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RESEARCH ARTICLE

Comparison of the effects of ethanol extract with aqueous extract of purple sweet potato tuber in expression of SOD-2, SOD-3, and eNOS on human vascular endothelial cells exposed by H2O2 in-Vitro

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Abstract

Aqueous extract of purple sweet potato tubers have antioxidant potential in animal and human vascular endothelial, through increased expression of superoxide dismutase. This study aims to prove the comparison between ethanol extract and aqueous extract of purple sweet potato tubers increasing the expression of superoxide dismutase -2, superoxide dismutase -3, and endothelial nitric oxide synthase in human vascular endothelium. This study was an experimental study of the vascular endothelial cells which underwent oxidative stress in vitro, which is protected by ethanol extract or aqueous extract of purple sweet potato tuber at various concentrations. Observation of the superoxide dismutase -2, superoxide dismutase -3, and endothelial nitric oxide synthase in endothelial cells is done using the method immunohistochemistry with monoclonal antibodies. The results showed an increase in superoxide dismutase - 2, superoxide dismutase -3, and endothelial nitric oxide synthase was significantly ($p < 0.05$) in endothelium protected by ethanol extract or aqueous extract of purple sweet potato tubers. The ethanol extract only showed its effect at concentration of 1.5625 µg/ml for superoxide dismutase -2 and superoxide dismutase -3. The effect of aqueous extract were at a concentration of 1.5625–3.125µg/ml. From these results it can be concluded that the ethanol extract or aqueous extract of purple sweet potato tubers can protect endothelial cells from oxidative stress through increased expression of superoxide dismutase -2 and superoxide dismutase -3. The ethanol extract requires much lower concentrations to show its effect compared with aqueous extracts.

Keywords: *Aqueous extract or ethanol extract of purple sweet potato, Superoxide dismutase, Human umbilical vein endothelial cells.*

Introduction

Solvents or methods used in the extraction of active ingredients of certain plants affect the potential of the resulting extract. Potential purple sweet potato tuber extract is also determined by the solvent used in the process as well as the extraction method used [1,2].

Solvents commonly used in the extraction process are: aqueous, ethanol, methanol, or solvent mixtures. Each solvent will produce extracts with varying levels of active ingredients so that the biological effects and potential generated also varies.

Anthocyanin is the active substance which is substantially present in purple sweet potato tubers [3] which is the pigment that are polar and will dissolve well in polar solvents. Results of research conducted with various solvents used, and extraction with ethanol gives the highest anthocyanin content than aqueous and other solvents [4]. Although almost all of the purple sweet potato tuber extract has good antioxidant properties, the ethanol extract demonstrated the ability to capture the highest free radical with 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method. Antioxidant potential of ethanol extract is 6 times higher than the aqueous extract [5].

In-vivo studies also demonstrated that potential ethanol extract of purple sweet potato tubers in preventing the influence of ultraviolet rays on the skin is better than the aqueous extract [6].

The difference between the aqueous extract with ethanol extract of purple sweet potato tuber in Bali is unknown, although the aqueous extracts were proven to increase Superoxide Dismutase (SOD) of endothelium [7] which raised the issue of whether the effect of ethanolic extract is better than the aqueous extract in enhancing the expression of SOD on human vascular endothelium in vitro.

Materials and Method

This study was an experimental study with post-test only control group design. The study was conducted in cultured human umbilical vein endothelial cells (HUVEC), with aqueous extract and ethanolic extract of purple sweet potato tuber (Ipoema batatas L.) as the test material. Observations on the expression of SOD-2, SOD-3 and, Endothelial Nitric Oxide Synthase (eNOS) were performed based on immunohistochemical method with monoclonal antibodies of SOD- 2, SOD-3 and, eNOS.

The aqueous or ethanolic dried extract were diluted by RPMI 1640 medium to obtain concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5625 µg/ml. The expression of SOD2, SOD-3, and eNOS were performed by using a single 96 micro plate wells. The HUVECs were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U / mL penicillin and 100 µg/mL streptomycin in a humidified incubator under 5% CO2 at 37°C. Briefly, cells at the mid-log phase were seeded in a 96-well plate at a density of 104 cells per well in 100 µL medium. RPMI1640 medium was added to the control and model groups. Then the original culture medium was removed and cells were washed with PBS twice. The antioxidant activity was evaluated by using HUVEC injury model induced by H2O2.

Cells were treated with H2O2 (1.250 µmol/L) for 2 hours. Then, 20 µL of MTT solution (5 mg/mL) was added to each well. After the evaluation of cell culture, the live cells in the cultures were taken back into the media given the concentration of H2O2 with 1.250 mol/L as a model of oxidative stress and given the test material with a concentration of aqueous or ethanolic extract of purple sweet potato tuber, were 50, 25, 12.5, 6.25, 3.125, and 1.5625µg/ml respectively, then incubated for 24 hours. After 24 h, live cells from each of the wells were taken with a special pipette and dripped and blotted on glass object to be painted with immunohistochemical techniques.

The slides were fixed for immunocytochemistry staining performed with the following steps. First the slides were washed with PBS and let stand for 5 minutes and the PBS was discarded and added with H2O2 and kept for 5 minutes. The slides were washed with PBS 4 times and then were dropped into 100 mL of Ultra V Block, and incubated for 5 minutes.

The slide was washed with PBS, and dropped with eNOS antibody, SOD-2, and SOD-3 (BIOS, USA) which were diluted in the ratio of 1: 200, respectively and incubated for 1.5 h in an incubator with a temperature of 250C. The remaining antibody was removed and washed with PBS 4 times. This slide was added with 100 µl Biotinylated Goat Anti-Polyvalent and incubated for 5 minutes at room temperature. The slide was washed with PBS 4 times and added with 100 µl Streptavidin Peroxidase and incubated for 5 minutes. Afterward, it was washed with PBS 4 times and added to the DAB for 5 minutes and then washed with aqua bidest.

The last step was staining with Meyer's Hematoxylin as counterstaining and covered with a coverslip. Slides are ready to be observed under microscope (Olympus CX41, Tokyo, Japan). On each slide 5 fields of views were observed with 40 times magnification. The data obtained were tested by ANOVA to determine the differences between groups. To prove the existence of the endothelium in the smear, observation was done in an inverted microscope with camera (Olympus DP 12, Tokyo, Japan).

Results

This study show the results of eNOS examination did not differ between the groups that were given the ethanol extract and aqueous extract at a concentration of 1.5625 microgram/ml. In the concentration of 3.125 microgram, the ethanol extract showed higher expression of eNOS significantly, but lower than the control group ($p < 0.05$).

The results of the examination of SOD-2 and SOD-3 showed a significant difference between the control group and a group given treatment at concentrations of purple sweet potato tuber extract at 1.5625 mg/ml–3.125 g/ml. Results of average calculation of endothelial cells that express eNOS, SOD-2, and SOD-3 are presented in Table 1 and Figure 1–5.

Table 1. Average Cells expressing SOD-2, SOD-3 and eNOS in Culture of Endothelial Cell In-vitro

Variabel	Control	H2O2	Ethanol Extract 1.5625 ug / ml.	Aqueous Extract 1,5625 µg/ml.	Ethanol Extract 3,125µg/ml.	Aqueous Extract 3,125µg/ml.
SOD-2	31 ±7,9	0	51,80±12,65	18,60±16,6	1,4±1,1	40,60±10
SOD-3	24,4± 13	2± 2,8	17,60±11	43,60 ± 4,2	9,40±5,7	36,20±6,8
eNOS	28,4± 1,1	0	30,20± 7,5	32,40± 1,3	21,20±5	1,2±0,8

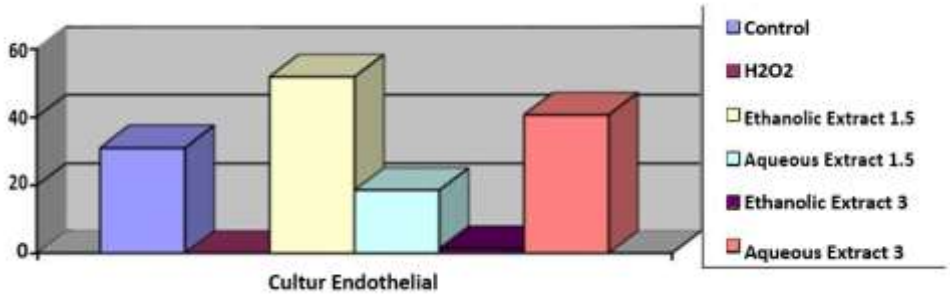


Figure 1 :Comparison of average SOD-2 Expression of Endothelial Cells with Ethanol Extract or Aqueous Extract at concentration of 1.5625 µg/ml and 3,125 µg/ml

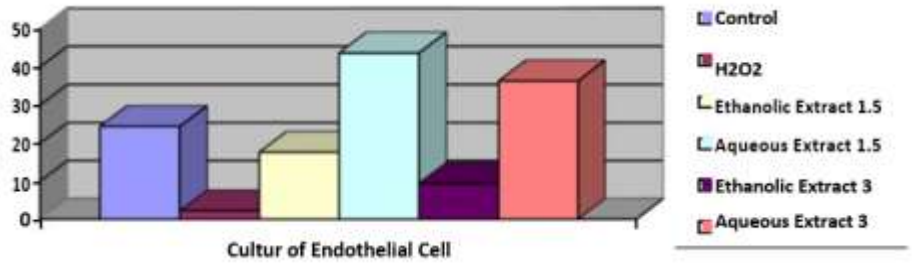
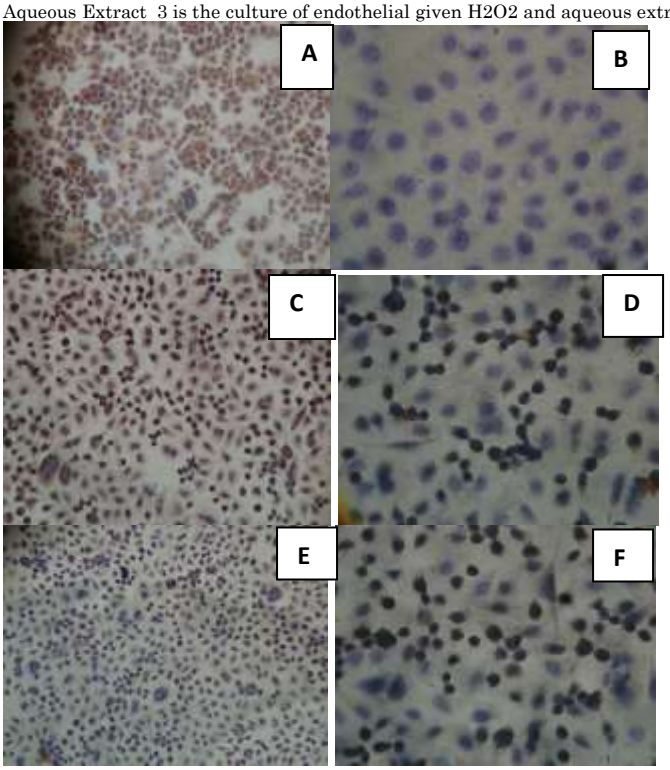


Figure 2: Comparison of average SOD-3 Expression of Endothelial Cells with Ethanol Extract or Aqueous Extract at concentration of 1.5625 µg/ml and 3,125 µg/ml

Caption:
Control is the culture of endothelial without treatment.
H2O2 is the culture of endothelial given H2O2 in the media (Oxidative Stress). Ethanolic Extract 1,5 is the culture of endothelial given H2O2 and ethanol extract of purple sweet potato tuber with a concentration of 1.5625 µg/ml.
Aqueous Extract 1,5 is the culture of endothelial given H2O2 and aqueous extract of purple sweet potato tuber with a concentration of 1.5625 µg/ml.
Ethanolic Extract 3 is the culture of endothelial given H2O2 and ethanol extract of purple sweet potato tuber with a concentration of 3.125 µg/ml.
Aqueous Extract 3 is the culture of endothelial given H2O2 and aqueous extract of purple sweet potato tuber with a concentration of 3.125 µg/ml.

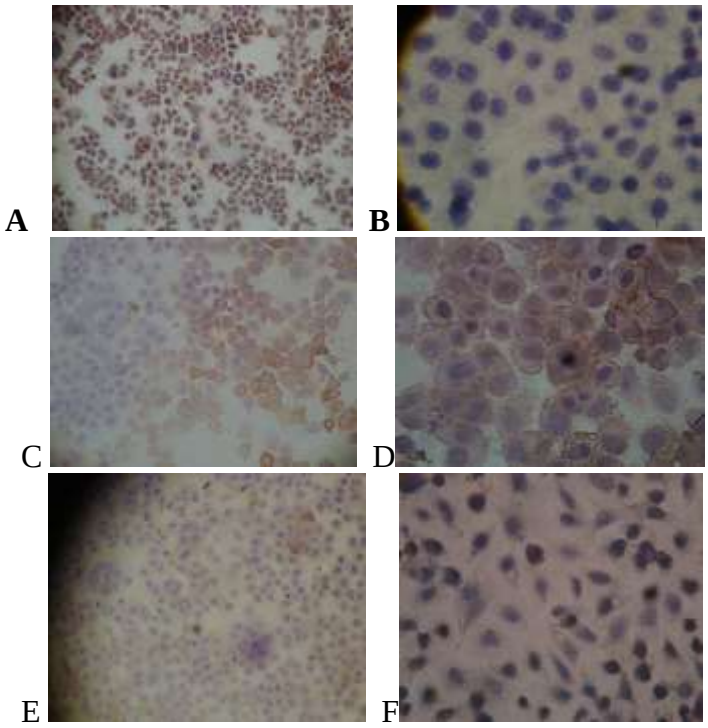


Caption:
A. Endothelial control, endothelial cells appear to express the SOD-2 positive with the color brown.
B.Endothelial given H2O2 (Oxidative Stress), cells appear pale blue, decrease of SOD-2 expression.
C. The endothelial cells given the low concentration of ethanol extract, seen an increase in SOD-2 in endothelial cells.

D. Endothelial given aqueous extract of low concentration occurs significant improvement.

E Endothelial given the concentration of ethanol extract 3.125, visible reduction in SOD2. F. Endothelial given water extract concentration of 3.125, appears to increase SOD2

Figure 3: Endothelial cells with SOD-2 Positive by immunocytochemistry



Caption:
A. Endothelial control, endothelial cells appear to express the SOD-3 positive with the brown color.
B.Endothelial given H2O2 (Oxidative Stress), cells appear pale blue, decrease of SOD-3 expression.
C. The endothelial cells given the low concentration of ethanol extract, seen an increase in SOD-3 in endothelial cells.
D. Endothelial given aqueous extract of low concentration occurs significant improvement.
E Endothelial given the concentration of ethanol extract 3.125, visible reduction in SOD3.
F. Endothelial given water extract concentration of 3.125, appears to increase SOD3

Figure 4: Endothelial cells with SOD-3 Positive by immunocytochemistry

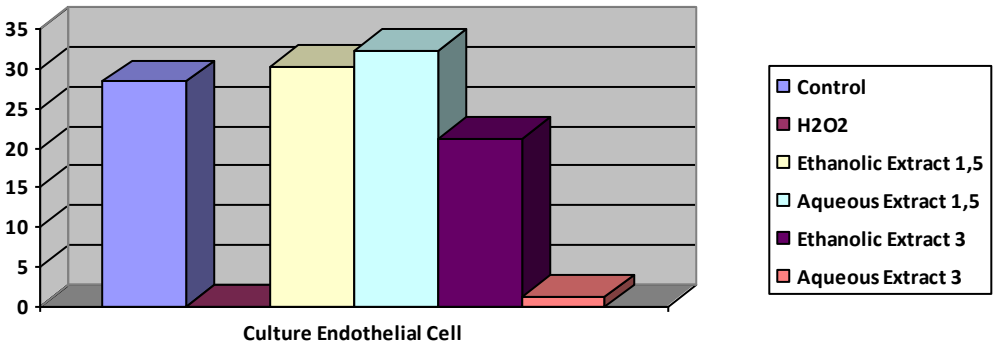


Figure 5: Comparison of average eNOS Expression of Endothelial Cells with Ethanol Extract or Aqueous Extract at a concentration of 1.5625 µg/ml and 3,125 µg/ml.
Caption:
Control is the culture of endothelial without treatment.
H2O2 is the culture of endothelial given H2O2 in the media (Oxidative Stress). Ethanolic Extract 1,5 is the culture of endothelial given H2O2 and ethanol extract of purple sweet potato tuber with a concentration of 1.5625 µg/ml.
Aqueous Extract 1,5 is the culture of endothelial given H2O2 and aqueous extract of purple sweet potato tuber with a concentration of 1.5625 µg/ml.
Ethanolic Extract 3 is the culture of endothelial given H2O2 and ethanol extract of purple sweet potato tuber with a concentration of 3.125 µg/ml.
Aqueous Extract 3 is the culture of endothelial given H2O2 and aqueous extract of purple sweet potato tuber with a concentration of 3.125 µg/ml.

Discussion

The expression of SOD-2, SOD-3, and eNOS in culture of endothelial cell decreases significantly after the administration of H2O2 for 24 hours. The aqueous extract or ethanol extract of purple sweet potato tubers can sustain the expression of SOD-2 and SOD-3 significantly at a concentration of 1.5625 microgram/ml–3.125 microgram / ml. The expression of eNOS can only be maintained at the concentration of aqueous extract of 1.5625 microgram/ml while the ethanol extract at a concentration of 1.5625–3.125 microgram/ml.

Giving H2O2 to the endothelium caused oxidative stress, resulting in decrease of SOD and eNOS, because H2O2 will cause the activation of NADPH oxidase, causing increased superoxide ion in endothelial cells [8]. Increased superoxide ions from application of H2O2 is accompanied by the activation of oxidant-generating enzymes that would otherwise be causing endothelial disfunction or disorders [9].

The aqueous extract or ethanol extract of purple sweet potato tuber can sustain expression of the SOD-2 and SOD-3 due to the

antioxidant properties of flavonoids such as anthocyanins [10,11,12]. As a powerful antioxidant in vitro [12, 13] and in-vivo. Anthocyanins can act as an antioxidant with a variety of mechanisms, for example through the effects of free-radical scavenging [14], with the increasing variety of antioxidant enzymes such as SOD-2 and SOD-3, through the activation of antioxidant response element [15]. Anthocyanins also can interact with transition metal ions (chelating) such as with Fe and Cu, thus preventing the formation of free radicals [16]. In this study eNOS increased at a low dose of the extract. Increased eNOS occurs through several mechanisms: redox changes in cells, as well as the increase of calcium in cells via activation of estrogen receptor [17].

The ethanol extract in this study at lower doses had increase of SOD. The ethanol extract is more potent because ethanol can attract active ingredient higher than other solvents [18-20], thus increasing SOD higher than the same dose of aqueous extract.

So the increase in SOD in the endothelium given aqueous extract or ethanol extract of purple sweet potato tubers in this study, is due to the antioxidant properties of anthocyanins comprising cyanidin and peonidin [21]. SOD is an endogenous antioxidant that is strong enough in outlining the superoxide ions, thereby reducing oxidative stress [22].

In all mammalian tissues, there are three types of SOD including the endothelium of blood vessels, which acts to overcome oxidative stress is mainly caused by superoxide ion. The three types of SOD are SOD-1 (Cu, Zn–SOD), SOD-2 (Mn–SOD) and SOD-3 (Cu, Zn–SOD). The SOD-3 is found in the extracellular fluid, on the surface of the endothelium and in the blood, has the greatest role in maintaining the function of the vsacular endothelium [23-25], while SOD-1 and SOD-2 are also present in cells their role in maintaining endothelial function is smaller than the role of SOD-3 [23-25].

The results are consistent with studies in mice given a purple yam extract increased expression of SOD in liver tissue of mice [26]. The results of this study are also consistent with studies conducted with anthocyanins from black rice can reduce superoxide ion and increase SOD in cells in vitro [27]. The results are consistent with studies of Blackberry extract containing flavonoids can protect endothelial cells from the umbilical influence of H2O2 in vitro, through increased production of SOD [28].

Conclusions

Ethanol extract of purple sweet potato tuber at low doses was better than aqueous extract in increasing the expression of SOD-2, SOD-3, and eNOS of human vascular endothelium that was exposed to H2O2 in vitro. At higher doses aqueous extract was better than ethanol extract in improving the expression of SOD-2 and SOD-3 in human vascular endothelium that was exposed to H2O2 in vitro

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