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Research Article

Antioxidant Activity of Unripe Sapodilla Fruit Extract (*Manilkara zapota* L.) through Nrf2 and SOD Expression in Type 1 Diabetic Mice

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ABSTRACT

This research aims to analyze the effect of unripe sapodilla fruit extract on endogenous antioxidant expression in T1DM BALB/c mice and its free radical scavenging activity. *Manilkara zapota* extract (MzE) is an aqueous extract of unripe sapodilla fruit and was obtained by maceration and freeze-drying process. This study used 25 male BALB/c mice with 7-weeks-old of age. They were divided randomly into five groups (n=5) before treatment. A single high dose (145 mg/kg BW) of streptozotocin (STZ) was intraperitoneally injected to induce type 1 diabetes mellitus (T1DM). MzE was given orally once each day for 14 days. Liver cells were isolated and immunoassay with anti-superoxide dismutase (SOD) and anti-nuclear factor erythroid 2-related Factor 2 (Nrf2), and then the results were analyzed by flow cytometry. Diphenylpicrylhydrazyl (DPPH) assay was performed to analyze free radical scavenging. Data were analyzed statistically with one-way ANOVA ($p<0.05$). The result showed that the glucose levels in diabetic mice after MzE administration were significantly lower than in the DM group. MzE treatment increased the expression of Nrf2 and SOD in diabetic mice. MzE could scavenge DPPH with the IC50 value obtained at 48.35 µg/mL, while ascorbic acid as a control could scavenge DPPH with the IC50 value at 22.24 µg/mL. The increase in the scavenging activity is in line with the increase in extract concentration. In conclusion, this study revealed that MzE can be an endogenous antioxidant enhancer by improving the expression of Nrf-2, SOD and can inhibit free radicals as an exogenous antioxidant in T1DM.

Keywords: Antioxidant, Free radical, Nrf2, SOD, Manilkara zapota

Introduction

Around 10.7 million people in Indonesia have diabetes mellitus (DM), according to data from the Ministry of Health in 2020. This number is predicted to increase to 16.6 million by 2045. This shows that DM is one of the most well-known metabolic disorders in the world. DM occurs because of a glucose metabolism disorder that causes an increase in blood glucose levels higher than average (hyperglycemia). One of the types of DM is type 1 diabetes mellitus (T1DM), which occurs because of pancreatic β-cell destruction that produces insulin to control glucose metabolism [1].

Reactive oxygen species (ROS) generation will rise in DM patients with hyperglycemia [2]. Oxidative stress is brought on by an irregular rise in ROS that is not counterbalanced by an increase in antioxidants. ROS are very reactive and can easily interact with biomolecules to disrupt cell structure and function [3]. High generation and levels of ROS will activate the nuclear factor kappa B (NF-κB) transcription factor, which will cause the release of pro-inflammatory cytokines [4].

One of the most significant endogenous antioxidants is superoxide dismutase (SOD). This en-

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dogenous antioxidant overcomes oxidative damage throughout the body. However, based on previous research, SOD expression in diabetes mellitus patients is known to be decreased, which in turn may worsen the oxidative stress conditions in diabetes mellitus [5]. In addition, a study with animals lacking Nuclear Factor Erythroid 2-related Factor 2 (Nrf2) revealed an increase in oxidative stress and blood glucose levels [6]. The transcription of detoxification enzymes such as Heme Oxygenase-1 (HO-1) and SOD are known to be induced by the transcription factor Nrf2, which has an important role in reducing ROS [7]. Therefore, SOD and Nrf2 expression are essential to maintain homeostasis condition.

Sapodilla (*Manilkara zapota* L.) is a plant commonly found in tropical countries such as Indonesia. Sapodilla fruit is known to exhibit antioxidant activities. Unripe and ripe sapodilla contains polyphenol compounds such as quercetin, catechin, epicatechin, gallocatechin, gallic acid, dihydromyricetin, and chlorogenic acid [8, 9]. These phytochemical compounds have antioxidant activities to decrease autooxidation from free radicals and inhibit lipid peroxidation [8]. Moreover, compared to ripe sapodilla fruit, the unripe sapodilla fruit contains proanthocyanidin, a compound known to have high antioxidant capabilities. Since the sapodilla fruit used in this study is unripe, it contains higher concentrations of polyphenols than ripe sapodilla fruits [10], and the polyphenol concentration will decrease with fruit ripening [11].

This study was conducted as scientific evidence related to the potential of unripe sapodilla fruit aqueous extract as an alternative therapy for T1DM. This study aims to analyze the effect of *Manilkara zapota* aqueous extract (MzE) on endogenous antioxidant expression in the T1DM model of BALB/c mice and its free radical scavenging activity.

Material and Methods

Herb extraction

Unripe sapodilla fruits (*Manilkara zapota*) (specimen number: 074/223A/102.7/2020) were collected from Nganjuk city. The samples were turned into powder and determined by Materia Medica, Batu City, Indonesia. Sapodilla fruit was extracted by dissolving its powder form with distilled water using a 1:10 ratio (herb: solvent, w/v). Homogenization was accomplished using a magnetic stirrer within the rotary shaker at 1080 rpm for 24 h at 25˚C. Whatman no. 1 paper was used to filter the residue to separate the water-soluble bioactive components. Freeze-drying was done to isolate the compounds.

Experimental design

The Animal Care and Use Committee at Brawijaya University assessed and approved each step of this experiment (Ethic No. 016-KEP-UB-2021). Male BALB/c mice 7-week-old (n=25) were used in this experiment, and they were obtained from Maulana Malik Ibrahim State Islamic University in Malang, East Java, Indonesia. All mice were housed in a pathogen-free facility in the biology department of Brawijaya University, supplied with a nutritional pellet diet and enough water. The mice were divided into five randomized groups after a week of acclimatization.

Normal mice (N) was a group of mice that were neither exposed to streptozotocin (STZ) nor given any MzE. The diabetic mice group (DM) was induced by STZ injection (BioWorld, USA). In treatment groups, mice were given injections of STZ before receiving one of three doses of MzE: 150 mg/kg BW (DM-D1), 250 mg/kg BW (DM-D2), and 400 mg/kg BW (DM-D3) (n=5 mice for each group). For 14 days, MzE was given orally once per day. Glucometer (EasyTouch) was used to measure body weight and blood sugar levels.

Induction of Diabetes Model

An injection of single high-dose streptozotocin (STZ) was used to induce type 1 diabetes mellitus (T1DM) (BioWorld, USA). STZ was intraperitoneally given at a dose of 145 mg/kg BW after a week of acclimatization. Before receiving the STZ injection, mice were fasted for 4 hours. STZ was dissolved in a citrate buffer with a pH of 4.5 for 10 minutes of usage. Four days following the injection of STZ, blood glucose levels were assessed using an Easy Touch glucometer (Bioptik Technology Inc., Taiwan). When the mice's blood glucose level was more than 200 mg/dL, they were classified as having T1DM.

Immunostaining and flow cytometry

The antibodies used in this study include anti-SOD-PerCP and anti-Nrf2-PerCP (BioLegend, USA). Phosphate buffer saline (PBS) was used to homogenize the isolated hepatocyte cells. The

cells were centrifuged at 2500 rpm, 10˚C for 10 min. As much as 1 mL of PBS was used to resuspend the obtained pellet. Then, 50 µl of the cell suspension was added to the microtube before being incubated with 50 µl of CytofixTM. The suspension was incubated at 4˚C for 20 minutes. A Wash buffer solution (BioLegend, USA) was added for permeabilization.

The cells were stained with intracellular antibodies (anti-SOD and anti-Nrf2) after centrifugation and then 400 µl of PBS was added to the sample for flow cytometry analysis (BD FACSCalibur, USA).

DPPH radical scavenging assay

The scavenging ability of MzE against free radicals was measured using 2,2-diphenylpicrylhydrazyl (DPPH) assay. MzE was divided in four different concentrations; 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL. Ascorbic acid was dissolved with water and used as a comparison in this assay. Ascorbic acid was divided with the same concentration as MzE; 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL. One hundred microliters of MzE and ascorbic acid were transferred into 96 well plates. This experiment was carried out in three replications (n=3) at each concentration. DPPH with 0.4 mM concentration was dissolved in ethanol. DPPH (100 µl) was added into the MzE and ascorbic acid well. The control solution was made by mixing water and 0.4 mM DPPH (1:1). A blank solution was made by mixing water and ethanol (1:1). Control and blank solution were transferred into a96 well plates with three replications (n=3). The 96 well plate was incubated for 30 minutes at room temperature and in a darkened room after adding DPPH. An ELISA reader with a 490 nm wavelength was used to read the outcome. The equation used to compute the percentage of DPPH radical scavenging activity is as follows:

$$\%\text{scavenging activity} = \frac{A_0 - A_1}{A_0} \times 100 \ldots (1)$$

A_0 is the absorbance of the control, while A_1 is the absorbance of the sample extract. Then % of inhibition was plotted against concentration (µg/mL), and from the regression value, IC_{50} was calculated.

Statistical analysis

Statistical analysis was conducted using SPSS 21 software for the analysis of variance (ANOVA) test with the Post Hoc Duncan Test. A value of $p<0.05$ was used in this investigation to determine whether the difference between groups was significant. The mean and standard deviation (SD) of all data was displayed.

Results and Discussion

STZ-injected mice succeeded as animal model of DM, which was showed by a significantly higher than normal initial glucose levels at 425 mg/dL (Figure 1). The levels of blood glucose in normal mice group were 135 mg/dL and remained consistently in the normal range for 14 days. The administration MzE successfully decrease glucose levels in all treatment groups after 14 days of treatment. MzE effectively reduced blood glucose levels in the DM-D1 and DM-D2 groups, were 285 mg/dL and 291 mg/dL, respectively. The decrease

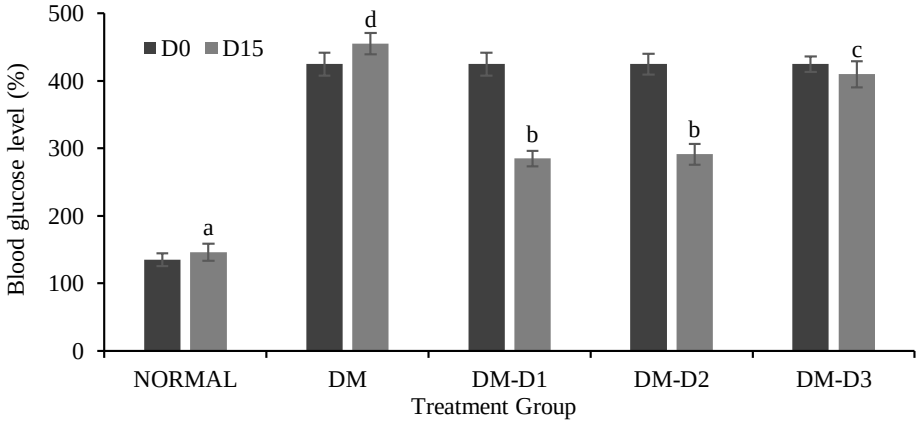
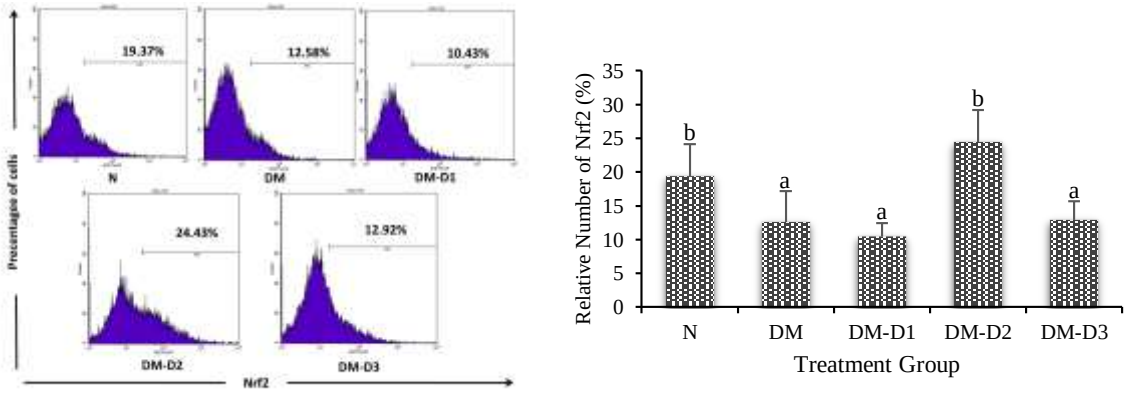


Figure 1. Blood glucose level before and after the treatment of MzE. The data are mean value ± SD of five mice in each group with a significant value $p<0.05$ (n=25).

in blood glucose levels occurred significantly compared to the DM group with blood glucose levels of 455 mg/dL. However, the decrease in blood glucose levels in DM-D1 and DM-D2 groups had not reached normal blood glucose. In the DM-D3 group, the blood glucose level after 14 days of treatment was at 410 mg/dL with no significant difference from the DM group, and it was also significantly different from the other groups.

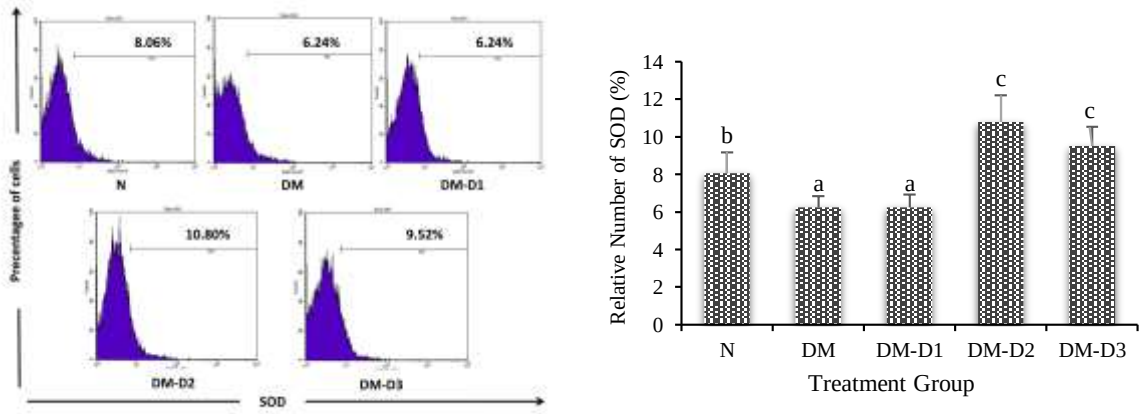
The decrease in blood glucose levels after treatment was related to antioxidant activity in diabetic mice. Antioxidant activity will control the ROS and reduce oxidative stress in diabetic mice [12]. MzE could induce antioxidant production and improve antioxidant activity through phenolic and flavonoid compounds. Phenolic and flavonoid compounds have a role in inducing insulin production that will reduce glucose levels in diabetic mice [13]. The phenolic and flavonoids also repair cell damage and reduce damage from oxidative stress and establish homeostasis in the body [14].

The expression of endogenous antioxidants in diabetic mice was correlated with Nrf2 and SOD expression levels (Figure 2 and Figure 3). The MzE has given antioxidant effects in diabetic mice by increasing the activity of Nrf2 and endogenous antioxidants such as SOD. The Nrf2 expression levels in the DM group was significantly reduced from 19.37% to 12.58% ($p<0.05$) compared to normal mice (Figure 2). The normal level had not been reached in the MzE-treated diabetic mice in DM-D1 (10.43%) and DM-D3 (11.84%), and they did not differ substantially from the DM group ($p<0.05$). The DM-D2 (24.43%) group showed increased expression of Nrf2 level higher than the DM group. The level of Nrf2 expression has



(a) (b)

Figure 2. The relative number of Nrf2 expressing cells in each mice group. (a). A plot of flow cytometry analysis and (b). Results of statistical analysis. The data are mean value ± SD of five mice in each group with a significant value $p<0.05$ (n=25).



(a) (b)

Figure 3. The relative number of SOD-expressing cells in each mice group. (a). A plot of flow cytometry analysis and (b). Results of statistical analysis. The data are mean value ± SD of five mice in each group with a significant value $p<0.05$ (n=25).

matched that of the normal group, and there is no discernible difference between the two groups ($p<0.05$).

The SOD expression was also elevated in diabetic mice after MzE treatment. SOD expression in the DM group was significantly lower than the normal group ($p<0.05$), decreasing from 8.06% to 6.24% (Figure 3). The SOD expression in DM-D2 (10.80%) and DM-D3 (9.52%) was markedly increased than normal and DM group after 14 days MzE administration. In the lowest dose of MzE treatment, SOD expression in the DM-D1 group did not increase, and it was not significantly different from the DM group ($p<0.05$). These findings suggested that MzE increased endogenous antioxidant activity in diabetic mice, which had an antioxidant effect. Due to its ability to raise Nrf2 and SOD expression, the dose used in the DM-D2 group has the strongest antioxidant impact.

Sapodilla contains phytochemical compounds with high antioxidant activity, including flavonoid and non-flavonoid compounds. Using phytochemical compounds from various compounds contained in plants is the right strategy for preventing and treating diabetes [15]. According to a previous study, unripe sapodilla is rich in flavonoids and polyphenols, especially tannin [10]. Tannin contents within the extract could increase endogenous antioxidant activity by restoring enzymatic antioxidant activity [16]. The endogenous antioxidant activity in this research was shown by the increased activity of SOD and Nrf2 as a transcription factors.

In order to reduce the generation of ROS, Nrf2 modulates the activity of endogenous antioxidants, phase II detoxifying enzymes, and other proteins involved in xenobiotic detoxification. The increase in heme oxygenase-1 (HO-1), (NAD(P)H quinone reductase 1) (NQO-1), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) is in line with the increase in Nrf2, a transcription and signaling factor for producing those enzymes [17]. Nrf2 expression will be increased and activated in normal conditions after exposure to ROS or oxidative stress conditions. Nrf2 is activated by changing cysteine residues from the Keap1 protein. This transcription factor will release and translocate to the nucleus, where it will bind to the antioxidant response element (ARE) with the assistance of a small Maf (sMaf) protein. Besides, Nrf2 promotes the transcription of detoxifying enzymes and antioxidants such as HO-1 and SOD to suppress ROS in the body [7]. However, there was a decrease in Nrf2 expression in the group of DM mice. The decreasing expression level of Nrf2 will cause a reduction in endogenous antioxidants and enhance ROS production. As a result, high ROS levels cause tissue damage and worsen the condition of hyperglycemia [18]. In this study, the increase of Nrf2 after MzE administration has shown the ability of MzE compounds to increase the activation of Nrf2 expression (Figure 2). Nrf2 activation increases the ability of the body to produce endogenous antioxidants and lower ROS production [12]. The use of Nrf2 activation could be the strategy to prevent and slow down organ damage in diabetic conditions [19].

SOD activity is needed to reduce superoxide and has a protective role in preventing tissue damage that causes the overproduction of ROS [20]. In this study, the SOD expression in diabetic mice was decreased. The decreased SOD expression is one of the markers of diabetes and various complication of diabetes [21]. The decreasing expression of SOD activity leads to increased ROS in the body, such as superoxide and other hydroperoxides that cause oxidative stress [18]. Based on that case, SOD should be enhanced to prevent oxidative stress and complications effect from diabetes. In this study, the increase of SOD after the administration of MzE has shown the ability of MzE compounds that could increase the activation of SOD (Figure 3). The expression of SOD could be enhanced by active compounds that can scavenge and increase the expression of SOD to reduce ROS levels. The MzE has active compounds that could increase antioxidant levels, such as tannin and chlorogenic acid. Tannin can restore enzymatic antioxidant activity [16] and chlorogenic acid can withstand the decrease of SOD expression [22].

Based on this study, an analysis of the DPPH scavenging ability of MzE with ascorbic acid as a control was carried out. The results showed that MzE could scavenge DPPH at a concentration of 0.4 mM. The DPPH scavenging activity of MzE and ascorbic acid was analogous to the increasing concentration of the samples. The scavenging activity of sapodilla extract and standard ascorbic acid were 67.17% and 79.64%, respectively. The IC_{50} from MzE extract was obtained at 48.35 μg/mL, and the IC_{50} from ascorbic acid was obtained at 22.24 μg/mL (Figure 4).

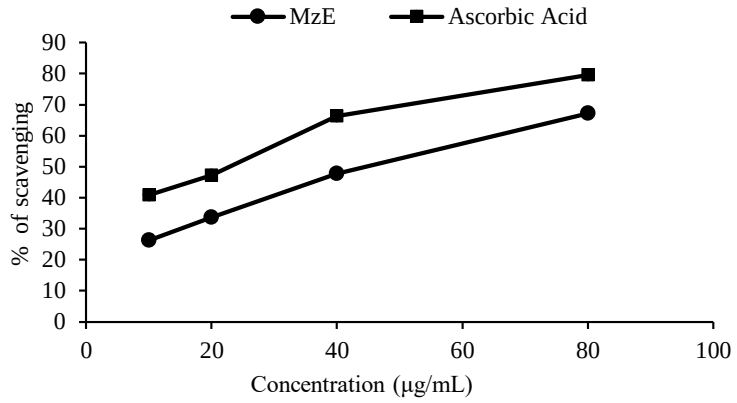


Figure 4. The DPPH scavenging activity of MzE using DPPH assay (n=3).

The DPPH scavenging activity in this research revealed the free radical scavenging activity (Figure 4). The IC50 value of MzE and ascorbic acid in this study shows a value of <50 µg/mL, categorized as a sample character with very strong antioxidant activity [23]. Based on the IC50 value, ascorbic acid has a lower IC50 value than MzE, showing that ascorbic acid has stronger antioxidant activity than MzE. The antioxidant activity of ascorbic acid is certainly stronger because it is a single bioactive compound that is reported to have antioxidant properties [24]. Meanwhile, MzE is a crude extract of various bioactive compounds where not all compounds act as antioxidants.

The decrease of ROS is not only done via endogenous antioxidants, it can also be eliminated by exogenous antioxidants. Due to the presence of phenolic and flavonoid compounds, MzE extract can scavenge free radicals in the body to reduce the effect of ROS. The phenolic and flavonoid compounds are reported that have roles as free radical scavengers and antioxidants [25]. Although the MzE needs a higher concentration to scavenge 50% of DPPH compared to ascorbic acid as a control, this assay proved that the MzE could ward off free radicals not only by playing a role in regulating endogenous antioxidants but also has the potential to inhibit free radicals acts as an exogenous antioxidant. It has more advantages because it is safer and as great as synthetic antioxidants [25].

Conclusion

In this study, we conclude that MzE can decrease blood glucose levels and has the ability as an antioxidant. The MzE could act as an endogenous antioxidant enhancer by improving the expression of endogenous antioxidants in T1DM that was decreased. The MzE can also scavenge ROS with the IC_{50} value at 48.35 µg/mL in the DPPH assay.

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