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Response Oxidative Stress of Ethanol Extract *Abelmoschus manihot* L Leaf on The Activity of Superoxide Dismutase Enzyme in Diabetic Wistar Rats.

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ABSTRACT

The objective of this study was to analyze the response of oxidative stress of ethanolic extract of Abelmoschus manihot L leaves on the activity of superoxide dismutase and glutation peroxide enzyme in diabetic wistar rats. The methods of this study was laboratory experimental to analyze response of oxidative stress which was using randomized pre and post-test control group design. Data was analyzed by using One Way Anova of variance and Duncan *post hoc test.* The results showed that the response of ethanol extract of Abelmoschus manihot L leaves on the mean of superoxide dismutase enzyme activity in the positive control group was respectively (55,89 ± 7,38)%, treatment group each of Abelmoschus manihot L leaf extract dose of 5, 10 and 15 mg/kgbw (48,78 ± 4,19)% ; (56,31 ± 6,93)% and (76,45 ± 2,54)%. Statistically, significant with $p<0.05$. Ethanol extract of Abelmoschus manihot L leaf dose of 5, 10 and 15 mg/kgbw give response to the increase of superoxide dismutase enzyme activity in diabetic wistar rats.

Keywords: Oxidative Stress, Abelmoschus manihot L, Superoxide dismutase, Diabetic

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INTRODUCTION

Cardiovascular complications in diabetes mellitus is one of the factors causing oxidative stress at the cellular level. Despite the administration of insulin or oral antidiabetic drugs, complications of diabetes are still difficult to overcome almost all over the world including Indonesia. Deaths from these complications are estimated to reach 40% of people with diabetes mellitus. This condition is triggered by the failure of β-pancreatic cells in insulin production and is followed by high glucagon plasma [1,2].

Diabetes mellitus is a multifactor disorder associated with proinflammatory cytokines characterized by the formation of excess reactive oxygen species (ROS) compounds [3,4]. ROS that occurs in people with diabetes mellitus will lead to decreased activity of enzyme Superoxide dismutase and Gluttion Peroxidase [5].

Superoxide dismutase (SOD) are enzymes to reduce H_2O_2 compounds that act as endogenous antioxidants in the case of superoxide ion production which is one of the reactive oxygen compounds in the mitochondria [6]. The antioxidant enzymes used to assess oxidative stress are superoxide dismutase, glutathione peroxidase and catalase. This condition triggers the occurrence of oxidative stress, ie the imbalance between free radicals with antioxidants in the body leads to histologic changes of pancreatic tissue and decreased activity of SOD enzymes. However, to anticipate the occurrence of excessive free radical accumulation, it is necessary to be given exogenous antioxidants from natural sources, one of which is leaf extract Abelmoschus manihot L.

According to Mamahit [7], reported that one of the traditional medicinal plants that have the potential to be developed as an antidiabetic drug is Abelmoschus manihot L leaf, because traditionally leaves Abelmoschus manihot L has been used to treat various diseases, such as diabetes, gastritis, heart disease, high blood pressure, osteoporosis, kidney disorders, seizures, and depression. The results showed that leaf extract of Abelmoschus manihot L contained several chemical compounds, among others; flavonoids, terpenoids, alkaloids, tannins, polyphenols, saponins and serotonin. While the isoflavone compounds that are derivatives of the flavonoid group are thought to have activity as exogenous antioxidants and function to increase the number of pancreatic-β cells through decreased blood glucose and cholesterol levels in diabetic wistar rats [9]. According to Gunawan *et al* [10] also stated that Euchresta horsfieldii lesch benn extracts of natural rich antioxidants can increase the activity of SOD enzymes in rat blood due to oxidative stress, thus protecting cells from oxidative stress attacks.

RESEARCH METHODS

Research design

This research used laboratory experimental method with randomized posttest control group design [8]. The in study used wistar rats, 3 months old with weight 200-250 g. The sample size in this study followed the Montgomery procedure [11], 25 wistar rats were grouped into five groups, one control group and four treatment groups dose of 5 mg/kgbw, 10 mg/kgbw and 15 mg/kgbw.

Preparation of Plant Extract

Abelmoschus manihot L plant is taken from the leaves and then cleaned and dried by putting the place open with open air circulation and not exposed to direct sunlight. Then milled with a blender until it becomes a powder. The dried leaves of Abelmoschus manihot L leaf weighed 6 kg and extracted maceration using ethanol solvent for 24 hours then evaporated using a rotary vacuum evaporator. Whereas the obtained residue was re-extracted using ethanol solvent. The ethanol extract was evaporated until ethanol extract was obtained, then the SOD enzyme activity were examined and saw pancreatic β-histopathologic cell profile using immunihistochemical method in diabetic wistar rats after alloxan induced 140 mg/kgbw.

Blood Investigation

The wistar rats to be taken for 12 hours (but the drinking was given as usual, then the rat weighed weighing) After anesthesia with inhalation of diethyl ether until a regular breathing rhythm was taken 3 ml of blood The collected blood was allowed to stand for 30 minutes at room temperature , then dizzying at 2700 rpm for 10 minutes Serum separated, taken and put into bottle and then closed Sample and stored at 4^0C The further activity of SOD enzymes is measured by the magnitude of the ability to neutralize the superoxide ions formed during oxidative stress.

Measurement of Superoxide Dismutase (SOD) Activity on Wistar Rats

A total of 0.06 ml of mouse blood was reacted with a mixture consisting of 2.70 ml of 50 mM sodium carbonate buffer containing 0.1 mM EDTA (pH 10), 0.06 ml xanthine 10 mM, 0.03 ml bovine serum albumin (BSA) 0,5% and 0.03 ml of NBT 2.5 mM. Furthermore, the addition of xanthine oxidase (0.04 units). The resulting absorbance after 30 minutes was measured at a wavelength of 560 nm. As the control used the solution used in the preparation of blood samples of PBS containing 11.5 g/L KCl. SOD activity (%) was calculated using the following equation: (1- (A / B)) x 100% [12, 13].

A = absorbance of sample solution and B absorbance of control solution

This SOD measurement method is used to measure the capture capacity of superoxide anion radicals. Superoxide anions are produced enzymatically by the xanthine-xanthine oxidase system. The method of analysis of SOD activity was carried out following Wijeratne *et al* [13] research procedure with slight modification.

Statistical Data Analysis

The data obtained in the statistical analysis with the steps as follows:

1. Analysis of normality of data of SOD level of trial rats in each group was analyzed by Shapiro-Wilk test, with significance level $\alpha = 0,05$
2. The homogeneity test of variance was analyzed using Levene's test to determine whether the variation in each homogeneous group
3. Comparative test was performed with One Way Anova because of normal and homogeneous distribution data, to see the difference between groups followed by LSD at significance level $\alpha = 0.05$
4. If there is a significant effect then the analysis is continued Duncan multiple comparison test to determine the average difference between treatments in the form of leaf extract of Abelmoschus manihot L with 5% significance limit.

RESULTS AND DISCUSSION

Abelmoschus manihot Leaf Extraction L

1000 g Leaf powder Abelmoschus manihot L was extracted by maceration using 96% ethanol solvent. The obtained filtrate was evaporated using a rotary vacuum evaporator until all ethanol evaporated to obtain 37.15 g of blackish brown ethanol extract. Furthermore, ethanol condensed extract is hydrolyzed with 2N HCl to separate the aglycone compound with glycine compound. The ethanol viscous extract was tested for its antioxidant activity by the 1,1-diphenylpycryl-2-hydrazil (DPPH) method to determine the ability to capture free radicals and see if there was any positive linear correlation to its ability to capture free radicals due to oxidative stress or reactive oxygen species (ROS). The results of antioxidant capacity to 1,1-diphenyl-2-picrilhidrazine showed that ethanol extract of Abelmoschus manihot L had IC_{50} of 31,29 ppm compared to standard ascorbic acid having IC_{50} of 12,89 ppm, this is probably caused by the compound which is antagonistic in crude extract capable of suppressing the ability to absorb free radicals, but after extracted with 96% ethanol free radical damping is very strong because the number of flavonoid compounds more than the crude extract so that the

antioxidant activity is stronger. According Sinaga [15] states that a compound is said to be very powerful antioxidants if the IC_{50} value is less than 50 ppm and weak if IC_{50} has a value greater than 500 ppm. Thus, the ethanol extract of leaf Abelmoschus manihot L has a very strong antioxidant activity. Furthermore, active antioxidant extracts are made of phytochemical screning to determine the class of compounds. Based on phytochemical test of ethanol extract of Abelmoschus manihot L positive contain flavonoid compound with a typical color intensity.

Analysis of Superoxide Dismutase Activity

The result of SOD activity analysis before and after giving leaf extract of Abelmoschus manihot L in each treatment group can be described in Figure 1 and Table 2.

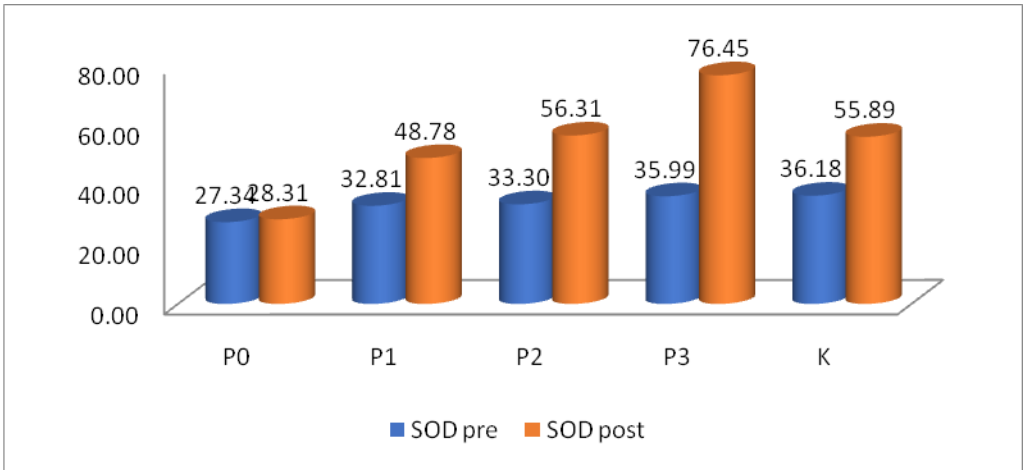

Figure 1: Superoxide Dismutase (SOD) pre and posttest Enzyme Activity

Table 2: Increase in SOD Enzyme Activity

Treatment	Before Giving Extract	After Giving Extract	Increase SOD Activity	% Increase SOD Activity
Negatif control (P0)	27.34	28.31	0.97	3.55
Abelmsoschus manihot L extract dose of 5 mg/kg/bw (P_1)	32.81	48.78	15.97	48.67
Abelmsoschus manihot L extract dose of 10 mg/kg/bw (P_2)	33.30	56.31	23.01	69.09
Abelmsoschus manihot L extract dose of 15 mg/kg/bw (P_3)	35.99	76.45	40.46	112.42
Positive control (K^+)	36.18	55.89	19.71	54.48

Table 3: Mean of SOD Enzyme Activity between Treatment Group after Leaf Extract Abelmoschus Manihot L

Treatment group	N	Mean SOD Activity (%)	SD	*P*
Negative control (P_0)	5	28.31	1.91	
Abelmoschus manihot L extract dose of 5 mg/kgbb (P_1)	5	48.78	4.19	
Abelmoschus manihot L extract dose of 10 mg/kgbb (P_2)	5	56.31	6.93	0,001
Abelmoschus manihot L extract dose of 15 mg/kgbb (P_3)	5	76.45	2.54	
Positive control (K^+)	5	55.89	7.38	

n = number of treatments, SD = standard deviation, *p* = significant value

The SOD enzyme is one of the intracellular antioxidant enzymes that play the role of capturing superoxide radicals (O_2.) Into H_2O_2 compounds whose radical nature is lower than superoxide [12]. In this study used alloxan as superoxide radical producing compounds. The expression of the SOD enzyme is marked by the number of superoxide radicals neutralized into H_2O_2 compounds that can be detected by a spectrophotometer at a wavelength of 560 nm.

Based on the data above shows that giving Abelmoschus manihot L leaf extract potency to increase activity of SOD in wistar mice after given alloxan dose of 140 mg/kgbw. The highest percentage increase in SOD activity was in group P_3 dose 15 mg/kgbw of 112.42%. This increase occurs because of the hemeostatic condition and has been in the normal range. The P_1 group gave a lower activity increase compared to P_2 and P_3, probably because the SOD activity was already at a sufficient level so that the Abelmoschus manihot L leaf extract did not have much effect. While giving the extract dose of 10 mg/kgbw (P_2) showed the percentage increase of SOD enzyme activity equal to 69.09% compared to the control group positive (*glibenclamide*) dose 5 mg/kgbw with percentage increase of SOD equal to 54.48%. It showed that giving leaf extract of Abelmoschus manihot L dose of 15 mg/kgbw/day for 21 days was able to increase the activity of SOD enzyme, where the active compound on leaf extract of Abelmoschus manihot L was able to react with free radical present in body and allegedly responsible induction of gene on the synthesis of antioxidant enzymes through Nrf2 translocation to the nucleus thus increasing the expression of antioxidant coding gene. According to Jawi [16] found that the anthocyanin compound contained in purple sweet potato tuber extract increases the expression of SOD enzyme by increasing protein Nrf2 so it is antioxidant indirectly.

The result of homogeneity test using Levene's Test showed homogeneous data with $p>0,05$, while One Way Anova test to the mean of SOD activity showed $p<0,05$, so the value indicated that the five treatments gave significant different effect.

Further test results using Post Hoc LSD Test showed that overall SOD enzyme activity in wistar mice gave significantly different effect with $p<0,05$. Furthermore, statistical analysis with Duncan test $\alpha = 0.05$ indicates that the treatment between groups within the subset is not significantly different, but between subset there is a real difference.

Profile of Histopathology β-Cells Pancreas Wistar Rats

Changes in morphology of pancreatic wistar β-cells with 400x magnification using *Aldehyde Funchsine-Nuclear fast red* staining from normal to diabetes due to alloxan induction dose 140 mg/kgbw can be seen in Figure 3.

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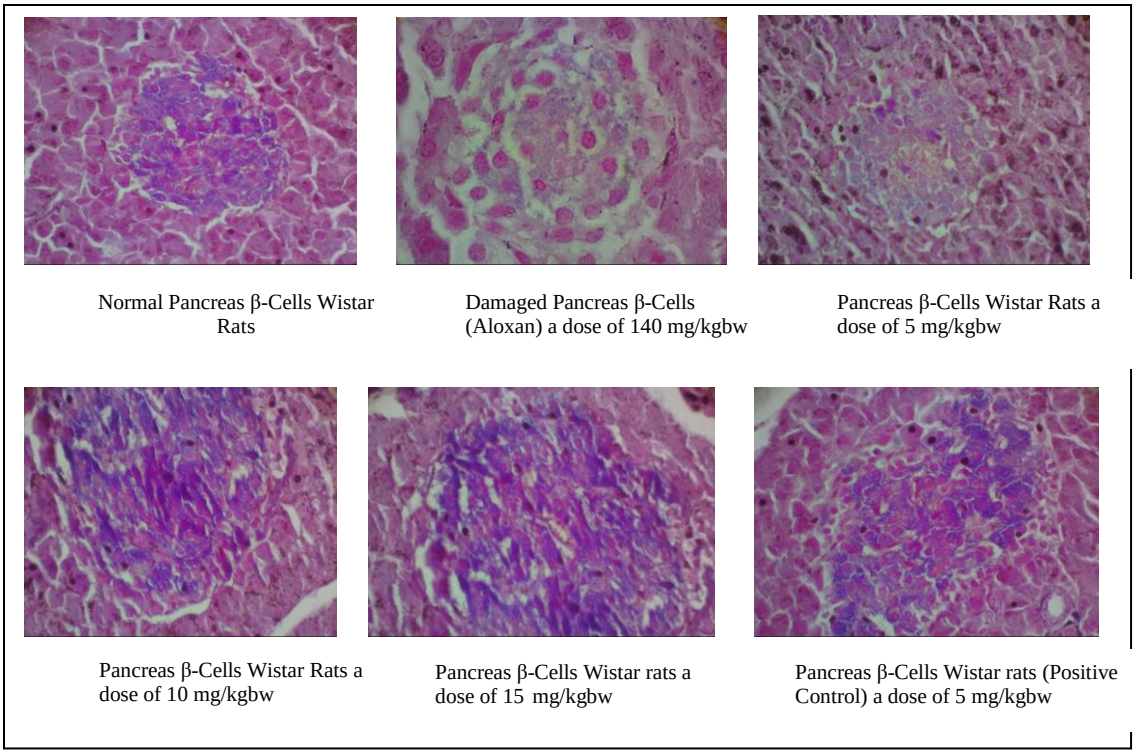

Figure 3: Profile of Histopathology Pancreatic β-cells with *Aldehyde Funchsine-Nuclear fast red*

In the figure above, shows that there has been a change in morphology and an increase in the number of pancreatic β-cells of wistar rats before and after dosage of n-heksan leaf extract of Abelmoschus manihot L. The increased number of pancreatic β-cells in wistar mice means that according to the theory that when cells suffered injury due to a stimulus then potentially reversible changes that can be returned as before. The mechanism of repair of pancreatic β-cells is the possibility of leaf extract of Abelmoschus manihot L dose of 10 mg/kgbw has greater insulin content than the treatment group dose of 5 mg/kgbw so that pancreatic β-cell damage can be repaired quickly and almost near normal. Similarly, the number of pancreatic β-cells in Langerhans Island due of Abelmoschus manihot L ethanol extract dose of 15 mg/kgbw more than the number of pancreatic β-cells after the administration of antidiabetic drug (*glibenclamide*) dose 5 mg/kgbw. The number of cytoplasmic granules in the β-cells has increased to near normal conditions so that the process of repairing pancreatic tissue can take place quickly. In contrast the pancreas β-cells of wistar mice degenerate to necrosis due to alloxan induction dose of 140 mg/kgbw. This is because alloxan selectively destroys pancreatic β-cells through the formation of reactive oxygen species (ROS) initiated by alloxan reduction and characterized by elevated blood glucose levels (diabetes), so that the number of pancreas β-cells is insulin-resistant.

Observation of pancreatic β-cells was performed quantitatively by calculating the number of pancreatic-β-cells wistar tissue in both groups of both the control group and the treatment group. The β-cells detected with *Aldehyde Funchsine-Nuclear fast red* staining and magnification 400x are shown Figure bluish purple cells on Langerhans island while the other cells are red. The result of β-cell count calculation on Langerhans island of pancreatic wistar mouse in control group and fifth field viewer treatment group was presented in Table 6.

Table 6: Mean Number of Pancreatic β-cells Wistar Rats in Langerhans Island

Treatment	Number Pancreatic β-cell
	Mean ± SD
Normal group	61.00 ± 1.414 f
Negative control (K^-)	7.60 ± 1.019 a
Positive control (*glibenclamide*)	35.40 ± 1.625 d
Abelmoschus manihot L extract dose of 5 mg/kg bw	11.80 ± 2,315 b
Abelmoschus manihot L extract dose of 10 mg/kg bw	26.60 ± 1,019 c
Abelmoschus manihot L extract dose of 15 mg/kg bw	57.00 ± 1.095 e

The mean number of pancreatic β-cells followed by different letters in the same column shows significantly different test results (p<0.05)

Furthermore, the data of normalist and homogeneous test results on the number of pancreatic wistar β-cells in both groups of both the control group and the 5 mg/kgbw, 10 mg/kgbw and 15 mg/kgbw treatment groups showed that all data were normally distributed and the variants homogeneous (p>0.05)

One way anova analysis showed that there was a significant difference between pancreatic β-cell count in negative control group (K^-) and treatment group after ethanol extract of Abelmoschus manihot L leaf dose of 5 mg/kgbw, 10 mg/kgbw and 15 mg/kgbw with p<0,05. In contrast, the positive control group (K^+) also had significantly different β-cell counts (p<0.05) with treatment group after ethanolic extract dose of 5 mg/kgbw, 10 mg/kgbw and 15 mg/kgbw.

CONCLUSION

Ethanol extract of Abelmoschus manihot L leaf dose of 5, 10, 15 mg/kgbw, can prevent oxidative damage through increased antioxidant activity of superoxide dismutase enzyme and glutation peroxidase in diabetic wistar rat. P_1 (48,78 ± 4,19)%; P_2 (56,31 ± 6,93)% and P_3 (76,45 ± 2,54)%. This difference was statistically significant with p<0.05.

Ethanol extract of Abelmoschus manihot L leaf dose of 15 mg / kgbw give the best response to the increase of superoxide dismutase enzyme activity in diabetic wistar rats, when viewed from the change of histopathologic structure of pancreatic β-cells formed on Lengerhans island.

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