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Aqueous Extract of Purple Sweet Potato Tuber Increases Sod And Decreases VCAM-1 Expression By Increasing Nrf2 Expression In The Aortic Endothelia Of Hypercholesterolemic Rabbits

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Abstract

Aqueous extract of purple sweet potato tuber are supposed to prevent oxidative stress and protect endothelial function by unknown mechanisms. The purpose of this study was to prove the ability of purple sweet potato tuber aqueous extract in preventing oxidative stress through increasing superoxide dismutase (SOD) via Nrf2 upregulation and decreasing VCAM-1 expression in rabbit aortic endothelium. This study was experimental laboratoric study, with randomized post-test only control group design. The rabbits were randomized into 3 groups (6 rabbits per group). The control group was treated with standard diet, treatment 1 was treated with high-cholesterol diet, and treatment 2 was treated with high-cholesterol diet plus aqueous extract of purple sweet potato tuber with the dose of 4 mL/kg bw/day. After 12 weeks of treatment, blood samples were obtained for the examination of SOD, MDA and total cholesterol level. The expression of Nrf2, SOD-2 and VCAM-1 in aortic endothelium was evaluated based on immunohistochemical methods. One-way ANOVA test were applied in data analysis, followed by path analysis. The results demonstrated significantly decreased MDA and increased blood SOD level, accompanied by statistically-significant increase in SOD-2 and Nrf2 expression, and decrease of VCAM-1 expression in the treatment group 2 compared to treatment group 1 ($p < 0.05$). It can be concluded that the purple sweet potato tuber aqueous extract prevents oxidative stress by increasing the expression of SOD-2, via Nrf2 protein up-regulation, resulting in decreased VCAM-1 expression in the aortic endothelia of high-cholesterol diet-fed rabbits.

Keywords: Purple sweet potato, oxidative stress, SOD-2, Nrf2, VCAM-1, rabbit aorta

1. Introduction

Oxidative stress may cause endothelial dysfunction and atherosclerosis. The link between atherosclerosis with oxidative stress and the role of antioxidant has been studied (Prior, 2003). The role of aqueous extract of purple sweet potato with high anthocyanin content as an antioxidant has been studied (Jawi *et al*., 2008), with a variety of unclear mechanisms (Ghosh and Konishi, 2007).

Anthocyanins can directly capture free radicals (Collins *et al*., 2003; Prior, 2003; Micallef *et al*., 2007), or increase endogenous antioxidants expressions through a variety of poorly-elucidated mechanisms (Johnson *et al*., 2009).

The most important endogenous antioxidant that is influenced by anthocyanins from different types of plants is the enzyme superoxide dismutase (SOD). Increased SOD expression by anthocyanins from certain plants, occurs via the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) (Han *et al*., 2007; Wang *et al*., 2010). The strength of anthocyanins in activating Nrf2 were highly varied, depending on the source of anthocyanins (Sterling, 2001). Activation of Nrf2 can also downregulate VCAM-1 expression, so that Nrf2 can inhibit the atherosclerosis (Chen *et al*., 2005).

The anthocyanin content of aqueous extract of purple sweet potato tuber is high (Suprapta et al., 2004), and anthocyanin has been proven as an antioxidant *in vitro* (Padda, 2006) and *in vivo* (Jawi *et al*., 2008; Jawi and Budiasa, 2011). The effect of purple sweet potato tuber-derived anthocyanin on the expression of SOD through Nrf2 activation has not been proven yet. Therefore, this study was aimed on proving the purple sweet potato tuber-derived anthocyanin antioxidative effects on SOD upregulation and anti-inflammatory effect by decreasing VCAM-1 expression in aortic endothelial of rabbits with oxidative stress induced by high-cholesterol diets for 90 days.

2. Materials and Methods

2.1. Animals Model

This study was an experimental laboratoric study, with randomized post-test only control group design. The samples in this study were 18 male local rabbits, 3-4 months of age, that were obtained from a local breeder in Bali. The samples were divided into 3 groups, with 6 rabbits per group. Group 1 was fed with standard diets as a negative control group. Group 2 was the group of rabbits given high-cholesterol diet, as a positive control

group. Group 3 was the group given high-cholesterol diet and also aqueous extract of purple sweet potato tubers with a dose of 4 mL/kg bw/day, by mixing with food, as the treatment group. The treatments were done for 12 weeks.

High-cholesterol diets for the rabbits were formulated in Pharmacology Department, Faculty of Medicine, Udayana University, consisting of a mixture of rabbit diets (Goldblend, with a composition of 12% water, 19% protein, 4% coarse crude fat, 5% crude fiber, 6.5% ash, 1.1% calcium, and 0.9% phosphorus), and 1 egg yolk per day for each rabbit.

2.2. Aqueous extract of purple sweet potato tubers

Aqueous extract of purple sweet potato tuber was made in the following manner: purple sweet potato tubers, 3-4 months of age, obtained from Balinese farmers, were washed with clean water and then peeled. Once peeled, the sweet potatoes are cut into chunks (thickness: 2 – 2.5 cm). The chunks were mixed with water at a ratio of 1 kg sweet potato to 1 liter of water and then blended and filtered with three layers of gauze. Filtrate obtained from screening was heated to boiling point. The content of anthocyanin of this material was 146 mg/mL.

2.3. Biochemical examination of blood

Post-test examinations were done after treatment for 90 days. Blood samples were taken from the veins in the rabbits' ears to quantify blood total cholesterol, SOD levels by RANDOX method, and MDA levels by TBARS method. The principle of RANDOX method is to look at the activity or rate of SOD in neutralizing superoxide ions formed during oxidative stress. This method uses xanthine and xanthine oxidase to form superoxide ions to be neutralized with a solution of INT that will produce a red color. SOD activity will be measured by the magnitude of the reaction barrier (expressed in U/g Hb).

2.4. Examination of endothelial Nrf2, SOD-2 and VCAM-1 expression in aorta

All rabbits were sacrificed by the administration of ether for anesthesia to obtain their aortae. Aortae were then identified and 5 cm long segments were sampled. The aortic segments, were soaked in PBS solution (pH 7.4) in sterile petri dish and stirred, so that the blood in the segments could be removed. Periaortic connective tissues and fats were also removed. Once cleaned, aortic segments were then dissected, making it easier to scrap the aortic endothelium with a scalpel. Scrapings were dissolved in PBS and centrifuged using a Sartorius Centrifuge 2-6E's, for 8 minutes at 2500 rpm. Supernatant was carefully removed, 0.25% trypsin solution (100 µL) was added into the remaining pellets. The solution was stirred until evenly mixed for 10 minutes to give time for complete separation of the endothelial cells from its anchorage. PBS (1.5 mL) solution was then added into the previous solution, stirred and centrifuged again for 5 minutes at 2500 rpm. Supernatant was discarded, and the pellet was mounted on polylysine-coated slide for histological examination.

Fixation with methanol PA was performed, and slide that had been fixed was immunocytochemically-stained with the following steps: Slide was washed with PBS and let stand for 5 minutes. After drained for 5 minutes, PBS was then discarded and treated with H_2O_2 for 5 minutes. The slides were washed with PBS 4 times and ultra V block (100 µL) was then added into the slide, and let the slide incubated for 5 minutes. After 5 minutes, the slides were washed with PBS, and dripped with 100 µL of rabbit Nrf2 antibody, rabbit SOD-2 antibody and rabbit VCAM-1 antibody (BIOSs) (diluted 1:200) (respectively for examination of Nrf2, SOD-2 and VCAM-1) and incubated for 1.5 hours in an incubator with a temperature of 25^0C. After 1.5 hours, the rest of the antibodies were removed and washed with PBS 4 times. Biotinylated goat anti-polyvalent (100µL) was further added and incubated for 5 minutes at room temperature. After 5 minutes washed with PBS 4 times, 100µL of streptavidin peroxidase was added and incubated for 5 minutes. Then, the slide was washed with PBS 4 times and added DAB, let stand for 5 minutes then washed with aquabides.

The last step was staining with counterstain and placing coverslip onto the slide. Slide was then ready to examined read under light microscope. Each slide was read in 10 field of view (40 times of magnification) with Olympus microscope (CX41 Olympus DP12, Philippines).

2.5. Statistical test

The data obtained were presented in mean ± SD. Data were then evaluated by Oneway-Anova, followed by path analysis. The results with p value <0.05 were accepted as statistically-significant results.

3. Results

3.1. Blood Total Cholesterol, SOD, and MDA Levels in Rabbits

The results of blood examination to investigate total cholesterol, SOD and MDA levels were presented in Table 1.

Table 1 Mean of Blood SOD, MDA and Total Cholesterol of The Rabbits

Groups	Mean±SD of T. Cholesterol (mg/dL)	Mean±SD of SOD (U/g Hb)	Mean±SD of MDA (mmol/L)
Control	101,200 ±2,00[a]	618,64 ±14,1[a]	0,99 ±0,1[a]
Treatment 1	212,400 ± 8,49[b]	395,48 ± 15,7[b]	8,22 ± 0,1[b]
Treatment 2	146,133 ± 4,34[a]	751,41 ± 23,7[c]	0,71 ± 0,1[c]

*Mean (n=6) followed by the same superscript letters within the same column are not significantly different according to least significance difference (LSD) at 5% level.
*Controls were standard diet-fed rabbits.
*Treatment 1 group was treated with high-cholesterol diet.
*Treatment 2 group was treated with high-cholesterol diet plus aqueous extract of purple sweet potato tuber.

Table 1, shows that there was significant decline in blood SOD level, increase in blood MDA level and total cholesterol ($p < 0.05$) in the group treated with high-cholesterol diet (treatment 1), compared to control group and the treatment 2. It was found that there was significant increase of blood SOD, decrease of blood MDA level and total cholesterol ($p<0.05$) in group given water extract of purple sweet potato tuber (treatment 2) compared with high-cholesterol diet-fed group. This study showed that hypercholesterolemia induced the increase of blood MDA level and decrease of blood SOD activity. Provision of aqueous extract of purple sweet potato tuber could prevent the increase of MDA and decrease of SOD activity due to hypercholesterolemia.

3.2. Nrf2 Protein and SOD-2 Expression in Aortic Endothelia of Rabbits

Microscopic examination (40 times of magnification, 5 fields of view) on the immunohistochemical preparations of aortic endothelial cells was done to investigate the expression of Nrf2 protein and SOD-2 in those cells. There results were presented in Table 2. These results indicate that the aqueous extract of purple sweet potato tuber can increase Nrf2 protein and SOD-2 expression in the aortic endothelial cells of high-cholesterol diet-fed. The photomicrographs of rabbit aortic endothelial cells treated with immunohistochemical methods using anti-Nrf2 and anti-SOD-2 monoclonal antibodies are presented in Figure 1 and Figure 2.

Table 2 Mean of Nrf2 and SOD-2 Expression in Aortic Endothelia of Rabbits

Groups	Mean±SD of Nrf2 (Cells/Field of View)	Mean±SD of SOD-2 (Cells/Field of View)
Control	13,8 ±0,8[a]	29,0 ±0,61[a]
Treatment 1	8,2 ± 1,2[b]	22,46 ± 1[b]
Treatment 2	23,5 ± 1,5[c]	34,76 ± 1,8[c]

*Mean (n=6) followed by the same superscript letters within the same column are not significantly different according to least significance difference (LSD) at 5% level.
*Controls were a standard diet-fed rabbits.
*Treatment 1 group was treated with high-cholesterol diet.
*Treatment 2 group was treated with high-cholesterol diet plus aqueous extract of purple sweet potato tuber.

Results of path analysis, the effects of aqueous extract of purple sweet potato tuber on the expression of SOD-2 in rabbit aortic endothelial, in general, the aqueous extract of purple sweet potato tuber increased SOD-2 expression in rabbit aortic endothelium with the total effect of 0.876, which consists of a direct effect of 0.237 and indirectly by 0.641, via Nrf2 up-regulation. The aqueous extract of purple sweet potato tuber also decrease the expression of VCAM-1 via Nrf2 up-regulation indirectly by -0,814.

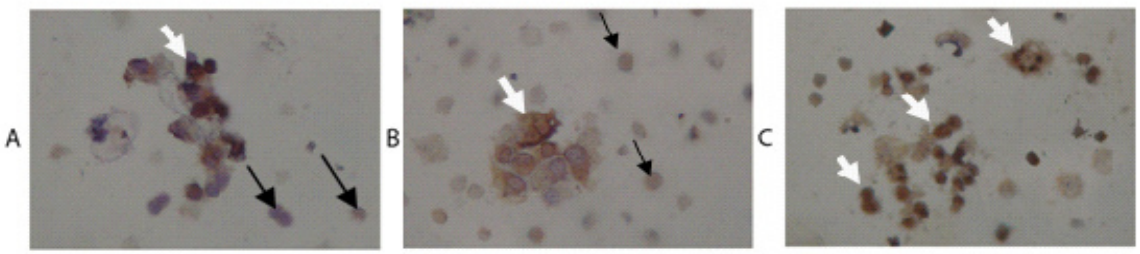

Figure description:

A. Control group, endothelial cells express the Nrf2 protein (white arrows), seen as brown color in the cytoplasm and nuclei of the cells, because of the Nrf2 localization in cytoplasm and nucleus. Endothelial cells that do not express Nrf2 have pale color (black arrows).

B. Treatment 1, a decrease in the number of endothelial expression of Nrf2, and more endothelial cells without Nrf2 (black arrows).

C. Treatment 2, endothelial cells appear to express Nrf2 with higher intensity than the control group.

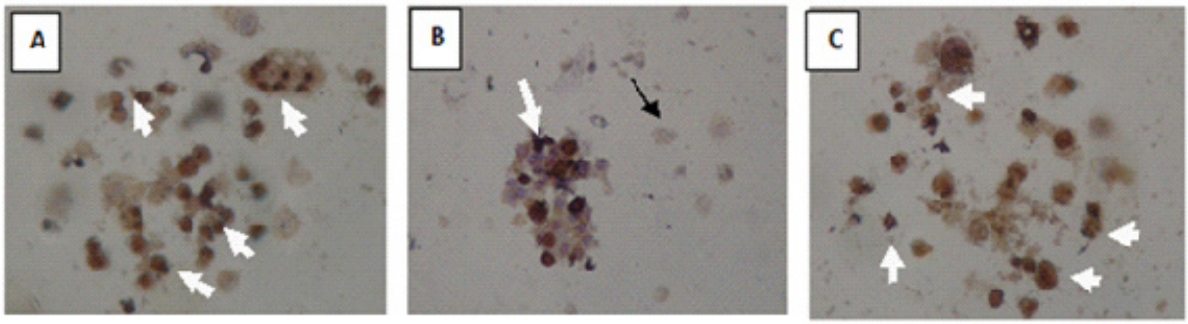

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Figure description:

A. Control group, it appears that some endothelial cells express SOD-2 (white arrows). The cytoplasms express brown colour, because SOD-2 is localized in the cytoplasms (the mitochondria). Endothelial cells that do not express SOD-2 are marked with black arrows, their cytoplasms appears pale in colour.

B. Treatment 1, there is a decrease in the number of SOD-2-expressing endothelial cells, and there are pale endothelial cells too.

C. Treatment 2, endothelial cells appear to have increased SOD-2 expression that is similar to those of the control group.

3.3. Aortic Endothelial VCAM-1 Expression

The results of light microscopic examinations (40 times of magnification, 5 field of view) for each of the sample is show in Table 2. The difference in VCAM-1 expression, elaborating the use of rabbit VCAM-1 antibody immunocytochemistry, is shown in Figure 6.

Table 3. Mean of Aortic Endothelial Cell and Mean of Aortic Endothelial Cells With VCAM-1 Expression of Rabbits

Groups	Mean±SD of Endothelial Cell (cells/5 fields of view)	Mean±SD of VCAM-1(cells/5 fields of view)
Control	66,5 ±2,00[a]	0,00 ±0,00[a]
Treatment 1	69,3 ± 2,49[a]	57,15 ± 2,7[b]
Treatment 2	67,6 ± 1,34[a]	9,3 ± 3,7[c]

*Mean (n=6) followed by the same superscript letters within the same column are not significantl different according to least significance difference (LSD) at 5% level.

*Controls were a standard diet-fed rabbits. *Treatment 1 group was treated with high-cholesterol diet. *Treatment 2 group was treated with high-cholesterol diet plus aqueous extract of purple sweet potato tuber.

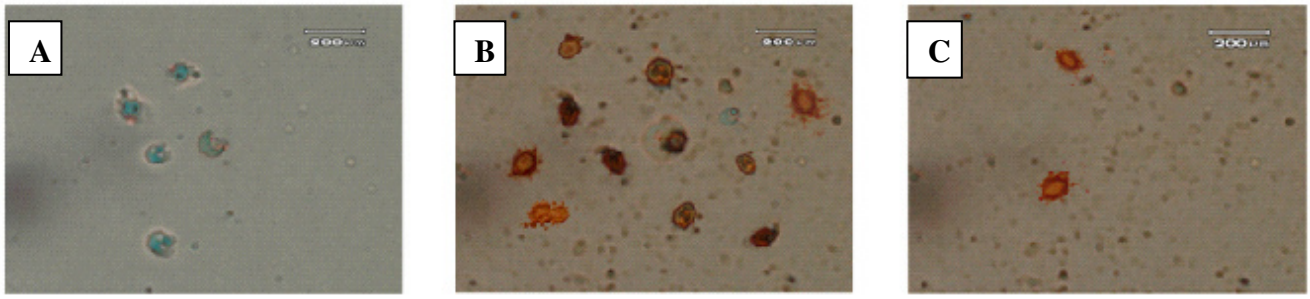

Figure 4.
VCAM-1-positive rabbit aortic endothelial cells in immunohystochemical studies using VCAM-1-specific monoclonal antibodies.

Figure description:

A. Control group, showed VCAM-1 negative (endothelial cells appeared blue).
B. Treatment 1 (Hypercholesterolemic group), showed VCAM-1 positive in most of endothelial cells, appeared brown.
C. Treatment 2 group, showed few endothelial cells with VCAM-1-positive (appeared brown).

4. Discussion

4.1. Blood Cholesterol, SOD and MDA Levels in the Rabbits

This study showed that blood MDA level increase significantly in hypercholesterolemic rabbits, because high level of blood total cholesterol or hypercholesterolemia may increase of reactive oxygen species (ROS) and cause oxidative stress (Cai and Harrison, 2000; Madamanchi *et al*., 2004). Increase of ROS such as superoxide ion could increase lipid peroxidation so can increase of blood MDA, as one of biomarkers of oxidative stress (Singhania *et al*.,2008; Abdelhalim, 2010). Treatment with aqueous extract of purple sweet potato tubers in this study also showed decrease of MDA level and increase of SOD activity in the blood significantly. The role of anthocyanin-rich aqueous extract of purple sweet potato tubers in this study (Suprapta, 2004; Lachman et al.,2009; Jiao et al.,2012), is a potent antioxidant (Kano *et al*., 2005; Padda, 2006; Jawi *et al*., 2008; Garcia-Alonso *et al*.,2009; Jawi and Budiasa, 2011). Anthocyanin could be an antioxidant that act via multiple mechanisms such as free-radicals scavenger (Lila, 2004), by increasing the range of antioxidant enzymes SOD, through the activation of the antioxidant response element (Shih *et al*., 2007). Anthocyanins may also interact with transition metal ions (act as chelating agent), such as with Fe and Cu, thus preventing the formation of free radicals (Gomes *et al.*, 2008).

The role of endothelial SOD in preventing oxidative stress is evident through diminished superoxide ion level. Giving SOD inhibitors can induce 2-folds increases in the superoxide ion level in the endothelium of blood vessels, thus SOD has essential role in preventing oxidative stress in blood vessels (Guzik *et al*., 2005). Flavonoids in certain foods can increase tissue SOD expression (Jeon *et al*., 2001; Jeon *et al*., 2002). Anthocyanins are a type of flavonoids, and it has been shown that anthocyanins from various plants can increase SOD and other antioxidants levels in the hepatic tissue (Kyu-Ho *et al*., 2006). Aqueous extract of purple sweet potato tubers in Bali is high in anthocyanin (Suprapta, 2004), thus the results are consistent with the findings that there is oxidative stress amelioration (MDA decrease) due to increase of SOD (Guzik *et al*., 2005; Kyu-Ho *et al*., 2006).

4.2. Nrf2 Protein and SOD-2 in Aortic Endothelial

In this study, high-cholesterol diet led to decreased expression of Nrf2 and SOD-2 (Mn-SOD/SOD-mitochondrial), on aortic endothelial observed by immunohistochemical staining, using Nrf2 and SOD-2 monoclonal antibodies, compared with the control group and the treatment 2 ($p < 0.05$). In the treatment 2 (the group given aqueous extract of purple sweet potato tuber) there was significant increase in the expression of Nrf2 and SOD-2 ($p < 0.05$) compared with the control group and the group treated with high-cholesterol diet (treatment 1).

SOD-2 is important in neutralizing superoxide ions there are formed in the mitochondria due to the mitochondrial electron transport chain, so that enzyme prevents oxidative stress and prevents mitochondrial dysfunction (Faraci and Didion, 2004). Role of SOD-2 enzymes in the blood vessels is estimated only 2% -12% of the total SOD, but is crucial in protecting the cell, because it is the first line of defense in preventing oxidative stress in mitochondria (Faraci and Didion, 2004; Heistad, 2006). The increase in endothelial superoxide ions caused by the presence of hypercholesterolemia would lead to an increase in mitochondrial superoxide ions, resulting in oxidative stress (Li and Shah, 2004). Oxidative stress in endothelial cells due to

hypercholesterolemia also causes an increase in peroxynitrite (ONOO-). Peroxynitrite will reduce SOD-2, thereby causing dysfunction of mitochondria, and increase in mitochondrial superoxide ions (MacMillan-Crow, Cruthirds, 2001).

Aqueous extract of purple sweet potato tuber containing anthocyanin (Suprapta, 2004), in this study, can prevent oxidative stress on aortic endothelial, resulting in increased expression of SOD-2, through activation of Nrf2 protein. Mild oxidative stress due to the provision of anthocyanins from aqueous extract of purple sweet potato tuber was able to break the bonds of Keap-1 protein with the Nrf2 protein which stimulate SOD-2 expression. In this study it was also proved that the increase of SOD-2 expression are the result of an increase in Nrf2 protein. Pathway analysis proved that the Nrf2 protein had a role in increasing the expression of SOD-2 by 3 folds compared to the increase through other pathway. So in this study it was proved that increase in the expression of SOD-2 in the aortic endothelial was facilitated by activating Nrf2 protein which is a transcription factor essential for the expression of antioxidant genes.

Genes of the 3 types of SOD (SOD-1, SOD-2 and SOD-3) are different, but the transcription factors that play a role in the expression of these three genes are almost the same Nrf2 protein. In this study, gene expression of SOD was measured as SOD enzyme in the blood that is SOD-3 and SOD-2 in the aortic endothelial which is the intracellular SOD. SOD-3 is an enzyme which is important in maintaining the vascular endothelium, thus endothelial function may be normal or protected from oxidative stress caused by superoxide ion (Lund *et al.*, 2009).

In addition to the role of various inducer to SOD gene transcription regulation, Nrf2 predominantly only affect SOD-3 and SOD-2 expressions, whereas its effect on SOD-1 is very low because the SOD-1 is a constitutive SOD (Miao and StClair, 2009).

4.3. Endothelial VCAM-1

Rabbits fed with high-cholesterol diets (treatment 1) showed higher expression of VCAM-1 in aortic endothelial cells significantly compared with treatment 2 and control group ($p < 0.05$). VCAM-1 is one of the adhesion molecules expressed by endothelial cells due to oxidative stress (Lee *et al*., 2001; Singhania *et al*., 2008; Hartvigsen *et al*., 2009; Zhiqing *et al*., 2010; Dong *et al*., 2011). Hypercholesterolemia in rabbits fed with high-cholesterol diets will cause oxidative stress, due to the activation of the enzyme NADPH oxidase, that increase ROS production (Madamanchi *et al*., 2004). Increased ROS will lead to an increase in the formation of uncoupled eNOS, that will enhance the formation of ions superoxide, resulting in impaired endothelial function (Stangl, 2010).

The increase of ROS caused by hypercholesterolemia, and increased superoxide ion will affect the endothelium and resulting in increased apoptosis, and increasing the expression of adhesion molecule VCAM-1 (Taniyama and Griendling, 2003; Gustavsson *et al*., 2010). These findings are consistent with studies in rabbits given high-cholesterol diets for 3 weeks. The diets increase the expression of VCAM-1 in the endothelial cell (Libby, 2003). Other researchers proved that there was increased VCAM-1 expression in the area of atherosclerotic artery in mice fed with high-cholesterol diets, compared with control mice. VCAM-1 was expressed on endothelial cell membrane after the mice were treated with high-cholesterol diet for one week. This study proved that the role of VCAM-1 is essential in early stage of atherogenesis, because VCAM-1 will stimulate rolling, strong adhesion of monocytes and lymphocytes to the endothelial area, that will have atherosclerosis (Dansky *et al*., 2001; Libby, 2003; Hansson, 2005). Increase in plasma cholesterol levels would increase the expression of VCAM-1 on endothelial cells, so there is a positive correlation between plasma cholesterol levels with the expression of VCAM-1 (Gustavsson *et al*., 2010).

Provision of aqueous extract of purple sweet potato tubers in this study was able to reduce the expression of VCAM-1 significantly ($p < 0.05$). Purple sweet potato tubers contain anthocyanin, which is a powerful antioxidant (Padda, 2006; Jawi *et al*., 2008), and can increase total antioxidant level (Jawi and Budiasa, 2011). Anthocyanins are also shown to increase endogenous antioxidants through activation of Nrf2 (Ping-Hsiao *et al*., 2007; Hwang *et al*., 2011). Anthocyanin found in purple sweet potato tuber water extract was able to reduce oxidative stress and improve endothelial function, thereby reducing the expression of VCAM-1 in this study. Protocatechuic acid, an anthocyanin metabolite, had been proven to reduce the expression of VCAM-1 and ICAM-1 in mice aortic endothelial cells (Wang *et al*., 2010). Anthocyanins may also act as anti-inflammatory agents by decreasing adhesion molecule genes expression via NF-κB inhibition (Shan *et al*., 2009; Speciale *et al*., 2010; Hwang *et al*., 2011), thereby reducing the expression of VCAM-1 (Xia *et al*., 2009).

5. Conclusion

Aqueous extract of purple sweet potato tubers increases blood SOD level and increases SOD-2 expression through Nrf2 protein upregulation in the aortic endothelial cells of high-cholesterol diet-fed rabbits. Therefore, the extract protects the endothelial cells of rabbits from superoxide-induced oxidative stress and decreases VCAM-1 expression in the aortic endothelial cells.

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