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Antioxidant Potential of Kersen LeavesSteeping (Muntingia calabura L.) Against Endogenous Enzyme Superoxide Dismutase (SOD) Levels in Diabetes Mellitus Rats

Running title: Kersen Leaves Steeping SOD

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Running title: Muntingia calabura SOD

Abstract

Oxidative stress occurs when the levels of free radicals and antioxidants in the body is not balanced. Free radicals can be formed as a result of an increase in blood glucose levels in diabetes mellitus that can cause damage to cells, tissues, and organs such as the liver, kidneys, heart. Antioxidants are necessary to dampen the negative effects of oxidants. Flavonoids on the cherry crop is antioxidative. This study was investigated the effectiveness of steeping cherry leaves (Muntingia calabura L.) in increasing levels of the enzyme SOD in Diabetes Mellitus Rats.The subjects of this study were 36 Sprague Dawley rats divided into 6 groups, namely group 1 (normal), group 2 (negative control), group 3 (positive control), group 4 (steeping cherry leaves 250 mg/200 grBW), group 5 (steeping kersen leaves 500 mg/200 grBW), and group 6 (steeping cherry leaves 750 mg/200 grBW). Groups 2-6 were induced with streptozotocin and nicotinamide for 5 days until the rats became Diabetes Mellitus (blood glucose>135 mg/dl) and then treated for 14 days. Data were analyzed using a paired t-test and One-Way ANOVA test. Statistical test results with paired t-tests showed significant differences in blood glucose levels before and after treatment (p=0.0001). In the One-Way ANOVA test, there was a different mean of SOD levels in each group (p=0.0001). The most effective to increase SOD levels is a dose of 750 mg/200 grBW.

Keywords: oxidative stress, kersen, diabetes, superoxide dismutase

Introduction

Diabetes mellitus occurs due to abnormalities in insulin secretion, insulin action, or both1. Insulin itself is produced by beta cells that are in the Langerhans islands of the pancreas. Damage to pancreatic beta cells can cause a state of hyperglycemia[1][2][3]. Hyperglycemia in diabetes is involved in the formation of free radicals. an imbalance between protective antioxidants (antioxidant defense) and increased free radical production is a precursor to oxidative damage known as oxidative stress[4][5][3].

Antioxidants are needed to reduce oxidative damage. Antioxidants are compounds that can reduce the negative impact of oxidants[6][7]. Antioxidants are divided into 2 based on their source, namely endogenous antioxidants and exogenous antioxidants. Endogenous antioxidants come from within the body itself, consisting of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase. Exogenous antioxidants are obtained from outside. An increased supply of sufficient antioxidants will help prevent clinical complications of DM[8][9][10].Diabetes medicine is needed to avoid serious complications. The chemicals contained in drugs, including diabetes drugs, have many side effects and the price is not cheap. The alternative that is needed by the community is natural DM treatment without any side effects, which is effective and affordable[11][12][13][14][15].

Kersen is a tropical fruit plant that is easy to find and belongs to the Elaeocarpaceae family. Kersen leaves contain a group of compounds or lignans including flavonoids, tannins, triterpene, saponins, and polyphenols which show antioxidative activity[16][17][16] Kersen leaves are usually just wasted and become trash. Even though cherry leaves contain ingredients that have the potential as antioxidants. So, it is necessary to do research related to the effectiveness of *kersen* leaf infusion.

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

Materials and methods

This studywas a laboratory experimental study to test the effectiveness of kersen steeping leaves (Muntingia calabura L.) on levels of the endogenous enzyme Superoxide Dismutase (SOD) in Diabetes Mellitus rats induced by Streptozotocin-nicotinamide (STZ-Na) with a post-test only design with a control group design. This research was conducted for 30 days using a white rat (Rattus novergicus) Sprague Dawley strain.

Selection and Description of Participants

The number of tested animals were thirty-six with six in each group. There were five groups, namely the normal group, the negative control group, the positive control group, the kersen leavessteeping group with a dose of 250 mg / 200 grBW, the group of kersen leaves steeping with a dose of 500 mg / 200 grBW, and the group of kersen leaves steeping with a dose of 750 mg / 200 grBW Blood samples were taken 3 times, namely before being induced by Streptozotocin-nicotinamide, after induction of Streptozotocin-nicotinamide, and after treatment to test fasting blood glucose levels of mice, while to measure the SOD enzyme, the rats' liver organs were collected through surgery.

The inclusion criteria of the tested animals used were male Sprague Dawley, ± 8 weeks old, and ± 150-200 grams body weight. White mice with inactive/inactive activity, died during the treatment period, were sick (appearance of dull hair, hair loss, or baldness), and experienced a weight loss of> 10% during the adaptation period in the laboratory were excluded from the study.

The independent variable was the steeping of kersen leaves (*Muntingia calabura* L.) with a dose of 250 mg / 200 grBW , 500 mg / 200grBW, 750 mg / 200 grBW, while the dependent variable is the level of the SOD enzyme. As a variablecontrolled factors were genetic, age, body weight, cage and feed conditions are the same.

Technical information

The materials used in this study were kersen leaves (*Muntingia calabura* L.) obtained from the laboratory page of the Center for Food and Nutrition Studies, the Center for Inter-University Center (PAU), Gadjah Mada University, streptozotocin, metformin found in pharmacies, fasting blood plasma of rats, nicotinamide, NaCl 0.9%, 0.1 M citrate buffer, distilled water, and rat liver tissue.The tools used in this study include digital scales for weighing rats, sonde for steeping rats, glass cups, syringes for taking blood glucose, gloves, masks, pots to boil water, filters, stoves, animal cages, centrifuge, microcapillary tube, spectrophotometer, and BioVision KIT.The research was carried out at the Laboratory of the Center for Food and Nutrition Studies at the Center for Inter-University Gajah Mada University (UGM). Samples were obtained from laboratory test animals of the Muhammadiyah University ofYogyakarta.

The implementation began with preparing cages, weighing the rats, and randomly divided them into 6 groups. Then the mice were adapted for 7 days. On the 7th day, the body was weighed to determine the dose of Streptozotocin-nicotinamide, and the first blood sample was taken to measure the levels of fasting blood glucose. On the 8th day, the rats were induced with nicotinamide 230 mg/kg BW, 15 minutes later continued with 65 mg/kg BW streptozotocin induction.

The second sampling was carried out 5 days after the induction of Streptozotocin-nicotinamide with parameters of GDP levels. Rats were diagnosed with diabetes mellitus if the blood glucose level was> 135mg / dl. After the rats were diagnosed with diabetes mellitus, the rats were weighed again for dosage determinationtreatment. Furthermore, preparations are made for the infusion of cherry leaves. The leaves used are dark green leaves, do not roll, and there are no insect bites. The leaves are taken from the courtyard of the Center for Food and Nutrition Studies at the Inter-University Center (PAU), Gadjah Mada University (UGM), dried in the sun to dry (brownish), then brewed with boiling water until the color resembles tea, before being given to mice, the brew is filtered. so that it separates from the leaves.

The treatment was given according to each group for 14 days, the normal group was not given any treatment, the negative control group was only given distilled water/rat/day, the positive control group was given metformin 0.09 mg / 200 grBW /rat/day, the treatment group 1 (P1) was given a dose of 250 mg / 200 grBW / rat/day, treatment group 2 (P2) was given infused leaves kersen dose of 750 mg / 200 grBW / rat/day. After 14 days of treatment, the blood glucose level was again measured.

Ethics

The animals in this study were treated with due regard to ethics in research with animal subjects. During the research, test animals were observed for their health status. When giving treatment, the actions that are injurious are accompanied by trained personnel to minimize the side effects of the treatment at the time of treatment.The animal handling and experimental procedure were approved by the Medical and Health Research Ethics Committee, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta, Yogyakarta.

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

Statistics

The data obtained were analyzed using the paired t-test for differences before and after induction and treatment. The OneWay ANOVA test to determine the significance of differences between research groups, followed by post-hoc-test and Tuckey means test.

Results and Discussion

The results of the GDP observations are shown in Table 1. From table 1 it is found that there is an increase in fasting blood glucose levels after the induction of Streptozotocin-nicotinamide.
Table 1. Average levels of GDP before and after induction of Streptozotocin-nicotinamide by paired t-test.

Groups	Fasting Blood Glucose (mg/dl) ± SD		p-value (paired -t-test)
	Pre-STZ	After STZ	
Normal	58.52 ± 1.53	58.81± 1.71	0.65
Negative	60.73 ± 2.26	213.32 ± 5.71	0.0001
Positive	59.47 ±1.62	206.82 ± 1.91	0.0001
P1(250 mg kersen)	62.24 ± 1.72	211 ± 4.26	0.0001
P2(500 mg kersen)	59.97 ± 1.91	207.52 ± 2.22	0.0001
P3(750 mg kersen)	58.83 ± 2.08	211.84 ± 3.18	0.0001

Table 1 shows that there are significant differences in blood glucose levels (except for the normal group) before andafter induction of Streptozotocin-nicotinamide. All rats were declared Diabetes Mellitus[18].

Table 2. The blood glucose levels before and after treatment with the paired t-test

Group	Means (mg/dl) ± SD		p-value (paired -t-test)
	Pre-STZ	After Treatmentt	
Normal	58.81 ± 1.71	59.21 ± 1.84	0.01
Negative	213.32 ± 5.71	214.22 ± 5.26	0.029
Positive	206.82 ± 1.91	99.25 ± 1.57	0.0001
P1(250 mg kersen)	211.00 ± 4.26	157.65 ± 1.88	0.0001

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

P2(500 mg kersen)	207,.2 ± 2.22	136.99 ± 2.35	0.0001
P3(750 mg kersen)	211.84 ± 3.18	103.11 ± 2.42	0.0001

Table 2 shows that there is a significant difference in blood glucose levels after treatment in all groups but in the negative control group, there was no decrease but an increase. To determine the significance of the difference in the effectiveness of the infusion of cherry leaves, the One Way Anova test was used.

Table 3. The difference in the average reduction in blood glucose levels with the one way ANOVA test

Group	Means ±SD (mg/dl)	p-value
Normal	-0.39 ± 0.09	
Negative	-0.90± 0.72	
Positive	107.56± 0.53	0.0001
P1 (250mg Kersen)	53.34 ± 3.36	
P2 (500mg Kersen)	70.53± 0.75	
P3 (750mg Kersen)	108.72 ±1.82	

Table 3 shows that the average difference in the reduction in blood glucose levels in this study is differentindicated by the value p = 0.0001 (p <0.05).

Table 4. The Average l of SOD enzyme levels in white rats (Rattus novergicus) after treatment

Group	Means±SD	*p-value*(One Way Anova)
Normal	73.13 ± 5.38	
Negative	15.30 ± 3.82	
Positive	66.32 ± 6.29	0.0001
P1 (250 mg Kersen)	23.12 ± 6.66	
P2 (500 mg Kersen)	45.92 ± 3.81	
P3 (750 mg Kersen)	61.22 ± 5.77	

Table 4 shows that there is a significant difference in the mean difference of the SOD enzyme in all experimental groups in the study indicated by a value of p = 0.0001 (p <0.05).

Table 5. The difference in SOD enzyme levels compared to the normal group

Group	Means of SOD (mg/dl)	Nilai *p* (One Way Anova)

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

Negative	57.82	
Positive	6.80	
P1 (250 mg Kersen)	50.00	0.0001
P2 (500 mg Kersen)	27.21	
P3 (750 mg Kersen)	11.90	

Table 5 shows that the difference in the levels of the SOD enzyme in all treatment groups compared to the normal group where the smallest difference was the positive control group followed by treatment group 3 (steeping kersen leaves 750 mg / 200 grBW) which means that these two groups are the closest to normal. . Meanwhile, the biggest difference was in the negative control group followed by the P1 group (250 mg / 200 grBW).

Table 1 shows significant differences in the five groups after the induction of Streptozotocin-nicotinamide with a value of $p = 0.0001$ ($p < 0.05$). All rat samples were declared diabetes mellitus type 2 with a GDP level> 135 mg / dl[18][19]. Streptozotocin is a nitrosourea derivative isolated from Streptomyces achromogenes which has anti-neoplasm activity and broad-spectrum antibiotics. Streptozotocin can directly damage the critical mass of Langerhans β cells or cause an autoimmune process to β cells so that it is more widely used in the manufacture of DM test animals[20][21][22].

Streptozotocin induces DM by damaging the DNA of pancreatic beta cells. In pancreatic beta cells, streptozotocin damages DNA through the formation of NO, hydroxyl radicals, and hydrogen peroxides. This DNA destruction stimulates ribosylation of poly ADP which in turn causes depletion of NAD + and ATP in the cell. As a result, insulin production is interrupted and the amount produced is reduced or can even cause cell apoptosis. The increase in ATP dephosphorylation will spur an increase in the substrate for the xanthine oxidase enzyme (pancreatic β cells have high activity against this enzyme), further increasing the production of uric acid xanthine oxidase catalyzing the reaction of the formation of active superoxide anions. Generation of superoxide anions will form hydrogen peroxide and superoxide radicals. NO, and reactive oxygen is the main causes of pancreatic β cell damage[20][19][23].

Meanwhile, the addition of nicotinamide induction to control excessive damage to pancreatic beta cells and protect beta-pancreatic cells in experimental animals due to the induction of streptozotocin. The white rats induced by streptozotocin at a dose of 65 mg/kg BW rats and nicotinamide 230 mg/kg BW of rats became Diabetes Mellitus within 5 days[20][19]

Table 2 shows significant differences in all test groups ($p < 0.05$) after being given treatment according to each group. From the results of the paired t-test after treatment, it was found that the GDP level decreased in the positive control group, the 250 mg / 200 grBW steeping group, the 500 mg / 200 grBW steeping group, and the 750 mg / 200 grBW steeping group. Meanwhile, the negative control group did not experience a decrease but an increase.

The dose assessment of the kersen leaves steeping on the blood glucoselevel and the SOD enzyme in this study was carried out by the One Way Anova test shown in table. From the One Way ANOVAblood glucose and the SOD enzyme level, the p-value = 0.0001 ($p < 0.05$) which means that the average reduction in blood glucoseand increased levels of SOD from the five treatments are different. To determine which infused dose was the most effective in reducing FBG levels and increasing FBG, a Post-Hoc analysis was performed. The results of the Post-Hoc test showed that the most effective decrease in the level of blood glucoselevel was the result of the kersen group of 750 mg / 200 grBW with the largest difference of decrease, namely 108.72 mg/dl, while the largest increase in the level of SOD was the most effective, namely the result of the kersen group of 750 mg / 200 grBW.

Muntingia calabura L. is known to contain flavonoids which can reduce blood glucose levels. Flavonoids are known to play a role in capturing free radicals or function as natural antioxidants[24][7]. This antioxidant activity allows flavonoids to capture or neutralize free radicals such as ROS associated with phenolic OH groups so that they can repair the damaged tissue in other words, the inflammatory process is inhibited[6][1][25]. Kersen leaves steeping was also proven to significantly reduce blood glucose levels in Diabetes Mellitus rats ($p < 0.05$), this is because the content of kersen leaves is a flavonoid. Flavonoids can act as antioxidants that can reduce oxidative stress, causing a protective effect on pancreatic beta cells and increasing insulin sensitivity[8][4][24].

ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

The results showed that the mean post-treatment blood glucose levels were negative control group, steeping 250 mg / 200 grBW and brewing group 500 mg / 200 grBW > 135 mg / dl while the metformin group and steeping group 750 mg / 200 grBW<135 mg / dl. The normal blood glucose levels of Sprague Dawley rats are 55-135 mg/dl[26]. This shows that the provision of metformin and 750 mg / 200 grBW of kersen leaves were effective in reducing levelsfasting blood glucose in diabetic rats.

Research on the effect of kersen on SOD enzyme levels is still very rare before. Table 5 shown that when compared with the normal group there was an increase in SOD levels after being given kersen leaf treatment. From the results of the Post Hoc test, the effective dose for increasing SOD levels, the dose of 750 mg / 200 grBW was the same as the most effective dose for reducing blood glucose levels, namely 750 mg / 200 grBW. Hyperglycemia in Diabetes Mellitus produces a lot of ROS and this condition will cause pancreatic beta cell dysfunction, that in the pancreatic beta cells whose function is impaired will experience a decrease in levels of antioxidant enzymes such as SOD, GPx, and catalase so they are prone to oxidative stress[16][4][27][28].So, if the blood glucose level decreases in Diabetes Mellitus, the SOD level in the blood will increase[11][29][30].This study shown that the leaves steeping is effective in increasing SOD levels in diabetic rats.

Conclusion

The conclusion of this study is that kersen leaves steeping is effective in increasing SOD levels in diabetic rats.

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ISSN:0975-3583,0976-2833 VOL12,ISSUE04,2021

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