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Fermented black rice bran extract inhibit colon cancer proliferation in WiDr cell lines

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Abstract

The objective of this study was to find the best inhibition of WiDr colon cancer cells growth using Fermented Black Rice Bran (FBRB) extract and the mechanism of colon cancer inhibition by fermented rice bran. In this study, BRB was fermented by *Rhizopus oligosporus* (FBROLI), *Rhizopus oryzae* (FBRO), respectively, for 0, 24, 48, 72, and 96 h to analyze changes profile of phenolic content, total flavonoid, and antioxidant activity during fermentation. The result of elected fermentation and non-fermented (0 h) (NFBR) analyzed cytotoxic activity on Vero normal cells and WiDr cells. Genes' mRNA expression of proliferation and apoptotic markers on WiDr cells was also analyzed that normalized the ACTB housekeeping gene. The results demonstrated that fermentation for 72 h produced the highest phenolic content, total flavonoids, and antioxidants activity with FBROLI extract, which had the highest inhibition of WiDr cells with IC_{50} value 650.7 µg/mL, while the FBRO with IC_{50} was 873.9 µg/mL, and NFBR with IC_{50} was 831.8 µg/mL. The mechanism of inhibition of WiDr cells through suppression of PCNA proliferation gene expression and increased apoptotic gene expression of p53, caspase-8, caspase-9, and caspase-7 with the highest increase of apoptotic gene expression obtained FBROLI extracts.

Keywords: fermentation; rice byproduct; *rhizopus*; WiDr; apoptosis.

Practical Application: The investigated FBRB extract has the potential as a functional food to inhibit the growth of colon cancer cells.

1 Introduction

Colon cancer is the third highest cause of death globally, reaching as high as 1,148,515 incidents in 2020. It is expected to increase by 2040, with an estimated 1,823,278 incidents around the globe (International Agency for Research on Cancer, 2020). Cancer begins with the mutation of DNA (Deoxyribonucleic Acid) in cells known as mutation cells. The mutated cells begin to replicate and uncontrollably divide at a high rate, thus damaging the surrounding tissue. This mutation is due to the damage of tumor suppressor genes and the oncogenic activation (Gamudi & Blundell, 2010). Colon cancer occurs in the inner lining of the large intestine.

Rice bran is a byproduct of the rice polishing process. It contains tricin, γ-oryzanol, ferulic acid, ß-sitosterol, tocopherols, tocotrienols, and phytic acid that might be able to inhibit the growth of colon cancer cells. The inhibition of colon cancer cell growth by bioactive compounds in rice bran is classified as preventive. On the contrary, chemotherapy agents, such as oxaliplatin, irinotecan, leucovorin, UFT, capecitabine, and 5-fluorouracil, are generally used to treat cancer patients, and are classified as curative. The use of these curative agents often disrupts the function of other organs (Focaccetti et al., 2015). Therefore, rice bran is likely to be developed as a functional food to prevent colon cancer.

The potential of cancer prevention in plant material is often related to their high antioxidant activity, which is determined by the number of free forms bioactive compounds present (Chen et al., 2015).

One effective technique to increase bioactive compounds in rice bran was found to be the solid-state fermentation technique. Fermentation using *R. oryzae* and *R. oligosporus* fungi were reported effectively for increasing bioactive compounds in rice bran (Zulfafamy et al., 2018; Razak et al., 2017; Ardiansyah et al., 2019). Martins et al. (2011) reported that the increase in the number of bioactive compounds is dependent on the type of inoculum used as well as the fermentation time.

Therefore, this study aimed to assess the inoculum and fermentation time to obtain rice bran while also examining cancer cells' highest antioxidant activity and cytotoxic activity. For comparison, the fermented and non-fermented black rice bran was examined for its cytotoxic activity on WiDr cancer cells and normal Vero cells. According to the authors' knowledge at

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present, no studies have been conducted to examine the relations of rice bran fermented with gene expression of colon cancer cells. Hence, in this study, the expression of apoptosis marker genes was also identified to determine the inhibitory growth pathways of WiDr cells, including p53, caspase 7, 8, and 9 genes, and PCNA proliferation marker genes. The findings of this study can contribute to increase the production of functional food products produced with black rice bran.

2 Materials and methods

2.1 Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH•), quercetin, gallic acid, Fetal Bovine Serum (FBS), Phosphate Buffer Saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI-1640), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Folin-Ciocalteu, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), tartaric acid, trypsin, Dimethyl Sulfoxide (DMSO), 100x antibiotic solution (100,000 U/I of penicillin), and 100,000 mg/L streptomycins were purchased from Merck (Darmstadt, Germany). The caspase genes p53, 9, 8, 7, PCNA, and housekeeping gene ACTB were purchased from Integrated DNA Technologies (Coralville, Lowa, USA). Butterfield's Phosphate-Buffered (BPB), Plate Count Agar (PCA), Potato Dextrose Agar (PDA), Nutrient Agar (NA), Buffered Pepton Water (BPW), and Violet Red Bile Glucose Agar (VRBGA) were purchased from Oxoid Ltd (Altrincham, CH, England).

2.2 Black rice bran sample

The black rice used in this study was Cempo Ireng variety obtained from the Cigudeg Local Farmers, Bogor regency, Indonesia. It was ground using a husker machine (Yanmar, HW-60A, Japan) and then polished using a rice polisher (type N-70F, Japan) for 90 s/200 g to obtain the bran.

2.3 Black rice bran fermentation

Rice bran was fermented with a single culture of *R. oligosporus* isolated from commercial Tempe starter culture (RAPRIMA, Indonesia) and *R. oryzae* IPBCC 88010 (IPB Culture Collection, Bogor, Indonesia). The rice bran fermentation method referred to Zulfafamy et al. (2018) with modification. The spore suspension used was 15% (10^6 spores/mL) of the weight of bran. The bran was incubated using an incubator (Heraeus, Jerman) at 30 °C with the fermentation time of 0, 24, 48, 72, and 96 h. After the fermentation was done, the bran was dried using a freeze dryer (Labconco, USA) for 24 h, then stored at -18 °C to be extracted.

2.4 Sample extract preparation

The sample extract preparation was conducted by referring to the method used by Salazar-Aranda et al. (2011) with a few modifications. 20 g of rice bran was weighed, and 120 mL methanol (1 : 6 w/v) was added. Subsequently, it was macerated using a magnetic stirrer for 2 h. The extract was filtered using Whatman filter papers (Grade 1, pore size 11 µm), and the extraction process was repeated twice. The filtrate result was evaporated with a rotary evaporator (IKA, Staufen im Breisgau, BW, Germany) at 38 °C with low pressure. After the evaporation, the filtrate was dried using a freeze dryer and stored in a refrigerator at 4 °C. During the analysis, the extract was dissolved in methanol (1 mg/10 mL).

2.5 Analysis of phenolic content

Phenolic content analysis was performed by referring to the method employed by Wanyo et al. (2014). A sample extract of 0.3 mL was added with 2.25 mL of a Folin-Ciocalteu reagent that had been diluted with demineralized water (1 : 10) and allowed to stand for 5 min. A 2.25 mL solution of Na_2CO_3 (60 g/L) was subsequently added. The solution was incubated for 90 min at room temperature. The absorbance of the sample was measured with a UV-VIS spectrophotometer (ThermoScientificTM GENESYSTM 150, Waltham, MA, USA) at 725 nm. The total phenolic compound was expressed in milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g extract).

2.6 Analysis of total flavonoid compound

Total flavonoid compound analysis employed the method used by Chahardehi et al. (2010). A sample extract of 2 mL was added to 2 mL of 2% $AlCl_3$ solution in methanol and incubated for 10 min at room temperature. The absorbance of the sample was measured using a UV-VIS spectrophotometer at 415 nm against a blank sample consisting of a mixture of 2 mL of sample extract and 2 mL of methanol without $AlCl_3$. The total flavonoid compound was expressed in milligrams of quercetin equivalent per gram of dry extract (mg QE/g extract).

2.7 Analysis of antioxidant activity

Analysis of antioxidant activity used Baba & Malik's procedure (Baba & Malik, 2015). 0.2 mL sample extract was added with 3.8 mL of DPPH solution in methanol (25 mg/L). The solution was incubated for 60 min at room temperature in dark conditions. The absorbance of the sample was measured using a UV-VIS spectrophotometer at 517 nm. Control absorbance consisted of 0.2 mL methanol and 3.8 mL DPPH. The ability of bran extract antioxidant activity in capturing DPPH free radicals was expressed in percentage, which was calculated through the following equation (Equation 1):

$$\text{Antioxidant activity}(\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{x} 100 \quad (1)$$

2.8 Culture cell

Vero cells (ATCC CCL-81) and WiDr cells (ATCC CCL-218) were obtained from the Microbiology and Immunology Laboratory, Primate Animal Study Center, IPB University, Bogor, Indonesia. Vero and WiDr cells were cultured in DMEM media equipped with 5% FBS and 1% penicillin 100,000 U/I, streptomycin 100,000 mg/L. The cells were then incubated at 37 °C with 5% CO_2 using a CO_2 incubator (Binder Inc. Johnson Ave, Bohemia, USA).

2.9 Analysis of Vero and WiDr cell cytotoxic activity

Cytotoxic activity of bran methanol extract was measured using MTT colorimetric testing (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) to measure the number of living cells. Confluent cells were sub-cultured by removing the media in a flask and replaced with 10 mL PBS and 5 mL trypsin (0.125%), then incubated at 37 °C with 5% CO_2 for 10 min. Cells separated from the flask wall were moved into a 15 mL tube and centrifuged at 1500 RPM for 5 min (Flexpin, Tomy Kogyo Co., Ltd, Tagara, Tokyo, Japan). The cells were then counted using a hemacytometer (Blaubrand, Brand Gmbh + Co. Kg, Wertheim, Germany).

Cells were grown into 96 well plates with a density of $5x10^3$ cells/well and incubated for 24 h at 37 °C. The media was discarded and replaced with new media containing extracts of NFBR, FBROLI, FBRO as much as 100 µL/well and incubated for 48 h at 37 °C. PBS was added to remove the remaining sample that could disturb the reading, and 100 µL of RPMI 1640 media (5% PBS, 1% Penicillin-Streptomycin, $NaHCO_3$ (2 g/L)) was incorporated to facilitate reading. The MTT reagent dissolved in PBS (5 mg/mL) was added at 10 µL/well and incubated for 4 h at 37 °C. Following the MTT reagent, 100 µL/well ethanol (96%) was added, and absorbance was measured at 595 nm using iMark™ microplate absorbance reader (Bio-Rad Laboratories Inc, Hercules, California, USA). The following formula refers to Matuszewska et al. (2018) to calculate the ability to inhibit 50% of cell growth by test extract (Equation 2):

$$\text{Inhibition}(\%) = \frac{\text{Absorbance of control cell} - \text{Absorbance of treated cell}}{\text{Absorbance of control cell}} \times 100 \quad (2)$$

2.10 Analysis of gene expression

WiDr cells were plated on a 6-well plate containing DMEM media with an initial density of 5×10^4 cells/well and incubated for 24 h at 37 °C with 5% CO_2. After the incubation, each cell was treated with a concentration of 1700 µg/mL NFBR test extract, 1400 µg/mL FBRO, and 1100 µg/mL FBROLI. 2-well plate contained only WiDr cells (untreated cell) as negative controls (C-). Subsequently, the cells were incubated at 37 °C with 5% CO_2 for 72 h. WiDr cells were then harvested and stored at -20 °C until the next testing process.

RNA extraction of both treated and untreated WiDr cells employed the Zymo Direct-zolTM RNA MiniPrep Plus (USA) protocol. The purity of RNA was checked by measuring the concentration using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). RNA was converted to cDNA following the SuperScriptTM III Reverse Transcriptase procedure (Invitrogen, Waltham, MA, USA). Relative quantification of gene expression was conducted using the Bio-Rad iQ5™ Real-Time PCR (Bio-Rad Laboratories Inc. Hercules, California, USA) equipped with SsoFast™ EvaGreen Supermix (Bio-Rad, USA), nuclease-free water (Bio-Rad, USA), gene primer of caspase apoptosis 7, 8, 9, p53, PCNA proliferation gene, and ACTB gene for normalization.

The Real-Time PCR was performed with 40 cycles consisting of enzyme activity at 95 °C for 30 s, denaturation in 95 °C for 10 s, and annealing for 20 s with optimum annealing temperature adjusted to the condition of the available equipment. Primary sequences and annealing temperatures can be seen in Table 1. The nucleotide sequence and annealing temperature of ACTB, p53, caspace-9, and caspace-7 genes refer to Laila et al. (2020), the caspase-8 gene refers to Borhani et al. (2014), and the PCNA gene refers to Saigusa et al. (2014).

Measurement of gene expression used the *delta-delta threshold* method ($2^{-\Delta\Delta CT}$) with the C_T target gene (example p53) normalized with C_T reference (ACTB gene). Sample A was a WiDr cell given rice bran extract, and Sample B was a negative control cell (C-). The following formula refers to Schmittgen & Livak (2008) to calculate gene expression (Equation 3):

$$2^{-\Delta\Delta CT} = 2^{-(C_T \text{target gene} - C_T \text{ reference})_A - (C_T \text{target gene} - C_T \text{ reference})_B} \quad (3)$$

2.11 Statistical analysis

Data obtained in this experiment were in the form of mean ± standard deviation (SD) for two replications of each sample (n = 2). Data were analyzed using the Generalized Linear Model (GLM) and further tested by Duncan Multiple Range Test (DMRT). The significance value was determined based on a 5% significance level using SPSS 22.0 software (Chicago, IL, USA).

Table 1. Primary sequencing and annealing temperature for Real-Time PCR.

Gene		Nucleotide sequence	Temp (°C)
ACTB*	F	5′-AGAGCTACGAGCTGCCTGAC-3′	57
	R	5′-AGCACTGTGTTGGCGTACAG-3′	
p53*	F’	5′-CCGCAGTCAGATCCTAGC-3′	55
	R	5′-AATCATCCATTGCTTGGGACG-3′	
Caspase-9*	F	5′-GAATGACGTGAAACACGACAG-3′	57
	R	5′-TTAACGGCATCCCCCACTTAG -3′	
Caspase-7*	F	5′-GCAGCGCCGAGACTTTTAG-3′	57
	R	5′-GCTGCAGTTACCGTTCCCAC-3′	
Caspase-8**	F’	5′-AGAGTCTGTGCCCAAATCAAC-3′	57
	R	5′-GCTGCTTCTCTCTTTGCTGAA -3′	
PCNA***	F	5’-GAAGCACCAAACCAGGAGAA-3’	50
	R	5’-TATCGGCATATACGTGCAAA-3’	

*Refer to Laila et al. (2020); **Refer to Borhani et al. (2014); *** Refer to Saigusa et al. (2014)

3 Result and discussion

3.1 Phenolic content, flavonoids, and antioxidant activity in fermented black rice bran extract

The phenolic content in rice bran was increased significantly ($p < 0.05$) at 72 h fermentation time in both *R.oligosporus* and *R. oryzae* inoculums (Table 2) by 22.5% and 14.7%, respectively. The increase was caused by enzymes, which were produced by fungi capable of releasing bonds of phenolic compounds in the substrate during the fermentation process. Some fungi enzymes reported to release phenolic in the substrate are β-glucosidase, cellulase, and α-amylase (Chen et al., 2020).

The phenolic compound in the substrate was known to be bound to glucose or other sugars. Therefore, the production of the β-glucosidase enzyme during the fermentation process by fungus helped in the release of these phenolic bonds-glycons (sugar groups) (Zheng & Shetty, 2000). The decomposition of these bonds was due to the need for glucose or other sugar groups to support the growth and reproduction of the fungi (Martins et al., 2011; Sánchez, 2009). The hydrolysis of these bonds during the fermentation process was also very beneficial to increase the availability of phenolic compounds in the rice bran.

On the contrary, rice bran that was fermented for more than 72 h showed a decrease in phenolic levels. The cause of the suspected decrease was because there was not glucose available in the rice bran. This unavailability of the substrate pushed fungi to degrade the released phenolic compound as a carbon source for its growth. Adetuyi & Ibrahim (2014) reported that microbes use the available free compounds as a substrate for their growth if the primary substrate for microbial growth is not available.

The phenolic content analysis results also showed that the rice bran fermented by *R. oligosporus* had an increased content of total phenolic compared to the rice bran fermented by *R. oryzae* IPBCC 88010. These results were confirmed by the results of the study of Cheng et al. (2013), where the highest phenolic content in fermented black soybean was produced from *R. oligosporus* BCRC 31996 with 2545.3 ± 33.5 (μg/g), whereas *R. oryzae* showed the result of 2088.3 ± 21.3 (μg/g). The results of the flavonoid compound analysis demonstrated a significant increase ($p < 0.05$) at 72 h fermentation time by *R. oligosporus* from 1.20 mg QE/g (0 h) to 1.54 mg QE/g (72 h) or an increase of 28.3% (Table 2). The bran fermented by *R. oryzae* did not show a significant increase statistically ($p > 0.05$) with flavonoid levels ranging from 1.28 mg QE/g (0 h) to 1.30 mg QE/g (72 h). The content difference was suspected due to the fungi type used. Martins et al. (2011) reported that the number of bioactive compounds is dependent on the fungi type inoculum, substrate, and fermentation conditions.

In this study, the analysis of antioxidant activity used DPPH as a free radical. The results showed a significant increase ($p < 0.05$) in antioxidant activity during the fermentation process (Table 2). The percentage increase for rice bran fermented by *R. oligosporus* was 60.2-72%, whereas rice bran fermented by *R. oryzae* showed an increase of 59.5-65.8%. Both types of fungi had the same potential to increase antioxidant activity; however, the bran fermented by *R. oligosporus* showed a higher antioxidant activity. The increase in antioxidant activity was determined by the presence of phenolic acid and flavonoid compounds, as they act as antioxidants by donating hydrogen atoms or transferring electrons (Procházková et al., 2011). They showed why fermented rice bran had a higher antioxidant activity ($p < 0.05$) than non-fermentation based on phenolic content and flavonoid.

3.2 Correlation between phenolic content, total flavonoids, and antioxidant activity in fermented black rice bran extract

The Pearson correlation coefficient value represented the evaluation of the relationship between phenolic content and flavonoids with antioxidant activity in fermented rice bran.

The results showed that phenolic content and antioxidant activity were positively correlated ($R = 0.901$) with very significant differences ($p < 0.01$) (Table 3). They also indicated that the range of phenolic content in rice bran played an active role in increasing its antioxidant activity, which was further strengthened by Akoglu's statement (Akoglu, 2018) that the

Table 2. Compounds of functional and antioxidant properties in fermented rice bran.

Fermentation time (h)	Phenolic content (mg GAE/g extract)		Total flavonoid (mg QE/g extract)		Antioxidant activity (%)	
	R.oligosporus	*R.oryzae*	*R.oligosporus*	*R.oryzae*	*R.oligosporus*	*R.oryzae*
0	6.50 ± 0.18^{a}	6.52 ± 0.23^{a}	1.20 ± 0.05^{ab}	1.28 ± 0.04^{c}	60.17 ± 7.06^{ab}	59.50 ± 2.51^{a}
24	7.12 ± 0.16^{c}	6.66 ± 0.20^{a}	1.18 ± 0.05^{a}	1.28 ± 0.03^{bc}	60.92 ± 1.56^{ab}	64.05 ± 0.92^{bcd}
48	7.68 ± 0.32^{d}	7.00 ± 0.17^{bc}	1.24 ± 0.07^{abc}	1.29 ± 0.04^{c}	68.89 ± 2.68^{ef}	65.20 ± 5.71^{cde}
72	7.96 ± 0.31^{e}	7.48 ± 0.11^{d}	1.54 ± 0.07^{d}	1.30 ± 0.03^{c}	72.01 ± 1.22^{f}	65.80 ± 0.99^{de}
96	6.73 ± 0.28^{ab}	6.71 ± 0.28^{a}	1.16 ± 0.05^{a}	1.29 ± 0.06^{c}	61.67 ± 1.60^{abc}	60.31 ± 1.99^{ab}

Data presented were mean ± standard deviation. n = 2 (duplicate). Numbers with different letters in the same column and row showed significant differences at the 5% test level.

Table 3. Correlations between phenolic, flavonoid, and antioxidant activity.

	Phenolic	Flavonoids	Antioxidants
Phenolic	1	0.591	0.901**
Flavonoids	0.591	1	0.710*
Antioxidants	0.901**	0.710*	1

*Significant correlation at the 0.05 level. **Significant correlation at the 0.01 level.

correlation coefficient of +0.9 and -0.9 interpreted a powerful interplay between the two variables.

In the evaluation results of the flavonoid correlation, the coefficient correlation of flavonoid with antioxidant activity was positively correlated (R = 0.710) with a significant difference ($p < 0.05$) (Table 3). It indicated that the flavonoid content in fermented rice bran was believed to be 95% able to increase antioxidant activity with a moderate interplay between the two variables. This finding was consistent with Ghasemzadeh & Ghasemzadeh's statement (Ghasemzadeh & Ghasemzadeh, 2011) which said that an increased antioxidant activity was influenced by the number of phenolic and flavonoid content. These compounds significantly contributed to capturing free radicals, reducing agents, and preventing the formation of oxygen singlets.

Hydroxyl groups have been reported to play an active role in capturing free radicals or chelating metal ions. Those hydroxyl groups were bound to the phenolic acid and flavonoid aromatic ring structure (Shahidi & Yeo, 2016; Kumar & Pandey, 2013). The known mechanism states that the hydroxyl group donates hydrogen atoms or electrons to hydroxyl and peroxynitrite radicals to reduce the action of these radicals and produce a more stable flavonoid radical product (Kumar & Pandey, 2013). Quercetin and isorhamnetin 7-glucoside of flavonoid content were found in rice bran fermented, which synergistically enhance antioxidant activity (Ardiansyah et al., 2021).

3.3 Analysis of the cytotoxic activity of fermented black rice bran extract

The sample extracts examined for the cytotoxic activity had the highest results of the phenolic content, flavonoids, and antioxidant activity at 72 h fermentation time. The highest cytotoxic activity in WiDr cells was FBROLI extract with IC_{50} value 650.7 µg/mL, followed by NFBR IC_{50} extract 831.8 µg/mL, and FBRO IC_{50} 873.9 µg/mL (Table 4). The lower IC50 value was shown in the FBROLI extract results, which implies that a 650.7 µg/mL concentration could inhibit 50% growth of the WiDr cell culture population.

The high cytotoxic activity at FBROLI extracts due to R. *oligosporus* fungus released more phenolic and flavonoid content from their bonds into the substrate. The free form of available content was responsible for antioxidants. The amount of free functional compounds was positively correlated with increased antioxidant activity, translated as an increase in anticancer bioactivity (Chen et al., 2015). The effectiveness of phenolic acids and flavonoids contents as anticancer was also determined by the position and number of free hydroxyl groups, aromatic ring structures, and unsaturated fatty acid chain bonds (Anantharaju et al., 2016).

Table 4. IC_{50} values in non-fermented and fermented black rice bran extracts.

Treatments	IC_{50} value (µg/mL)	
	Vero Cell	WiDr Cell
NFBR	1139.5	831.8
FBRO	1001.9	873.9
FBROLI	811.1	650.7

Data presented were one replication of each sample.

Cytotoxic activity of rice bran extracts on normal Vero cells showed a lower cytotoxic activity, as seen on the higher IC_{50} values of Vero cells compared to WiDr cells (Table 4). It indicated that rice bran extract was more effective in inhibiting the proliferation of WiDr cells than Vero cells. In addition, rice bran extract was also proved safe against normal human cells.

In this study, the cytotoxic activity of rice bran extracts on WiDr cells was categorized as weak. The determination of cytotoxic activity was based on the statement of Atjanasuppat et al. (2009) that the activity of an extract of natural ingredients was determined from the IC_{50} value, where ≤ 20 µg/mL was considered as active; > 20-100 µg/mL as moderate; > 100-1000 µg/mL as weak; and > 1000 µg/mL as inactive.

3.4 Gene expression analysis of fermented black rice bran extract

The results depicted an increased p53 gene expression in WiDr cells treated with NFBR, FBRO, and FBROLI extracts (Figure 1A). The highest p53 gene expression was shown in WiDr cells, which were given FBROLI extract. This increase was presumably due to the higher number of phenolic and flavonoid contents available. Thus, it had more potential to have an increased p53 gene expression compared to FBRO and NFBR treatments. The potential of phenolic and flavonoid contents in regulating gene expression associated with tumor control was also widely reported (Xavier et al., 2011; Zhong et al., 2010).

More than 50% of cancer patients are marked by mutations in the p53 gene (Basu, 2018). It supported the hypothesis of the reason why the p53 gene in WiDr colon cancer cells shown by negative control (C-) (Figure 1A) was relatively lower than other treatments, albeit it was not statistically significant ($p = 0.113$). p53 gene expression testing is often done to detect cancer cells as it plays a vital role in stopping the development of progressive cancer.

The increased p53 gene expression positively affected another apoptotic marking gene expression, namely caspase-9 (Figure 1B), although it was not statistically significant ($p = 0.153$). The increase in caspase-9 expression was due to active p53 inducing the transcription of puma and noxa BH3 proteins, thereby activating Bax, another pro-apoptotic family (Ashkenazi, 2008). Bax in the cytoplasm enters the mitochondrial membrane by damaging the membrane, resulting in membrane permeability changes and promoting the release of cytochromes c from the mitochondria. The release of cytochrome c factors from mitochondria into the cytoplasm are able to activate caspase-9 by collaborating cytochromes c factors with Apaf-1 apoptotic proteins (Ashkenazi, 2008). Caspase-9 is commonly used to detect cancer inhibitory mechanisms of intrinsic pathways and are known as mitochondrial pathways.

The results also showed increased caspase-8 gene expression in the NFBR, FBRO, and FBROLI treatments (Figure 1C). Caspase-8 is an apoptotic marker involved in the extrinsic pathway

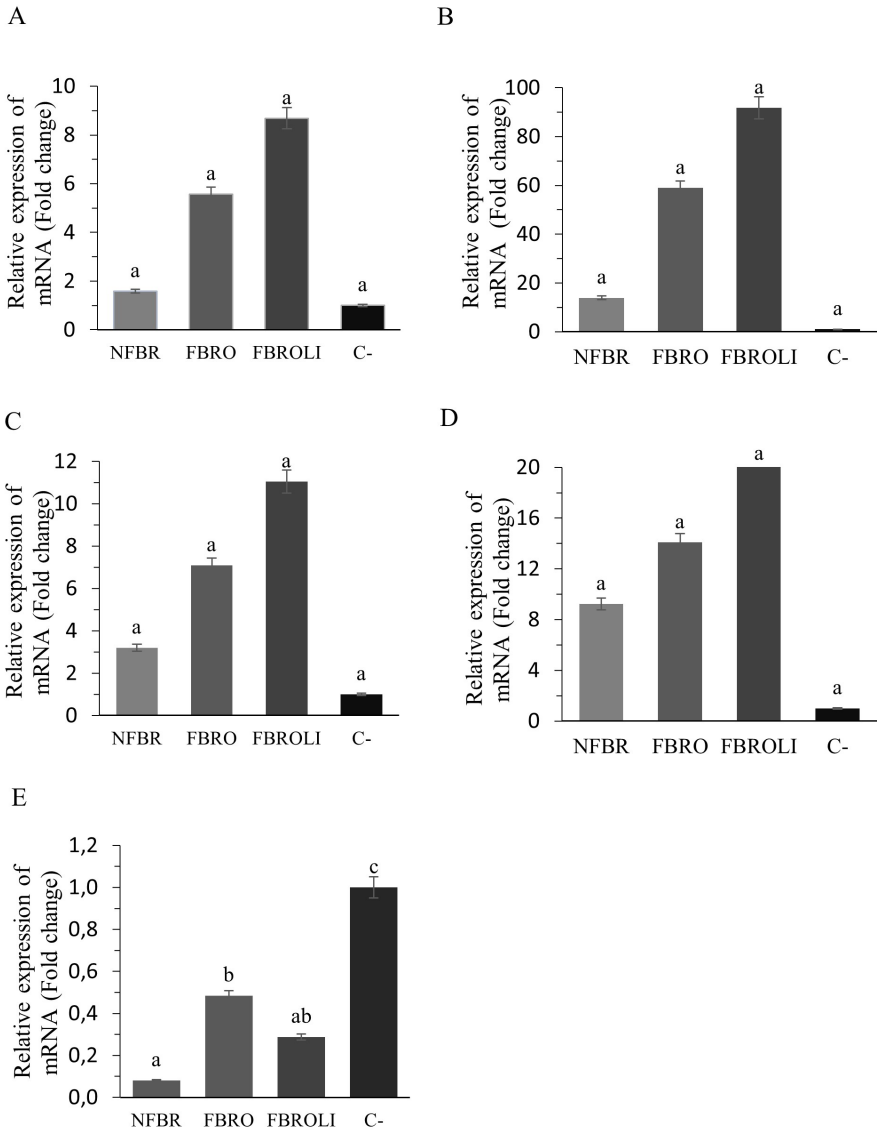

Figure 1. Evaluation of gene expression of p53 (A); caspase-9 (B); caspase-8 (C); caspase-7 (D); and PCNA (E) on WiDr colon cancer cells treated with NFBR extract 1700 µg/mL; FBRO 1400 µg/mL; and FBROLI 1100 µg/mL. C- was used as a comparison (WiDr without administration of sample extracts) (n = 2). Different superscripts in each bar represent a significantly difference ($p < 0.05$).

apoptotic cascade. The increase of caspase-8 gene expression was presumably due to the return of increased sensitivity of the WiDr cells that were resistant to TRAIL resulting in apoptosis. A factor believed to be the cause of cancer cell resistance to TRAIL was the deficiency of mortality receptors on the cell surface (Jin et al., 2004) or the low expression of DR4 and DR5 mortality receptors, and loss of function as a receptor of mortality due to mutations (Van Geelen et al., 2004; Zhang & Fang, 2005). WiDr cells were reported to be resistant to TRAIL-induced apoptosis. One of the causes was the low expression of TRAIL receptors (Zhang et al., 2019). It strengthens the suspicion why the result in C- (Figure 1C) showed a low caspase-8 gene expression compared to other treatments, even though the difference was not statistically significant ($p = 0.175$).

The return of increased sensitivity was believed to be the active role of the bioactive compounds present in rice bran. It supported the result of Kong et al. (2009), where the administration of cycloartenyl ferulate, a bioactive compound of rice bran, could increase the sensitivity of colon cancer cells SW480 to TRAIL-induced apoptosis. It was proved by the increase in the number of DR4 and DR5 mortality receptors and the activity of caspase-8 and 10, thus increasing effector caspase 3,6 and 7.

In the caspase activation, both intrinsic and extrinsic pathways end at effector caspase-7. Caspase-7 is significant as an executor of apoptosis due to its involvement in the fragmentation of DNA chromosomes, cross-linking of proteins, and degradation of core and cytoskeleton proteins (Elmore, 2007). The results showed increased caspase-7 gene expression in the NFBR, FBRO, and FBROLI treatments (Figure 1D). However, it did not show statistically significant differences with controls ($p = 0.646$). This result demonstrated that one of the mechanisms inhibiting the

development of WiDr colon cancer cells by rice bran was with the help of the apoptotic pathway.

The activation of caspase-7 originates from a linear pathway. Cytochrome c with Apaf-1 activates caspase-9, in turn activates the caspase-7, and caspase 8 releases molecules in the cytoplasm, resulting in cleaving and ultimately stimulates the effector caspase-7 (Ashkenazi, 2008). This linear pathway strengthened the reason why WiDr cells that were given extracts of NFBR, FBRO, and FBROLI also showed an increase in expression of the caspase-7 gene. This result was in accordance with the evaluation results in both genes of caspase-9 and caspase-8, which also showed increased expression. It was confirmed by the research results Lim et al. (2007), where it was found that the flavonoid content of the luteolin compound did stimulate not only the activation of the initiator caspase-9, but also the effector caspase-3 and caspase-7 on HT-29 cells. Turktekin et al. (2011) also reported that the administration of the flavonoid content of apigenin to colon HT-29 cancer cells induced apoptosis by regulating the increased expression of caspase-8 and 3.

In this study, activation of effector caspases on WiDr cells might be due to bran bioactive compounds' involvement in reactivating functions key of apoptotic regulators. PCNA (Proliferating Cell Nuclear Antigen) is widely used to detect cancer because of its involvement in replicating DNA. The PCNA activity was found to be higher in colorectal carcinoma patients. The PCNA activity could be expressed 3 to 4 times higher than the normal tissue (Poosarla et al., 2015). The higher the stage of cancer, the higher the PCNA activity. The resulting study showed that the PCNA gene was expressed significantly higher ($p < 0.05$) in C- compared to NFBR, FBRO, and FBROLI (Figure 1E).

On the other hand, the evaluation results on WiDr cells treated with NFBR, FBRO, and FBROLI extracts showed relatively lower PCNA gene expression than C- (Figure 1E). Decreased expression of PCNA genes was likely due to the binding of PCNA by p21 (cyclin-dependent kinase inhibitor) in its operational condition. Consequently, DNA replication was stopped, and cell cycle progression was inhibited (Singh et al., 2017). The inhibition of PCNA activity was very beneficial as it inhibited growth in cancer cells. Some researchers widely reported the activity of this p21 protein, regulated by the p53 tumor suppressor gene. Hence, PCNA was freely regulated by an activated p53 gene (Singh et al., 2017), and bioactive compounds in rice bran were believed to take over in restoring the normal function of p53.

4 Conclusions

The increase in the bioactive compound was influenced by the type of inoculum and the fermentation time. The best results with the highest phenol content, flavonoids, and antioxidant activity were obtained in the fermented FBROLI extract within 72 h. It also had the highest inhibition of WiDr colon cancer cell growth. Its inhibitory mechanism was observed, including suppression of PCNA proliferation gene expression and increased expression of apoptotic genes through extrinsic and intrinsic pathways. The highest increase in apoptotic gene expression was obtained from FBROLI extracts.

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