The Influence of Ethanolic Root Extracts of *Ruellia tuberosa* L. on Pancreatic Protease Activity and MDA Level of Rats (*Rattus norvegicus*) Induced by MLD-STZ

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Abstract. Diabetes mellitus (DM) is a metabolism disorder, indicated by increasing of blood glucose levels, due to lack of insulin secretion or insulin resistance. This study aimed to determine the influence of ethanolic root extracts of *Ruellia tuberosa* L on protease activity and MDA levels on pancreatic diabetic rats. This experiment used 20 rats in randomized design, that were divided into five groups: negative control, positive control, and three groups of treatments, with doses of 250, 375, and 500 mg/kg bw, respectively for 21 days. The positive control group and therapeutic group were induced by MLD-STZ (multiple-low dose streptozotocin) at dose of 20 mg/kg bw for 5 consecutive days, in order to induce diabetic states. Protease activity and MDA levels were determined by spectrophotometric method. The results showed that ethanolic root extracts of *Ruellia tuberosa* L. at dose of 250 mg/kg bw was the best dose to decrease significantly protease activity and MDA level in the pancreas of diabetic rats with the percentages of 52.11% and 50.54%, respectively.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that indicated by high blood glucose levels that caused by impairing insulin secretion from pancreatic β–cells. Type I diabetes mellitus often affects children but does not rule out the possibility for adults. The damage of pancreatic β–cells is caused by virus infection, genetic disorder or induction of toxic agents such as steptozotocin and alloxan [1].

Streptozotocin (STZ) (2-deoxy-2-(3-methyl-3-nitrosoureao)-1-D-glucopyranose) is glucose analogue that is taken up by pancreatic β–cells through GLUT2 transporter, where it causes β–cells death due to DNA fragmentation. Possible mechanism of the diabetogenic action of STZ is attributed by its ability to act as nitric oxide (NO) donor in pancreatic cell that inhibits aconitase activity leading to DNA alkylation and damage. β–cells are particularly sensitive to damage by nitric oxide and free radicals because of their low levels of free radical scavenging enzymes. The increase of free radicals can be marked by the increase of malondialdehyde (MDA) levels. The damage of pancreatic β–cells lead to hyperglycemia that is accumulated of glucose in cell and even in tissue triggering the inflammation., consequently, the activity of protease will increase [2].

Currently available medication for diabetes mellitus are by administration of insulin and synthetic drugs, including sulphonylurea and biguaride derivatives. However, such medications can cause adverse side effects such as hypoglycemia, obesity, and renal dysfunction [3]. Therefore, it is important to study natural products from food and/or plants as potential anti-diabetic compounds. Due to less side effects compared to synthetic drugs, currently 80% of the world population depends on plant-derived medicine for the first line of primary health care [4]. One of the plants that commonly used for diseases therapy is *Ruellia tuberosa* L. This plant has been proposed to have potency as anti-diuretic, antidiabetic, antipyretic, analgesic, antihypertensive, and antidotal agents. A previous study explored the leaves part of *R. tuberosa*, and reported that leaves of *R. tuberosa* contain phenol compounds (0.36 mg/g), saponins (0.10 mg/g), glycosides (0.59 mg/g), flavonoids (0.75 mg/g) and vitamin (K, C) and carotenoid content [5]. The bioactive compounds of *R. tuberosa* root obtained from GC-MS revealed 25 compounds including stigmasterol 8.89%, sitosterol 3.99%, cholesterol 2.24% and lupeol by 68.14%. Triterpenoid is the major bioactive compounds contained in the *R. tuberosa* root can act as antioxidants [6], which can decrease ROS [7].
R. tuberose leaves and stems are already widely for hyperglycemia treatment. Water extract of R. tuberose leaves can lower blood glucose levels of diabetic rat. The utilization of R. tuberose root has not yet applied for hyperglycemia therapy and there is no scientific research on the hypoglycemic effect; hence the extract of R. tuberose root has IC\textsubscript{50} of 10.06 μg/mL [7]. Based on the description above, this study has been conducted to determine the effect of R. tuberosa root parts to decrease the MDA level and protease activity in pancreas of diabetic rat induced by MLD-STZ.

2. Experimental Methods
2.1. Animal Model
The animal model of male Wistar white rats were purchased from Institute of Biosciences, Brawijaya University, Malang. The experimental animals have received a certificate of ethics from Research Committee of Brawijaya University, No: 873-KEP-UB-2018. The materials used in this research were Ruellia tuberosa L roots which purchased from Materia Medica, Batu City, East Java. Other materials were purchased from Sigma-Aldrich: ethyl alcohol (pure, d = 0.789 g/mL), glacial acetic acid (pharmaceutical secondary standard), HCl (37%, analytical grade), H\textsubscript{2}SO\textsubscript{4} (99.99%, analytical grade), NaCl (powder, ≥99.5%, analytical grade), sodium citrate solution, citrate buffer solution pH 4.5. Other materials used were streptozotocin (Bioworld), PBS solution (Phosphate Buffer Saline), 1% PBS-azide solution, 10% trichloroacetic acid (TCA), and MDA reagent. Instruments used was UV-Vis spectrophotometer (1601, Shimadzu).

2.2. Preparation of Diabetic Rats
The rats were adapted in the animal house for a week prior to experiment. Rats were divided into five groups: (I) negative control group; (II) diabetic group; and (III, IV, V) therapy groups, 4 rats in each group. A 100 mg of streptozotocin was dissolved in 3 mL of citrate buffer at pH 4.5. A dose of 20 mg/kg bw was intraperitoneal injected to rats in groups II, III, IV, V for 5 consecutive days, while rats in group I (negative control) were injected with PBS only [8].

2.3. Extraction of R. tuberosa Root
Root powder was macerated with ethanol:water (1:1), the volume was 7.5 times the weight of the powder. Maceration solution was stirred every 1 h in the first 5 h, and then allowed to stand for 48 h. The extract obtained was decanted to separate from R. tuberosa root. Solvent in the extract was evaporated with a rotary evaporator at 40 °C, 100 rpm, until the concentrated extract obtained.

2.4. Therapy with Ethanolic Root Extracts of R. tuberosa for Group III, IV, V
Rats in group III, IV, V were treated with extract of R. tuberosa root at a dose of 250 mg/kg bw, 375 mg/kg bw and 500 mg/kg bw per day, for 21 consecutive days. Changes in blood glucose levels were monitored per week during the treatments. At the end of the assay, rats were sacrificed, and the pancreatic organ was collected for further analysis.

2.5. Measurement of Malondialdehyde (MDA) Levels
Measurement of MDA levels was conducted by spectrophotometry method, using TBA reagents. The pancreatic homogenates from five groups group 1, II, III, IV, V were prepared based on previous method [9]. The pancreatic supernatants were measured their absorbance using a UV-Vis spectrophotometer at the 530 nm.

3. Results and Discussion
Diabetic rats have been successfully prepared by intraperitoneal injection of MLD-STZ. All diabetic rats of groups II, III, IV, V showed blood glucose levels above 200 mg/dL, that was 542 mg/dL, while the negative control was 134.25 ± 20.77 mg/dL (normal standard of blood glucose level is 50 – 135 mg/dL) [10]. The high blood glucose level related with free radicals such as reactive oxygen species. The imbalance of free radicals and endogenic antioxidant causes oxidative stress lead to occur lipid peroxidation. The number of lipid peroxidation can be marked by MDA level. The group III, IV, V were then therapies by ethanolic extract of R. tuberosa root and the results of MDA level on pancreas of diabetic rats are displayed at Table 1.
Table 1 MDA levels on pancreatic rats from group I, II, III, IV, V after 21 d of therapy with root extracts of *R. tuberosa*

| Group                          | MDA levels (μg/mL)* |
|--------------------------------|---------------------|
| I. negative control            | 1.263 ± 0.136*      |
| II. Positive control           | 4.137 ± 0.145*      |
| III. Therapy 250mg/kg bw       | 2.046 ± 0.181      |
| IV. Therapy 375 mg/kg bw       | 2.502 ± 0.132      |
| V. Therapy 500 mg/kg bw        | 3.006 ± 0.150      |

*different letters (a-e) show significant statistical different effect in each group (p <0.05)*

Based on Table 1, negative control group showed MDA level of 1.263 ± 0.136 μg/mL which is significantly different compared to positive control group. MDA level in normal rat as a result of byproduct of normal metabolism and phagocytosis of damaged cells. The number of free radicals in negative control is not higher than the number of endogenic antioxidants lead to scavenge the free radicals and inhibit lipid peroxidation. On the other hand, MDA level in positive control showed the highest value because of STZ activity. The induction of MLD-STZ causes the increase of free radicals on rat pancreas triggering oxidative stress and lipid peroxidation. Lipid peroxidation on cell membrane due to reaction between free radicals and *poly unsaturated fatty acid* (PUFA) leads to produce MDA which is toxic to cell [11].

The therapy of *R. tuberosa* root extract with the dose of 250 mg/kg bw significantly decreased pancreatic MDA level compared to positive control group, and closed to the value of negative control group. The decrease of MDA levels is suggested as a result of improvement of pancreatic β cells. *R. tuberosa* root extracts contain phytosterol compounds, that have functions as antioxidant and can scavenge free radicals. Moreover, phytosterol compounds will activate GLUT4 and enhance insulin secretion into the bloodstream that will improve the process of glucose uptake [12]. In contrast, at group therapy 375 and 500 mg/kg bw showed the MDA level slightly increased since excessive amounts of phytosterol compounds, that may be act as pro-oxidants, instead of antioxidants.

In addition, in diabetic state, increasing MDA level also affects protease activity. The therapies effect of ethanolic extract of *R. tuberosa* root to protease activity of diabetic rat pancreas are listed in Table 2.

Table 2 Protease activity on pancreatic rats from group I, II, III, IV, V after 21 d of therapy with root extracts of *R. tuberosa*

| Group                          | Protease activity* (μmol/mL min) |
|--------------------------------|----------------------------------|
| I. negative control            | 0.021 ± 0.004*                   |
| II. Positive control           | 0.071 ± 0.005*                   |
| III. Therapy 250 mg/kg bw      | 0.034 ± 0.005*                   |
| IV. Therapy 375 mg/kg bw       | 0.046 ± 0.002*                   |
| V. Therapy 500 mg/kg bw        | 0.056 ± 0.001*                   |

*different letters (a-e) show significant statistical different effect in each group (p <0.05)*

Under normal conditions, protease functions to help the apoptosis process. Cells will undergo apoptosis after a certain life span. Proteins in damaged or dead cells may no longer be needed that will be degraded by the protease. Protein degradation is the process of maintaining or protecting cells or maintaining cells where unwanted or damaged proteins are broken down into amino acids that can be reused for cell regeneration [13]. Based on Table 2, the negative control group showed protease activity values of 0.021 ± 0.004 μmol/mL min. This value is used as a standard for determination of increasing protease activity of positive control.

The increase in protease activity of 238.09% is a result of streptozotocin induction which can cause an inflammatory process in the rat pancreas which will activate pro-inflammatory cells and release of protease enzymes. STZ is a toxic chemical in pancreatic β cells. Streptozotocin enters β cells by glucose transporter molecules (GLUT2) and causing DNA alkylation (addition of alkyl groups on DNA). DNA damage induces poly ADP-ribosylation activation, poly ADP-ribosylation causes depletion of cellular
NAD$^+$ and ATP. Increased dephosphorylation of ATP after administration of streptozotocin produces a substrate for xanthine oxidase that produces superoxide radical formation (O$_2^•$). As a result, hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH $•$) are also produced. Furthermore, streptozotocin release a number of toxic nitric oxide (NO) which inhibits aconitate activity and contributes in DNA damage [1].

The appearance of ROS activates Nuclear Factor kB (NF-kB), that is a transcription factor which regulates the expression of inflammatory genes and immune genes, which play an important role in the inflammatory process. NF-kB plays a role in regulating the expression of immune genes, and inflammatory responses, such as proinflammatory cytokines, chemokines, inflammatory enzymes and adhesion molecules. NF-kB can induce inflammatory cells such as macrophages and neutrophils which function as immune defense cells. In addition, neutrophils will also release protease enzymes (proteolytics) in response to inflammation. The activity of protease enzymes is used to measure the level of inflammation. The higher the value of protease activity, the higher the level of tissue damage. Therefore, the protease activity value of the diabetic group is higher compared to the negative group [14].

Protease activity in the therapy group 250 mg/kg bw, 375 mg/kg bw and 500 mg/kg bw decreased respectively by 52.11%, 35.21% and 21.12%, against positive control. The decrease of protease activity was in line with decreasing of blood glucose levels, it showed that the administration of ethanolic extract of $R$. tuberosa has the potential antioxidants to scavenge free radicals. The mechanism reaction is supposed that O-H bound of stigmasterol will be homolytically broken up and radical H$•$ binds to the ONOO radicals derived from STZ to form HNO$_3$. At the end of reaction, the remain H$•$ is possible to bind others H$•$ derived from $β$-sitosterol or campesterol, leading to H$_2$ production. Therefore, the compounds of stigmasterol, $β$-sitosterol and campesterol may act synergistically to reduce free radicals. However, high doses of ethanolic root extracts of $R$. tuberosa is not significant in reducing protease activity due to the excess of antioxidants may be turned as pro-oxidants similar to free radicals.

4. Conclusion

The ethanolic root extract of $R$. tuberosa at a dose of 250 mg/kg bw was the best treatment dose to decrease the blood glucose level, pancreatic malondialdehyde level and protease activity of diabetic rats. The flavonoid and phytosterols contained in the $R$. tuberosa root extracts may be responsible for the anti-diabetic actions. Further research is needed to investigate molecular mechanisms of flavonoids and phytosterols acting as free radical scavengers and stimulator of insulin secretion and also the toxicity of $R$. tuberosa extract.

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