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Original Research Articles

Therapeutic Effect of Red Spinach (*Amaranthus tricolor L.*) Extract on Pancreatic MDA Levels Rats (*Rattus norvegicus*) Exposed to MLD-STZ

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Abstract

Background: Several studies have reported that diabetes mellitus (DM) in patient can cause complications to death that occur due to oxidative stress conditions. Red spinach extract was found to be rich in antioxidant compounds. However, there has been no further research on the ability of red spinach to lower blood glucose levels and prevent oxidative stress, which can be seen from the levels of malondialdehyde (MDA; a marker of oxidative stress) in the DM body.

Objective: This study aims to determine whether there is a change in blood glucose levels and MDA levels in DM animal models between the groups that were given red spinach extract and not.

Methods: This study used white rats (*Rattus norvegicus*) which were divided into 5 groups: C(-) group in which the rats were not induced by diabetogenic agent and were not treated; C(+) group, the rats were made DM induced by multiple low dose streptozotocin (MLD-STZ); and T1, T2, and T3 groups were exposed to MLD-STZ and treated with red spinach extract (*Amaranthus tricolor L.*) at a dose of 200 mg/kgBW, 300 mg/kgBW and 400 mg/kgBW, respectively. Blood sugar levels were checked by using glucometer digital. Meanwhile, MDA levels were measured by thiobarbituric acid (TBA) test using protein isolates from the pancreas of each rat.

Results: The results showed that the average MDA levels in the C-, C+, T1, T2, and T3 groups were 1.759 ± 0.08 , 2.280 ± 0.15 , 2.303 ± 0.11 , 1.927 ± 0.06 , and 1.801 ± 0.04 . While the average blood sugar levels in the C-, C+, T1, T2, and T3 groups were 114.4 ± 8.82 , 464 ± 72.78 , 421.2 ± 37.60 , 140.6 ± 20.19 , and 176 ± 13.06 .

Conclusion: It can be concluded that the administration of red spinach extract therapy in DM model rats was able to reduce both glucose levels and MDA levels. It is also believed to be able to prevent oxidative stress in cells which causes tissue damage.

Keywords: Diabetes mellitus; red spinach; MDA level; blood glucose

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INTRODUCTION

Diabetes mellitus (DM) is one of the four priority non-communicable diseases that are targets for follow-up by world leaders. This disease, which is characterized by soaring glucose levels in the blood, or what is commonly known as hyperglycemia, has an ever-increasing number of cases over the last few decades. Not only does it increase the risk of complications, it also increases the risk of death.

The prevalence of diabetes increased sharply in low-income countries, including Indonesia, compared to high-income countries¹. In Indonesia, diabetes mellitus is one of the main causes of death in Indonesia with the percentage of deaths increasing from year to year². This fact is also supported by data recorded at the International Diabetes Federation (IDF) in 2017, Indonesia is in the sixth position in the world with the highest prevalence of DM with 10.3 million DM sufferers. In 2045, the International Diabetes Federation predicts that there will be 16.7 million people with diabetes in Indonesia³.

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Chronic hyperglycemia is associated with complications of diabetes. Several signaling pathways can be altered by experiencing hyperglycemia in different tissues, resulting in oxidative stress. Signaling pathways that are directly triggered by hyperglycemia play an important role in diabetic complications due to the production of Reactive Oxygen Species (ROS). The accumulation of ROS causes oxidative stress, up to cell death⁴. In addition to excessive ROS production, there was also an increase in malondialdehyde (MDA) levels which are the main product of lipid peroxidation by ROS. MDA is also referred to as a marker of oxidative stress. The presence of MDA has been widely reported in diabetic patients⁵.

DM is a lifelong disease, but it can be controlled with a healthy lifestyle such as medical therapy and physical activity simultaneously with pharmacological interventions. Two pharmacological interventions for diabetes, namely with oral antihyperglycemic or oral antidiabetic drugs and/or a mixture. Modern antidiabetic drugs such as metformin, glibenclamide, and sulfonylureas are often complained of because they have some side effects. Side effects that appear such as diarrhea, vomiting, stomach, weight loss, can cause hypoglycemia.⁶⁻⁸ Treatment with herbal plants that are believed to have a smaller risk of side effects than modern medicines.

Several previous studies have shown that the increase in blood sugar is emphasized by therapy using herbal plants, one of which is the administration of red spinach extract (*Amaranthus tricolor L.*)⁹. Red spinach is considered superior to other types of spinach because it contains high antioxidants¹⁰. This antioxidant content is able to capture free radicals / ROS that exist in DM conditions. However, the relationship between red spinach extract therapy and its ability to reduce MDA levels in DM conditions is still not widely known. In this study, the effect of red spinach (*Amaranthus tricolor L.*) extract on the level of oxidative stress in the animal's body will be identified quantitatively through pancreatic MDA protein levels and blood sugar monitoring.

MATERIALS AND METHODS

Preparation of Experimental Animals

This study used healthy male white rats (*Rattus norvegicus*) Wistar strain with a body weight of \pm 150-250 grams aged 2-3 months, as many as 25 mice were adapted for 2 weeks in the Lab. Pharmacology, Binawan University. All rats were divided into 5 groups, including: (1) healthy group/C(-), not given any preoperative treatment; (2) group of rats with DM type 1 / C(+), group of rats induced by MLD-STZ alone without any therapy; and the other groups (3), (4), & (5) were the therapeutic rat group (T1, T2, and T3). Groups T1, T2, and T3 were groups of rats receiving therapy with red spinach extract (*Amaranthus tricolor L.*) at a dose of 200 mg/kgBW, 300 mg/kgBW, 400 mg/kgBW, respectively, after the rats were declared DM due to MLD-STZ induction.

Preparation of Red Spinach Leaf Extract

About 5 kg of fresh red spinach (*Amaranthus tricolor L.*) were washed and air-dried to obtain dry red spinach leaves. Then the dried red spinach leaves are blended /

ground to obtain red spinach powder. Dried red spinach powder was macerated with 70% ethanol solvent with 1:6 ratio, then the macerate was concentrated using a vacuum rotary evaporator and filtered to obtain a thick extract. The ethanol extract of red spinach leaves was made at a dose of 200 mg/kgBW, 300 mg/kgBW, 400 mg/kgBW.

Thick extract was screened for phytochemicals to determine the active compounds in it. Phytochemical screening was carried out by adopting several methods that have been common and have been widely used in previous studies. The identification of alkaloid compounds was carried out using the Dragendorff test and Wagner's test. Saponin compounds were tested using the foam method. The presence of tannins and phenols in the extract was tested using gelatin and salt. The flavonoid compounds in the extract were tested using Shinoda's test/ Mg-hydrochloride reduction test. The identification of triterpenoid and steroid compounds was carried out by Salkowski's test and Libermann-Burchard's test. Meanwhile, to identify the presence of glycosides, the Keller-Killani test was carried out¹¹.

Measurement of Blood Glucose Level

Measurement of blood glucose levels of experimental animals was carried out 3 times during the study, namely: 1) Before injection of MLD-STZ, as the initial blood glucose level, 2) After induction of MLD-STZ (blood glucose levels exceeding 135 mg/dL rats were declared DM), and 3) after therapy with red spinach extract. Measurement of blood sugar levels using a glucometer stick and a digital glucometer (FamilyDr. Blood Glucose AGM-513S) according to the instructions for use.

Preparation of MLD-STZ Solution

Streptozotocin (STZ) 100 grams was dissolved with 1.5 mL of citrate buffer pH 4.5 and homogenized with a vortex. STZ solution was stored at 4 °C as stock to be injected into experimental animals with dosage conditions adjusted to the body weight of the experimental animals. The STZ dose used was 20 mg/kg BW, 5 times, which was given once a day for 5 consecutive days (multiple low dose) by intraperitoneal¹².

MDA for the Measurement

0.2 gram of the organ was cut into small pieces and then crushed in a cold mortar placed on an ice block and added 1 ml of 0.9% NaCl. The homogenate was then transferred to a small test tube and centrifuged at 8000 rpm for 20 minutes and the supernatant was taken. The supernatant was taken 100 L, added with 550 L distilled water, 100 L TCA, 250 L 1N HCl, and 100 L Na-Thio. For each addition of reagent, the mixed solution was homogenized with a vortex, then centrifuged at 500 rpm for 15 minutes. The supernatant was separated and transferred to a new test tube. The solution was incubated in a water bath at 100 °C. The absorbance of the sample was measured maximally for the TBA test and plotted on a standard curve that had been made to calculate the concentration of the sample¹³.

Data Analysis

The research data were analyzed using the Shapiro-Wilk normality test and the Levene homogeneity test.

Then, it was continued with the One-way Anova comparative test and the post hoc test-Tukey.

RESULTS

Phytochemical screening is the initial stage to identify chemical compounds contained in an extract. Based on the results of phytochemical tests from red spinach extract (*Amaranthus tricolor L.*), it was found that several secondary compounds contained in red spinach extract were alkaloids, flavonoids, saponins, tannins, steroids, phenolics, and glycosides. As summarized in Table 1., red spinach extract showed a positive reaction with the 7 secondary compounds above, and did not show any reaction to the triterpenoids in the extract.

Table 1. Phytochemical Test Results of Red Spinach Leaf Extract

No.	Secondary Compounds	Results
1	Alkaloids	+
2	Saponins	+
3	Tannins	+
4	Phenolic	+
5	Flavonoids	+
6	Triterpenoids	-
7	Steroid	+
8	Glycoside	+

(+) : contained in the extract

Measurement of blood glucose levels was carried out at three different times with 3 repetitions per rat. The first measurement is on all rats in each group. The mean blood sugar obtained in the C-, C+, T1, T2, and T3 groups, respectively, were 120.2 ± 11.26 mg/dL, 130.0 ± 4.80 mg/dL, 111.6 ± 7.70 mg/dL, 118.0 ± 16.48 mg/dL, and 114.2 ± 11.39 mg/dL, as shown in Table 2. The second measurement of glucose levels was carried out on the day after stz induction, so that measurements were carried out only in the group induced by STZ which was C+, T1, T2, and T3. The average blood sugar obtained in groups C+, T1, T2, and T3 respectively were 402.4 ± 72.57 mg/dL, 410.8 ± 30.99 mg/dL, 407.0 ± 46.32 mg/dL, and 404.8 ± 42.55 mg/dL. The third blood sugar measurement was on the 7th day of therapy with red spinach extract. To see the latest blood sugar comparison, the blood sugar measurement at the third time was carried out in all groups including the C- group. The average blood sugar data obtained were 114.4 ± 8.82 mg/dL, 429.6 ± 65.99 mg/dL, 421.2 ± 37.60 mg/dL, 140.6 ± 20.19 mg/dL, and 176.0 ± 13.06 mg/dL which data were from groups C-, C+, T1, T2, and T3 respectively.

Determination of MDA levels was carried out using the Thiobarbituric acid test method. The sample used for the determination of MDA levels was protein isolate from the pancreas of each rat in all groups. All rats were dissected and protein isolated from their pancreas organs on day 8 after the therapy group was finished given red spinach extract for 7 consecutive days. Based on the results of the study, the average concentration of MDA obtained by the Thiobarbituric acid test method in the pancreas of rats in groups C-, C+, T1, T2 and T3 respectively was 1.759 ± 0.08 μ g/mL, 2.280 ± 0.15

μ g/mL, 2.303 ± 0.11 μ g/mL, 1.927 ± 0.06 μ g/mL dan 1.801 ± 0.04 μ g/mL (Table 3).

Table 2. Comparison of Blood Glucose Levels of All Groups

Groups	Blood Glucose level (mg/dL)		
	Before MLD-STZ induction	After 5 days of MLD-STZ induction	After 7 Days of Therapy
C-	120.2 ± 11.26	-	114.4 ± 8.82 **
C+	130.0 ± 4.80	402.4 ± 72.57	429.6 ± 65.99 *
T1	111.6 ± 7.70	410.8 ± 30.99	421.2 ± 37.60 *
T2	118.0 ± 16.48	407.0 ± 46.32	140.6 ± 20.19 **
T3	114.2 ± 11.39	404.8 ± 42.55	176.0 ± 13.06 **

* Sig. $p < 0.05$ compared to C-

** Sig. $p < 0.05$ compared to C+

Table 3. Comparison of MDA Levels of All Groups

Groups	MDA Concentration (μ g/mL)		Post Hoc Test Result
	Average	stdev	
C-	1.759	0.08	**
C+	2.280	0.15	*
T1	2.303	0.11	*
T2	1.927	0.06	**
T3	1.801	0.04	**

* Sig. $p < 0.05$ compared to C-

** Sig. $p < 0.05$ compared to C+

DISCUSSION

In previous studies, it was known that red spinach extract contains several secondary metabolite compounds including antioxidants¹⁰. This is in line with the results of the phytochemical test of the ethanolic extract of red spinach which reacted positively to several secondary compounds, one of which was flavonoid compounds.

Flavonoid compounds were identified using concentrated Mg and HCl. The formation of a red color after the addition of concentrated Mg and HCl in the extract of red spinach (*Amaranthus tricolor L.*), this indicates that the sample contains flavonoids (Table 1.). Flavonoid compounds will be reduced with Mg and HCl to produce a red or yellow color¹⁴.

The results of this study showed that the average blood glucose of C(+) group induced by MLD-STZ and not treated had a significant increase in blood sugar at 429.6 mg/dL compared to blood glucose levels in C(-) group, namely 114.4 mg/dL. This is in accordance with previous studies that normal blood glucose in mice ranges from (111.72 ~ 155.60) mg/dL, while in diabetes mellitus, blood glucose levels are above normal, and generally it is said to be diabetes if blood glucose > 201 mg. /dL¹⁵. So, it can be concluded that C(+) group is already in DM condition.

STZ has high cell specificity compared to other diabetogenic agents. This compound belongs to the group that is soluble in air and if it reacts in the body it will be toxic to cells, thus affecting blood glucose¹⁶. The action of STZ on the islets of Langerhans is accompanied by changes in insulin and glucose in the blood. This is because STZ can interfere with glucose oxidation and decrease insulin biosynthesis and secretion. The intracellular action of STZ causes DNA changes in pancreatic cells and acts as a nitrogen oxide (NO) radical donor and produces Reactive Oxygen Species (ROS) which will increase the number of free radicals in the pancreas. Excessive NO radicals will react with superoxide radicals to form peroxynitrite which is toxic to pancreatic cells¹⁶. This situation is very capable of damaging beta cells and inhibiting insulin secretion which results in an increase in the amount of blood glucose. Blood sugar levels that exceed normal are an indication of diabetes¹⁷. A similar study stated that giving STZ, in low doses (MLD-STZ, Multiple Low Dose Streptozotocin), in experimental animals given i.p (intraperitoneal) injection at a dose of <40 mg/kg BW for 5 consecutive days caused hyperglycemia / DM¹⁸.

Meanwhile, in the therapy group, which was a group of mice induced by MLD-STZ, and treated with extracts at a dose of 200 mg/kg BW, 300 mg/kgBW and 400 mg/kgBW, respectively, for groups T1, T2, T3, there were groups who experienced a decrease in glucose levels, and there was also a group whose blood glucose levels did not decrease.

Based on statistical data from Post Hoc Test-Tukey (Table 2.) it is known that the average blood sugar of the T2 and T3 groups decreased significantly ($p < 0.05$) compared to the C(+) group, while the average blood sugar of the T1 group was not different. significantly compared to the C(+) group. These results indicate that therapy given using red spinach extract at a dose of 200 mg/kgBW had no effect in lowering blood sugar in DM rats, while therapy in DM rats using red spinach extract at a dose of 300 mg/kgBW and 400 mg/kgBW could reduce blood sugar levels to normal

The same trend was also seen in the MDA levels of the pancreas of rats (Table 3). There was a significant ($p < 0.05$) decrease in MDA levels in DM rats when treated with red spinach extract at doses of 300 mg/kgBW and 400 mg/kgBW which were in groups T1 and T2 compared to MDA levels in C(+) group. Meanwhile, in DM rats that were only treated with red spinach extract at a dose of 200 mg/kgBW, the mean pancreatic MDA levels in the groups were not significantly different ($p < 0.05$) compared to C(+) group.

Hyperglycemia conditions that change several signaling pathways, causing complications of diabetes⁴. In this condition, there is an increase in the number of ROS, AGEs, MGO which causes oxidative stress. The increase in MDA levels in the C(+) group of rats occurred due to an increase in the amount of excess ROS in DM conditions that trigger oxidative stress to complications. The main target of ROS is lipid. One of the most widely produced decomposition products of lipid oxidation is malondialdehyde (MDA). These compounds are formed through the biosynthesis of prostaglandins, such as endoperoxidase of polyunsaturated fatty acids (PUFA). Changes in MDA

levels indicate a change in ROS activity. Increased ROS production affects the increase in MDA levels.

In this study, the increase in MDA that occurred in the T2 and T3 groups was able to be suppressed by antioxidant therapy from red spinach (*Amaranthus tricolor L.*) extract at a dose of 300 mg/kgBW and 400 mg/kgBW. However, administration of 200 mg/kgBW red spinach (*Amaranthus tricolor L.*) extract therapy was not sufficient to suppress MDA formation in the T1 group, thus failing to reduce MDA and blood sugar levels in that group.

The flavonoid compounds contained in red spinach extract act as free radical scavengers with proton donors. Singlet electrons from ROS received by flavonoids through proton donors on the hydroxyl group of flavonoids will form flavonoid radicals^{19,20}. However, the unpaired electron is delocalized by the resonance of the benzene structure of the flavonoid compound and maintains the stability of the compound²¹. So that radical flavonoid compounds have lower radical activity or even are not reactive. The decrease in the number of ROS was followed by a decrease in the activity of MDA production and the activation of blood sugar controlling proteins so that glucose levels in the blood could be controlled properly.

CONCLUSION

Based on this study, it is known that therapy with red spinach extract at a dose of 200 mg/kgBW has not been able to reduce blood sugar levels and pancreatic MDA in rats. Meanwhile, therapy using red spinach (*Amaranthus tricolor L.*) extract at a dose of 300 mg/kgBW 400 mg/kgBW in DM rats was able to significantly reduce blood glucose and pancreatic MDA of rats. Using these two doses is believed to also be able to inhibit the oxidative stress pathway in DM animal model.

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