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The Effect of Anthocyanins Black Rice Bran Extract (ABRiBE) on Colorectal Cancer Cell Proliferation and ABCA1 Gene Expression in the HT-29 Cell Line

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Abstract

Background: Anthocyanins inhibit colorectal cancer cell proliferation and cholesterol levels regulation by interacting with the adenosine triphosphate-binding cassette subfamily A member 1 (ABCA1) protein, expressed at reduced levels in colon cancer. Anthocyanins can also increase the efflux of cholesterol from cancer cells and are present in Indonesian native black rice bran. Therefore, this study investigated the effect of Anthocyanin Black Rice Bran Extract (ABRiBE) on the mRNA expression of the ABCA1 gene and the proliferation of colorectal cancer cells in the human HT-29 cell line. The hypothesis is that ABRiBE inhibits HT-29 cell proliferation and increases ABCA1 gene expression.
Methods: This experimental study on the HT-29 cells used anthocyanins extracted from black rice bran powder through ethanol acidified with citric acid. The MTS assay measured the inhibitory concentration (IC_{50}) of 50% cell proliferation inhibition using 12 extract concentrations and control groups for 24 and 48 hours. The ABCA1 gene expression in groups above the antiproliferative IC50 value and the control group was analyzed using the quantitative Polymerase Chain Reaction (qPCR) method.
Results: The IC_{50} of black rice bran extract was 1.57 and 1.35 mg/mL for 24 and 48 hours of incubation, which decreased HT-29 cell proliferation with values of (r= 0.97, p=0.001 and r=0.95, p=0.001), respectively. Extract exposure of the 2 concentrations above the IC_{50} values of 1.7 and 2.0 mg/mL increased ABCA1 gene expression in HT-29 cells, by 1.16 and 2.32 fold compared to the control group.
Conclusion: The ABRiBE inhibited HT-29 cell proliferation and increased the ABCA1 gene expression.

Keywords: Anthocyanins; ABCA1 Gene; Black Rice Bran Extract; HT-29 Cell Line; Proliferation

1. Introduction

Colorectal cancer is among the top ten cancers contributing to cancer-related deaths. In 2020, a total of 1,931,590 incidences and 935,173 mortalities of colorectal cancer were reported (Sung *et al.*, 2021), with both increasing by 130,613 and 54,173 from 2018, respectively (Bray *et al.*, 2018). Globally, colorectal cancer ranks third and second in incidence and mortality compared to other cancers (Sung *et al.*, 2021). The increase can be influenced by changes in dietary patterns (Sung *et al.*, 2021), such as the consumption of red or processed meat, a high-fat diet, and alcohol(WCRFI, 2018).

According to Katona and Weiss (2020), chemoprevention can help reduce the incidence and mortality of colorectal cancer. The agents used for chemoprevention can either be drugs or natural substances, such as anthocyanins, which are being studied for their potential to prevent the development of colorectal cancer (Katona and Weiss, 2020). Anthocyanins are natural pigments that belong to the flavonoid family and exist in a glycosylated form (Martin *et al.*, 2017; Khoo *et al.*, 2017). Anthocyanins are natural pigments that give fruits, flowers, and whole-grain rice red, purple, blue, brown, and black coloring (Martin *et al.*, 2017; Khoo *et al.*, 2017). The high anthocyanins content is primarily found in berries, such as grapes, currants, and tropical fruits like dragon fruit skin (Khoo *et al.*, 2017). In Indonesia, one of the foodstuffs with high anthocyanins is black rice, the primary staple food of the population. The country is the largest producer of pigmented rice, especially black rice, after China and India (Prasad *et al.*, 2019). Cempo Ireng is an Indonesian black rice with the highest total anthocyanin content compared to other varieties, with 428.38 mg/100g. This is almost equivalent to the anthocyanin content of blueberry*Vaccinium corymbosum* (CVAC5.001 cultivar) (430 mg/100g) (Kristamtini and Wiranti, 2017; Peña-Sanhueza *et al.,* 2017). Thus, black rice can be an alternative source of ri ch anthocyanins, particularly in areas where blueberries are not readily available.

Most in vitro studies on colorectal cancer cell lines use extracts containing anthocyanins from berries. Afrin *et al.* (2016) found that freeze-dried extract of black raspberry, administered at doses of 0.6 and 1.2 mg/mL, demonstrated anticancer activity on H T-29 cells after 48 hours of incubation. Meanwhile, this study is a novel contribution to the field due to limited in vitro examinations on human colorectal cancer cell lines using ABRiBE. Anthocyanins affect colon cancer chemopreventive through the mechanism of antioxidant, antiproliferation, induction of apoptosis, anti-invasive activity, gene demethylation, anti-inflammation, and microbiota (Shi *et al.,* 2021). In HCT 116 and HT-29 cell lines, cyanidin-3-O-glucoside and delphinidin-3-O-glucoside as anthocyanin's single compounds reduced EGFR (Shi et al., 2021; Mazewski *et al*., 2018).

The level of ABCA1 in human colorectal cancer cells is lower than the ABCA1 level in healthy cells (Lo Sasso *et al.,* 2017; Hlavata *et al.,* 2012). Previous reports have shown that anthocyanins can increase the mRNA expression of the ABCA1 gene through the Peroxisome Proliferator-Activated Receptor α (PPARα) and Liver X Receptor α (LXRα) pathways by promoting intracellular cholesterols efflux (Xia *et al.,* 2005). Intracellular cholesterols accumulation is associated with the pathogenesis of colorectal cancer (Smith and Land, 2012). According to Xia *et al.* (2005), anthocyanins can increase cholesterols efflux through the ABCA1 transporter protein. The high mRNA and protein expression of ABCA1 may reduce tumor formation in human colorectal cancer cell lines (Pasello *et al.,* 2020). Mazewski *et al.* (2018) also discovered that increased ABCA1 protein by anthocyanins exposure inhibits cancer cell proliferation. The increase of anthocyanin-modulated mRNA expression of the ABCA1 gene can enhance the elimination function of ABCA1 protein-mediated cholesterols and reduce Akt-dependent survival signaling that contributes to anticancer activity (Smith and Land, 2012). Subfamily A of ABC transporters for tumorigenesis affects the stage of cancer initiation and progression (Hlavata *et al.,* 2012; Pasello *et al.,* 2020).

The effect of ABRiBE on cell proliferation in colorectal cancer cell lines requires the determination of an IC_{50}. Although several studies have been conducted using anthocyanin-rich foods on cell lines (Afrin *et al*., 2016), limited experiments have used black rice bran extract. Additionally, little information is available on the impact of ABRiBE on cell proliferation and ABCA1 gene expression in human colorectal cancer cell lines. Therefore, this study aimed to investigate the effects of ABRiBE on the proliferation of colorectal cancer cells and the mRNA expression of the ABCA1 gene using the HT-29 cell line.

2. Materials and Methods

The study was approved by the Health Research Ethics Committee, Ethics Number: KET−841/UN2.F1/ETIK/PPM.00.02/2020, Faculty of Medicine Universitas Indonesia and Cipto Mangunkusumo Hospital (HREC−FMUI/CMH).

2.1. Black Rice Bran Extraction

The Cempo Ireng black rice used in the study was obtained from a local farmer, Mr. Murji, located in Cigudeg, Bogor, West Java, Indonesia. The materials prepared for extraction included black rice bran powder obtained from grinding, 96% ethanol, and 20% citric acid (w/v). The procedures and reagents used for extraction have been declared safe by the National Agency of Drug and Food Control (NA-DFC) of Indonesia, as per Quality Requirements for Health Supplements No. 17 of 2019. The black rice bran and ethanol-citric acid mixture (1:10, w/v) were macerated for 24 hours at room temperature in a dark room. The black rice bran powder was homogenized in the first two hours and the last two hours of the 24 hours of maceration time. The extract was filtered using Advantec® Toyo Qualitative Filter Paper No. 5B (Advantec Toyo Kaisha, LTD., Tokyo, Japan), and the filtrate was evaporated at a temperature of 50°C using an IKA® HB10 Vacuum Rotary Evaporator (VirtualExpo Group, UK)at a speed of 30 rpm with an IKA® RV10 Digital Vacuum Rotary Evaporator (VirtualExpo Group, UK)until the ethanol had completely evaporated. Subsequently, the extract was dried using a freeze dryer for 72 hoursand stored in a freezer at -20°Cuntil used.

2.2. HT-29 Cell Line Propagation

The human colorectal cancer cell line (HT-29), on its 21st passage, was obtained from the Research Center for Virology and Cancer Pathobiology at the Faculty of Medicine, Universitas Indonesia and Dr. Cipto Mangunkusumo General Hospital. The HT-29 cell line was propagated using protocols from the Molecular Biology and Proteomics Core Facilities (MBPCF) at the Indonesia Medical Education and Research Institute (IMERI) and the Culture Unit Protocol at the Integrated Laboratory of the Faculty of Medicine, Universitas Indonesia. The cells were cultured in 90% Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, and 1% amphotericin. Furthermore, the cells were incubated at 37°C in 95% air/5% Carbon Dioxide (CO_2) and a water-saturated atmosphere. They were grown up to 80% confluence and divided into 72 wells using a 96-well plate for antiproliferative activity examination. Subsequently, the cells were calculated at $1x10^4$ per well. The cells for analysis of gene expression were divided into 9 wells using a 12-well plate and were calculated at $25x10^4$ per well.

2.3. Determination of the Antiproliferative Activity of HT-29 Cells

The effect of ABRiBE on colorectal cancer cell proliferation was determined using the MTS assay with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS), catalog number selected: G5421 (Promega Corporation, Wisconsin, USA). The assay was performed following the Culture Unit Protocol established by the Integrated Laboratory of the Faculty of Medicine at Universitas Indonesia. The frozen extract of black rice bran was thawed and diluted to 12 concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.0, 2.4, 2.8, and 3.2 mg/mL. There were 3 groups on the antiproliferative activity of colorectal cancer cells examination, namely the experiment, the negative control, and the blank groups. Each group was conducted in triplicate, where the experimental group consisted of plates filled with $1x10^4$ HT-29 cells per well and exposed to black rice bran extract. All groups were incubated for 24 and 48 hours independently after exposing the extract. The absorbances were read by spectrophotometric at wavelength 490 nm and the cell viability was calculated using the formula below

$$\text{Cell viability/cell proliferation activity (\%)} = \frac{A_E - A_B \times 100\%}{A_{NC} - A_b} \quad (1)$$

in which,

A_E= The absorbance value of the experiment group at the concentration χ

A_{NC}= The absorbance value of the negative control group

A_B= The absorbance value of the blank group

The cell viability was calculated per each extract concentration intervened to HT-29 cells.

The antiproliferative activity of colorectal cancer cells was the inhibition of colorectal cancer cell proliferation. It was determined using the result from the formula in equation (1) above.

The formula for calculating the antiproliferative activity of the colorectal cancer cells was:

Inhibition/antiproliferative activity (%)=$100\%_{NC}-\%_{Conc.\chi}$ cell viability at concentration χ (2)

where:

$100\%_{NC}$= 100% of cell viability resulted from the negative control group

$\%_{Conc.\chi}$ = % Cell viability at concentration χ (% the result from equation (1) calculation)

The value of the inhibition percentage was used to determine the Inhibitory Concentration (antiproliferative IC).

2.4. IC_{50} Determination

The IC_{50} value was obtained by inputting the inhibition percentage and the IC into the Microsoft Excel Spreadsheet Software. The antiproliferative IC_{50} value was obtained from the linear regression line equation, y = a+bχ. The determination of antiproliferative IC_{50} value referred to Culture Unit Protocol, Integrated Laboratory Faculty of Medicine, Universitas Indonesia. The linear regression line equation emanated from the linear trendline. The antiproliferative IC_{50} value was also calculated manually from y = a+bχ, which details were:

χ = Concentration of ABRiBE (mg/mL)

y = Inhibition/ antiproliferative activity (%)

$$a = Constantan\ (a = \frac{\Sigma y.\Sigma x^2 - \Sigma x.\Sigma xy}{n\Sigma x^2 - (\Sigma x)^2})$$

$$b = Slope\ coefficint\ (b = \frac{n(\Sigma xy) - \Sigma x.\Sigma xy}{n\Sigma x^2 - (\Sigma x)^2})$$

The antiproliferative IC_{50} value was based on 24 and 48 hours of incubation time independently after exposing the extract.

2.5. HT-29 Cell Line's Population Doubling Time (PDT)

The PDT of the HT-29 cell line used the serial culture technique to determine the current PDT of the HT-29 cell line at the 21st passage. The PDT of the HT-29 cell line was carried out through several methods that referred to Culture Unit Protocol, Integrated Laboratory Faculty of Medicine, Universitas Indonesia. Those steps are explained below.

2.5.1. Culture of HT-29 Cells

A total of 1.2 x 10^5 HT-29 cells were inserted into a 12-well plate containing a complete DMEM, consisting of the full medium composition of 10% FBS, 1% penicillin-streptomycin, 1% amphotericin, and 1% gentamicin. This was carried out with 3 repetitions, and the examination was conducted for 24, 48, 72, 96, 168, 192, 216, 240, and 264 hours. Cells incubation was carried out in an incubator with a temperature of 37°C and 5% CO_2. The cells were harvested from the culture before serial culture and PDT calculation. Harvesting the culture was repeated according to the amount of examination time.

2.5.2. PDT Calculation

The PDT was calculated on the following formula:

$$PDT = \frac{\Sigma t}{n} = \frac{\Sigma t}{\frac{(log Ne - log\ No)}{log\ (2)}} = \frac{\Sigma t \times 0.301}{(log\ Ne - LogNo)} \quad (3)$$

in which,

∑t= Total observation time

n= The number of doubling cells population

N_e= The number of viable cells at the end of observation

N_0= The number of viable cells at the beginning of the observation

The experiment was conducted from 3 groups, consisting of 2 groups using the concentration above antiproliferative IC_{50} value and the control group. Each group was conducted in triplicate.

2.6. mRNA Expression of ABCA1 Gene

2.6.1. 2.6.1 RNA Extraction and cDNA Synthesis

A total of $25x10^4$ HT-29 cells per well were prepared for mRNA expression analysis of the ABCA1 gene. HT-29 cells were treated with black rice bran extract and incubated for 48 hours. RNA was extracted from the lysate homogenate using the QIAamp® RNA Blood Mini Kit (Qiagen, Hilden, Germany). The purity and RNA concentration was determined using NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific, UK) at wavelength 260 and 280 nm. The RNA purity at A_{260}/A_{280} of 2 and the final concentration of RNA as high as 100 ng/ 20 µL was used for cDNA synthesis. cDNA was synthesized using SensiFAST™ cDNA Synthesis Kit (Bioline Ltd, UK).

2.6.2. Relative Quantification of RT-qPCR

The primer for qPCR was designed by the Integrated Laboratory of the Faculty of Medicine, Universitas Indonesia, Dr. Cipto Mangunkusumo General Hospital, namely the human HRPT1 gene as a reference gene and the ABCA1 gene as the target gene (Table 1).

Table 1. The primer pair sequence of target and reference genes and their amplicon sizes.

Gene Name	**Accession** Number	Primary Sequence (Forward)	Primary Sequence (Reverse)	Amplicon Size
Human HRPT1	NM_024529.5	CCAGTACCAAGACCAGTTTCTC	GGTGGTAGCTGCAGGAATTAT	93 bp (base pair)
Human ABCA1	NM_005502.4	GGTGGTGTTCTTCCTCATTACT	CCGCCTCACATCTTCATCTT	112 bp

Relative quantification using RT-qPCR was performed with the QuantiTect® SYBR® Green PCR Kit (Qiagen, Hilden, Germany). The relative quantification by RT-qPCR was optimized and measured by Livak' method, i.e. 2^-(ΔΔCt) equation as fold gene expression.

The reaction mixture consisted of 2x Q uantiTect® SYBR® Green PCR Master Mix, 10 µ M forward primer, 10 µM reverse primer, 2.0 µL of c DNA template, and RNase-free water. The initial activation stage of PCR was carried out at a temperature of 95°C for 15 minutes. Denaturation, annealing, and extension were performed 40 times at 94°C for 15 s econds, 58°C for 30 s econds, and 72°C for 30 seconds. The final extension was carried out at 72°C for 3 m inutes. A melting curve was performed after the amplification, which was obtained from a slope of 72 to 95 degrees.

2.7. Statistical Analysis

This study was analyzed descriptively and inferentially. The data were processed using Microsoft Excel Spreadsheet Software and Statistical Package for Social Science (SPSS) software version 23. The linear regression line equation was obtained from a linear trendline, available at Microsoft Word trendline layout. The r-value was obtained from the formed chart of a linear regression line equation. The p-value for the correlation of black rice bran extract and antiproliferative activity was analyzed with linear regression using SPSS software.

3. Results

3.1. ProliferativeActivityofHT-29Cells

The influence of black rice bran extract was determined through the percentage value of colorectal cancer cell proliferation activity in each concentration of ABRiBE.The freeze-dried black rice bran extract consists of 12 c oncentrations used to determine the percentage value of colorectal cancer cell proliferation activity: 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.0, 2.4, 2.8, and 3.2 mg/mL. The percentage of colorectal cancer cell proliferation activity was calculated based on i ncubation time after exposing the extract to the cells for 24 a nd 48 hours, independently.

The effect of exposing various concentrations of ABRiBE into the cells tended to lower the proliferation of colorectal cancer cells. Exposing the black rice bran extract into the cells exhibited the derivation trend of colorectal cancer cell proliferation at both incubation times (Figure 1). This study found that a longer incubation time of 48 hours compared to 24 hours after administering the extract improved ABRiBE in reducing the proliferation of colorectal cancer cells, especially at higher concentrations of HT-29 cells. After 48 hours of incubation, the extract showed a significant decrease in the proliferation of colorectal cancer cells compared to the 24 hours of intervention. The difference in proliferation between the two incubation times was observed at extract concentrations above 1.5 mg/mL.

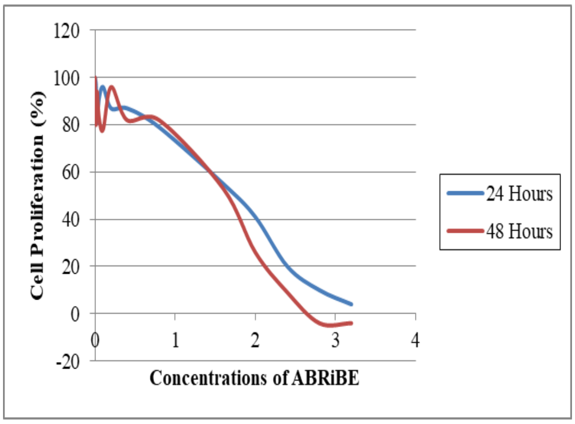

Figure 1. The trend of colorectal cancer cell proliferation activity after exposing the ABRiBE.

3.2. AntiproliferativeActivityofHT-29Cells

The percentage of colorectal cancer cell proliferation inhibition was calculated based on incubation time after independently exposing the extract to the cells for 24 a nd 48 hours (Table 2). The results were based on equation (2).Antiproliferative activity of HT-29 cells was determined from three groups namely the experiment group, the negative control group, and the blank group. The experiment group was the plates filled with $1x10^4$ HT-29 cells per well and exposed to black rice bran extract. Each extract concentration was intervened to well containing HT-29 cells. The negative control group was the plates filled with $1x10^4$ HT-29 cells per well and did not expose to black rice bran extract. The blank group contained only medium.

Table 2. Percentage of inhibition of colorectal cancer cell proliferation after exposing the ABRiBE.

Extract Concentrations (mg/mL)	24 Hours (%)	48 Hours (%)
Control	0	0
0.0125	3	20
0.025	19	6
0.05	9	17
0.1	4	22
0.2	13	4
0.4	13	18
0.8	21	18
1.6	45	47
2.0	59	74
2.4	80	91
2.8	90	104
3.2	96	104

3.3. Antiproliferative IC_{50}

The IC_{50} value is the extract concentration that can suppress cell proliferation by 50% of the total cells and is obtained from the linear regression line equation, y = a+bχ.This study obtained the antiproliferative IC_{50} value from the linear regression line equation for each incubation time, 24 and 48 hours independently after exposing the extract.

The antiproliferative IC_{50} value at the 24 hours of incubation time was obtained by inserting the value 50 into y in the linear regression line equation formed, y = 29,014χ + 4,4442 (Figure 2). 50 = 29.014χ + 4.4442. Therefore, the antiproliferative IC_{50} value based on 24 hours of incubation time was 1.57 mg/mL, which was the χ value from the equation calculation. The r-value was 0.95, indicating that the concentration of ABRiBE had a very strong positive correlation with antiproliferative activity. The p-value was 0.001, showing that there was an influence of the various concentrations of ABRiBE on the antiproliferative activity.

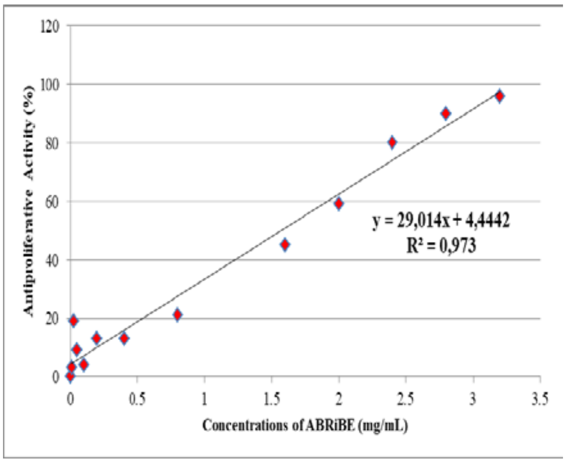

Figure 2. Antiproliferative IC_{50} is based on the incubation time of 24 hours.

The linear regression line equation of the antiproliferative IC_{50} formed at the 48 hours of incubation time was y = 31.828x + 7.1181 (Figure 3). Therefore, the antiproliferative IC_{50} was 1.35 mg/mL, which was the x value from the equation calculation. The r-value was 0.97, indicating that the concentration of ABRiBE had a very strong positive correlation with antiproliferative activity. Meanwhile, the p-value was 0.001, showing that there was an influence of the various concentrations of ABRiBE on the antiproliferative activity.

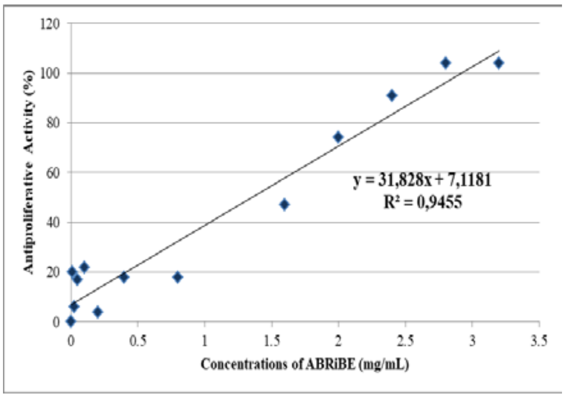

Figure 3. Antiproliferative IC_{50} is based on the incubation time of 48 hours.

3.4. PDT of HT-29 Cells

The current PDT of the HT-29 cell line at the 21st passage was 68.74 hours (Figure 4). PDT was examined through 2 groups using the concentration above the antiproliferative IC_{50} value and the control group. PDT value resulted from $\frac{\Sigma t}{n}$ calculation.

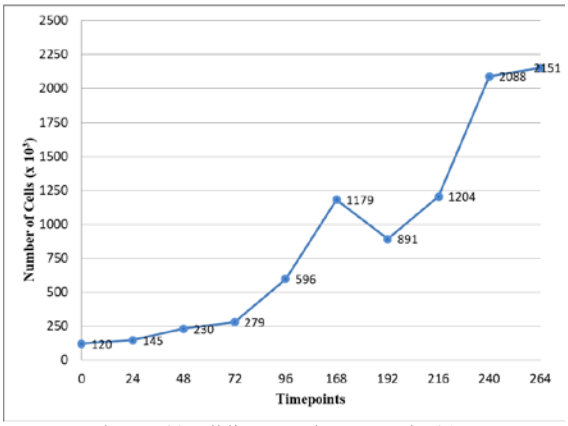

Figure 4. The HT-29 cell line growth curve at the 21st passage.

3.5. mRNA expression of ABCA1 gene

The mRNA expression analysis of the ABCA1 gene used 2 concentrations above the IC_{50} value as the treatment group, namely 1.7 mg/mL and 2.0 mg/mL. HT-29 cells totaling $25x10^4$ per well were treated with black rice bran extract and incubated for 48 hours.There were 3 groups in the gene expression analysis, consisting of 2 treatments and a control group. The RNA purity of each group at 2 wavelengths, 260 and 280 nm, was 2. The value of 2 on RNA purity proved that it is pure. The final concentration of RNA for cDNA synthesis was 100 ng/20 µL, which indicated 100 ng RNA in 20 µL of the final total volume.

There was an increase in the mRNA relative expression of the ABCA1 gene of the HT-29 cells at both concentrations after exposing the ABRiBE compared to the control group. The results of this relative quantification are in a fold gene expression. The results showed that exposing HT-29 cells to 1.7 and 2.0 mg/mL ABRiBE led to a 1.16 and 2.32 fold increase, respectively, in the mRNA relative expression of the ABCA1 gene compared to the control group (Figure 5).

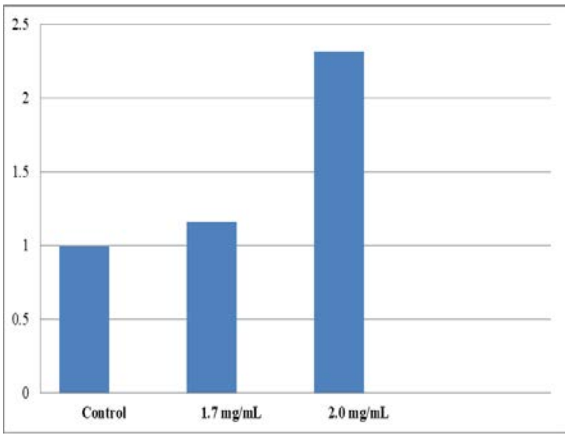

Figure 5. The mRNA relative expression of the ABCA1 gene

4. Discussion

A total of 12 concentrations of ABRiBE were dissolved in ethanol and 20% citric acid, namely 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.0, 2.4, 2.8, and 3.2 mg/mL. Meanwhile, 8 concentration ranges, including 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 mg/mL were used to obtain an IC_{50} of 0.2 mg/mL from the B16-F10 cell line after exposing the anthocyanins blueberry extract dissolved in 70% ethanol and acidified using HCl 0.1% (Wang *et al.,* 2017). Thus, if the extract in this study was acidified with HCl, the concentration range became narrower, and the IC_{50} produced was also smaller. This made the selection of black rice bran powerful and provided more satisfactory results in reducing the cell proliferation of colorectal cancer and increasing the expression of ABCA1 mRNA.

Colorectal cancer cell proliferation tended to decrease after exposing various concentrations of ABRiBE. The use of the HT-29 cell line model by Zhao *et al*. (2019) showed that anthocyanins were able to inhibit the cell proliferation of colorectal cancer. It was discovered that at 48 hours of exposing various concentrations of ABRiBE, the proliferation of colorectal cancer cells significantly reduced compared to 24 hours, with a high concentration of the extract exposed on HT-29 cells. Various concentrations of anthocyanins blueberry extract exhibited an increasing antiproliferative activity and lowered cell proliferation as well as the B16-F10 melanoma cell line with 48 hours of incubation time after exposing extract decreased than 24 hours (Wang *et al*., 2017). Indonesian Cempo Ireng black rice bran extract exhibited cell proliferation or viability decreased and antiproliferative activity increased on T47D cells (human epithelial breast cancer cell line) and HeLa cells (human cervical cancer cell line), which were incubated for 24 and 48 hours with various concentrations of extract (Pratiwi *et al*., 2019 and Pratiwi *et al*., 2015). Paudel *et al*. (2014)also reported that citric acid significantly contributed to the antiproliferative activity of the berry extract. However, the mechanism of citric acid in controlling colon cancer cell proliferation had not been elucidated. Based on previous investigations, 70% (v/v) ethanol and 5% (w/v) citric acid were found to be the most effective solutions in extracting bioactive compounds with antiproliferative effects on HCT-15 and HepG2 cancer cell lines from black sorghum bran extract as well as commercial sumac sorghum bran extract. These solutions were effective in inhibiting cancer cell proliferation in both cancer cell lines (Cox *et al.,* 2019).

The antiproliferative IC_{50} value of the extract was determined by the concentration that can suppress cell proliferation by 50% of the total cells. Based on the results, an antiproliferative IC_{50} value of 1.57 mg/mL was obtained after exposure to the ABRiBE at an incubation time of 24 hours . This is not significantly different from the study conducted by Konczak *et al*. (2012) where the IC_{50} values of quandong fruit extract, Davidson plum, rabbiteye blueberry, and southern highbush blueberry containing anthocyanins exposed to HT-29 cells were 1.88, 1.35, 1.51, and 0.93 mg/mL, respectively. Mazewski *et al*. (2018) also reported that antiproliferative IC_{50} values of black lentil, sorghum, and red grape exposed to HT-29 cells were 1.40, 1.70, and 2.00 mg/mL, respectively. According to Konczak *et al*. (2012) and Mazewski *et al*. (2018) the IC_{50} value was determined using an incubation time of 24 hours after exposing the extract. In this study, the black rice bran extract was more potent in its influence on the antiproliferation of HT-29 cells. This was shown by a lower IC_{50} value of 1.57 mg/mL compared to the other two potent extracts, namely sorghum and red grape with values of 1.70 and 2.00 mg/mL, respectively according to Mazewski and 1.88 mg/mL Quandong fruit, as stated by Konczak(Mazewski *et al.,* 2018, Konczak *et al.,* 2012).

All the 11 foodstuffs extracted by Mazewski *et al*. (2018) including black rice were exposed using a concentration of 2.5 mg/mL in HT-29 cells, which was the IC_{50} of red corn extract. Meanwhile, only 3 extracts with the highest percentage of colorectal cancer cell proliferation inhibition were examined for IC_{50}. The IC_{50} of black rice was not examined because the inhibition percentage of HT-29 cells was small, but it had the highest total anthocyanin content and Total Phenolic Content (TPC) among other extracts. The 3 essential parameters that influenced the high of colorectal cancer cells inhibition according to Mazewski *et al*. (2018) were the content of delphinidin-3-O-glucoside, TPC, and total condensed tannin. These parameters showed a strong correlation with the inhibition of colorectal cancer cells (r=0.69; r=0.87; r=0.77, respectively). Black rice contained cyanidin-3-O-glucoside and peonidin-3-O-glucoside (Yawadio *et al.,* 2007). Black rice was not the most potent foodstuff in inhibiting HT-29 cell proliferation in the study by Mazewski. This was because black rice did not have delphinidin-3-O-glucoside and high total condensed tannin, which is a family of phenolics. The high content contained in black lentil was TPC and condensed tannin. However, the IC_{50} yielded by black rice bran extract in this study was more potent than sorghum and red grape extract. These two extracts were potent among others with the highest inhibition percentage. The smaller IC_{50} value in black rice bran extract made it more potent compared to others, showing that the ABRiBE was potent. This is based on the principle that the lower the IC_{50} value, the more potent the influence of the extract on the inhibition of colorectal cancer cell proliferation.

Only pure anthocyanins such as delphinidin-3-O-glucoside had not been able to inhibit colorectal cancer cells (Mazewski *et al.,* 2018; Zhang *et al.,* 2005). However, crude extracts containing various anthocyanins and other phenolic compounds had a potential synergistic effect in inhibiting colorectal cancer cells (Mazewski *et al.,* 2018). The inhibition process required extract with high concentration, particularly advanced colorectal cancer cells such as HT-29. HT-29 cells required higher concentration due to the resistance of the cancer cells(Mazewski *et al.,* 2018). Mazewski *et al.*(2018) used an extract concentration of 1.0 mg/mL to intervene on HCT 116 cells, which are early developmental colorectal cancer cells. The IC_{50} values of red corn extract in the HCT 116 and HT-29 cell lines were 0.1 and 2.5 mg/mL, respectively (Mazewski *et al.,* 2018).

In this study, the antiproliferative IC_{50} value based on incubation time of 48 hours after exposing the ABRiBE was 1.35 mg/mL, and a comparative analysis was not discovered for time. The inhibition of cancer cells by 50% of the total cells, namely antiproliferative IC_{50}, can be achieved by giving a lower extract concentration of 1.35 mg/mL. Higher IC_{50} of the extract was associated with less antiproliferative effect. Concentrations of ABRiBE were very strong positive correlation with antiproliferative activity at both incubation times of 24 and 48 hours. ABRiBE exerts a greater effect on colorectal cancer cells that have had a longer (48 hours) incubation period after being exposed to the extract. The antiproliferative IC_{50} values in colorectal cancer cells after exposing anthocyanins-rich extracts varied based on the types of human cancer cell lines, the anthocyanins sources used, and the cells' incubation time after exposing the extracts.

PDT of HT-29 cells at the first passage was approximately 23 hours, with the viable cells plated at $5x10^4/cm^2$ (ATCC, 2012). The results showed that the PDT of the HT-29 cell line at the 21st passage was 68.74 hours. This indicated that the higher the passages of the cell line are, the longer the PDT is. The purity of nucleic acids and proteins was determined from the ratio of the maximum absorbance at wavelengths of 260 and 280 nm, respectively (Matlock, 2015). The absorbance at both wavelengths in each group was 2; therefore, the RNA was declared pure (Matlock, 2015). The final concentration for cDNA synthesis ranged from 10-100 ng from the initial total RNA concentration (Affymetrix, 2015). The final concentration for cDNA synthesis was 100 ng, with a final total volume of 20 µL (Affymetrix, 2015).

ABCA1 gene mRNA expression increased in HT-29 cells after exposing the ABRiBE. Similarly, Du *et al.* (2015) discovered that anthocyanins increased the expression of ABCA1 mRNA on HK-2 cells. The increased expression also occurred in MPM cells after exposing the anthocyanins-rich extract (Xia *et al.,* 2005). This indicated that anthocyanins exposed to cell lines can increase the expression of the ABCA1 mRNA. It had been suggested that anthocyanins activate PPARα and LXRα to increase mRNA expression of ABCA1 gene. It also reduces cholesterols accumulation by increasing intracellular cholesterols efflux through the ABCA1 transporter protein (Xia *et al.,* 2005; Du *et al.,* 2015). Generally, intracellular cholesterols levels are regulated by LXRα with mediating ABCA1 mRNA expression, which binds to the LXR element as the promoter of the ABCA1 gene (Venkateswaran *et al.,* 2000). LXR is a member of the PPAR nuclear receptor superfamily that regulates mRNA expression of the ABCA1 gene and plays an essential part in lipid metabolism (Schmitz and Langmann, 2005; Nakaya *et al.,* 2011). Anthocyanins-rich black rice extract of 31.3 g/100 g detected in rat plasma reduced triacylglycerol, LDL cholesterol, and cholesterol level (Zawistowski *et al.,* 2009; Ahuja *et al.,* 2008). An investigation conducted by Tsuda *et al*. (2006)on human adipocytes using anthocyanins also found an increased UCP2 gene expression. Wang *et al.*(2012) demonstrated that the cyanidin-3-O-β-glucoside of pure anthocyanins was gut microbiota dependent. The gut microbiotas converted cyanidin-3-O-β-glucoside from anthocyanins to protocatechuic acids. Protocatechuic acids intervened in MPM and THP-1 cells increased mRNA expression of the ABCA1 gene by suppressing miR-10b level, thereby increasing the cholesterols efflux from macrophages (Wang *et al.,* 2012). ABCA1 mRNA and protein were also found in macrophages, which played a key role in cholesterols efflux from macrophages and the other cells (Tall *et al.,* 2002).

The expression of higher ABCA1 mRNA and protein reduced cholesterols accumulation through PPARα and LXRα pathways (Du *et al.,* 2015). Anthocyanins also increased the mRNA expression of the ABCA1 gene through these pathways (Du *et al.,* 2015). During this process, PPAR-α functioned as the first regulator of the reverse cholesterol transport pathway through the ABCA1 protein-mediated cholesterols efflux regulation (Pasello *et al.,* 2020). It activates LXRα, and LXRα activates LXR, which acted as a cholesterol homeostasis sensor in a heterodimeric complex with Retinoid X Receptor (RXR) (Pasello *et al.,* 2020). LXR and RXR were activated by oxysterol and 9-cis-retinoic acid, respectively (Pasello *et al.,* 2020). The direct repeat response element (DR-4) binds to the LXR receptor and the heterodimeric receptor pair, LXR, and RXR. Subsequently, DR-4, LXR, and RXR increase ABCA1 mRNA expression, which escalates the ABCA1 transporter protein. ABCA1 protein played a role in cholesterols efflux (Pasello *et al.,* 2020) and acted a transporter carrying out the cholesterols from intracellular to extracellular, which was accumulated in the tumor cells. The ABCA1 protein mediated the biogenesis of High-Density Lipoprotein (HDL) and transported cholesterols (ABCA1 protein-cholesterol binding) across the plasma membrane to Apolipoprotein A1 (ApoA1). ApoA1 is the major lipoprotein component of HDL (Attie, 2007). However, the molecular mechanism by which the ABCA1 protein mediates the cellular binding of ApoA1 and nascent HDL is not well understood. Some studies proved that ApoA1 interacted directly with the extracellular domain-specific conformation of the ABCA1 transporter protein in the early process of HDL formation (Gulshan *et al.,* 2016; Ishigami *et al.,* 2018). HDL was catalyzed by the Lecithin Cholesterol Acyltransferase (LCAT) enzyme. Cholesterols succeeded in leaving the cells grabbed by HDL to form HDL-cholesterol (HDL-C) (Lyu *et al.,* 2020). Furthermore, the cancer cell proliferation decreased due to the release of cholesterols accumulated from intracellular to extracellular through this mechanism(Xia*et al*., 2005; Du *et al*., 2015). Increased ABCA1 protein expression can inhibit colorectal cancer cell proliferation. The high ABCA1 mRNA expression increased intracellular cholesterols efflux mediated by ABCA1 transporter protein causing a decrease in colorectal cancer cell proliferation. Colorectal cancer exhibits a loss of cholesterols efflux function. The decreased or loss of cholesterols efflux function by ABCA1 transporter protein led to tumorigenesis through high intracellular cholesterols levels(Smith and Land, 2012). Several pathways of increasing intracellular cholesterols were observed in cancer, which included low ABCA1 mRNA and protein expression. Intracellular cholesterols accumulation was associated with colorectal cancer pathogenesis (Smith and Land, 2012). The accumulation of cholesterols in tumor cells (Smith and Land, 2012) can cause an increase in the proliferation of cancer cells. The mechanism of cholesterols accumulation in cells tumor with increased cancer cell proliferation is still unknown. However, it can be associated with a risk factor for colorectal cancer is a high-fat diet (WCRFI, 2018).

The ABCA1 mRNA and protein were expressed low in colon cancer compared to healthy cells (Lo sasso *et al.,* 2013). It was found that anthocyanins-rich foodstuffs significantly increased ABCA1 mRNA and protein expression as well as suppress cholesterols accumulation in the aorta and liver (Wu *et al.,* 2013; Zern *et al.,* 2003).The mechanism of the influence of anthocyanin-rich foodstuffs on colorectal cancer in increasing the ABCA1 mRNA and protein expression still needs further investigation. The increase of ABCA1 mRNA expression modulated by anthocyanins is thought to improve cholesterols elimination function mediated by ABCA1 protein and reduce Akt-dependent survival signaling. These factors contribute to anticancer activity by the ABCA1 mRNA and protein (Smith and Land, 2012). Therefore, further study is recommended to determine the influence of anthocyanins on ABCA1 mRNA and protein to prevent colorectal cancer.

5. Conclusion

This study showed that the proliferation of colorectal cancer cells can be reduced after exposure to various concentrations of ABRiBE. The extract also increased the mRNA expression of the ABCA1 gene in HT-29 cells, suggesting that ABRiBE has an impact on the antiproliferative activity of colorectal cancer cells through ABCA1 mRNA expression. The strength of this study is a strong correlation between various concentrations of ABRiBE intervention and antiproliferative activity. Moreover, further biomolecular study is needed to determine the relationship between the ABCA1 gene and PPARα and LXRα in the HT-29 cell line with the use of ABRiBE. It is also recommended to further investigate this topic in an animal model using the extract.

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