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Ethanolic Extract of Black Rice 'Sembada Hitam' Bran Did not Show Cytotoxic Effect on HeLa Cell (*Cervical Cancer Cell Line*)

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Abstract

Black rice (*Oryza sativa* L.) contains various phenolic compounds such as anthocyanins which act as antioxidants. Antioxidants inhibit cell proliferation and induced apoptosis of cancer cells as well as protect cells from damage that triggers cancer. 'Sembada Hitam' is a new cultivar of black rice in Indonesia that has not been studied for its anticancer potential. This study aimed to study the cytotoxic activity and apoptosis induction of the ethanolic extract of black rice (*Oryza sativa* L. 'Sembada Hitam') bran against HeLa cells. The method used was the cytotoxicity test with the MTT assay and the apoptosis test with the AO-PI double staining method. The test used various concentrations of black rice bran extract, DMSO as solvent control, and doxorubicin as a positive control for 24 and 48 hours. Data analysis was performed using one-way ANOVA ($p = 0.05$) followed by the Tukey HSD test. The results showed that ethanolic extract of black rice 'Sembada Hitam' bran has no cytotoxic activity against HeLa cells ($p > 0.05$). Moreover, extracts at the concentrations of 250 and 500 µg/mL only induced apoptosis of HeLa cells by 11.62 ± 0.50 and 11.49 ± 9.88% respectively. These results indicate that the ethanolic extract of black rice 'Sembada Hitam' bran has no potential to be developed as an agent for cervical cancer therapy.

Keywords: apoptosis, cytotoxic, Hela cell, Oryza sativa L. 'Sembada Hitam'

Abstrak

Beras hitam (*Oryza sativa* L.) mengandung berbagai senyawa fenolik seperti antosianin yang berperan sebagai antioksidan. Antioksidan menghambat proliferasi sel dan menginduksi apoptosis sel kanker serta melindungi sel dari kerusakan yang memicu kanker. 'Sembada Hitam' merupakan padi hitam kultivar baru di Indonesia yang belum diteliti potensi antikankernya. Penelitian ini bertujuan untuk mempelajari aktivitas sitotoksik dan induksi apoptosis ekstrak etanolik bekatul beras (*Oryza sativa* L. 'Sembada Hitam') terhadap sel HeLa. Metode yang digunakan adalah uji sitotoksisitas dengan MTT assay dan uji apoptosis dengan metode pewarnaan ganda AO-PI. Pengujian menggunakan berbagai konsentrasi ekstrak bekatul beras hitam, DMSO sebagai kontrol pelarut, dan doxorubicin sebagai kontrol positif selama 24 dan 48 jam. Analisis data dilakukan dengan menggunakan *one way* ANOVA ($p = 0.05$) dilanjutkan dengan uji Tukey HSD. Hasil penelitian menunjukkan bahwa ekstrak etanol bekatul beras hitam 'Sembada Hitam' tidak memiliki aktivitas sitotoksik terhadap sel HeLa ($p > 0,05$). Selain itu, ekstrak pada konsentrasi 250 dan 500 µg/mL hanya menginduksi apoptosis sel HeLa masing-masing sebesar 11.62 ± 0.50 dan 11.49 ± 9.88%. Hasil ini menunjukkan bahwa ekstrak etanolik bekatul beras hitam 'Sembada Hitam' tidak berpotensi untuk dikembangkan sebagai agen terapi kanker serviks.

Kata kunci: *apoptosis, sitotoksik, sel Hela, Oryza sativa L. 'Sembada Hitam'*

INTRODUCTION

Non-communicable diseases play an important role in the majority of global deaths. Cancer is considered as the most important cause of death and reduces life expectancy in every country around the world in the 21st century (Bray *et al*., 2018). According to the data, there were 604,127 cases of cervical cancer worldwide and there were 341,831 patients who died from cervical cancer (Singh *et al*., 2020). In Southeast Asia, there were 68,558 new cases of cervical cancer in 2020, with 38,491 deaths. Indonesia had the highest prevalence of cervical cancer cases in Southeast Asia in 2020, with 36,633 new cases and 21,003 deaths (Zhao *et al*., 2022). The number of cases of cervical cancer begins to increase in women by 20-29 years, reaches a peak by 55-64 years, and decreased over 65 years (Kashyap, Krishnan, Kaur, & Ghai, 2019).

Various treatments for cervical cancer include surgery, radiation, chemotherapy, and immunotherapy. However, radiotherapy and chemotherapy have limitations. Radiotherapy rays show decreasing in its effectiveness as the tumor size increases. Whereas, chemotherapy drugs have failed to induce apoptosis of cancer cells which may lead to decreased immune function (Li *et al*., 2008). Cervical cancer cells also showed resistance to radiation and chemotherapy treatments (M. K. Kim *et al*., 2011; T. Kim *et al*., 2006). Chemotherapy agents such as doxorubicin, cisplatin, and paclitaxel are cytotoxic and affect normal cells (Jenie, Handayani, Susidarti, & Meiyanto, 2020). The use of cisplatin showed a higher kidney toxicity profile than doxorubicin (Chen *et al*., 2008). In addition, the long-term use of doxorubicin in cancer therapy causes resistance. There is overexpression of P-glycoprotein (Pgp) which causes doxorubicin to be pumped out of the cell and decreased the concentration of doxorubicin in the cell. These conditions decreased the cytotoxic potential of doxorubicin (Sarmoko, 2012). The chemotherapeutic agent paclitaxel also causes cancer cells to become resistant. Cervical cancer therapy raises various side effects during and after therapy, including diarrhea, constipation, lymphedema, menopausal symptoms, poor body image, sexual dysfunction, dyspareunia, and excessive fatigue. All of these side effects affect the patient's emotional and psychosocial (Maree & Ntinga, 2015).

Excess reactive oxygen species (ROS) accumulated in cell cause DNA damage, abnormal DNA, and associated with the pathogenesis of various types of cancer. Antioxidant compounds protected cells from oxidative damage thereby inhibiting carcinogenesis. Antioxidants cause growth inhibition and induced cell death in cancer cells (Thanuja & Parimalavalli, 2018). Therefore, the development of antineoplastic herbal drugs is a strategic choice to increase and assist the sensitivity of cancer therapy (Ismaryani, Salni, Setiawan, & Triwani, 2018).

Natural antioxidant compounds suppress the growth of cancer cells. Antioxidants inhibited proliferation and induced apoptosis in cancer cells by inhibiting the formation of free radicals and protecting cells from damage that triggers cancer. Antioxidants can prevent tumor and cancer growth by eliminating free radicals and reducing cellular DNA damage due to oxidative stress, as well as inhibiting cell transformation caused by gene mutations (Thanuja & Parimalavalli, 2018).

Various studies showed that black rice contains various phytochemical compounds used as antioxidants, anticancer, anti-inflammatory, antibacterial, antidiabetic, anticholesterol, and antiallergenic. Phytochemical compounds in black rice are polyphenols, tocopherols, γ-oryzanols, flavones, flavonols, carotenoids, and flavonoids which are dominated by anthocyanins (Forster *et al*., 2013; Phetpornpaisan, Tippayawat, Jay, & Sutthanut, 2013). The anthocyanin content of ‘Sembada Hitam’ black rice grown in the lowlands reaches 345.75 mg/100 g (Kristamtini, Wiranti, & Sutarno, 2018). The bioactivity of phytochemical compounds in black rice has been shown to have the potential to cure metabolic diseases and trigger apoptosis in cancer cells (Deng *et al*., 2013). The antioxidant compounds cyanidin 3-glucoside and peonidin 3-glucoside in 'Cempo Ireng' black rice showed induced apoptosis > 50% compared to doxorubicin (21.29%) in HeLa cells (Pratiwi, Tunjung, Rumiyati, & Amalia, 2015).

More than 200 varieties of black rice are found worldwide (Zhang, Kai, Xia, Wang, & Ai, 2020), 'Sembada Hitam' is one of the varieties. 'Sembada Hitam' is a new black rice cultivar planted

and developed in the Sleman and Bantul regions, Yogyakarta, Indonesia. Currently, the potential of the ethanolic extract of 'Sembada Hitam' has been known to reduce the risk of preeclampsia. The concentration of 'Sembada Hitam' 2.5 µg/mL contains high antioxidants which significantly reduced MDA levels in HUVEC-induced preeclampsia for 24 hours. The preeclampsia-induced MDA level of 8,7085 µM decreased to 5,0895 µM. Comparable to control MDA levels in normal pregnancy conditions (Christanto *et al*., 2020). The study also reported that the ethanolic extract of 'Sembada Hitam' rice bran has a potential antiangiogenic effect in the preeclampsia treatment (Christanto *et al*., 2021). However, research related to the potential of 'Sembada Hitam' has not yet been studied, especially regarding its anticancer potential. According to the studies, black rice bran 'Sembada Hitam' contains a lot of antioxidants, specifically those which belong to the anthocyanin group. Thus, we suspect that the ethanolic extract of black rice bran 'Sembada Hitam' has a cytotoxic effect on HeLa cells. Furthermore, we also hypothesize that the ethanolic extract of black rice bran 'Sembada Hitam' can trigger apoptosis in HeLa cells. Therefore, it is necessary to research and study the potential of 'Sembada Hitam' related to cytotoxic activity and induction of apoptosis on cervical cancer cells.

METHODS

Extraction of Black Rice Bran 'Sembada Hitam'

Black rice 'Sembada Hitam' is reported to have flag leaves that average 27.44 ± 3.09 cm in length and 1.72 ± 0.06 cm in width. The panicle density of 'Sembada Hitam' is 6.67 ± 0.87 grains/cm, and the panicle length is about 26.94 ± 0.92 cm. According to Widyaningtias *et al*. (2020), this cultivar has a compact panicle type, slender grain shape, slender rice shape, and purple rice color. Black rice 'Sembada Hitam' bran were obtained from the local farmer at Ngaglik, Sleman, Yogyakarta, Indonesia. Black rice 'Sembada Hitam' is harvested by farmers around July – August. Then the black rice is air-dried and stored at room temperature. Rice bran was obtained by scraping twice the outer layer of whole black rice and sieved with a 60 mesh sieve. 10 mg of rice bran was extracted with 100 mL of ethanol acidified with HCl 1N (85 mL of absolute ethanol : 15 mL of HCl) by maceration for 48 hours at room temperature and stirring occasionally. The extract was filtered using Whatman No.1 paper and the filtrate was remacerated with 50 mL of solvent containing HCl 1N (85 mL of absolute ethanol : 15 mL of HCl) twice overnight. The filtrate was evaporated with a fan until it formed a paste (Christanto *et al*., 2021).

Cytotoxicity Test Using MTT Assay

HeLa cells were cultured with density 1 x 10^4 cells/well in a 96-well plate using DMEM and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 hours. After incubation, the cell was treated with the various concentration (7.8125; 15.625; 31.25; 62.5; 125; 250; 500; and 1000 µg/mL) of ethanolic extract black rice 'Sembada Hitam' bran. The usage of concentrations ranging from lowest to highest is selected to determine the range of extract concentrations that can prevent cell proliferation. A chemical is considered to have cytotoxic activity against cells if its IC_{50} value is lower than 1000 µg/mL. According to Tunjung and Sayekti (2019), the cytotoxic activity of a compound is categorized into three categories: extremely strong (IC_{50} value below 10 µg/mL), strong (IC_{50} value between 10 and 100 µg/mL), and weak (IC_{50} value between 100 and 500 µg/mL). Therefore, in this study, the extract concentration was chosen from 7.8125 µg/mL to 1000 µg/mL. Besides the extract treatment group, the positive control group was treated using doxorubicin (1.25; 2.5; 5; and 10 µg/mL). Solvent control group was treated using 1% DMSO, and cell control group was not treated with any chemical except culture media. The cells were incubated for the next 24 and 48 hours with three replications in each group.

Cytotoxicity was performed by colorimetric quantitative MTT (3- (4,5- dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide) assay. After incubation, 100 µL/well of 5 mg/mL MTT (dissolved in 10 mL PBS) was added and the cells were incubated for 4 hours followed by the addition of 100 µL/well of SDS (10% in 0.1 N HCl) as a stopper reagent. The absorbance was measured at 595 nm using BIO-RAD Microplate Reader. The percentage of cell viability was calculated based on

the following formula (Kamiloglu, Sari, Ozdal, & Capanoglu, 2020):

$$\% \text{ Cell Viability} = \frac{\text{Mean Optical Density sample}}{\text{Mean Optical Density blank}} \times 100\%$$

The determination of the IC_{50} value in this study was carried out using the probit analysis method.

Apoptosis Test Using AO-PI Staining

HeLa cells were treated in a 24-well plate with a density of 5 x 10^4 cells/well and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 hours. The cells were treated with the combination of black rice bran selected concentration, doxorubicin, and 1% DMSO for 24 hours with three replications for each group. The cell suspension was mixed with 10 µL of acridine orange and propidium iodide AO/PI (1:1) on a slide. Detection of live cell morphology and apoptosis was done using AO/PI staining. Observations using a confocal microscope (Carl Zeiss/Axio Observe 21) were carried out by dividing each coverslip into four areas of view. Each field of view is divided into nine grids, three specific areas are selected for cell counting. The selection of grid boxes in each field of view is equivalent based on the grid number. Apoptotic cells were counted in a population of at least 200 cells per grid and presented as percent (%).

Statistical Analysis

The data were analyzed using one-way ANOVA and followed by the Tukey HSD test if there is a significant difference ($p < 0.05$). In this study, IBM SPSS ver. 25 software was used for the statistical analysis. Data is presented in the form of bar charts and graphs.

RESULTS AND DISCUSSION

In this study, black rice brans 'Sembada Hitam' was obtained from local farmers in Sleman district, Special Region of Yogyakarta, Indonesia. Black rice bran was extracted by maceration method using absolute ethanol acidified with 1N HCl. 10 mg of black rice bran was macerated in 100 mL of 100% ethanol and HCl at an 85 : 15 volume ratio, yielding 1.864 g of extract in the form of a thick purple paste.

The results showed that the percentage of viable cells at 24 hours of incubation with 1% DMSO treatment was 100 ± 11.62%. It indicates that the 1% DMSO solvent had no toxicity on HeLa cells. These findings are consistent with the literature, which demonstrates that 1% DMSO in culture does not impair the growth of HeLa cells. Most cell lines can tolerate 0.5% DMSO whereas some cells can withstand up to 1% DMSO (Yuan *et al*., 2014). The percentage of viable cells treated with doxorubicin at a concentration of 1.25 µg/mL to 10 µg/mL decreased from 64.68 ± 9.93% to 27.80 ± 1.35% (Figure 1.). Similar findings were reported in the previous study, which found that the viability of HeLa cells decreased as doxorubicin concentration increased (Xia *et al*., 2018). The proliferation rate of HeLa cells also declined with increasing doxorubicin dosage.

The MTT assay cytotoxic test results with a 24-hour incubation period showed that the percentage of HeLa cell viability treated with black rice bran extract 'Sembada Hitam' with the lowest concentration of 7.8125 µg/mL was 110.38 ± 11.50%. HeLa cell viability reached the highest level of 129.19 ± 12.48% in the extract treatment with the highest concentration of 1000 µg/mL. The extract treatment with a concentration of 250 µg/mL of 105.97 ± 4.25% had the lowest percentage of viable HeLa cells. At all doses, the percentage of HeLa cell viability treated with black rice bran extract after a 24-hour incubation period was higher than 100%. According to statistical analyses, there was no significant difference in HeLa cell viability between the concentrations of black rice bran extract (Figure 1.).

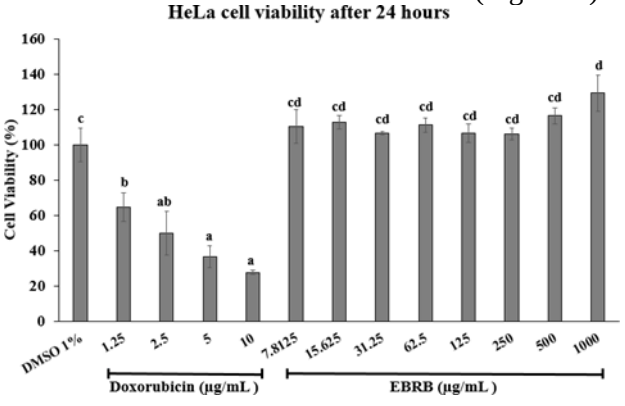


Figure 1. Black rice ethanolic extract was not cytotoxic to HeLa cells at an incubation time of 24

hours. EBRB: Ethanolic extract of black rice bran (n = 3).

Cytotoxicity test results showed that 'Sembada Hitam' black rice bran extract had no potential to reduce cell viability against HeLa cells after 24-hour incubation. Therefore, a cytotoxicity test was carried out with a prolonged incubation of 48 hours to determine the effect of incubation time on HeLa cell viability. The results showed that the percentage of viable cells at 48 hours of incubation with 1% DMSO treatment was 100 ± 22.17%. The doxorubicin group demonstrated that the 0.5 µg/mL concentration had the lowest percentage of HeLa cell viability which was 21.51 ± 4.90%. (Figure 2).

The black rice bran 'Sembada Hitam' extract treatment group at a concentration of 7.8125 µg/mL had a 76.63 ± 8.96% cell viability. Furthermore, HeLa cell viability was greater than 70% at the 1000 µg/mL concentration of black rice bran extract treatment, which was 74.50 ± 15.28%. The number of viable cells after a 48-hour incubation period was still rather high, ranging from 74 to 182%. According to the ISO 10993-5 standard, cellular response to an extract is considered non-cytotoxic to the cells tested when cell viability is more than or equal to 70% (Tan *et al*., 2016). Based on these conditions, it can be concluded that the black rice bran 'Sembada Hitam' extract 'Sembada Hitam' has no anti- cervical cancer potential and is not cytotoxic to HeLa cells after incubated for 24 and 48 hours (Figure 2.).

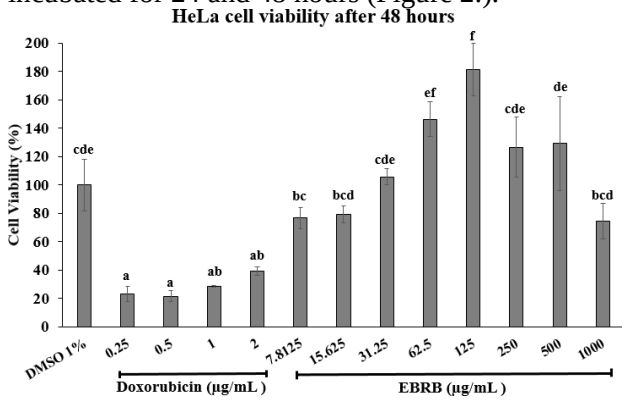
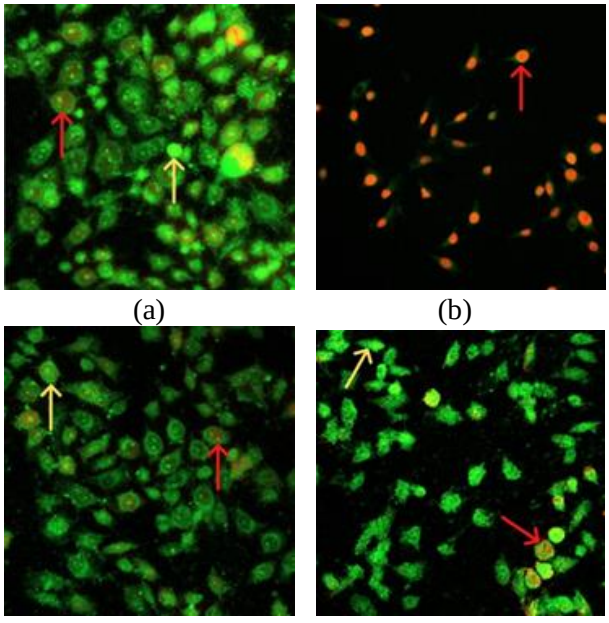


Figure 2. Black rice ethanolic extract was not cytotoxic to HeLa cells at an incubation time of 48 hours. EBRB: Ethanolic extract of black rice bran (n = 3).

According to the cytotoxic test of black rice bran 'Sembada Hitam' extract on HeLa cells, there was a reduction in viability, particularly in cells that were incubated for 48 hours, although it did not demonstrate a cytotoxic effect since cell viability was still over 70%. However, further investigation into the possible cell death pathway that occurs is still required. To investigate whether the ethanol extract of 'Sembada Hitam' rice bran triggers the apoptotic pathway, a double staining method of apoptosis test was conducted using the acridine orange-propidium iodide (AO-PI) dye. Due to the permeability of the acridine orange dye, it may pass across both live and dead cell membranes. Acridine orange may bind to double- stranded DNA and emits a green fluorescence in viable cells and a reddish-orange fluorescence in dead cells. Unlike acridine orange, propidium iodide dye only crosses damaged or dying cell membranes and interacts with nucleic acids to turn cells glow red. Administration of black rice bran 'Sembada Hitam' extract causes morphological changes and induces apoptosis in HeLa cells. Figure 3. showed the morphological changes and apoptosis of HeLa cells.



(a) (b)

(c) (d)

Figure 3. Double staining test of HeLa cells with treatment of (a) DMSO 1%, (b) doxorubicin 0.5 µg/mL, (c) black rice bran ethanolic extract 250 µg/mL, and (d) 500 µg/mL. Red arrows indicate apoptotic cells.Yellow arrows indicate normal cells. 40 x 10 magnification.

According to Figure 3., in all treatment groups, there were HeLa cells underwent apoptosis, as marked by the presence of an orange nucleus. Nuclear shrinkage, membrane blebbing, and fragmentation of cell organelles and DNA were also observed in apoptotic HeLa cells. It is similar to earlier studies that showed that when cells stained with AO-PI, viable cells with intact membranes appear green, while apoptotic cells show orange nuclei, membrane blebbing, shrunken cells, and fragmented organelles (Kwan *et al*., 2016; Rahmawati & Maryati, 2021).

As seen in Figure 3b., almost all HeLa cells in the doxorubicin 0.5 µg/mL treatment group underwent apoptosis characterized by the appearance of an orange nucleus. Doxorubicin is a drug widely used in cancer treatment since it is highly effective at preventing the development of cancer cells and triggering apoptosis or programmed cell death (H. Kim, Kim, & Lee, 2009). The anti-cancer drug doxorubicin inhibits the proliferation of cancer cells and induces apoptosis through several pathways, including upregulating the pro-apoptotic protein *Bax* and downregulating the anti-apoptotic protein *Bcl-2* to promote cell death (Larasati, Kusharyanti, Hermawan, Susidarti, & Meiyanto, 2011). Previous studies have shown that doxorubicin alters the structure of the mitochondrial membrane in HeLa cells, inhibits oxidative phosphorylation to reduce ATP synthesis, and causes an energy crisis in the mitochondria that kill HeLa cells (Bellance *et al*., 2020). Due to its ability to trigger Chkl phosphorylation, doxorubicin is known to lead to cell cycle arrest in the S phase. Caspase 3 activation, DNA fragmentation, and elevated oxygen species reactivity caused by doxorubicin activity are known to induce apoptosis in HeLa cells (Larasati *et al*., 2011).

The 1% DMSO treatment group (Figure 3a) was dominated by viable HeLa cells marked with fluorescent green stained cells. In that group, some of the cells that had undergone apoptosis were also found. The treatment group of black rice bran extract at concentrations of 250 µg/mL (Figure 3c.) and 500 µg/mL (Figure 3d.) showed similar results where viable HeLa cells accounted for about 11.62 ± 0.50% and 11.49 ± 9,88%, respectively. The viability of HeLa cells after treatment with the black rice bran 'Sembada Hitam' extract was relatively high, as indicated by observations of apoptosis and the results of the MTT Assay. Based on the double staining test, it is known that black rice bran 'Sembada Hitam' extract cannot induce apoptosis in HeLa cervical cancer cells.

Black rice bran extract 'Sembada Hitam' is well known for containing several phenolic compounds, especially anthocyanins such as peonidin-3-glucoside, cyanidin-3-glucoside, malvidin-3-glucoside, cyanidin-3-rutinoside, and other anthocyanin compounds (Prasad, Sharavanan, & Sivaraj, 2019). Polyphenols, tocopherols, gamma oryzanol, and carotenoids have also been found in black rice (Forster *et al*., 2013; Phetpornpaisan *et al*., 2013). However, based on the results of this study, black rice bran 'Sembada Hitam' extract is not cytotoxic, does not inhibit cell growth, and shows relatively low apoptosis induction in HeLa cancer cells. It is possibly due to the low concentration of anthocyanins peonidin 3-glucoside and cyanidin 3-glucoside. In comparison to the local variety 'Cempo Ireng' (428.38 mg/100 g), Tugiyo umur panjang black rice (298.93 mg/100 g), and hairless Magelang black rice (288.53 mg/100 g), black rice 'Sembada Hitam' has a lower anthocyanin level (284.05 mg/100 g) (Kristamtini & Wiranti, 2017).

The anthocyanin levels in the black rice bran 'Sembada Hitam' extract may have been influenced by the extraction method and solvent selection utilized in this research. According to a previous study, the ethanol solvent is less effective in the anthocyanin extraction process (Taghavi, Patel, & Rafie, 2022). Ethanol concentrations of around 50 to 70% were the most effective for extracting

cyanidin-3-glucoside from black rice. Increasing the concentration of ethanol solvent above 70% can significantly reduce the levels of cyanidin-3-glucoside in the extract (H. J. Kim, Wee, & Yang, 2015). Cyanidin-3-glucoside extracted with 95% ethanol solvent had a concentration of 9.35 ± 0.46 mg/g, or only 25% of those from cyanidin-3-glucoside extracted with 50% ethanol solvent (H. J. Kim *et al*., 2015). The concentration of cyanidin-3-glucoside that may have been contained in the extract in this research was relatively low since the extraction method used 100% ethanol as a solvent.

The maceration method is also less efficient in extracting active compounds since crude extracts are obtained, requiring the purification and isolation of active compounds through fractionation. According to a previous study, anthocyanin compounds fractionated from Chinese black rice bran were cytotoxic to MCF-7, MDA-MB-231, and MDA-MB-453 breast cancer cells (Hui *et al*., 2010). Other studies have also shown that the ethanolic fraction of 'Woja Laka' black rice bran can inhibit cell proliferation in WiDr cells (Rukmana, Soesilo, Rumiyati, & Pratiwi, 2017). Based on previous studies shows that black rice bran fractionation could separate the bioactive components, especially anthocyanins, allowing them to work more efficiently in reducing cancer cell proliferation.

Anthocyanin levels in extracts are influenced by external factors such as pH, temperature, light, and storage time (Zozio, Pallet, & Dornier, 2011). In this study, the preparation of a stock solution of black rice bran ethanol extract 'Sembada Hitam' was not carried out immediately before the treatment of HeLa cells but was made within four months. As a result, the antioxidant activity may be affected by a decrease in the quantity and quality of anthocyanins. Previous studies showed that the antioxidant activity of extracts containing anthocyanins reached its highest after four weeks of storage and dropped after three months (Le *et al*., 2019). Therefore, the treatment of HeLa cells with the ethanolic extract of black rice bran ‘Sembada Hitam’ must be carried out as soon as the extraction procedure is complete and no later than three months after the extract has been obtained. The anthocyanin stability of Robusta coffee extract decreased during storage, based on previous research (Sukatiningsih, Windrati, & Yudistira, 2012). In this study, the black rice bran 'Sembada Hitam' extract was stored and used for four months, resulting in the possibility that airborne bacteria might have contaminated the extract and altered its pH. The quantity and quality of anthocyanins in black rice bran extract decreased due to long-term storage and pH alterations, making the extract ineffective in killing HeLa cancer cells or inducing apoptosis.

In this research, apoptotic cells were counted manually using ImageJ after AO-PI staining. Further investigation using other techniques, such as flow cytometry, is required to improve the reliability in determining the percentage of cell apoptosis.

CONCLUSION

From this study, it is known that HeLa cells treated with black rice bran ethanolic extract 'Sembada Hitam' for 24 and 48 hours have cell viability above 70%. It shows that the ethanolic extracts of black rice bran 'Sembada Hitam' is non-cytotoxic and does not inhibit the growth of HeLa cancer cells. The black rice bran 'Sembada Hitam' extract also showed relatively low apoptosis induction, limiting its potential as a potential treatment for cervical cancer. It is necessary to optimize the 'Sembada Hitam' black rice bran extraction process by selecting the appropriate solvent and extraction method. Further studies regarding extract storage also need to be carried out since external factors such as pH, temperature, and storage duration affect anthocyanin levels.

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