2). The active compounds contained in the extract cause abnormal membrane structures and loss of cell adhesion to adjacent cells or extracellular matrix (Lu *et al.*, 2012).

Cytotoxic effect of the extract fractions on HeLa cells

The methanolic extract fractions of black rice bran can be separated into 6 fractions with R_f values ranged from 0 - 0.97 with each specific colour (Table 4). Most fractions from preparative TLC showed the cytotoxic activities on HeLa cells with IC_{50} values lower than methanolic extract (408.13 $\mu\text{g}/\text{mL}$) (Table 5). HeLa cells are more sensitive to fractions F5, F3, F2 and F4 with the IC_{50} values of 86, 97, 97 and 111 $\mu\text{g}/\text{mL}$ respectively, compared to Fraction F1 and F6.

Table 4. Fractions of black rice bran methanolic extract

PTLC Fraction	R_f	Mass (g)	Colour
F1	0.81 - 0.97	0.1566	Dark yellow
F2	0.75 - 0.81	0.0902	Red
F3	0.53 - 0.75	0.1881	Brown
F4	0.44 - 0.53	0.3046	Dark violet
F5	0.13 - 0.44	0.5091	Black
F6	0 - 0.13	0.8019	Dark brown

Table 5. The IC_{50} values of PTLC fractions to HeLa cells after 48 hours incubation

Fraction	IC_{50} ($\mu\text{g}/\text{mL}$)
F1	180.57 \pm 7.86
F2	96.83 \pm 7.78
F3	91.71 \pm 4.49
F4	111.47 \pm 3.29
F5	85.95 \pm 5.56
F6	131 \pm 0.063

Table 6. Apoptotic and necrotic cells percentage caused by PTLC fractions of black rice bran methanolic extract on HeLa cells

Treatments	Cells Percentage (%)		
	Live	Apoptotic cells	Necrotic cells
Media control	87.85 ± 0.36 ^f	9.3 ± 0.68 ^a	2.86 ± 0.32 ^a
F2	33.88 ± 1.02 ^c	52.09 ± 1.30 ^d	14.03 ± 0.28 ^b
F3	49.15 ± 0.014 ^d	32.78 ± 0.41 ^c	18.08 ± 0.40 ^c
F4	29.22 ± 0.028 ^b	55.16 ± 0.19 ^d	15.63 ± 0.16 ^b
F5	51.85 ± 0.13 ^e	30.18 ± 0.59 ^c	17.97 ± 0.72 ^c
<i>Doxorubicin</i>	3.85 ± 0.11 ^a	21.29 ± 0.61 ^b	74.87 ± 0.71 ^d

Table 7. Cyanidin 3-glucoside and peonidin 3-glucoside concentration in the methanolic extract and fractions of black rice bran

Sample	Cyanidin 3-glycoside			Peonidin 3-glycoside		
	RT*	Area	conc. (µg/mg)	RT*	Area	conc. (µg/mg)
Extract	12.81	660,331.07	1.89	13.80	393,357.64	0.843
F4	12.85	65,738.26	0.14	nd	0	0
F2	nd	0	0	13.89	4,050.64	0.012
F5	nd	0	0	nd	0	0

RT* = Retention Time

nd = not detected

Apoptosis induction on HeLa cells by fractions containing cyanidine 3-glucoside and peonidin 3-glucoside

Fractions of the methanolic extract of black rice bran showed higher cytotoxic activities on HeLa cells compared to the methanol crude extract, but lower than doxorubicin, a common drug for cancer chemotherapy. To confirm whether the cause of cell death is due to apoptosis or necrosis, flowcytometry was performed.

The results demonstrated that all fractions of F2, F3, F4 and F5 induced apoptosis more effectively (> 50%) compared to doxorubicin (21.29%) (Table 6). Sugimoto *et al.* (2002) also reported that low-dose of doxorubicin (40 nmol/L) induced 90% of necrosis and 8% of apoptosis in Jurkat cells. Furthermore, the percentages of apoptosis induction by fractions F2 and F4 are higher than F3 and F5. These results are consistent with Hui *et al.* (2010) suggesting that compounds in the black rice bran induced apoptosis rather than necrosis in HeLa cells.

In addition, we measured the concentration of anthocyanin compounds,

especially cyanidin 3-glucoside and peonidin 3-glucoside. Previous studies reported that anthocyanin compounds have cytotoxic activity and induce apoptosis on several lines of cancer cells (Chen *et al.*, 2005). Cyanidin 3-glucoside (1.89 µg/mL) and peonidin 3-glucoside (0.84 µg/mL) were identified in the methanolic extract of black rice bran, consistent with Xia *et al.* (2010). Results also showed that fractions F2 contain only peonidin 3-glucoside whereas F4 only contain cyanidin 3-glucoside (Table 7). In contrast, there were no cyanidin and peonidin detected in fraction F5, while F3 was excluded because the R_f of this fraction was out of the range of R_f flavonoid standard.

Based on these results, we suggest that the apoptosis induction in HeLa cells most probably is caused by cyanidin 3-glucoside and peonidin 3-glucoside containing fractions. Cyanidin 3-glucoside was detected in fraction F4 (0.14 µg/mL) and peonidin 3-glucoside in F2 (0.012 µg/mL). However, both compounds were not detected in fraction F3 and F5 of methanolic extract of black rice 'Cempo Ireng' bran.

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