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Research Article

Extract of Black Rice (*Oryza sativa* L. 'Sembada Hitam') Bran Protect Cytotoxicity of Hydrogen Peroxide on Vero Cells in a Short Time Incubation

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ABSTRACT

Oxidative stress induced by hydrogen peroxide (H_2O_2) can lead to cellular damage, contributing to degenerative diseases and aging. Black rice bran is a functional food known for its antioxidant properties, which are crucial in reducing the adverse effects of oxidative stress and maintaining redox balance. In this study, we aimed to investigate the protective effect of the extract of black rice bran (EBRB) 'Sembada Hitam' on Vero cells against H_2O_2 toxicity. To evaluate the protective effect, a co-culture method was employed, and cell viability was assessed using the MTT assay. Additionally, cell growth was examined through trypan blue staining. Vero cells were exposed to different concentrations of H_2O_2 and EBRB for a 24-hour period. The results demonstrated that EBRB at concentrations of 15.625, 250, and 500 µg/mL exhibited a protective effect on Vero cells exposed to H_2O_2 at concentrations of 100, 200, and 400 µM, respectively. Notably, when Vero cells were treated with EBRB at concentrations of 250 or 500 µg/mL for five days in conjunction with H_2O_2 exposure at concentrations of 200 or 400 µM for 24 hours, a significant decrease in cell viability was observed on day 3. Based on the collective findings, it can be concluded that EBRB has the potential to protect Vero cells against H_2O_2 -induced toxicity, but primarily during a short-term incubation period. Overall, this study highlights the protective properties of EBRB against H_2O_2 -induced cellular damage and emphasizes the importance of further investigations to fully elucidate the underlying mechanisms and potential long-term effects of EBRB on cell viability.

Keywords: Black rice (Oryza sativa L.) 'Sembada Hitam' bran, Cell growth, Cell viability, Hydrogen peroxide, Vero cells

Introduction

The cellular defense system in the body may experience various endogenous or exogenous stressors. Endogenous stressors occur internally due to natural processes like metabolism, while exogenous stressors include carcinogens, UV exposure, oxidative stress, chemicals, and inflammation. Endogenous and exogenous stressors may cause degenerative diseases such as cancer, nervous system disorders, and aging [1, 2].

Oxidative stress in cells was triggered by the overproduction of reactive oxygen species (ROS) within the body. ROS accumulation causes DNA mutation and disruption of cellular homeostasis in normal tissue. An imbalance of antioxidant and free radical production inside the body causes oxidative pressure, which leads to cell and tissue damage. Oxidative stress induced by hydrogen peroxide exposure (H_2O_2) may cause protein and DNA damage, mitochondrial dysfunction, aging, apoptosis, necrosis, and autophagy [3–6].

Cell damage can be effectively neutralized by enhancing cellular antioxidant defense, preventing oxidative stress in cells or tissues [7]. Typically, endogenous antioxidants such as catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) may protect cells. Antioxidant enzymes can prevent cellular damage caused by ROS. However, the defense system of endogenous antioxidants can sometimes not remove all the intracellular ROS. Thus, exogenous antioxidants are

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necessary to support endogenous antioxidant activities. Exogenous antioxidants can be found in natural products such as grain [8], corn silk [9], and black rice [10].

Black rice contains minerals, protein, phenolics, anthocyanins, non-fatty saturated acids, and vitamins, the composition of which depends on the type of cultivars and growing location [11]. Bioactive compounds such as flavonoids, ɣ-oryzanol, polyphenols, and carotene in black rice are known as antioxidants [12, 13]. Several studies have demonstrated that black rice can potentially reduce oxidative stress caused by chemical exposure, such as H_2O_2 and TBHP (*tert*-butyl hydroperoxide). Furthermore, it was reported that black rice extract treatment led to a significant increase in fibroblast WI-38 cell viability against H_2O_2 exposure [14]. Pre-treatment of HepG2 cells with an extract of black rice 'Heugjinjubyeo' bran against TBHP-induced oxidative stress showed a strong protective effect. This was evidenced by decreased cell cytotoxicity, modulation of caspase 3 activity, reduction in ROS generation, prevention of ERK ½ activity, and activation of Akt [15]. Black rice extracts demonstrate a protective effect against TBHP-induced oxidative stress in HepG2 cells [16]. A previous study also reported that glutinous black rice at a concentration of 0.31 – 2.5 mg/mL against oxidative stress caused by H_2O_2 on HT-29 cells showed a cytoprotective effect [17]. In line with the previously reported cytoprotective effect of EBRB ‘Sembada Hitam’ on NIH3T3 cells exposed to hydrogen peroxide (H_2O_2), which showed significant protection at concentrations starting from 7.81 mg/mL [18], this study aims to investigate the protective effect of EBRB 'Sembada Hitam' on Vero cells against H_2O_2-induced toxicity.

Material and Methods

Black rice bran extraction

Black rice bran ‘Sembada Hitam’ was brought from Mr. Gunarto, a farmer from Ngaglik, Sleman, Yogyakarta, Indonesia. The extraction method followed from the previous study with modifications in sieve size [19]. Using the maceration method, 10 g of black rice bran was sieved using a 40-mesh size and then extracted with 100 mL of ethanol acidified with 1N HCl (85 mL absolute ethanol: 15 mL HCl). The extraction process was conducted 48 hours with intermittent stirring at room temperature. Subsequently, the obtained EBRB was filtered using Whatman paper No.1. Then, the filtrate of EBRB was remacerated twice overnight. The extract was evaporated with a fan until it became a paste.

Vero cell line culture

Vero cells were obtained from the Laboratory of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. This research was conducted under Ethical Clearance No: KE/FK/1302/EC/2021, issued by the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Vero cell culture was cultured in disk (size 90×15 mm^2) using DMEM containing 2% penicillin-streptomycin, 10% fetal bovine serum (FBS), and 0.5% amphotericin B (fungizone). Cells were harvested after reaching 80% confluency.

Cytotoxicity assay of hydrogen peroxide induction on Vero cells

1×10^4 cell/well cultured in 96-well microplate using DMEM and incubated at 37°C with 5% flow of CO_2 for 24 hours. Then, after washed using PBS, cells were treated with the various concentrations (12.5, 25, 50, 100, 200, and 400 µM) of hydrogen peroxide, three replications each, and cells were re-incubated for the next 24 hours. Vero cells without H_2O_2 treatment were used as a control. Cytotoxicity of H_2O_2 was evaluated by MTT assay. After re-incubation, cells were observed under an inverted microscope. Cells were washed with PBS 100 µL/well once, and solution reagent (0.5% MTT) was added and re-incubated for 4 hours, followed by 100 µL/well SDS (stopper reagent). The absorbance of the samples was measured at 595 nm using a Microplate Reader, BIO-RAD. The percentage of cell viability was calculated based on the formula as given in Equation 1 [20]:

$$\frac{\text{Absorbance of treatment-Absorbance control medium}}{\text{Absorbance control cell-Absorbance control medium}} \times 100\% \quad \text{Eq. 1}$$

Cytotoxicity assay of EBRB ‘Sembada Hitam’ on Vero cells

Vero cells were cultured at a density of 1 x 10^4 cells/well in a 96-well microplate using DMEM and incubated at 37°C with 5% CO_2 for 24 hours. Following incubation, cells were washed once with 100 µL/well of PBS. Various concentrations (7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and

1000 µg/mL) of EBRB were applied to the Vero cells, with three replicates for each concentration, and the cells were incubated for an additional 24 hours. The control group was treated with 1% DMSO. The cytotoxicity of EBRB was assessed using the MTT assay. After the incubation period, cells were observed under an inverted microscope, washed once with 100 µL/well of PBS, and then treated with 0.5% MTT solution for 4 hours. Subsequently, 100 µL/well of SDS (stopper reagent) was added. The absorbance and percentage of cell viability were measured using the formula in Eq. 1 as mentioned above.

Cytoprotective Assay of EBRB 'Sembada Hitam' on Vero cells against H_2O_2

Vero cells were seeded at a density of 1 x 10^4 cells/well in a 96-well microplate using DMEM. The cells were then incubated at 37°C with 5% CO_2 for 24 hours. After the 24-hour incubation period, the cells were washed once with 100 µL/well of PBS. Cells were induced by selected concentrations of H_2O_2 combine with selected concentration of EBRB. Three replicates of Vero cells were treated with different combinations of EBRB concentrations (15.625, 250, or 500 µg/mL) and hydrogen peroxide concentrations (100, 200, or 400 µM). The cells were then re-incubated for 24 hours. Untreated Vero cells and cells treated with 0.5% DMSO were used as controls. After the re-incubation, the cells were observed under an inverted microscope. They were washed with PBS and then re-incubated with 0.5% MTT solution for 4 hours. Next, 100 µL/well of SDS (stopper reagent) was added, and the absorbances of the samples were measured and calculated using the formula described in Eq. 1 above.

Cell growth assay on Vero cells induced by H_2O_2 combine with EBRB 'Sembada Hitam'

Vero cells at a density 1 × 10^5 cells/well cultured in 6-well microplates and incubated with 5% flow of CO_2 at 37°C for 24 hours. After incubation, selected concentration of H_2O_2 (200 or 400 µM) were treated in the Vero cells for 24 hours and combine with selected concentration of EBRB (250 or 500 µg/mL) for 24 hours with two replications. 0.5% DMSO treatment and Vero cells without treatment were used as control. The cells were cultured for the next 5 days (day 5), with a single passage method after 3-days culturing, using medium which contain of EBRB with the concentration as mentioned above. To analyze cell proliferation assay, the cells were counted at days 3 and 5 after the trypsin addition with trypan blue staining.

Statistics

The results were described as mean ± standard deviation (SD) using statistics of ANOVA with the value of $p < 0.05$ and followed by the Duncan test.

Results and Discussion

Cytotoxicity assay of hydrogen peroxide on Vero cells

Naturally, ROS has several important biological functions such as, regulate vascular homeostatic, cell differentiation, cell proliferation, gene expression [21], oxidative biosynthesis, and immune mechanism [22]. Moreover, ROS may act as a second messenger and influence specific signaling transduction pathway [22–24]. However, redox balance in the body can be disrupted due to excessive level of ROS leading to oxidative stress. Oxidative stress mediated by ROS may cause oxidative reaction which destroy lipids, nucleic acids, proteins, and increase apoptosis levels on cells [14, 25–27]. Oxidative stress is associated with inflammation, apoptosis, and autophagy [28]. Further, it may induce aging [29] and contribute on the onset of degenerative diseases such as cancer [29], diabetes, cardiovascular diseases, hypertension [30, 31], nervous system disorders [32], and obesity [33].

H_2O_2 is a strong oxidant. Its molecule produces OH• via Fenton reaction [34]. Under normal condition, low H_2O_2 concentration is produced as a by-product which is beneficial to cells [22]. H_2O_2 causes oxidative damage because it is a main ROS generated intracellularly throughout pathological and physiological processes [5, 6, 35, 36]. H_2O_2 readily permeates cells membranes and reacts with intracellular ions to cause intracellular damage [33]. In this study, we applied several concentrations of H_2O_2 in Vero cells as a model to induce oxidative stress. H_2O_2 enter to the cell as a free radical that can generate different free radicals within cell [37, 38].

Our findings indicate that exposure to H_2O_2 for 24 hours at concentrations ranging from 12.5 to 400 µM resulted in a decrease in Vero cell viability, ranging from 100 ± 25.268% to 57.912 ± 8.361%. Statistical analysis revealed that H_2O_2

concentrations of 50 and 100 µM exhibited a significant difference compared to the control, as well as the H_2O_2 concentration of 12.5 µM ($p < 0.05$). However, there was no significant difference observed between H_2O_2 concentrations of 25, 200, and 400 µM ($p < 0.05$). Notably, H_2O_2 exposure at concentrations of 50 to 400 µM resulted in a decrease in cell viability by approximately 30 to 40%. (Figure 1). Our result suggested that H_2O_2 decrease Vero cells viability in a dose-dependent manner. We predict it may be caused by low antioxidant enzyme activity in Vero cells due to H_2O_2 exposure.

Previous studies demonstrated that Vero cells induced by H_2O_2 significantly increase intracellular ROS activity [33, 39], decrease the cell's viability [33, 39, 40], reduce survival rate to 66.67% in zebrafish [33], and increase the chromatin condensation of nucleus [39]. Several studies have reported that treatment with H_2O_2 at a concentration of 0.1 mM for 24 hours resulted in a reduction in melanocyte cell viability [41]. It has been reported that exposure to H_2O_2 at concentrations ranging from 100 to 1000 µM for 24 hours significantly reduced mouse hepatocyte viability in a dose-dependent manner [36]. H_2O_2 induction to PC12 cells at the concentration of 200 µM also decreased cell viability [42]. Increasing doses of H_2O_2 exposure for 4 hours at concentrations ranging from 60 to 1200 µM resulted in a dose-dependent decrease in cell viability, with the viability decreasing to 46.45 ± 0.82% from an initial value of 100.00 ± 0.93% [43]. Previous studies have reported that exposure to H_2O_2 at concentrations ranging from 25 to 600 µM for 24 hours leads to a dose-dependent decrease in cell viability in both Nampt Tg-MEF and wild-type cells [44]. A study reported that H_2O_2 exposure at the concentration of 200 µM to AGS cells caused damage and decreased endogenous antioxidant activity such as GSH, SOD, and catalase activities compared to the H_2O_2 untreated group [43]. Moreover, a study also evaluated that H_2O_2-induced oxidative stress on cells in vitro depends on exposure concentration, volume of medium, length of the exposure time, and cell density [45]. Both previous studies and our results indicate that H_2O_2 is highly toxic to Vero cells, as it significantly decreases cell viability.

Cytotoxicity assay EBRB 'Sembada Hitam' on Vero cells

Protecting cells from oxidative stress is very important. When the body cannot handle the damage, an exogenous antioxidant is needed to mitigate the dangerous impact of oxidative stress. Various studies have investigated the use of exogenous antioxidants, including those derived from pigmented rice, to mitigate the effects of oxidative stress on cells. Pigmented rice, such as red, black, and purple rice, contains different pigment colors and varying levels of anthocyanins and proantho-

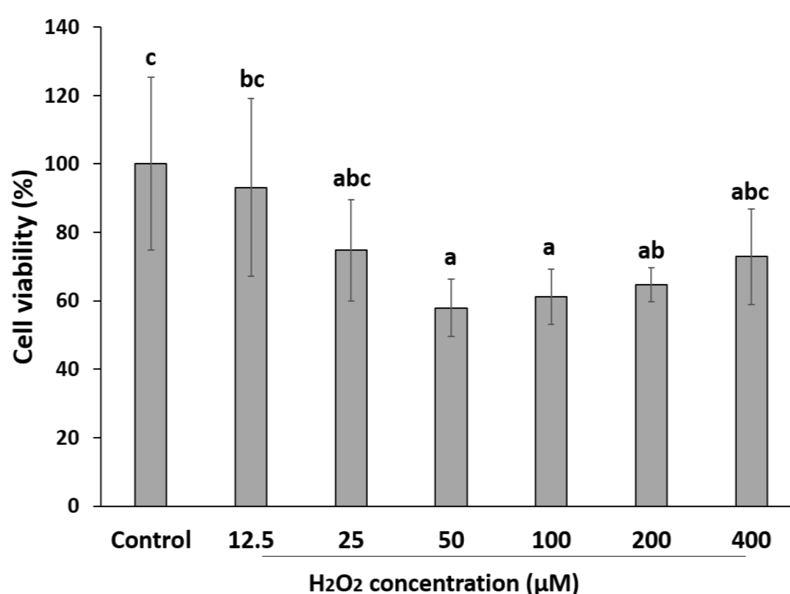

Figure 1. Exposure of H_2O_2 for 24 hours tends to decrease Vero cells viability. Significant difference of the value ($p < 0.05$).

cyanidins [46].

Colored rice has a higher antioxidant activity than white rice and contains more phenolic compounds [47]. Previous studies reported that antioxidants from pigmented rice, including black rice [15–17, 48] and red rice [17, 48], can reduce the harmful effect of oxidative stress. We used one of the potentially natural ingredients in protecting cells affected by oxidative stress H_2O_2, which is black rice. Black rice was known to possess antioxidant and biological activities. This pigmented rice is rich in phytochemical compounds such as flavonols, phenolic acid, carotenoids, γ-oryzanols, caffeic acid, malvidin, polyphenols, ferulic acid, and flavonoids is dominated by pelargonidine-3,5-diglucoside, malvidin, anthocyanins, tocotrienols, phytic acid, and tocopherols [10, 12, 13, 49, 50]. It was expected that antioxidant components of EBRB 'Sembada Hitam' can protect Vero cells against H_2O_2 exposure.

In our study, we used EBRB, a pigmented rice extract. Prior to the main experiments, we evaluated the potential toxicity of EBRB on Vero cells by treating them with various concentrations (7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL) and assessing cell viability using the MTT assay. The results showed a significant dose-dependent increase in Vero cell viability after 24 hours of EBRB treatment, ranging from 67.611 ± 3.034% at 7.8125 µg/mL to 84.814 ± 10.492% at 1,000 µg/mL (Figure 2). These values were significantly different from the control group ($p < 0.05$). Since we proved, there was no cytotoxicity effects of EBRB against Vero cells. Moreover, EBRB triggered Vero cell viability in a dose-dependent manner. We assumed that there were increasing bioactive compounds level mainly phenolic and flavonoid compounds along with increasing EBRB concentration. Flavonoids protect cells from oxidative stress by inhibiting the enzymes that are involved in superoxide anion production, such as protein kinase and xanthine oxidase, and preventing metal ions from forming free radicals [51, 52].

Our results similar with several studies that has been reported using pigmented rice in various cells type. It was reported that extracts of red rice (Hom Dang, Hom Red Rose) and black rice (Klam Doi-saked, Hom Dum Sukhothai II) have nontoxic effects against A375 cells [53]. A previous studies showed that EBRB 'Cempo Ireng,' 'Woja Laka,' and 'Toraja' have IC50 values 1874.14 ± 169.56, 1295.2 ± 37, and 1232.07 ± 165.51 µg/mL, respectively, on Vero cells. Thus, it has been reported to show no toxic activity [54]. Methanolic extract of 'Cempo Ireng' one of black rice cultivar found in Indonesia also showed nontoxic effect against Vero cells with IC_{50} value more than 1000 µg/mL [55]. Previous studies also showed that EBRB is not toxic to HUVEC cells [10]. Moreover, a study showed that EBRB did not have the toxicity effect in Vero cells with an IC50 value of 1139.5 µg/mL [56]. Cytotoxicity is classified into four categories based on IC_{50} values. IC_{50} value less than 10

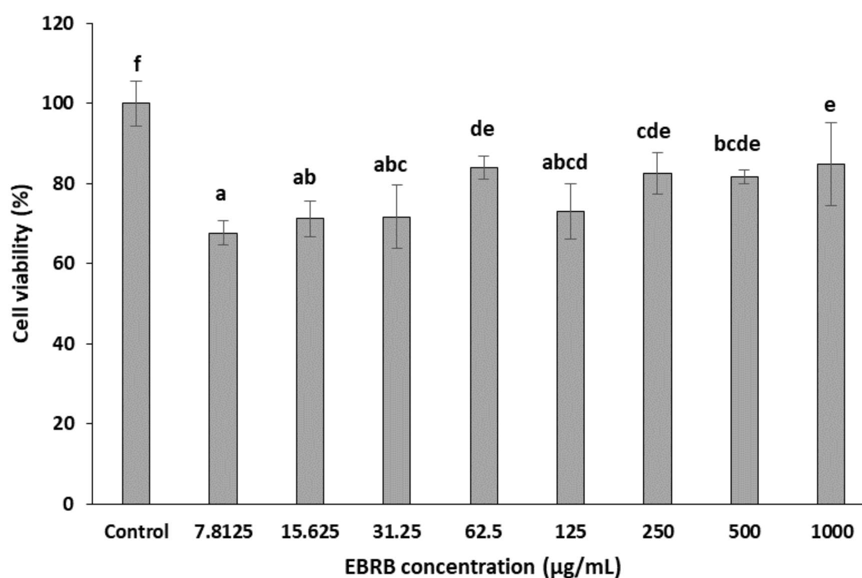

Figure 2. 24-hour EBRB treatment did not show cytotoxicity effect on Vero cells. Significant difference of the value ($p < 0.05$).

µg/mL was classified into powerful cytotoxicity effect. In contrast, strong cytotoxic effects have IC_{50} between 10 - 100 µg/mL, moderate toxic effect category with IC_{50} values of 100 – 500 µg/mL [54, 57], and IC_{50} value more than 1000 µg/mL was categorized as no toxicity [55]. Our findings, in line with previous studies, indicate that EBRB does not exhibit cytotoxic effects on Vero cells. This could be attributed to the antioxidant activity in black rice bran, which promotes the growth of Vero cells. Building upon these results, our next objective is to investigate whether the EBRB extract possesses a cytoprotective effect on Vero cells when exposed to H_2O_2 induction. Based on these results, we evaluated further the potency of EBRB to protect Vero cells induced by H_2O_2 exposure.

Cytoprotective assay of EBRB 'Sembada Hitam' on Vero cells against H_2O_2

To evaluate the protective effect of EBRB against H_2O_2-induced oxidative stress, Vero cells were treated with H_2O_2 in combination with EBRB for 24 hours. Cell viability was assessed using the MTT assay (Figure 3). Previous studies have focused on the impact of severe oxidative damage on the cellular redox system, often resulting in around 50% cell viability. In these conditions, exogenous antioxidants have been shown to enhance antioxidant enzyme activity and mitigate oxidative stress effects [37, 58–63].

Therefore, H_2O_2 concentrations of 100, 200, and 400 µM were selected for this study, while EBRB concentrations of 15.625, 250, and 500 µg/mL were chosen based on the viability results of Vero cells treated with the extract. Our results demonstrated that EBRB effectively protected Vero cells against H_2O_2-induced damage. The percentage of Vero cell viability consistently exceeded 100%, indicating no cell death. Statistical analysis revealed no significant differences between the various combinations of H_2O_2 and EBRB concentrations ($p < 0.05$). The results revealed that EBRB at the concentration of 15.625, 250, and 500 µg/mL may protect Vero cells from H_2O_2 exposure at quite high concentrations (100, 200, and 400 µM) (Figure 3).

Several studies have demonstrated the protective effects of pigmented extracts against H_2O_2-induced cell damage. In a previous study, it was reported that anthocyanins present in black sticky rice extract exhibited dose-dependent antioxidant effects, inhibiting lipid and protein oxidation in the presence of H_2O_2, at concentrations ranging from 100 to 1000 mg/L. Another research highlighted the potential of black glutinous rice extract to reduce oxidative stress in HepG2 cells and regulate the expression of low-density lipoprotein gene receptors [64]. A previous study also reported that pre-treatment extract of red rice 'Hom Dang,' 'Hom Red Rose' and black rice 'Klam Doi-saked' at the concentration of 10 mg/mL able to prevent oxidative stress of A375 cells with decreased viability up to 70% caused by H_2O_2

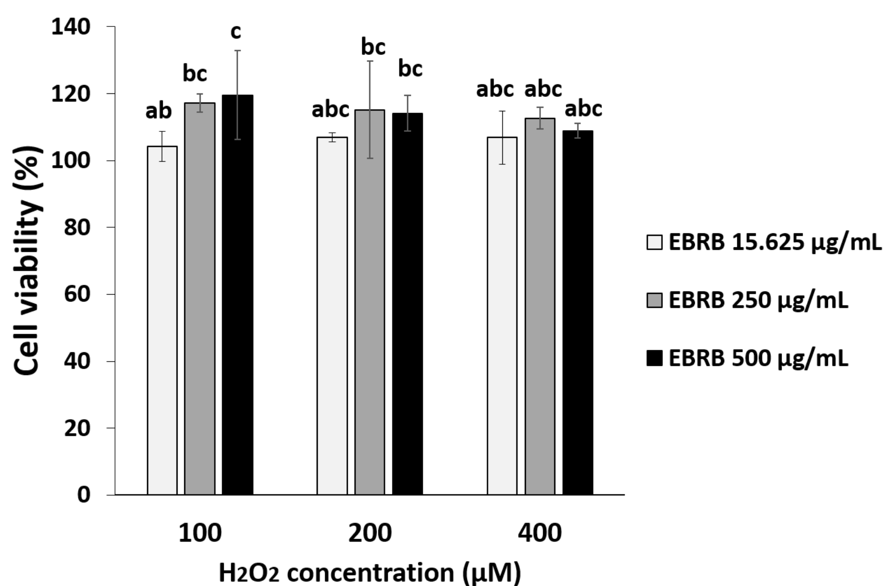

Figure 3. EBRB has protective effect on Vero cells when exposed to H_2O_2 at concentrations of 100, 200, and 400 µM for 24 hours. Significant difference of the value ($p < 0.05$).

exposure at the concentration of 80 µM. Extract of 'Hom Dang' and 'Klam Doi-saked' cultivars able to protect A375 cells due to *t*-BHP exposure [53]. This suggests that the flavonoid compounds present in the extract may exhibit a positive correlation with increased oxidative stress due to their high scavenging activity against hydroxyl radicals. Furthermore, the extract of black rice variety 'Kam Muang' has been reported to possess anti-inflammatory properties, reducing inflammation in HL-60 cells, as well as mitigating oxidative stress induced by superoxide and nitrogen monoxide [48]. A study found that H_2O_2 exposure for 24 hours decreased the viability of dermal fibroblast. Furthermore, pre-treatment with anthocyanin extracted from black rice at a concentration of 25 µg/mL for 2 hours has been shown to effectively prevent cell death and increase cell viability [65]. Based on these studies, it can be inferred that the bioactive compounds present in EBRB 'Sembada Hitam' are effective in protecting Vero cells. This is supported by the observation that the viability of Vero cells remains consistently above 100% even with increasing concentrations of H_2O_2 exposure. Thus, the cell viability mechanism of our results is probably similar to previous studies, which reported that extract of pigmented rice, especially extract of black rice, has a protective effect against oxidative stress by modulating antioxidant enzyme activity and ROS production.

Previously, some studies reported that treatment of pigmented rice extract on HepG2 cells has cytoprotective effect against oxidative damage [21, 23] by increasing the antioxidant enzyme activity such as catalase and SOD, and decreasing the ROS level [24]. Black rice extract also reported able to protect induction of oxidative damage in HepG2 cells via regulating antioxidant enzyme activities, GSH level, MDA generation, and ROS production [16]. Black rice extract was increase cells viability in fibroblast WI-38 induced by H_2O_2 [14].

Other studies evaluated antioxidant activity that can reduce the effect of free radicals, which might be by neutralizing free radicals by donating electrons, accepting radicals by eliminating the unpaired electrons, or stabilizing the harmful molecules [34]. It is indicated that in our results, the mechanism for cytoprotective effect of EBRB against H_2O_2 toxicity in Vero cells might be via antioxidant enzyme activity such as GPx, SOD, and catalase, which play a role as free radical scavenger to prevent toxic effect of H_2O_2. Similar with previous research which stated that superoxide radicals catalysed by SOD into H_2O_2 and O_2 [22]. GPx using glutathione as a substrate to remove H_2O_2. Catalase protect cells from H_2O_2 accumulation via breakdowns H_2O_2 became O_2 and H_2O. A study reported that quercetin enters into cells for promoting the production of antioxidant enzymes and subsequently catalysing as well as degrading ROS by initiate the antioxidant pathway of cells [62], and acts on superoxide anion radical or other free radicals (external and intracellular) [38], then scavenging radicals [59, 60]. PIG1 and PIG3V cells induced by H_2O_2 compared with control showed significantly decrease level of ROS, while after 24 h expose to paeoniflorin increased level of CAT and SOD [41]. Nrf2/HO-1/JNK signalling pathway activated by paeoniflorin protects H_2O_2-induced cell damage in melanocytes. We assumed that antioxidant activities from EBRB might be a direct response to oxidative stress by removing and or removing the free radicals with the role of SOD, catalase, and GPx that contribute to protecting the Vero cells via catalyze breakdowns, and removal of H_2O_2 from the cells. In short time incubation (24 hours) the EBRB potential to protect cell viability seem promising. Thus, we prolong EBRB treatment for up to 5 days on Vero cells induced by H_2O_2. Interestingly, prolonged treatment causes a decrease in Vero cell viability.

Cell growth assay on Vero cells induced by H_2O_2 with EBRB 'Sembada Hitam'

To investigate the potential of EBRB to prolong cell survival, we extended the exposure period to 5 days and performed a cell growth assay using trypan blue staining on day 3 and day 5. The selected concentrations of 250 or 500 µg/mL of EBRB were combined with 200 or 400 µM of H_2O_2 (Figure 4). Previous research has shown that quercetin, when administered at low concentrations and for short durations, can increase the viability of IPEC-J2 cells. However, the increase in cell viability becomes slower as the treatment duration is extended [38]. Other studies have reported that exposure to quercetin at a concentration of 5 µg/mL for 9 hours resulted in a decrease in cell viability. However, there was no significant difference observed compared to a 48-hour treatment [38]. Furthermore, it has been observed that treatment with quercetin at a concentration of 1.25 µg/mL for 3 hours, in combination with H_2O_2 at 750 µM for 1 hour, resulted in the maintenance of

IPEC-J2 cell viability at around 70%. However, when the duration of H_2O_2 treatment was extended to 2 hours, it led to a decrease in cell viability [38]. Interestingly, after 3 hours of H_2O_2 treatment, cell viability increased again up to 80%. It indicates that quercetin treatment against H_2O_2 exposure showed a decreasing or increased cell viability dependent on the dose and time of exposure. Another study found that paeoniflorin (PF) treatment for 24 hours could not restore cell damage caused by H_2O_2 [41]. However, if the treatment was extended for 48 hours, that compound could increase PIG1 and PIG3V melanocyte viability. Previous studies reported that vitamin C treatment may increase HUVEC cell viability compared to HUVEC cells, which were exposed to H_2O_2 for 72 hours (cell viability decrease) [67]. It was also known that chlorogenic acid combined with *Lactobacillus plantarum* 2142 exposures for 1 hour was able to increase IPEC-J2 cells viability [68]. However, cell viability significantly decreases after prolonged chlorogenic acid combined with Lp 2142 exposure for 4 and 24 hours. Our results also suggested that H_2O_2 exposure on Vero cells for 24 hours with EBRB and prolonged treatment until day five could not maintain cell survival. The control group also showed decreasing cell growth probably because of longer 0.5% DMSO exposure in media used as control (Figure 4).

The decrease in Vero cell growth observed in this study may be attributed to the prolonged treatment time, which could potentially cause the transformation of polyphenolic, flavonoid, and other bioactive compounds in EBRB into pro-oxidants that threaten cell survival. Pro-oxidants generated from phenolic compounds are primarily derived from the generation of phenoxyl radicals, which can react with oxygen to produce O_2 and H_2O_2, especially in the presence of transition metal ions. Consequently, elevated levels of cellular reactive oxygen species (ROS) can lead to lipid peroxidation, apoptosis in both normal and cancer cells, DNA damage, and cytotoxicity [17, 51]. Prolonged treatment with drugs or chemicals can lead to continuous drug absorption by cells. The metabolism and utilization of intracellular drugs can become challenging, resulting in high intracellular drug concentrations and a slow proliferation rate in IPEC-J2 cells [38].

A study reported that redox status of cell and extract concentrations can influence the role of bioactive compounds in rice bran, which can act as antioxidant or pro-oxidant [23]. A study on H9c2 (2-1) cells revealed that this cells were susceptible to cytotoxicity or had an overdose of natural antioxidant caused by high concentrations of black rice extract and H_2O_2 (exhibit pro-oxidant properties, has been reported) that probably threaten viability of cell and survival [21]. The harmful effect caused by H_2O_2 after treatment with extract and H_2O_2 may have further reduced H9c2 (2-1) cells viability. Based on these studies, we concluded that Vero cells response against protective effect of EBRB 'Sembada Hitam' treated by H_2O_2 only in short-time exposure. Short-time treatment of extract combined with H_2O_2 showed a significant increase in the Vero cell's viability.

Conclusion

The research focused on investigating the potential cytoprotective effects of EBRB (pigmented rice extract) on Vero cells and its ability to counteract H_2O_2-induced toxicity. The study yielded several significant findings. Firstly, the experiments revealed that EBRB did not exhibit cytotoxic effects on Vero cells. On the contrary, it promoted cell growth, suggesting its safety and potential therapeutic benefits. Furthermore, the results demonstrated a significant cytoprotective effect of EBRB against H_2O_2-induced toxicity in Vero cells. Even when exposed to varying concentrations of H_2O_2, the cell viability remained above 100%, indicating that EBRB effectively protected the cells from oxidative stress. The cytoprotective effect of EBRB was dose-dependent, meaning that higher concentrations of EBRB provided more excellent protection against H_2O_2-induced toxicity. This suggests that the bioactive compounds, such as flavonoids and anthocyanins, present in EBRB may contribute to its antioxidant properties and the observed cytoprotective effects. It was also noted that prolonged exposure to H_2O_2 or extended treatment with drugs or chemicals can harm cell viability and proliferation. This highlights the importance of considering the duration of therapy in experimental designs to ensure accurate assessment of cellular responses.

In addition, the presence of phenolic compounds in EBRB was found to generate pro-oxidants in the presence of transition metal ions. This can lead to increased levels of reactive oxygen species (ROS), lipid peroxidation, DNA damage, and ultimately cytotoxicity. These

findings emphasize the need for careful evaluation of the potential pro-oxidant effects of phenolic compounds in various contexts. Overall, the study concludes that EBRB has the potential to serve as a safe and effective cytoprotective agent against oxidative stress-induced toxicity in Vero cells. Further investigations are warranted to elucidate the underlying mechanisms of EBRB's protective effects and to optimize the concentration and duration of treatment for maximum cytoprotection.

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