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Anti-inflammatory of Purple Roselle Extract in Diabetic Rats Induced by Streptozotocin

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Abstract

The pathogenesis of diabetes mellitus involves a low-level inflammatory process due to the increase of blood glucose. In this research, testing of extracted rosella was done on Spraque Dawley rats inducing by streptozotocin. The rats were divided into six groups i.e normal rats (SG), diabetic rats group (DiW, DiR1, DiR2), preventive rats (PR1) and group were diabetic rats given glibenclamide (DiG). Analysis of inflammatory (TNF-α and IL-6) was performed on the spleen of rat using the ELISA technique. The results showed that roselle extract tended to decrease levels of the inflammatory TNF-α in diabetic rats, but could not be able to reduce the levels of IL-6.

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Keywords: Roselle, inflammatory, antioxidant, TNF-α, IL-6

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder syndrome of carbohydrates, fats, and proteins caused by reduced insulin secretion or decreased tissue sensitivity to insulin. DM can be classified into two types, namely type 1 and type 2 of diabetes mellitus. DM type 2 is also called (non insulin-dependent diabetes mellitus, NIDDM), which is caused by decreasing in the sensitivity of target tissues to the metabolic effects of insulin.

Of all cases in diabetes, approximately 90% that tends often to be found is diabetes mellitus type 2. In both types of diabetes, metabolism of all major nutrients becomes disorder. Resistance or absence of insulin will reduce the efficiency of the use and uptake of glucose by most of the body cells, excepting the brain cell. In DM, disorders of carbohydrate metabolism can cause hyperglycemia. Disorders in either the secretion or activity of insulin in

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diabetes mellitus could be caused by non-enzymatic glycation mechanism, which is the processes of glucose chemically binding to the free amino group on the protein without helping of enzymes, as well as increasing of inflammation. Inflammation is a physiological response of the body against damage or disturbance outside factors. Hyperglycemia condition caused the response of inflammatory compounds that mediated by cytokines. The presence of cytokines will damage the insulin sensitivity and glucose balance [1]. Inflammation occurs after increasing blood glucose that is marked by an increase of various markers of inflammation, such as high sensitivity C reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-18 (IL -18) [2,3]. Inflammation can be triggered through increase of ROS(reactive oxygen species) during diabetes mellitus. ROS can activate NF-κB, which is a transcription factor that regulates the expression of proinflammatory genes such as TNF-α, IL-6 and C reactive protein. In addition, the condition of diabetes can increase the availability of free fatty acids due to lipolysis process. The increase of free fatty acids will activate the immune system for releasing cytokines IL-6, TNF-α, IL-1β. It also explains the link between obesity and the increase of inflammation.

TNF-α and IL-6, inflammatory compounds, are released by adipose cells and immune cells (neutrophils, macrophages), and muscle cells. Nearly 30% of IL-6 is released by visceral adipose tissue. TNF-α plays a role in apoptosis of microvascular in DM type 1 and 2, was involved in the pathogenesis of diabetic nepropathy and retinopathy [4]. Inflammation causes not only insulin resistance which can worsen the condition of diabetes but also dysfunction of β cells [5,6].

Consumption increase of natural antioxidants can suppress the excess of inflammation [7,8,9,10]. The purpose of this study was to determine the capability of the extracted roselle toward decreasing levels of inflammatory compounds existing in diabetic rats.

Benefits of the research was to provide information about Roselle as anti-inflammatory properties that will contribute to the roselle functions on other degenerative diseases such as obesity, heart disease and atherosclerosis.

MATERIALS AND METHODS

Materials

The main used material was purple petals of roselle (*Hibiscus sabdariffa* Linn) obtaining from roselle plantation in Leuwiliang, Bogor. Male rats from Sprague Dawley strain (200-250g / head) with a-2-month age were used as experimental animals. Rats were obtained from BPPOM, Jakarta. inflammatory compounds were analyzed using immunochemical method (using a commercial kit of tumor necrosis factor alpha (TNF-α) from Biorbyt, UK with the catalog no. orb50113 and interleukin 6 (IL-6) from Biorbyt, UK with catalog no.orb50053, and BCA kit for protein content analysis. The main equipments for measuring the protein content of spleen fractions, and measurement of IL-6 and TNF-α were a micropipette (Eppendorf) and a microplate reader.

Methods

Extraction of roselle

Extracted Roselle was made by boiling dry roselle as much as 1% in water for 10 minutes and then filtered. Extracted roselle was concentrated using a vacuum evaporator for 10 times.

Experimental Design

Grouping for experiment was divided into six groups which one group comprised of four rats. Six groups for experiment consisted of (1) Group of rats was not induced with streptozotocin and given distilled water (SG); (2) Group of rats was induced with streptozotocin and given a-dose-0.45 mg/kg bw of glibenclamide suspending in distilled water (DiG); (3) Groups of rats were induced with streptozotocin and given distilled water (DiW); (4) Group of rats was fed extracted roselle for 11 days and then induced with streptozotocin before giving extracted roselle for a-21-day treatment period (PR1); (5) Group of rats was induced with streptozotocin and given roselle extract (72 mg / day / 200 g wb) (DiR1); and (6) Group of rats was induced with streptozotocin and given roselle extract (288 mg / day / 200 g wb) (DiR2).

Rats were injected intraperitoneally with a-35-mg / kg wb-dose of streptozotocin in 0.1 M cold citrate buffer (pH 4.5) [11]. After 72 hours of injection, the tail of rats was given alcohol 70% and tested blood glucose levels using a blood glucose test meter (Accu check). A rat will suffer diabetes if its blood glucose level > 200 mg/dl

Before being given treatment, rats adapted during the first seven days. During adaptation period, all rats were treated with the same standard diet. After seven days, treatment rats were given treatment based on six selected groups for 21 days. Drinking was given ad libitum. Feed for a rat was given more or less 25 g per day. A rat was weighted two days once. Residual feed was measured every day for checking how much treatment feed consumed every day.

The roselle extract was administered 2 ml/day orally. During treatment period, glucose levels were measured 2 or 3 times in all groups of treated rats. At the end of the treatment period, all rats were anaesthetized with ether, and the body was cleaned by alcohol. Further, the peritoneal was opened, and blood was sampled by using the syrinx. Rat spleen was washed in a PBS (phosphate buffered saline) solution, then drained and weighted. The organs were then wrapped in aluminum foil and stored in a freezer at -20ºC [12].

Analysis Method

Insulin

Insulin assay was transformed in a commercial kit (Biorbyt ORB54820, USA) using a sample of mouse blood plasma. This assay is a two site ELISA. The microplate was precoated with a monoclonal antibody against insulin. Standard and samples were added into the wells and co-incubated with a monoclonal antibody conjugated to horseradish peroxidase (HRP) enzyme. After washing step to remove any unbound substances, TMB substrate was added and colour develops in proportion to the amount of insulin bound initially.

Sample Preparation for Inflammatory Compounds Analysis.

A number of spleen samples (approximately 0.1 g) was weighted and added to 1 ml of PBS solution. Samples were milled until smooth, then centrifuged 4500 rpm 4 ° C for 10 minutes. The resulting supernatant was analyzed for levels of the protein. Measurement of protein levels was conducted using Bichinconinic acid (BCA). TNF alpha was analyzed using the kit catalog orb50113 of Biorbyt UK, while IL6 was analyzed using a kit catalog orb50053 of Biorbyt UK.

Biorbyt's mouse TNF-α and IL-6 ELISA Kit were based on standard sandwich enzyme-linked immune-sorbent assay technology. Mouse TNFα and IL-6 specific-specific monoclonal antibodies were precoated onto 96-well plates. The mouse specific detection polyclonal antibodies were biotinylated. The samples test and biotinylated detection antibodies were added to the wells subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow was proportional to the mouse TNFα amount of sample captured in plate. Inflammatory compound was determined using previous method [13]

RESULT AND DISCUSSION

Diabetes was characterized by increased levels of blood glucose. The blood glucose data were derived from the calculation of average blood sugar level on 4 rats during the treatment period as shown in Figure 1. In Figure 1, 7th day was the induction day with STZ and after 2-3 days rats were hyperglycemic indicated by an increase of blood glucose >200 mg/dl.

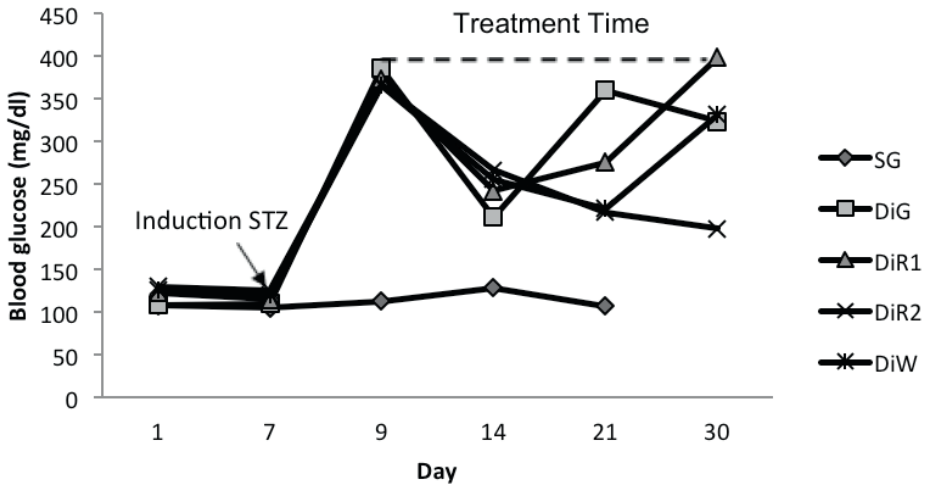

Figure 1.Average of blood glucose of treated rats (day 1 first level of blood glucose ; day 7, STZ induction, the 9th-21th day treatment time with roselle (DiR1,DiR2); with glibenclamide (DiG); control (SG) and negative (DiW).

Figure 1 showed that rats with given roselle extract 72 mg/ml/200g wb (DiR1) had a decrease of blood glucose level on the 14th day, but then increased again until the end of the research period. Meanwhile rats with given roselle extract 288 mg/ml/200g wb (DiR2) had a decrease of blood glucose level from the 14th day to the end of the research period. Negative control rats (DiW) had decreased of blood glucose level on the 7th day and the 14th day, but the blood glucose level increased again until the end of the research period. Rats that fed glibenclamide drug (DiG) had a decrease of blood glucose level on the 14th day. However, this blood glucose level was found to be increase on the 21th day before decreasing again at the end of the research period.

Figure 2 showed blood glucose of preventive rats group (PR1). The rats had hyperglycemia 2 to 3 days after. Once the conditions of hyperglycemia (blood glucose >200 mg/dl) were obtained, the rats were given roselle again until the 23rd or 24th days. These rats were found to suffer from hyperglycemia from the 13th days to 18th days before decreasing in blood glucose until the end of the experiment.

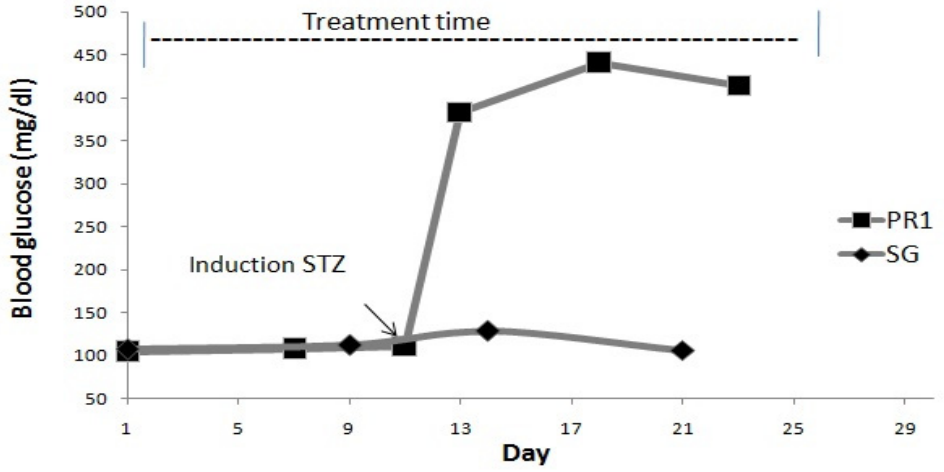

Figure 2. Average blood glucose of rats (PR1), given roselle extract 72 mg/ml/200g wb for 10 days,. Day 11th was the induction time with STZ. After 3 days rats had hyperglycemia and were continued to receive roselle until day 23.

Table 1 showed an increase of insulin in the group of rats with roselle dose 1 and dose 2 (DiR1 and DiR2), but this increase did not correlate with a decrease in blood glucose levels (Figure 1). Insulin increase was not able to lower blood glucose levels. This indicated the occurrence of insulin resistance that was characterized by type 2 diabetes mellitus. Insulin resistance is a decrease in the ability of insulin to bring glucose on the muscle cells and adipose cells, and to reduce the ability of the liver in producing glucose [4].

Table 1. The average of inflammatory compounds on rat spleen cells and content of insulin in rats plasma

Treatment	Protein content (µg/g)	*TNF- α* (pg/µg)	*IL-6* (pg/ µg)	Insulin (ng/ml)
SG	49250	1.0569±0.0874[a]	1.7298±0.4412 [a]	0.1968 ± 0.0486[ab]
DiW	89498	1.7404± 0.1479 [a]	3.4731± 0.9200 [a]	0.1286 ± 0.0337 [a]
PR1	86194	1.5537±0.3533 [a]	3.2595± 0.9443 [a]	0.3516 ± 0.1528 [ab]
DiG	52826	1.3646 ± 0.5846 [a]	2.7845± 0.9988 [a]	0.3504 ± 0.1853 [ab]
DiR1	85258	1.3534 ± 0.3998 [a]	-	0.4433 ± 0.1802[b]
DiR2	79955	1.1975 ± 0.3519 [a]	3.2801±0.2917 [a]	0.2918 ± 0.1083 [ab]

Values (Mean± SE) with different superscripts in a colom differ significantly (P<0.05); n=4
*control normal (SG); negative (DiW);; roselle 72 mg/day/200g wb (DiR1); roselle 288 mg/day/200g wb (DiR2); preventive rats (PR1); glibenclamide (DiG).

Insulin resistance can be caused by damage of either insulin receptors or of insulin itself [14]. One of the causes of insulin resistance is inflammatory compounds such as IL-6 and TNF-α that increased in diabetes. According to Sattar et al. [15], TNF-α and IL-6 would increase on rats with either obesity or resistant insulin conditions. IL-6 induced a number of glucocorticoid receptors, increased circulation of glucagon concentration and

adipose paracrine effect to decrease insulin action. TNF-α may function as a mediator of insulin resistance because this cytokine can damage the insulin receptor (IR) and insulin receptor substrate (IRS) and then it can inhibit insulin signal. TNF-α stimulated the expression of SOCS (suppressor of cytokine signal) which binded either IRS1 or IRS2 and mediated damage [16]. As a result, insulin could not take glucose into the muscle cells and adipose tissue, therefore glucose levels in blood plasma would increase. As its compensation, the β cells of the pancreas would produce excessively insulin and caused hyperinsulinemia. This condition then can lead to vascular inflammation and finally insulin resistance [4,6].

Table 1 showed TNF-α compounds data were not significantly different, but the levels of TNF-α roselle group 1 (DiR1) and group 2 rats roselle (DiR2) tended to be lower than groups of negative rats (DiW). The content of TNF-α on group of glibenclamide rats (DiG) (1.3646 ± 0.5846 pg /µg) was similar to group of rats with roselle 1 (1.3534 ± 0.3998 pg/µg). Table 1 showed IL-6 data did not differ significantly. However, group of diabetic rats (DiW, PR1, DiG and DiR2) tended to have a higher level of IL-6 than of normal rats (SG).

Diabetic rats (DiW) had the least amount of insulin and the highest level of TNF-α and IL-6 among other groups of rats. This is likely because inflammatory compound TNF-α leads to β-cell apoptosis. High concentration of inflammatory cells may cause apoptosis in β-cell which can lead to either lowering in number of cells of insulin-producer or insulin decrease [6,17].

Group of diabetic rats (DiW) had higher levels of both IL-6 and TNF-α than other groups. Groups of diabetic rats had hyperglycemia (blood glucose> 250 mg / dl). This condition causes high oxidative stress due to the excessive production of free radicals (ROS) that has not been supported by antioxidant defenses in the body. According Virgolici et al. [18] the increased levels of inflammation were associated with the increased oxidative stress. Diabetic conditions could increase inflammatory compounds such as TNF-α and IL-6. The increase of inflammatory compounds related to the increase in BMI (Body Mass Index) and insulin resistant [3]. Other studies mentioned the plasma of people who were obese and got type 2 diabetes mellitus had the high concentrations of IL-6 [19].

On the diabetic condition (DiW), an increase of superoxide can activate protein kinase C (PKC) [19]. PKC plays a role to activate cytokines. This causes an increase of cytokines levels in the study of TNF-α and IL-6 in the group of diabetic rats. Chang et al. [9] furthermore stated that diabetes mellitus may activate NF-κB (Nuclear factor kappa B) that was responsible for the production of several inflammatory compounds in which the expression of genes was regulated by proinflammatory cytokines, growth factors, and adhesion molecules.

Table 1 showed the rat groups of roselle (DiR1 and DiR2) tended to a lower value of TNF-α than the group of diabetic rats (DiW) Glibenclamide rat groups (DiG) had the same compounds of TNF-α as the group of rats DiR1 and DiR2. In this study, glibenclamide also has the ability to decrease the levels of inflammatory TNF-α. Table 1 showed all rats with diabetes had an increased level of inflammatory compounds IL-6, but the provision of rosella extract did not give effect to a decrease of the inflammatory compounds level.

TNF-α decreasing in the group of diabetic rats with roselle (DiR1 and DiR2) was expected because of the content of antioxidants that existed in roselle extract. Roselle contains anthocyanins, vitamin C, tannins, steroids, hidroxyquinon phenols and saponins. According to Giriwono et al. [7], 10% fermented barley supplementation

containing polyphenolic compounds might decrease 8OHDG levels on the plasma, and inhibited the increased levels of NO (nitric oxide) in plasma that associated with an increased inflammatory system.

The antioxidant activity of polyphenolic compounds existing on fermented barley would inhibit the propagation of ROS that would induce inflammation and organ damage due to induce of microbial lipopolysaccharide. Antioxidants on fermented barley could either suppress inflammatory cytokine levels on plasma (IL-6, IL-1β, TNF-α) or lower concentrations of NO plasma. Extracted roselle is also anti-inflammatory, analgesic, and has antipyretic activity on experimental animal [20]. The decreasing level of inflammatory compounds was also caused by increasing levels of liver antioxidant enzymes as reported by Ebaid et al. [8] that whey protein given to diabetic rats led to increasing levels of glutathione in the liver and decreasing inflammatory compounds. According to Okoko and Ibiba [21], extracted roselle can increase the enzyme of glutathione peroxidase (GSH) and catalase as well as lower the levels of TBARS (thiobarbituric Acid Reactive Substances). The research done by Chang et al. [9], the antioxidant compound found in grapes and berries were resveratrol that have ability of resveratrol as an anti-inflammatory of gene expression through suppression of gene expression on the strip of NF-κB and gene expression of cytokine IL-1β. Resveratrol was given to diabetic rats that were induced by streptozotocin. They further stated that resveratrol can prevent an excessive production of ROS due to its ability to capture free radicals. Research by Rytter [10] showed that giving α-carotene, β-carotene, lutein and lycopene may reduce levels of IL-6 on patients of diabetes.

This study showed that giving extracted rosella tended to have ability for decreasing inflammatory compounds. A decrease of inflammatory compounds could be expected for increasing insulin receptor sensitivity and repairing blood sugar taking into cells. According to Donath et al. [17] a decrease of inflammation in pancreatic β cells closely associated with the increase proinsulin synthesis to be insulin and improved insulin sensitivity and pancreatic β cell mass.

Conclusion

Diabetes causes elevated levels of inflammatory compounds. Groups of diabetic rats were given a dose of 72 mg roselle / day / 200 g BW and 288 mg / day / 200 g BW tended to be lower levels of TNF-α. However, the provision of rosella in diabetic rats did not affect the levels of IL-6. Antioxidant compounds contained in roselle can suppress or reduce levels of free radicals and cause decreased levels of TNF-α. This shows that the alleged roselle tend to reduce the level of inflammation, so that the impact of continuous severity due to inflammation can be suppressed.

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