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Anti-Inflammatory evaluation of black rice extract inhibits TNF- α , IFN- γ and IL-6 cytokines produced by immunocompetent cells

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ABSTRACT

Black rice has been known for its many beneficial effects to our body because it contains several chemical compounds that act as anti-inflammatory agents. This study aims to compare the anti-inflammatory activity of ethanol extract and aqueous extract of black rice. Anti-inflammatory activity test was performed *in vitro* on splenocytes of diabetes mellitus mice model as the control of inflammation. Cells were cultured in Roswell Park Memorial Institute medium with 10% fetal bovine serum, anti-CD3 and lipopolysaccharide and black rice extract 50, 100 and 200 $\mu\text{g}/\text{mL}$. In day 3, cells were harvested, stained and analyzed by flow cytometry. The result shows that the ethanol extract and aqueous extract of black rice have anti-inflammatory activity. 50 $\mu\text{g}/\text{mL}$ black rice aqueous extract has the most extensive anti-inflammatory activity indicated by increased T_{reg} cells, decreased nuclear factor kappa B activity on CD4^+ , CD8^+ T cells, decreased production of TNF- α by CD4^+ T cells, decreased production of IL-6 and IFN- γ by macrophages.

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Anthocyanin; black rice; cytokine; flow cytometry; inflammation

Introduction

Inflammation is one of the main responses of the immune system against infection and irritation. It is promoted by chemical factors such as histamine, bradykinin, serotonin and the prostaglandins. These chemical factors are released by inflammation-mediating cells that act in the immune system to protect tissue from infection. Prolonged inflammation (called chronic inflammation) can lead to various diseases such as autoimmune diseases, diabetes mellitus, cardiovascular diseases, pulmonary diseases and cancer (Dwijayanti, Djati, & Rifa'i, 2015; Yen et al., 2006). Therefore, chronic inflammation must be treated immediately to prevent these dangerous diseases. Aggarwal, Shishodia, Sandur, Pandey, and Sethi (2006) note that chronic inflammation is related to cytokine, chemokine and adhesion molecule expression. Pro-inflammatory cytokines, such as IL-1, TNF- α , IFN- γ , IL-6, IL-8 and IL-11, play roles in mediating chronic inflammation and are produced predominantly by

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activated macrophages and CD4⁺ T cells and mediated by the activation of nuclear factor kappa B (NF- κ B) (Dwijayanti, Widodo, Ibrahim, & Rifa'i, 2016; Liang, Zhou, & Shen, 2004).

Rice is a staple food in many Asian countries. Generally, there are three major kinds of rice grown in this region, white rice (*Oryza sativa*), brown rice (*O. glaberrima*) and black rice (*O. sativa* var. *indica*). Black rice has an abundance of phenolic compounds compared to white rice, a number of which are associated with the antioxidant activity. Phytochemical extracts of red rice and black rice extracts contain an alkaloid, flavonoids, terpenoids, tannins, quinones and nine types of anthocyanin.

A recent study showed that anthocyanin is one of the most powerful antioxidants in rice. The pigment of black rice is the strongest among the other colors of rice. This pigment is also rich in flavonoids, and the levels are five times more than in white rice. Flavonoids are phenolic compounds that act as antioxidants and prevent damage by free radicals (Dwijayanti et al., 2016). Both anthocyanins and flavonoids can donate hydrogen ions, capture free radicals directly, repair DNA, repair damaged cells and stimulate the activity of immunocompetent cells. According to Min, Ryu, and Kim (2010), black rice may have the ability to exhibit antioxidant, anti-inflammatory, anti-degranulatory, anti-anaphylactic, anti-scratching and anti-allergic effects.

As describe before, black rice is generally consumed directly as staple food so in this study we used a crude extract, both aqueous and ethanol, to be more applicable in the community. Ethanol is a semipolar solvent that can dissolve polar and non-polar compounds in black rice so that the active substance suspected of more than the aqueous extract that is able to dissolve only polar compounds. However, there are a few reports comparing the anti-inflammatory effects between aqueous and ethanol extract of organic black rice native to Indonesia. Therefore, we investigated the anti-inflammatory effects of Indonesian organic black rice against TNF- α , IFN- γ and IL-6 cytokines produced by immunocompetent cells *in vitro*.

Method

Splenocyte cells' culture

The spleen was isolated from both healthy mice and a mice model of diabetes mellitus (DM), and then washed with phospho-buffered saline (PBS). Cells were isolated from the spleen by crushing it in PBS. The homogenates were centrifuged at 2500 rpm, 10°C, for 5 min. Supernatant was discarded, and the pellet resuspended in 1 mL of medium. The splenocytes were then cultured in plate culture, in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), and 1% Penicillin and Streptomycin and 2-Mercaptoethanol (2-ME) at 1 μ L/10 mL medium. The stimulant anti-CD3 and lipopolysaccharide (LPS) were added to the medium at 10 μ L and 2 μ L/mL medium, respectively. The cells were divided into four treatment groups: normal group; DM group; ethanol extract of black rice (BE) group with doses of 50, 100, 200 μ g/mL; aqueous extract of black rice (BA) with doses of 50, 100, 200 μ g/mL.

Flow cytometry analysis

After the incubation period, the cells were harvested and centrifuged at 2500 rpm, 10°C, for 5 min. The pellet was resuspended in 1 mL of PBS and divided into four microtubes and

centrifuged. The supernatant was discarded and pellets were stained with conjugated antibodies. The combinations of antibodies were: (1) Fluorescein isothiocyanat (FITC)-conjugated rat anti-mouse CD4, Phycoerythrin (PE)-conjugated rat anti-mouse CD25 and PE/Cy5-conjugated rat anti-mouse CD62L; (2) FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8 and PE/Cy5-conjugated rat anti-mouse NF- κ B; (3) FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse IFN- γ and PE/Cy5-conjugated rat anti-mouse TNF- α and (4) FITC-conjugated rat anti-mouse CD11b, PE-conjugated rat anti-mouse IFN- γ , PE/Cy5-conjugated rat anti-mouse NF- κ B and APC-conjugated rat anti-mouse IL-6. Cells were stained with extracellular antibodies and then incubated for 20 min in the ice box. A total of 50 μ L of cytosol/cytoplasm fixative solution was added and incubated for 20 min in the ice box. Alternatively, 500 μ L of washperm washing solution was added and then centrifuged. The supernatant was discarded, while pellets were stained with intracellular antibodies and then incubated for 20 min in the ice box. A total of 500 μ L of PBS was added to both the cells that had been incubated with either the extracellular or the intracellular staining procedure. Each sample was transferred into a flow cytometry cuvet and then the amount of binding analyzed by flow cytometer.

Data analysis

Data were analyzed using BD cellQuest PRO™ software then tabulated and analyzed statistically. One-way ANOVA was used for statistical analysis with a significance level of 0.05, and then followed by the Tukey's test for pairwise comparisons.

Result

Pro-inflammatory cytokine

TNF- α , IFN- γ and IL-6 are included among the group pro-inflammatory cytokines that can cause chronic inflammation (Dwijayanti et al., 2015). As shown in Figure 1, this study showed that the relative amount of TNF- α and IFN- γ secreted by CD4⁺ T cells, and IL-6 and IFN- γ secreted by macrophages (CD11b⁺), was significantly increased ($p < .05$) in inflammatory conditions when compared to the control. Aqueous extract of black rice (BA) at a concentration of 50 μ g/mL decreased the relative amount of TNF- α secreted significantly ($p < .05$) compared to inflammatory conditions and equivalent to normal conditions (Figure 1(A)). BA at 200 μ g/mL also decreased the relative amount of the cytokine TNF- α significantly ($p < .05$) compared to inflammatory conditions, but these numbers were not equal to normal conditions ($p > .05$). Figure 1(B) shows that all concentrations of both the ethanol and aqueous extracts decreased the amount of IFN- γ cytokines in inflammatory conditions significantly. But they were still lower ($p < .05$) when compared with normal conditions. Furthermore, the ethanol extract of black rice (BE) at a concentration of 100 μ g/mL and BA at a concentration of 50 and 200 μ g/mL decreased the relative number of macrophages producing IL-6 and IFN- γ (Figure 1(C, D)).

Nuclear factor kappa B (NF- κ B)

The occurrence of inflammation was characterized by an increase in NF- κ B transcription factors (Liang et al., 2004). According to Aupperle et al. (2001) and Lawrence (2009), the

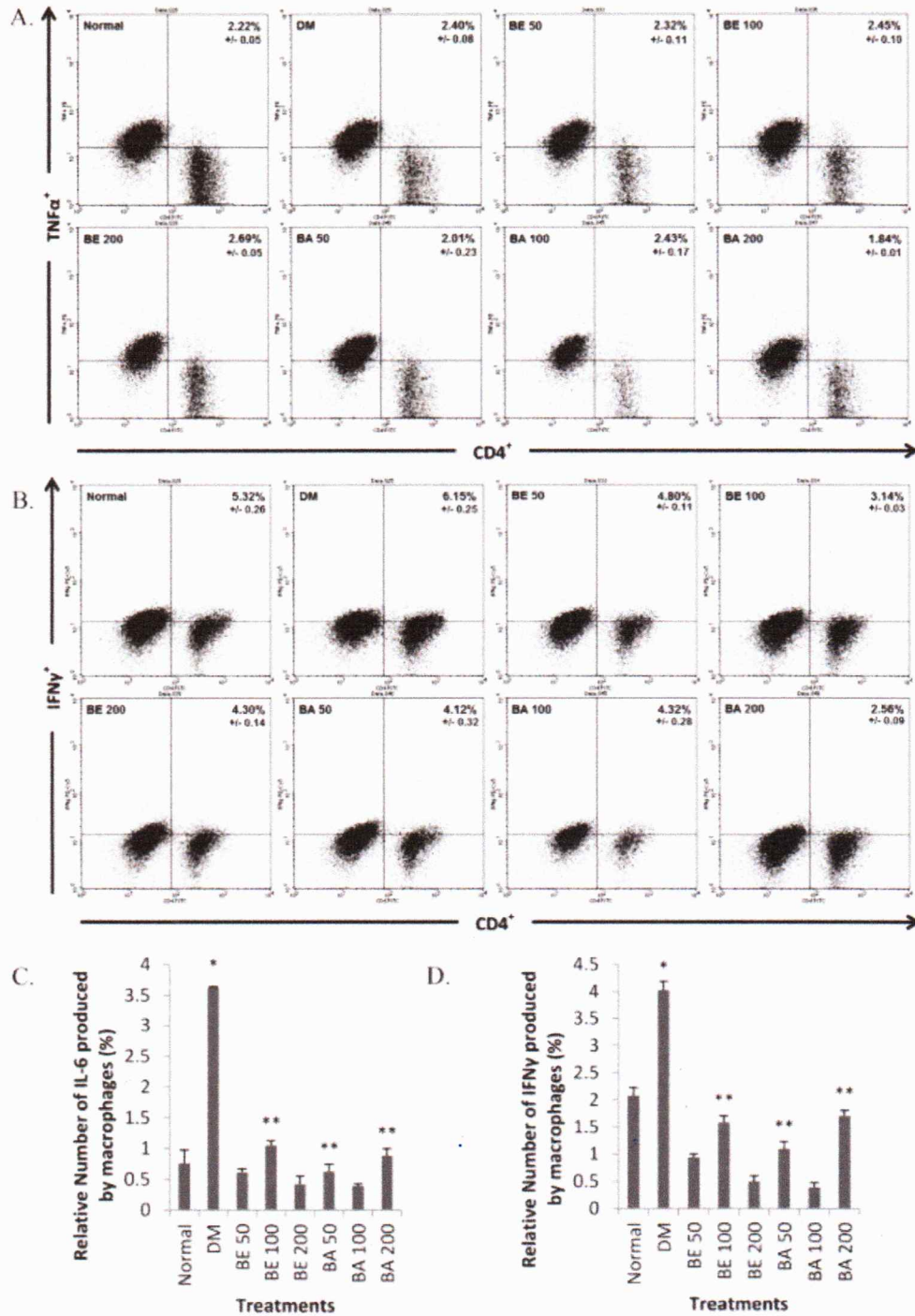


Figure 1. Stimulation of black rice extract was able to decrease the expression level of pro-inflammatory cytokine: (A) TNF- α produced by CD4⁺ T cell; (B) IFN- γ produced by CD4⁺ T cell; (C) IL-6 produced by macrophages; (D) IFN- γ produced by macrophages. Spleen was isolated from healthy mice and a mice model of DM then cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for three days. On day 3, cell cultures were harvested and analyzed using flow cytometry. The pro-inflammatory cytokines were presented in relative number. Data are mean \pm SD in each group with p -value $< .05$. * $p < .05$ vs. normal; ** $p < .05$ vs. DM.

NF- κ B pathway is considered as the initiator of pro-inflammatory pathway because NF- κ B promotes the expression of pro-inflammatory genes, including cytokines, chemokines and adhesion molecules. As shown in Figure 2, the relative amount of NF- κ B in CD4⁺ T cells (A), CD8⁺ T cells (B) and macrophages (CD11b⁺) (C) in splenocytes of DM mice was significantly increased ($p < .05$) compared with normal cells. BE at a concentration of 50 μ g/mL and BA at a concentration of 50 and 100 μ g/mL reduced the relative amount of NF- κ B in both T-cell types. BE at a concentration of 100 μ g/mL decreased NF- κ B only in CD8⁺ T cells. BE at a concentration of 200 μ g/mL increased the expression of NF- κ B in both the T-cell types.

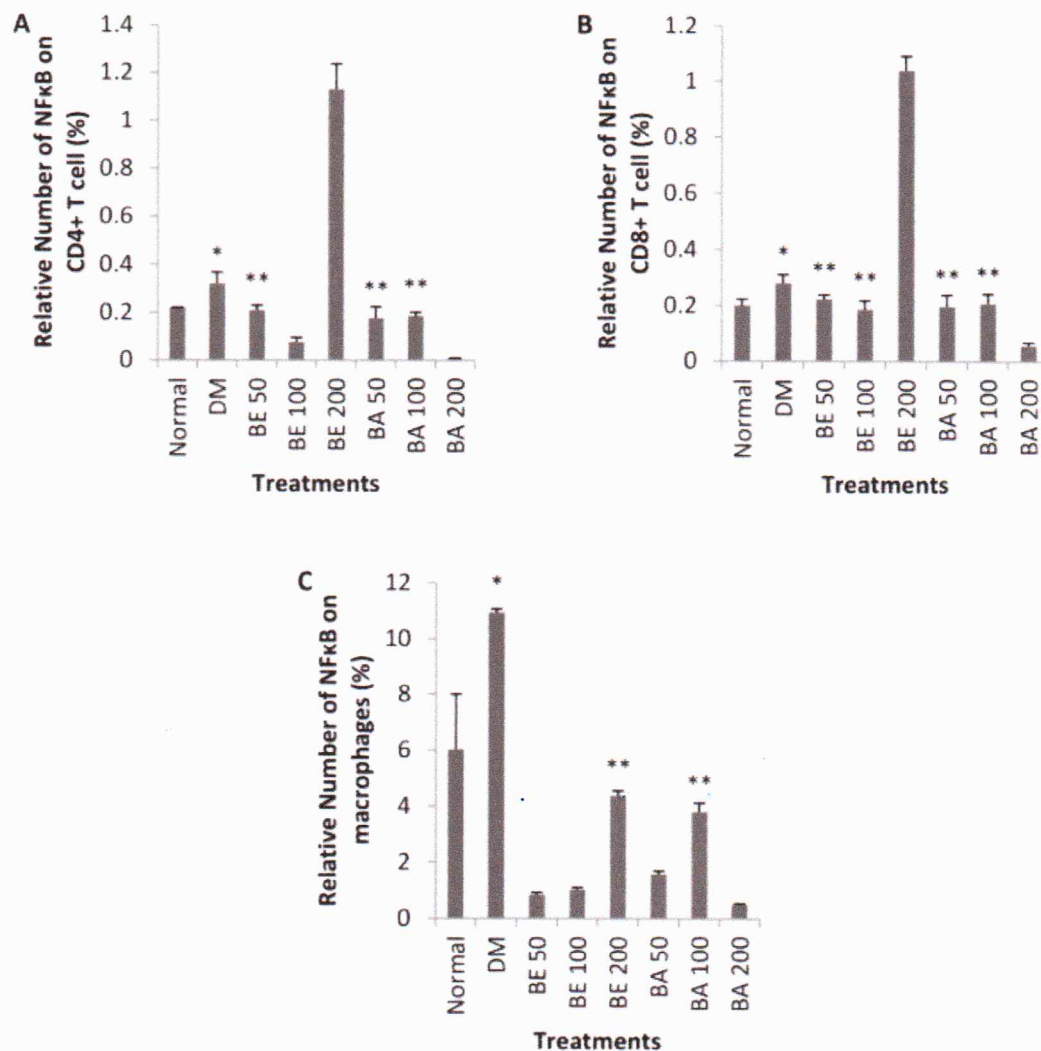


Figure 2. Stimulation of black rice extract was able to decrease the expression level of NF κ B on: (A) CD4⁺ T cell; (B) CD8⁺ T cell; (C) macrophage. Spleen was isolated from healthy mice and a mice model of DM then cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for three days. On day 3, cell cultures were harvested and analyzed using flow cytometry. The pro-inflammatory cytokines were presented in relative number. Data are mean \pm SD in each group with p -value $< .05$. * $p < .05$ vs. normal; ** $p < .05$ vs. DM.

BE at a concentration of 200 µg/mL and BA at a concentration of 100 µg/mL showed a decrease in the relative amount of NF-κB expression by macrophages that was equivalent to normal conditions (Figure 2(C)). This shows that BE at a concentration of 200 µg/mL and BA at a concentration of 100 µg/mL play an essential role in the process of quieting inflammation, which is mediated by macrophages through the inhibition of their NF-κB pathway, although BE at a concentration of 100 µg/mL and BA at concentrations of 50 and 200 µg/mL did not significantly affect the NF-κB pathway inhibition. Hansen et al. (2012) explains that inflammation is mediated by the NF-κB transcription factor as it activates C-jun kinase, and then activating protein 2 (AP2) acts as signal transducer and activator of STAT3/4/5 transcription. Figure 1 we suspect that BE at a concentration of 100 µg/mL and BA at a concentration of 50 and 200 µg/mL were targeted by one of the three transcription factors mentioned above.

Regulatory T cells (T_{reg})

T_{reg} cells are a type of T cells that play an important role in the immune system. T_{reg} cells regulate the performance of T helper cell (Th) and T cytotoxic cell (Tc) effectors. This regulation causes effector cells to not work reactively and not secrete cytokines (Rifa'i, 2014; Sakaguchi, Wing, Onishi, Prieto-Martin, & Yamaguchi, 2009). Under these conditions, T_{reg} cells are one of the cells that contribute to the quieting of the inflammatory process. In this study, the number of T_{reg} cells decreased significantly in inflammatory conditions (DM) compared to controls ($p < .05$). All concentrations of both types of extracts tended to increase the number of T_{reg} cells significantly ($p < .05$). However, only BA at a concentration of 50 µg/mL was not significantly different from normal conditions (Figure 3).

The impact of black rice extract on spleen cell viability

Viability cell in culture condition was observed by monitoring the percentage of living splenocytes by flow cytometry. This study proved that black rice extract, both ethanol (BE) and aqueous (BA) extract, have no toxicity effect although applied with highest dose (200 µg/mL) because both relative and absolute numbers of cells at all of doses were not significantly different compared to the control (Figure 4).

Discussion

It has long been known that black rice is rich in anthocyanins. Anthocyanins are the most popular antioxidant, anti-inflammatory and anti-cancer (Im et al., 2016; Liu, Chen, Li, & Sun, 2016). Anthocyanins are polar-solvents-soluble because of the hydroxyls and sugars attached. Therefore, generally it is extracted by methanol, ethanol or water (Hou, Qin, Zhang, Cui, & Ren, 2013; Welch, Wu, & Simon, 2008). The polarities of solvents determine the type and amount of compounds that can be extracted from the material. They will extract the same or similar polarity compounds. Furthermore, the different type and amount of compounds will result in different activities. Thus we want to compare the anti-inflammatory activity of ethanol extract and aqueous extract of black rice and in this study we have proved that there are some different activities between them.

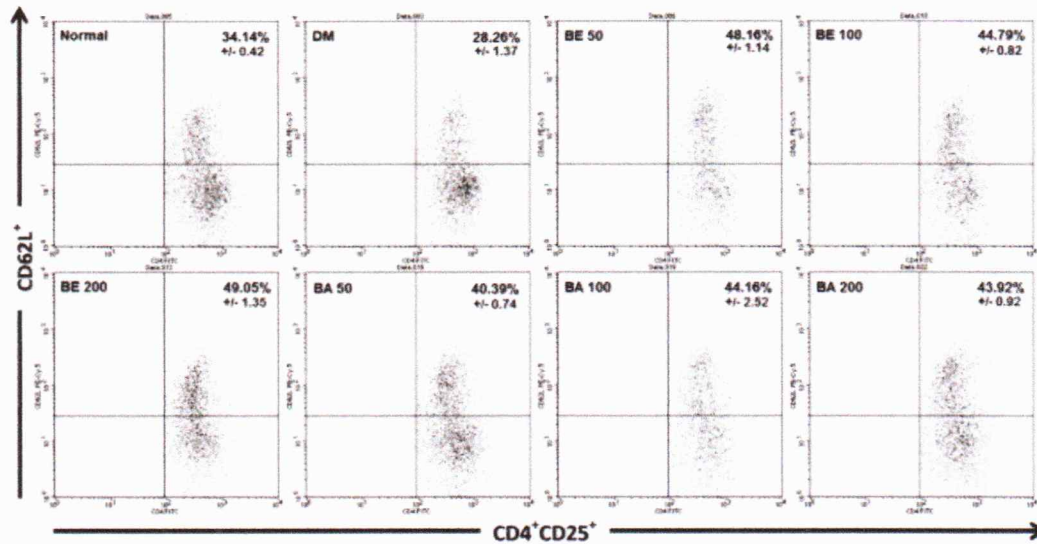


Figure 3. Stimulation of black rice extract was able to increase the relative number of Regulatory T cell (T_{reg}). Spleen was isolated from healthy mice and a mice model of DM and then cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for three days. On day 3, cell cultures were harvested and analyzed using flow cytometry. Percentages of $CD4^+ CD25^+$ cells positively expressed CD62L molecule were presented in relative number. Data are mean \pm SD in each group with p -value $< .05$.

The most common free radical present in inflammatory situations is a radical form of oxygen commonly known as ROS (reactive oxygen species). Increased ROS in the body will cause oxidative stress (Hansen et al., 2012). Lamb and Goldstein (2008) describe oxidative stress causing an inflammatory response through activation of JNK, NF- κ B and p38MAPK. Therefore, antioxidant compounds may be associated with an anti-inflammatory

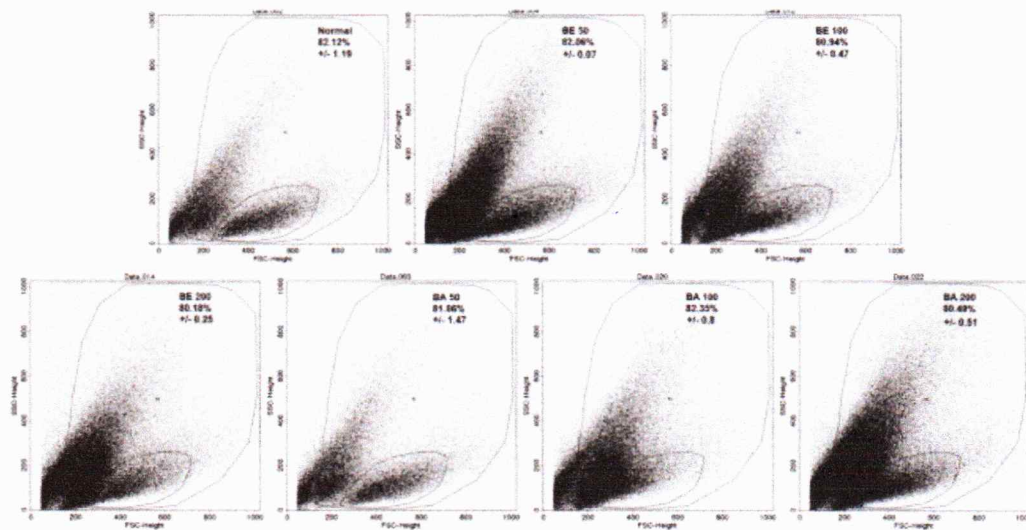


Figure 4. Black rice extract did not have cytotoxicity effect on splenocyte. Spleen was isolated from healthy mice and then cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for three days. On day 3, cell cultures were harvested and analyzed using flow cytometry. Percentages of living cells were presented in relative number. Data are mean \pm SD in each group with p -value $< .05$.

response. BE at a concentration 50 µg/mL resulted in a decrease in NF-κB in CD4⁺ and CD8⁺ T cells. BE 100 µg/mL reduced NF-κB in CD8⁺ T cells and decreased the production of cytokines IL-6 and IFN-γ by macrophages (CD11b). BE 200 µg/mL specifically suppressed NF-κB in macrophages, because at this concentration NF-κB in CD4⁺ and CD8⁺ T cells increased. BA at 50 µg/mL had the most extensive anti-inflammatory activity among five other treatments; at this concentration T_{reg} cells increased, NF-κB in CD4⁺ and CD8⁺ T cells decreased, production of cytokine TNF-α by CD4⁺ T cells decreased and production of cytokines IL-6 and IFN-γ by macrophages also decreased. BA 100 µg/mL inhibited NF-κB in CD4⁺ T cells, CD8⁺ T cells and macrophages. BA 200 µg/mL reduced cytokines IL-6 and IFN-γ, produced by macrophages. According to Rifa'i and Widodo (2014), increasing the production of IFN-γ in DM mice resulted from activated immune cells. Consistent with that found by Koarada, Wu, Olshansky, and Ridgway (2002), IFN-γ level increased as a result of autoreactive CD4⁺ T cells. However, it does not rule out the possibility that the decreasing levels of pro-inflammatory molecules can be directed to improving the Th1/Th2 ratio. The balance of Th1 and Th2 ratio are thought to be responsible for coordinating the immune system (Kidd, 2003).

Min et al. (2010) showed that black rice has an anti-inflammatory activity by inhibition of pro-inflammatory cytokines such as TNF-α and IL-1β and inflammatory mediators such as NO, prostaglandin E2, iNOS and COX2. According to Park, Kim, and Chang (2008), the anthocyanins in black rice are cyanidin 3-O-glucoside, peonidin 3-O-glucoside, malvide 3-O-glucoside, 3-O-glucoside pelargonidin and delphinidin 3-O-glucoside. The dominant anthocyanins are cyanidin 3-O-glucoside (95%) and peonidin 3-O-glucoside (5%). Min et al. (2010) further explains that cyanidin 3-O-glucoside can prevent IκBα phosphorylation and MAPK. IκBα is one type of NF-κB inhibitor that is phosphorylated in stress conditions. When phosphorylated, IκB is degraded, affecting NF-κB activation, automatically moving NF-κB into the nucleus of the cell. Caamano and Hunter (2002) further explain that NF-κB targets genes including those that encode cytokines such as IL-1, IL-2, IL-6, IL-12, TNF-α, LTα, LTβ and GM-CSF; adhesion molecules such as intracellular adhesion, vascular cell adhesion and endothelial leukocyte adhesion molecules; chemokines such as IL-8; acute phase proteins such as SAA; as well as enzymes such as iNOS (inducible nitric oxide synthase) and COX-2 (Cyclooxygenase-2). Under these conditions, the presence of black rice components that prevent phosphorylation of IκBα will also prevent activation of genes that play a main role in the inflammatory process.

Conclusion

This study demonstrated that both ethanol extract and aqueous extract of black rice have anti-inflammatory activity. Aqueous extracts, especially at the concentration of 50 µg/mL, had the most extensive anti-inflammatory activity among the other five treatments, indicated by increased T_{reg} cells, decreased NF-κB in T cells CD4⁺ and CD8⁺, decreased production of TNF-α cytokine by T cells CD4⁺ and decreased production of IL-6 and IFN-γ cytokine by macrophages.

Disclosure statement

No potential conflict of interest was reported by the authors.

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