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Antioxidant and Antiproliferative Activity of Black Rice Extract Against Colon Cancer Cells *In-Vitro* and *In-Silico*

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Abstract

Black rice is less popular for healthy use and not widely available in different parts of Indonesia. Only a few scientific reports are available on the health benefits of black rice, specifically with an integrated *in-vitro* and *in-silico* method for varieties present in the market, showing a need for investigations regarding bioactivity. Therefore, this analysis examines the bioactivity of black rice extracts of Cempo Ireng variety *in-vitro* and *in-silico* as a source of antioxidant and antiproliferation for colon cancer cells, along with determination of the compound content. The novelty was the integration of *in-vitro* and *in-silico* methods to explore the natural antioxidant sources and the inhibition of colon cancer cell proliferation from black rice of Cempo Ireng variety. Moreover, antioxidant activity was analyzed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted to determine antiproliferative activity, which was further examined *in-silico* with metabolites from black rice extract identified using gas chromatography–mass spectrometry (GC-MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS). The results indicated black rice extract had antioxidant activity to scavenge radicals. The activity had IC_{50} of 4.67 µg/mL for black rice chloroform (BRC) extract and 52.45 µg/mL for black rice ethanol (BRE) extract. Additionally, black rice extract can effectively inhibit the proliferation of colorectal cancer cells (8.28% - 9.10%). Metabolite groups detected in the extract included organic acids, flavonoids, terpenoids, phytosterols, amino acids, purines, organic alcohols, and organic bases. Campesterol and 24 - methylenecycloartanol were metabolites that had the best activity in inhibiting cancer treatment target proteins *in-silico*. This study showed the health benefits of black rice, suggesting the development of a functional food to support digestive health.

Keywords: Antioxidant, Black rice, Cempo Ireng, Colorectal cancer, Food, Functional food, *In-silico*, *In-vitro*, Nutraceuticals

Introduction

Rice (*Oryza sativa* L.) is a staple food that can serve as the core of life for Asian people [1]. Currently, 70% of the world population consumes rice, which has become an essential food source in addition to cereals in America and Europe [2]. Different varieties based on species, subspecies, and pigment (white rice and rice with red or black pigment) classifications are cultivated worldwide as an income source for households, particularly in Asia and Africa [3]. The major content of rice consists of carbohydrates, fat, protein, vitamins, and

minerals, as well as a mixture of other compounds including anthocyanins, which can provide color to pigmented rice [4].

According to Ayurveda, rice is used to revitalize energy in the body, eliminate toxic compounds, prevent premature aging, and avoid skin diseases, functioning as a nutritious and medicinal food [1,3]. Compounds contained in pigmented rice, such as phenolic compounds, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, and gamma aminobutyric acid, possess antioxidant, antiarthritic, antitumor, antidiabetic, anti-inflammatory, and antibacterial activities [5]. Previous *in-vitro* analysis detected pigmented Indian rice could inhibit free radicals by 88% - 93% [6]. Local Korean rice has cellular antioxidant activity, which can reduce free radical levels in colorectal adenocarcinoma cells [7]. Consumption of defatted rice bran is capable of modulating the gut microbiota to improve colon health and reduce the risk of colorectal cancer [8]. Another report disclosed that pigmented and non-pigmented rice showed potential as an antidiabetic agent through the α-glucosidase inhibition by 24% - 87% [9].

In Indonesia, rice is used as a mixture of traditional medicines in the form of a bath scrub for smoothening and brightening skin color by rubbing it on the surface [10]. This food material is processed into a mixture of traditional medicines called jamu beras kencur, which increases body resistance and reduces digestive tract disorders [11]. Moreover, beras kencur compounds contribute to the potential *in-vitro* anticancer and antibacterial activity of the jamu product [12].

White rice is often used as a raw material for traditional medicine than pigmented rice. Previous analysis reported that the pigmented rice bioactivity was better than white rice [7,13]. Black rice is a pigmented rice of different varieties grown in Indonesia [14], with less recognition as a functional food source than the red type, even though the health benefits are similar or better [15-17]. The lower demand is due to the longer growing period and the color of black rice, which leads to being less attractive for consumption [18]. Black rice cv. Sintoheugmi, Japonica type, possesses antioxidant activity and contains cyanidin compounds, γ-oryzanol, folate groups, tocopherol, tocotrienol, lutein, polyunsaturated fatty acids, and phenol groups [19]. Analysis using LC-QTOF-MS/MS found primary metabolites including sucrose, glucose, as well as free and conjugated amino acids in black Rice Berry variety [20].

Cempo Ireng variety is a local black rice variety available on the market originally from Yogyakarta, Central Java, Indonesia [21]. The nutrition content includes carbohydrate (24.90%), protein (9.41%), fat (2.49%), and anthocyanin (31.64 mg/kg), with a glycemic index of 60 [22]. Additionally, secondary metabolites contained are flavonoids, alkaloids, and terpenoids [23]. The bran of Cempo Ireng variety provides antioxidant activity as a free radical scavenger [24]. This black rice from local farmers in Cigudeg, Bogor, West Java, has antiproliferative activity against colon cancer cells through the apoptosis mechanism [25,26]. Previous investigations reported the potential to be a functional food with properties as an antioxidant and alternative treatment for colon cancer.

This analysis examines the bioactivity of black rice extract of Cempo Ireng variety *in-vitro* and *in-silico* as a source of antioxidant and antiproliferation for colon cancer cells. The profiling of metabolites contained in the extract was carried out using GC-MS and LC-HRMS. Docking analysis using human proteins which are treatment targets for suppressing cancer cell growth was performed to verify antiproliferative compound activity in the extract. The results are expected to show the health benefits of rice, specifically Cempo Ireng variety. This information can be used in the form of a basic scientific tool for developing black rice as a functional food.

Materials and methods

Black rice (Cempo Ireng variety) planted in Yogyakarta, Indonesia, at coordinates of 7°15’24”-7°49’26” S and 110°24’19”-110°28’53” E was purchased from a local market. Fibroblast NIH/3T3 non-cancer cell lines and WiDr colorectal cancer cells were from the Cell Culture Laboratory, Research Center of Raw Materials for Drugs and Traditional Medicine-LAPTIAB, National Research and Innovation Agency (BRIN), Puspiptek Serpong in South Tangerang, Banten, Indonesia. The cells used were stored at −80 °C and growth regeneration was carried out until attaining 80% - 90% confluence before analysis. The extraction solvents were ethanol (Merck, Germany) and

chloroform (Merck, Germany). The free radical used was 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) and ascorbic acid solution (Sigma-Aldrich, USA) served as a positive control of antioxidant. Materials used in antiproliferative activity assay were the Dulbecco's minimal Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, UK), foetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, USA), penicillin-streptomycin (Gibco, Thermo Fisher Scientific, USA), amphotericin (Gibco, Thermo Fisher Scientific, USA), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), phosphate buffered saline (PBS) (Sigma-Aldrich, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Thermo Fisher Scientific, USA), and doxorubicin (CKD OTTO Pharmaceuticals, Indonesia). Moreover, all chemicals and reagents applied were of absolute grade.

Black rice extraction

Black rice was extracted through maceration by soaking for 72 h [27], where each sample (200 g black rice) was ground until homogeneous. During the execution of this method, ethanol and chloroform were the solvents used in a separate experiment at 1:3 w/v ratio with the sample (200 g of black rice in 600 mL of solvent). The filtrate obtained after filtering was evaporated at 60 °C applying a rotary evaporator (Büchi R-100, Flawil-Switzerland) with vacuum conditions of 474 mbar (chloroform) and 175 mbar (ethanol). Results were obtained as black rice ethanol (BRE) and black rice chloroform (BRC) extracts. The yield was calculated based on the ratio of both extract and black rice weight (200 g).

Antioxidant activity assay

DPPH in this analysis as the free radical [28], where 1 mL of samples (10, 50, 100, 150, and 200 µg/mL) was combined with 1 mL DPPH (0.1 mM). After incubation for 30 min at room temperature, absorbance was measured at 517 nm. DPPH solution without sample was applied as a negative control and ascorbic acid solution (1; 2.5; 5; 7.5; 10 µg/mL) was the positive control. Free radical reaction inhibition of DPPH from samples and positive control was showed as a percentage inhibition and calculated based on the difference the ratio in absorbance of negative control and sample to absorbance of negative control. The lower the absorbance value, the higher the percentage of sample free radical inhibition. In this test, IC_{50} values were calculated from percentage inhibition versus sample concentrations.

Antiproliferative cancer cell assay

MTT assay was applied to determine antiproliferative activity against cancer cells [27]. WiDr colorectal cancer and fibroblast (NIH/3T3) non-cancerous cells were used. Cells were grown in high-glucose Dulbecco's minimal Eagle's medium containing 10% FBS, 1% penicillin-streptomycin, and 0.5% amphotericin until 80% - 90% confluence. These were planted in 96 wells plate at 10,000 cells per well in 100 µL medium and incubated for 24 h (CO_2 5%; 37 °C). The 96-well plate containing control medium (without cells and sample), negative control, sample, and positive control were used for the analysis process. Samples were dissolved in DMSO at 62.5, 125, and 250 µg/mL concentrations. Approximately 100 µL of these were mixed with cells on a plate and incubated for 24 h (CO_2 5%; 37 °C), then each well was washed using PBS before adding 0.5 mg/mL MTT. Post incubation for 4 h, purple formazan crystals formed from the reaction of MTT with mitochondria of surviving cells, and the reaction was stopped by giving 10% SDS. Purple color absorbance was measured at 570 nm after overnight incubation. Negative control was a cell culture without samples, doxorubicin served as positive control (0.15, 0.625, 1.25, 2.5, 5 µg/mL), and blank solution was a control medium. All analyses were conducted in triplicate using an aseptic method. Cell viability was showed as percentage and calculated based on ratio of difference in absorbance between samples and blank solution to difference in absorbance of negative control and blank solution. The higher the absorbance value, the higher the live cells percentage. Percentage of proliferation inhibition was estimated as difference in cell viability in a sample from maximum cell viability (100%).

Metabolite profiling

Metabolite profiling of BRE and BRC was conducted using chromatography-mass spectrometry [27]. Volatile metabolites in samples were detected using GC-MS (Agilent Technologies, model 7890,

USA). Agilent J&W Ultra 2 GC (Agilent Technologies, type 19091B) column (30 m×200 μm×0.11 μm) was applied in this analysis. Samples (10 μL) were injected in 250 °C split mode, an 8:1 split ratio, and a 26,443 psi pressure, while helium (He) was an eluent at a 1.2 mL/min flow rate. Ion source and quadrupole temperatures were 230 and 150 °C, respectively. Mass spectrum detection range was 20 - 500 m/z, and Wiley W8N08.L was a mass spectral database used to identify metabolites based on metabolite peaks and retention times. Metabolites contained in sample were those with a mass spectrum similarity of 95% or more to the database. Metabolite concentration was determined from the area under the peak.

LC-HRMS was a method used to detect non-volatile metabolites in this study with UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS (Thermo Fisher Scientific, USA) and a C18 Accucore column (100 mm×2.1 mm×1.5 μm) (Thermo Fisher Scientific, USA) as the main instrument. Before analysis, 5 mg samples in 1 mL methanol were filtered through a 0.2 μM nylon membrane and injected (2μL) into the instrument. The eluents used were H_2O - 0.1% formic acid (A) and acetonitrile - 0.1% formic acid (B). Flow rate was 0.2 mL/min with gradient of 5% eluent B for 0 - 1 min, 5% - 95% eluent B for 1 - 25 min, 95% eluent B for 25 - 28 min, and 5% eluent B for 28 - 33 min. Spectrum detection was carried out using 100 - 1,500 m/z in positive ionization mode. Compound Discoverer 3.2 (Thermo Fisher Scientific, USA) was a mass spectral database used to identify metabolites based on metabolite peaks and retention times. Metabolites contained in the samples were those with a mass spectrum similarity of 95% or more to the database. Metabolite concentration was determined from the area under the peak.

Docking analysis

Selected ligands applied in docking analysis were metabolites contained in black rice extract identified using GC-MS and LC-HRMS. Furthermore, the 3D structures were obtained from PubChem chemical database (https://pubchem.ncbi.nlm.nih.gov/). All ligands were tested for bioavailability based on Lipinski's rules using bioinformatics and computational biology web services (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp) to evaluate physicochemical properties and drug-likeness (molecular weight < 500 Da, Log P < 5, H-bond donors < 5, H-bond acceptors < 10, 40 < molar refractivity < 130) [29,30].

Yet Another Scientific Artificial Reality Application software (YASARA version 19.9.17) was used for receptor preparation, ligand preparation, and docking analysis in this study. Water and unwanted ligands were removed from receptor. Hydrogen atom was added and co-crystal ligand was separated from receptor. Docking analysis was performed using 2R0U (Chek1), 3U9W (Leukotriene A4 hydrolase), and 4LXD (Bcl-2) receptors. 3D receptors structures source was from protein data bank (https://www.rcsb.org/). Validation process was conducted before docking analysis by re-docking receptors with co-crystal ligands. M54, 28P, and 1XV were the co-crystal ligands of 2R0U, 3U9W, and 4LXD, respectively. Moreover, grid box was positioned in the middle of the co-crystal ligand binding site on the receptors with a size of 1 - 7 Å and using AMBER14 force field. The selected grid box size for docking analysis had the smallest root means square distance (RMSD) value ≤ 2 Å [31].

Docking results were evaluated with binding affinity energies of each ligand, and YASARA software was applied to assess the ligand binding quality to the receptors based on Vina method [29,31]. 2D and 3D interaction figures were generated by BIOVIA Discovery Studio Visualizer V21.1.0.20298.

Results and discussion

BRC and BRE yields

Different values of yield were obtained for the 2 samples used in this study. The 200 g of BRC and BRE produced yields of 7.12% (14.24 g) and 5.18% (10.36 g), respectively (**Table 1**).

Table 1 Yield from the extraction process using different solvents.

Solvent	Yield (%)
Chloroform	7.12
Ethanol	5.18

Antioxidant activity

Each concentration of BRC and BRE inhibited free radicals (DPPH), with the inhibition being proportional to the increase in the extract concentration. This showed that BRC and BRE served as radical scavengers with inhibition values of 49% - 53% and 42% - 61%, respectively. The highest concentration of BRC (200 µg/mL) showed significant inhibition compared with other concentrations, as signified by different letter notations. In BRE, the highest sample concentration of 200 µg/mL was not significantly different from concentrations of 50 - 150 µg/mL, as represented with the same letter. Only the lowest BRE (10 µg/mL) concentration was significantly different from the highest concentration (200 µg/mL). The inhibitory effect of BRC and BRE was higher at concentrations of 10 and 50 - 200 µg/mL (**Figure 1**). The IC_{50} value of BRC was 4.67 µg/mL and BRE had 52.45 µg/mL, suggesting that BRC was more active as an antioxidant. Other results showed samples lower antioxidant activity when than ascorbic acid (IC_{50} = 1.06 µg/mL) as a positive control.

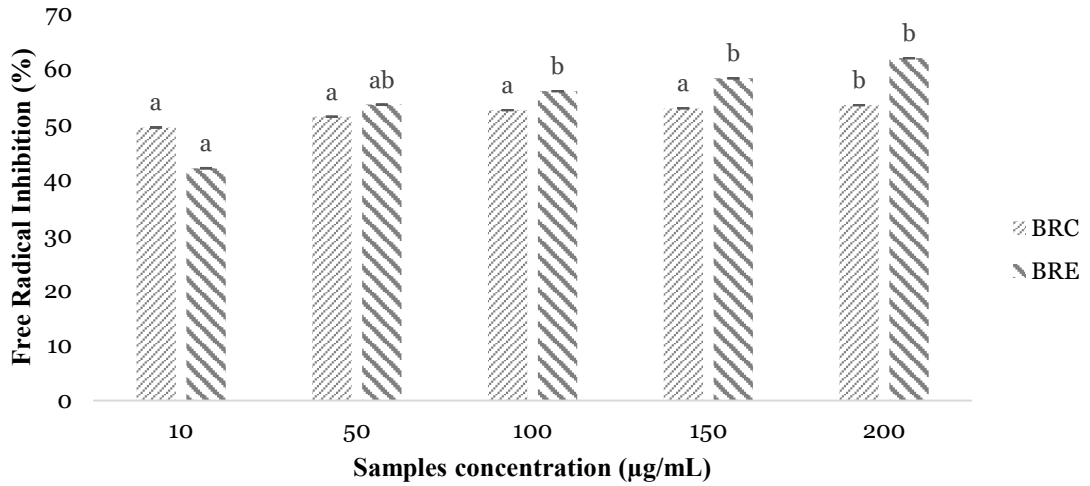


Figure 1 Antioxidant activity of black rice extract. BRC: Black rice chloroform extract, BRE: Black rice ethanol extract. The results of triplicate experiments have been presented with standard deviation. Different letter notations imply significant differences ($p < 0.05$).

Antiproliferative activity against cancer cells

The results indicated samples had antiproliferative activity against WiDr cells. At low concentrations, each extract did not prevent cancer cell proliferation (**Figure 2(A)**). Cancer cell growth increased by 6% - 8% at the lowest concentration of 62.5 µg/mL. BRC measuring 125 µg/mL inhibited cancer cell proliferation by 11.2%, while at the same concentration of BRE, cancer cell growth increased by 1.78%. At 250 µg/mL, cancer cell proliferation was inhibited by 9.10% (BRC) and 8.28% (BRE). In this analysis, sample inhibition was weaker, compared doxorubicin as a positive control which inhibited 84% at 5 µg/mL. All extracts cannot prevent the proliferation of non-cancerous cells, only BRC inhibited normal cell growth by 1.84% at a concentration of 250 µg/mL (the highest concentration in the test), and BRE did not inhibit cell proliferation at any concentration (**Figure 2(B)**). These results showed that both extracts inhibited cancer cell proliferation at high concentrations and were non-toxic to normal cells.

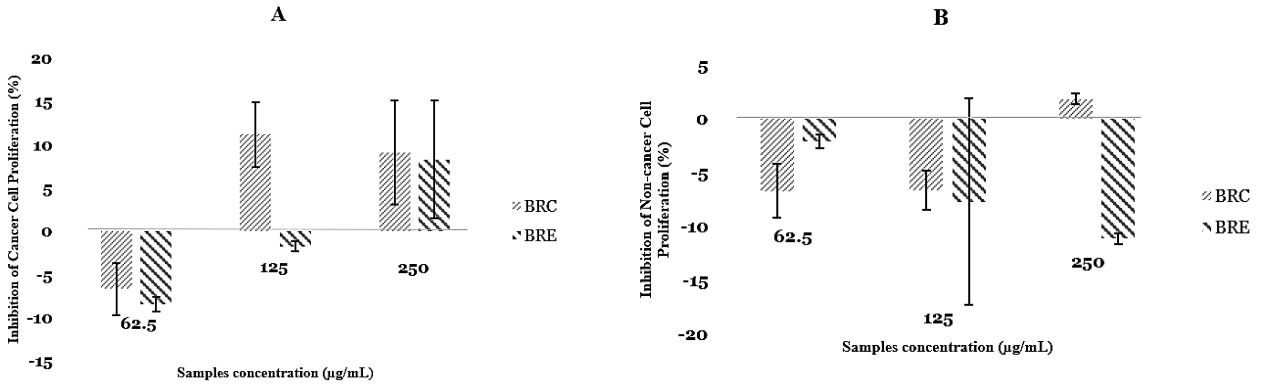


Figure 2 Antiproliferative activity of black rice extract at different concentrations against WiDr cells (A) and non-cancer cells (B). BRC: Black rice chloroform extract, BRE: Black rice ethanol extract. The results of triplicate experiments have been presented with standard deviation.

Metabolite profiling

A total of 20 metabolites were found in BRC applying GC-MS (7 metabolites) and LC-HRMS (13), as presented in **Figure 3**. These consisted of organic acids, alcohols, phytosterols, terpenoids, bases, amino acids, purines, and flavonoids. Hexadecanoic acid was the metabolite with the highest content (54.30%) in BRC and the lowest was Linoleoyl ethanolamide (0.30%) (**Table 2**).

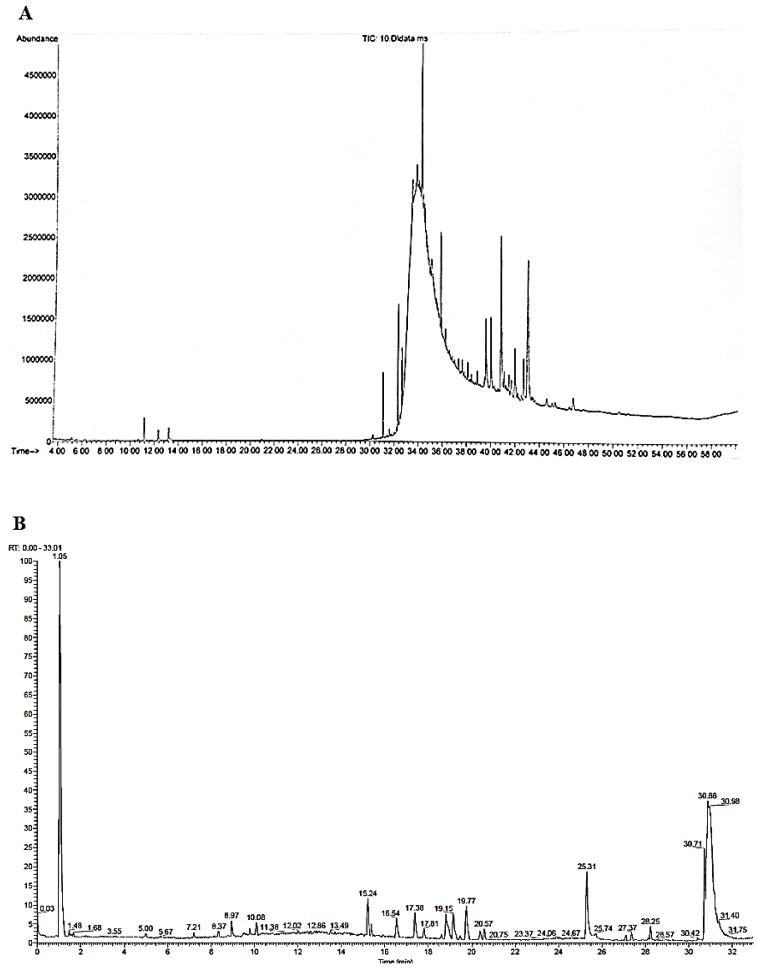


Figure 3 Chromatogram of BRC extract in GC-MS (A) and LC-HRMS (B) analysis.

Table 2 Metabolites content in BRC.

Metabolites	Method	Group	Formula	RT (min)	Metabolites content (%)	Similarity index (%)
Hexadecanoic acid	GC-MS	Organic acid	$C_{16}H_{32}O_2$	33.395	54.30	98
Linoelaidic acid	GC-MS	Organic acid	$C_{18}H_{32}O_2$	34.023	6.30	95
9,17-octadecadienal	GC-MS	Organic alcohols	$C_{18}H_{32}O$	34.230	6.78	95
Campesterol	GC-MS	Phytosterol	$C_{28}H_{48}O$	39.649	1.79	99
Stigmasterol	GC-MS	Phytosterol	$C_{29}H_{48}O$	40.056	1.50	97
Gamma-sitosterol	GC-MS	Phytosterol	$C_{29}H_{50}O$	40.911	4.15	99
24-methylenecycloartanol	GC-MS	Terpenoid	$C_{31}H_{52}O$	42.028	1.29	99
Choline	LC-HRMS	Organic base	$C_5H_{13}NO$	1.047	24.40	96
Hexitol	LC-HRMS	Organic alcohol	C6 H14 O6	1.069	1.35	95
Valine	LC-HRMS	Amino acid	$C_5H_{11}NO_2$	1.089	5.24	97
Adenine	LC-HRMS	Purine	$C_5H_5N_5$	1.096	0.58	98
Xylosyl adenine	LC-HRMS	Purine	$C_{10}H_{13}N_5O_4$	1.127	0.48	99
Leucine	LC-HRMS	Amino acid	$C_6H_{13}NO_2$	1.631	1.30	99
Phenylalanine	LC-HRMS	Amino acid	$C_9H_{11}NO_2$	2.446	0.73	96
Quercitrin	LC-HRMS	Flavonoid	$C_{21}H_{20}O_{11}$	6.497	1.06	97
Tectoridin	LC-HRMS	Flavonoid	$C_{22}H_{22}O_{11}$	7.156	0.74	97
Isoquercetin	LC-HRMS	Flavonoid	$C_{21}H_{20}O_{12}$	8.806	0.33	96
isorhamnetin 3-O-glucoside	LC-HRMS	Flavonoid	$C_{22}H_{22}O_{12}$	9.521	0.40	96
Quercetin	LC-HRMS	Flavonoid	$C_{15}H_{10}O_7$	11.599	0.24	99
Linoleoyl ethanolamide	LC-HRMS	Organic acid	$C_{20}H_{37}NO_2$	22.433	0.30	96

Different 7 and 4 metabolites in BRE were detected using GC-MS and LC-HRMS method, respectively (**Figure 4**). The detected metabolites included organic acids, phytosterols, terpenoids, organic bases, amino acids, and flavonoids. Hexadecanoic acid was the metabolite with the highest content (25.24%) in BRE and the lowest was hesperetin (0.37%) (**Table 3**).

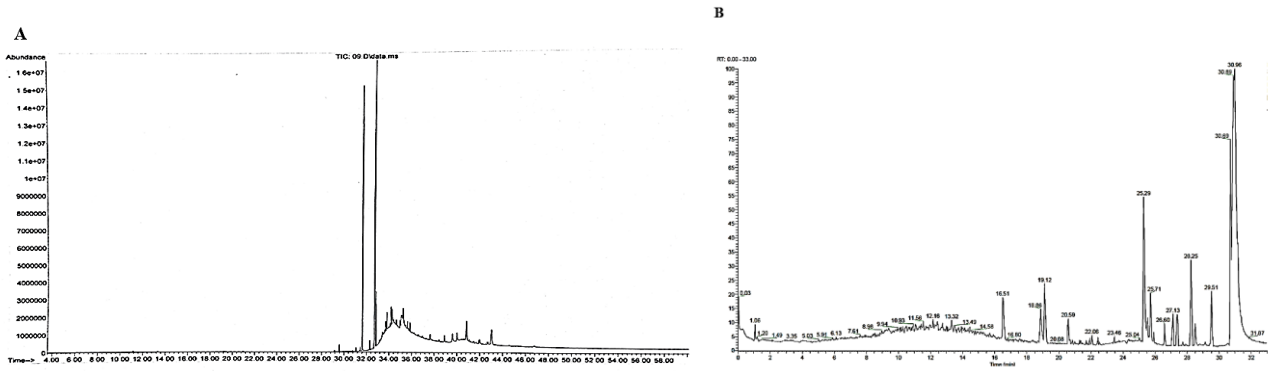


Figure 4 Chromatogram of BRE extract in GC-MS (A) and LC-HRMS (B) analysis.

Table 3 Metabolites content in BRE.

Metabolites	Method	Group	Formula	RT (min)	Metabolites content (%)	Similarity index (%)
Hexadecanoic acid	GC-MS	Organic acid	$C_{16}H_{32}O_2$	31.665	25.24	99
Linoleic acid	GC-MS	Organic acid	$C_{18}H_{32}O_2$	32.733	21.96	99
Oleic acid	GC-MS	Organic acid	$C_{18}H_{34}O_2$	32.768	21.20	99
Campesterol	GC-MS	Phytosterol	$C_{28}H_{48}O$	39.636	1.78	99
Stigmasterol	GC-MS	Phytosterol	$C_{29}H_{48}O$	40.036	1.27	96
Gamma-sitosterol	GC-MS	Phytosterol	$C_{29}H_{50}O$	40.870	4.52	99
24-methylenecycloartanol	GC-MS	Terpenoid	$C_{31}H_{52}O$	43.05	4.65	99
Choline	LC-HRMS	Organic base	$C_5H_{13}NO$	1.053	2.21	96
Valine	LC-HRMS	Amino acid	$C_5H_{11}NO_2$	1.072	0.40	97
Hesperetin	LC-HRMS	Flavonoid	$C_{16}H_{14}O_6$	12.884	0.37	95
Linoleoyl ethanolamide	LC-HRMS	Organic acid	$C_{20}H_{37}NO_2$	22.430	1.28	96

Eight metabolites were identified in BRC and BRE, which included campesterol, choline, hexadecanoic acid, gamma-sitosterol, stigmasterol, valine, 24-methylenecycloartanol, and linoleoyl ethanolamide. Three were identified only in BRE (linoleic acid, hesperetin, and oleic acid) and 12 in BRC, namely hexitol, isoquercetin, xylosyl adenine, quercetin, 9,17-octadecadienal, phenylalanine, leucine, linoelaidic acid, isorhamnetin 3-O-glucoside, tectoridin, quercitrin, and adenine. Chloroform was assumed to be a more effective solvent in black rice extraction process than ethanol. In this study, organic acids were the most frequently identified metabolites (23%) in black rice. Other metabolite groups detected were phytosterols (19%), flavonoids (19%), amino acids (13%), terpenoids (7%), organic alcohols (7%), purines (6%), and organic bases (6%) (**Figure 5**).

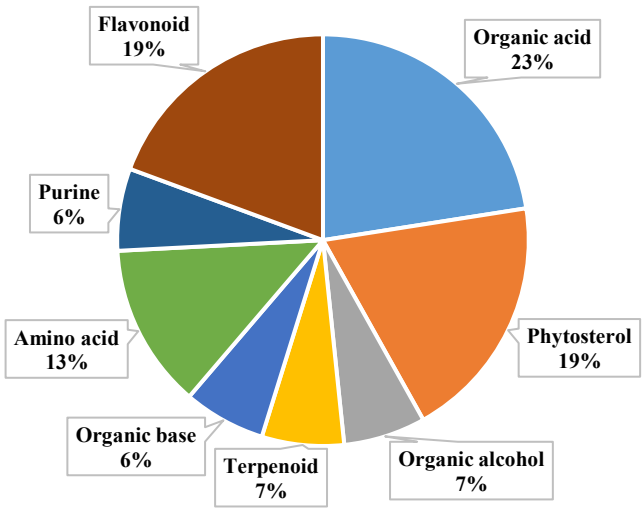


Figure 5 Metabolite groups were detected in BRC extract and BRE extract.

Ligand bioavailability

A total of 23 metabolites were identified in samples that became ligand candidates in the docking analysis. Initially, the bioavailability of these metabolites was predicted based on the 5 Lipinski's rules. Eleven metabolites did not comply with Lipinksi's rules, while the other 12 complied and qualified for docking analysis (**Table 4**).

Table 4 Selected metabolites for molecular docking analysis based on Lipinski's rules.

Metabolites	MW (Da)	LogP	H bond donors	H bond acceptor	Molar refractivity
Adenine*	135.054	–0.327	1	2	32.65*
Campesterol	400.700	1.418	1	1	105.23
Choline*	103.099	–0.554	1	1	21.28*
Gamma-sitosterol	414.70	1.418	1	1	107.66
Hesperetin	302.078	2.388	3	6	73.69
Hexadecanoic acid	256.42	0.637	1	2	57.57
Hexitol*	182.07	–3.507	6*	6	34.95*
Isoquercetin*	464.095	–0.691	8*	12*	104.64
Isorhamnetin 3-glucoside*	478.110	0.101	7*	12*	106.75
Leucine*	131.09	0.245	1	3	27.68*
Linoelaidic acid	280.40	1.292	1	2	68.28
Linoleic acid	280.40	1.292	1	2	68.28
Linoleoyl ethanolamide	323.281	0.234	2	3	78.78
24-methylenecycloartanol	440.70	1.266	1	1	111.24
9,17-octadecadienal*	264.40	0	0	1	0*
Oleic acid	282.50	1.046	1	2	66.17
Phenylalanine	165.70	1.285	1	3	42.82
Quercetin*	302.042	2.010	5*	7*	74.05
Quercitrin*	448.099	0.151	7*	11*	102.94
Stigmasterol	412.70	1.971	1	1	109.03
Tectoridin*	462.115	0.430	6*	11*	105.68
Valine*	117.07	0.164	1	3	24.44*
Xylosyl adenine	267.096	–2.203	3	8	57.67

*Did not comply with Lipinski's rule

Validation of docking analysis

Validation results showed that each receptor had a different gridbox size, with RMSD value of ≤ 2 Å (**Table 5**). The ligand shape changes before and after re-docking were not significantly different (**Figure 6**). The best grid box sizes for the 2R0U, 3U9W, and 4LXD receptors were 4.0, 4.0, and 5.0 Å with RMSD values of 0.14 Å (–10.43 kcal/mol), 1.34 Å (–10.39 kcal/mol), and 0.39 Å (–10.62 kcal/mol), respectively.

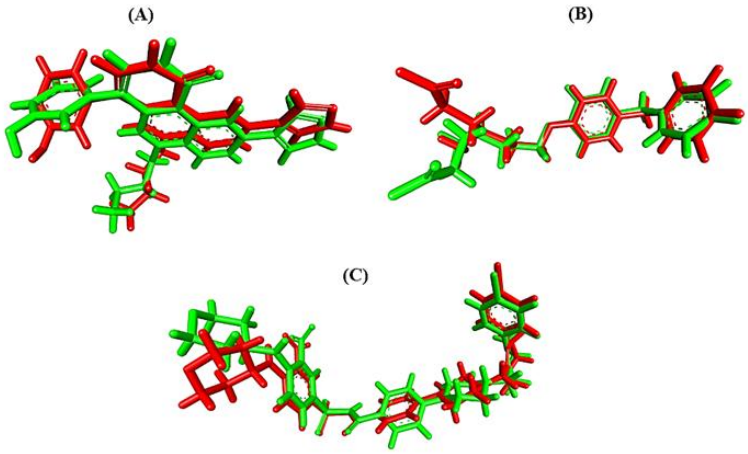


Figure 6 The results of validation analysis (redocking) of co-crystal ligands. (A) Receptor 2R0U ligand, (B) Receptor 3U9W ligand, and (C) Receptor 4LXD ligand. Red: Before redocking, green: After redocking.

Table 5 Validation analysis results of each receptor.

PDB ID	Gridbox (Å)	Gibbs free energy (kcal/mol)	RMSD (Å)
2R0U	4.0	–10.43	0.14
3U9W	4.0	–10.39	1.34
4LXD	5.0	–10.62	0.39

Docking analysis of 2R0U receptor

Docking results for the 2R0U (Chek1) receptor showed that the binding energy for each ligand ranged from –5.83 to –9.34 kcal/mol. Campesterol had the strongest binding energy in this interaction (–9.34 kcal/mol) but was weaker than the co-crystal and receptor interaction binding energy (–10.43 kcal/mol). The interaction types of ligands and receptors were H-bond, hydrophobic, van der Waals, and electrostatic. Cys87 is an amino acid residue often included in H-bond interactions. Leu15, Gly16, Val23, Lys38, and Glu55 are amino acid residues widely included in the hydrophobic and van der Waals interactions in each ligand (**Table 6**). Campesterol and 2R0U receptor interactions comprise 19 amino acid residues. Seven of these, namely Leu15, Gly16, Val23, Lys38, Glu55, Leu84, and Asp148), are binding sites for Chek1 with hydrophobic and van der Waals interactions (**Figure 7**).

Table 6 The docking analysis results of 2R0U receptor and ligand.

Metabolites	Gibbs free energy (kcal/mol)	H-bond interacting residues	Hydrophobic bond interacting residues	van der Waals bond interacting residues	Electrostatic bond interacting residues
M54 (co-crystal ligand)	–10.43	Glu85, Cys87, Glu134, Asn135[b]	Leu15[b], Val23[b], Ala36, Lys38[b], Leu84[b], Leu137	Gly16[b], Glu55[b], Val68, Leu82, Tyr86, Ser88, Gly90, Ser147, Phe149	Glu91, Asp148[b]
Campesterol	–9.34	-	Leu15[b], Val23[b], Ala36, Lys38[b], Leu82, Leu84[b], Leu137	Gly16[b], Glu55[b], Asn59, Val68, Tyr86, Cys87, Ser88, Gly90, Glu91, Ser147, Asp148[b], Phe149	

[b] protein binding site

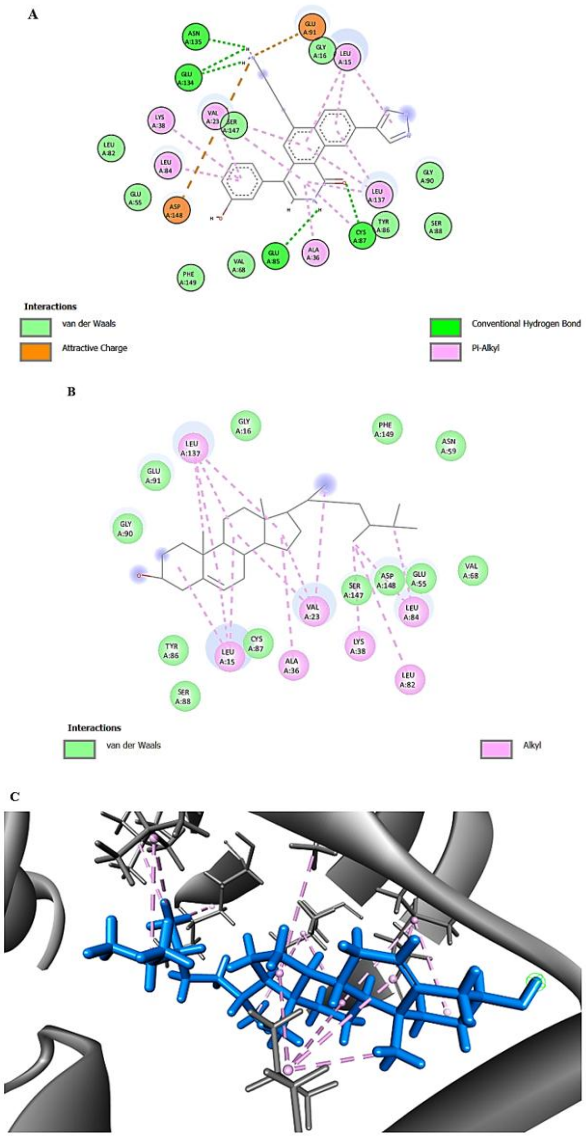


Figure 7 Co-crystal ligand and campesterol interactions with 2R0U receptor. (A) 2D interaction of co-crystal ligand and 2R0U receptor, (B) 2D interaction of campesterol and 2R0U receptor, (C) 3D interaction of campesterol and 2R0U receptor. (In the 2D view, the ligand is shown with line. In the 3D view, blue is the campesterol, and gray represents the receptor).

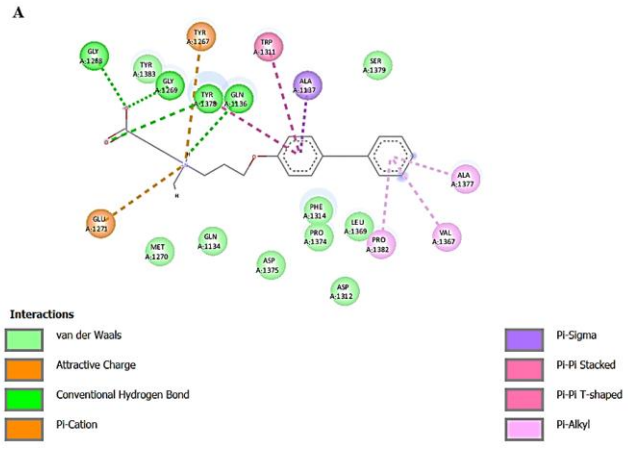
Docking analysis of 3U9W receptor

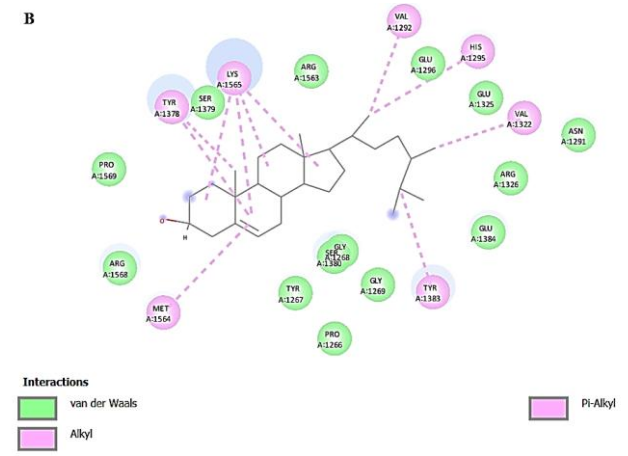
The 3U9W (leukotriene A4 hydrolase) receptor results showed that the binding energy for each ligand ranged from –7.83 to −10.48 kcal/mol. Campesterol had the strongest binding energy in this interaction (–10.48 kcal/mol), which was stronger than the co-crystal ligand and receptor binding (–10.39 kcal/mol). The interaction types of ligands and receptors were H-bond, hydrophobic, van der Waals, and electrostatic. All ligands had hydrophobic and van der Waals interactions. Gly1269 and Glu1296 are amino acid residues often included in H-bond interactions. Tyr1267, His1295, His1299 and Lys1565 are widely included in hydrophobic and van der Waals interactions in each ligand (**Table 7**). Campesterol and 3U9W receptor interaction comprises 21 amino acid residues, of which 8 including Pro1266, Tyr1267, Gly1268, Gly1269, His1295, Arg1563, Met1564, Lys1565 are binding sites. Meanwhile, only one (Glu1296) was an active site for leukotriene A4 hydrolase with hydrophobic and van der Waals interactions (**Figure 8**).

Table 7 Docking analysis results of 3U9W receptor and ligand.

Metabolites	Gibbs free energy (kcal/mol)	H-bond interacting residues	Hydrophobic bond interacting residues	van der Waals bond interacting residues	Electrostatic bond interacting residues
28P (co-crystal ligand)	–10.39	Gln1136[b], Gly1268[b], Gly1269[b], Tyr1378	Ala1137, Trp1311, Val1367, Ala1377, Pro1382	Met1270[b], Asp1312, Phe1314, Gln1134[b], Leu1369, Pro1374, Asp1375, Ser1379, Tyr1383	Tyr1267[b], Glu1271[b]
Campesterol	–10.48		Val1292, His1295[b], Val1322, Tyr1378, Tyr1383, Met1564[b], Lys1565[b]	Pro1266[b], Tyr1267[b], Gly1268[b], Gly1269[b], Asn1291, Glu1296[a], Glu1325, Arg1326, Ser1379, Ser1380, Glu1384, Arg1563[b], Arg1568, Pro1569	

[a] protein active site, [b] protein binding site





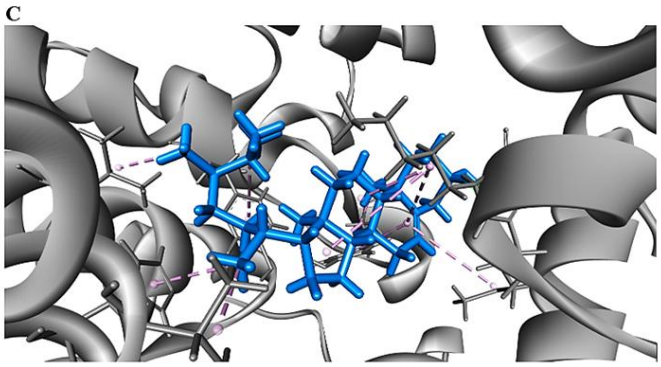


Figure 8 Co-crystal ligand and campesterol interactions with 3U9W receptor. (A) 2D interaction of co-crystal ligand and 3U9W receptor, (B) 2D interaction of campesterol and 3U9W receptor, (C) 3D interaction of campesterol and 3U9W receptor. (In the 2D view, the ligand is shown with the line. In the 3D view, blue is the campesterol, and gray represents the receptor).

Docking analysis of 4LXD receptor

Ligands and 4LXD (Bcl-2) receptor interactions showed a binding energy of –5.29 to –7.79 kcal/mol. The 24 - methylenecycloartanol had the strongest binding energy in this interaction (–7.79 kcal/mol). Meanwhile, the interaction of the co-crystal ligand with the receptor showed a binding energy of –10.62 kcal/mol. The interaction types of ligands and receptors were H-bond, hydrophobic, van der Waals, and electrostatic. Asp108 and Arg143 are amino acid residues widely included in all types of interactions between ligands and receptors (**Table 8**). These residues are binding sites for Bcl-2 and have van der Waals interactions with 24-methylenecycloartanol (**Figure 9**).

Table 8 Docking analysis results of 4LXD receptor and ligands.

Metabolites	Gibbs free energy (kcal/mol)	H-bond interacting residues	Hydrophobic bond interacting residues	van der Waals bond interacting residues	Electrostatic bond interacting residues
1XV (co-crystal ligand)	–10.62	Gly142	Ala97, Phe101, Tyr105, Phe109, Met112, Val130, Leu134, Ala146, Tyr199	Asp100, Glu133, Asn140, Trp141, Val145, Glu149, Phe150, Val153, Phe195	Asp108[b], Arg143[b]
24 - methylenecycloartanol	–7.79		Phe101, Tyr105, Met112, Leu134, Ala146	Asp108[b], Phe109, Val130, Arg143[b], Phe147, Glu149, Phe150, Val153	

[b] protein binding site

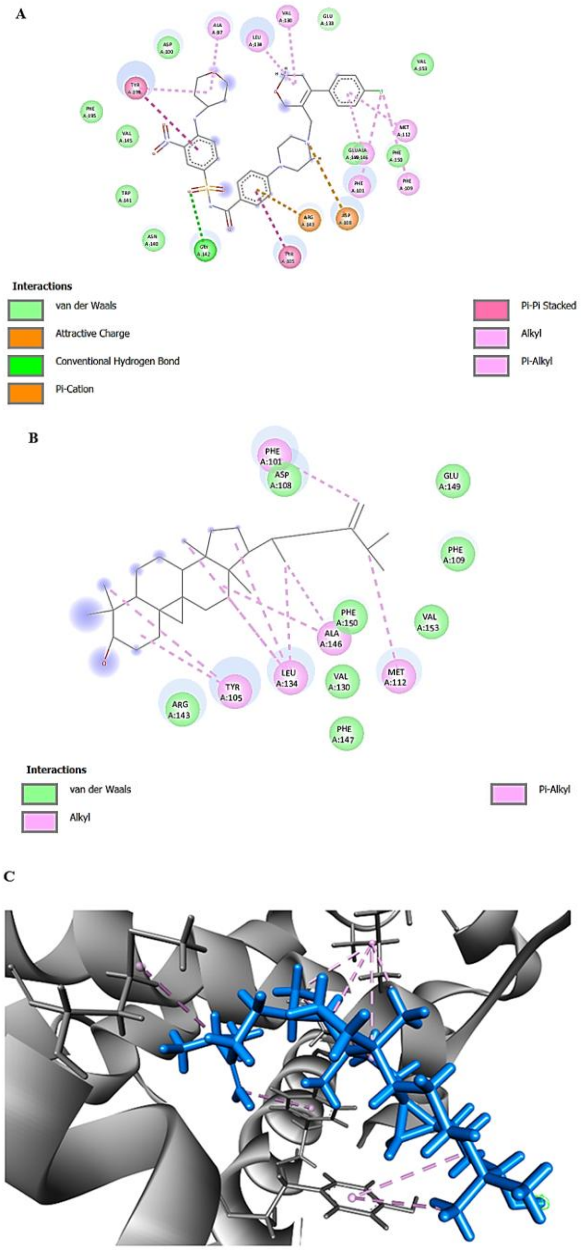


Figure 9 Co-crystal ligand and 24 – methylenecycloartanol interactions with 4LXD receptor. (A) 2D interaction of co-crystal ligand and 4LXD receptor, (B) 2D interaction of 24-methylenecycloartanol and 4LXD receptor, (C) 3D interaction of 24-methylenecycloartanol and 4LXD receptor. (In the 2D view, the ligand is shown with the line. In the 3D view, blue is the 24-methylenecycloartanol and gray represents the receptor).

Yield was the amount of extract obtained from black rice depending on the technique and solvent used [32,33]. The basic conventional extraction method used in this study was maceration [34]. BRC and BRE extraction processes used different solvents including chloroform (nonpolar) and ethanol (polar), respectively. Variations in solvents can produce differences in the yield obtained during the extraction process [35]. BRC yield (7.12%) was higher than BRE yield (5.18%), which was confirmed by metabolite identification analysis using GC-MS and LC-HRMS. Total metabolites detected in BRC (20) was higher than the number found in BRE (11).

Hexadecanoic acid was a metabolite from fatty acid group identified with the highest concentration in both black rice extracts, which was assumed essential in antioxidant and antiproliferative activities of colon cancer cells. Fatty acids, including unsaturated fatty acids, contain double bonds that can donate electrons to free radicals, thereby acting as antioxidant [36]. Other

studies reported that n-hexadecanoic acid scavenged free radicals with high inhibition [37]. This corresponds with DPPH reaction mechanism as a stable free radical at room temperature and changes color from purple to yellow when receiving or sharing electrons from antioxidant compounds [38]. Hexadecanoic acid prevents colon cancer cell proliferation through an apoptotic mechanism induced by enhanced ROS levels [39]. Reaction of ROS with the mitochondrial membrane causes membrane polarization, leading to apoptosis transduction signals and cascade reactions of pro-apoptotic molecules in cancer cells [40]. The presence of free radicals in normal cells was associated with increased oxidative stress conditions and damaged signaling pathways, leading to cancer formation [41]. Antioxidant compounds can help reduce oxidative stress, preventing the formation of cancer cells [42]. Therefore, hexadecanoic acid contained in black rice may be a good antioxidant and anti-colon cancer agent.

BRC antioxidant activity was higher compared to BRE, as signified by the lower IC_{50} value of BRC. Furthermore, the result was supported by the number of yields and metabolites identified in BRC such as flavonoid, terpenoid, and phytosterol, which were higher than BRE. The observation indirectly suggested that antioxidant activity of an extract depended on the active compounds content in the extract, specifically secondary metabolites such as phenols and flavonoids [43]. The IC_{50} value of black rice extract showed that antioxidant activity of the extract was still weaker than the positive control, ascorbic acid (IC_{50} = 1.06 µg/mL). Previous studies reported that the IC_{50} value of ascorbic acid in antioxidant tests using DPPH method ranged from 0.2 - 22.5 µg/mL [44,45]. The value obtained for an extract was attributed to many metabolites that did not entirely have a synergistic reaction mechanism as an electron donor to free radicals, providing a higher IC_{50} than ascorbic acid existing in the form of a single compound [46,47]. However, black rice antioxidant activity in this analysis was higher (42% - 61%) than the value of Melik Jawa and Toraja varieties, which scavenged free radicals by 33% - 38% [48]. Activity variations occurred due to differences in black rice varieties, growing locations, and extraction methods, leading to distinction in the profile and content of black rice metabolites [49].

Extracts inhibited WiDr cell proliferation at the highest concentration (250 µg/mL), suggesting that black rice was not efficient in preventing the cancer cells growth at low concentrations. In the test using non-cancerous cells, black rice extract did not inhibit fibroblast (NIH/3T3) growth except at the BRC (250 µg/mL) highest concentration suggested to have a toxic effect, but the hypothesis should be further investigated. Secondary metabolites like flavonoids, terpenoids, and alkaloids contained in black rice can prevent the cancer cells growth by damaging the signaling process and apoptosis, depending on the concentration of the compounds [50-52]. This is consistent line with the mechanism of doxorubicin as a positive control in antiproliferative activity test for cancer cells, which can inhibit growth through apoptosis, DNA damage, and cell cycle signal disruption [53]. The results of antiproliferative activity test showed that black rice extract promoted cancer cell growth at the lowest concentration. More analysis is essential to determine the black rice active components supporting cancer cell proliferation at low concentrations.

Antiproliferative activity observed in this study corresponded with previous investigations showing that black rice of Cempo Ireng variety, Cigudeg accession, Bogor, West Java, had antiproliferative activity against colon cancer cells *in-vivo* by suppressing the expression of the proliferating cell nuclear antigen (PCNA) gene, considered as a marker for colon cancer cell proliferation. The reported Cempo Ireng variety induced apoptosis by increasing the expression of caspase-3 and caspase-8 genes [54]. Furthermore, *in-vitro* antiproliferative activity was supported by *in-silico* analysis showing that the metabolites contained in black rice had a strong interaction with the target proteins of colon cancer treatment, namely Chek1, leukotriene A4 hydrolase, and Bcl-2.

Chek1 is a serine/threonine-specific protein kinase that causes DNA damage repair and supports tumor growth [55]. Similarly, leukotriene A4 hydrolase is a key modulator in the cell cycle associated with the formation of several cancer cells [56]. Bcl-2, an anti-apoptotic protein, plays a role in the development of resistance to treatment in colorectal cancer [57]. Campesterol is a group of phytosterols identified in both black rice extracts as anticancer agent that interferes with signal transduction in cancer cells, activates pro-

apoptotic molecules and induces cycle arrest in the G0/G1 phase [58]. *In-silico* study results showed that campesterol metabolite had the strongest interaction, based on binding energy of Chek1 (2R0U) and leukotriene A4 hydrolase (3U9W). This result was supported by the bond position between campesterol and both proteins at binding and active sites (**Tables 6** and **7**). Other results showed 24-methylenecycloartanol as a group of terpenoids contained in black rice extract that had the strongest interaction with Bcl-2 (4LXD) through a bond located in binding site position of Bcl-2 (**Table 8**). Previous investigations reported that terpenoids were natural anticancer agents with a mechanism of inhibiting protein synthesis by blocking ribosomes, inhibiting protein kinases, and increasing caspase regulation [59,60]. Based on the current results, black rice extract is speculated to prevent the proliferation of colon cancer cells by disrupting cell cycle, as well as inhibiting protein kinases and anti-apoptotic proteins.

In-vitro and *in-silico* evaluation results of black rice of Cempo Ireng variety showed many health benefits, similar to other pigmented types. These provide basic information for the development of black rice as a colon cancer treatment drug or a functional food with antioxidant effect on digestive tract health to prevent colon cancer. New information related to the mechanism of inhibiting colon cancer cell proliferation is provided from black rice, based on the target proteins Chek1, leukotriene A4 hydrolase, and Bcl-2. Additionally, the results serve as a reference for designing colon anticancer drugs with active compounds using campesterol and 24-methylenecycloartanol against the inhibition of Chek1, leukotriene A4 hydrolase, and Bcl-2 protein.

The limitations of this study included the use of only one variety of black rice and the inability to explain the differences in bioactivity that might occur due to the use of many varieties. Antioxidant activity was not measured with various methods, limiting the test scope to DPPH free radical scavenging. Additionally, the molecular mechanism of *in-vitro* antiproliferative activity was not investigated, and the mechanism of inhibiting cancer cell proliferation due to the administration of black rice extract remained unknown. More studies should focus on identifying antioxidant and antiproliferative mechanisms of black rice using several varieties, multiple solvents of different polarities, and experimental animals for easier execution of the clinical trial phase.

Conclusions

In conclusion, black rice extract of Cempo Ireng variety showed potential as a natural antioxidant and inhibitor of colon cancer cells. This extract had no toxic effect on non-cancerous cells at low concentrations. Additionally, hexadecanoic acid, campesterol, and 24-methylenecycloartanol contained in black rice were found to play an important role in bioactivity as antioxidant and antiproliferation against colon cancer cells.

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