Anti-diabetic properties of root extracts of *Ruellia tuberosa* L: effects on serum enzyme activity

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Abstract. This study aims to determine the anti-diabetic properties of the root extracts of *Ruellia tuberosa* L to enzymes (amylase, protease, and lipase) activities in serum of diabetic rats. Rats were divided into five groups: control, diabetic, and treatment groups with doses of 250, 375, and 500 mg/kg body weights. All treatment groups received root extracts of *R. tuberosa* L for 21 days by oral administration. Results from LC-MS study revealed that the flavonoids compounds including sorbifolin, cirsimaritin, cirsimarin, and cirsiliol 4'-glucoside, were detected in the extracts. The animal study results showed decreases in blood glucose levels by 54.56%, 37.70%, and 16.79%, for treatment doses of 250, 375, and 500 mg/kg body weights, respectively. All enzymes activities increased in diabetic rats, and after treatment the enzyme activities decreased. The decreases in protease activity were 52%, 36%, and 20% for doses of 250, 375, and 500 mg/kg body weights, respectively. In addition, the amylase and lipase activities also showed similar trends with decreasing activities to 63% and 51%, 42% and 37%, 16% and 21%, for treatment doses of 250, 375, and 500 mg/kg body weights, respectively.

Keywords: *Ruellia tuberosa* L, diabetes, serum, protease, amylase, lipase

1. Introduction

Based on our previous research, *Ruellia tuberosa* L n-hexane root extracts had anti-diabetic activity by conducting an *in vivo* study [1]. These included reduce blood glucose levels, reduce malondialdehyde (MDA) levels, and repair on the liver of histopathologic profiles [1]. Moreover, *R. tuberosa* L. hydroethanolic root extracts also had anti-diabetic activity from their positive effects on the pancreatic of diabetic rats [2]. Phytochemical investigation of this study revealed that *R. tuberosa* L. roots extracted with ethanol and water contained mostly flavonoid compounds [3].

In the current work, *R. tuberosa* L. roots are extracted using maceration technique, followed by identification and characterization of the resulted extracts, as previously mentioned in our paper [3]. The anti-diabetic properties of root extracts were determined by their effects on serum enzyme activity. The enzymes investigated were digestive enzymes: amylase, lipase, and protease.

Amylase plays a role in catalyzing the process of starch hydrolysis producing simple molecules such as glucose so that the body can be converted into energy [4]. Lipase is an enzyme that hydrolyzes ester bonds such as triglycerides to free fatty acids and glycerol [4]. Pancreatic damages in diabetic condition may lead to exocrine and endocrine inflammation or pancreatitis [5]. Progressive pancreatic inflammation causes irreversible pancreatic damage and results in exocrine and endocrine dysfunction.
In addition, pancreatic inflammation is also associated with an increase and decrease in amylase and/or lipase activity [6, 7]. Increased protease enzyme activity can also an indicator of inflammation in people with diabetes. Increased protease activity can be affected by advanced glycosilation end products (AGEs) and release of cytokines. Release of cytokines due to increase of protease activity causes the formation of an immune response activate the inflammatory system; whereas AGEs are formed through the replication process. Increased AGEs damages cells because of disturbing intracellular and extracellular proteins [8, 9].

2. Materials and Methods
2.1. Chemicals and Instrumentation
Chemicals were purchased from Sigma-Aldrich: ethyl alcohol (pure, d = 0.789 g/mL), glacial acetic acid (pharmaceutical secondary standard), HCl (37%, analytical grade), H₂SO₄ (99.99%, analytical grade), FeCl₃ (97%, reagent grade), NaCl (powder, ≥99.5%, analytical grade), KMnO₄ (≥99.0%, reagent grade), streptozotocin (BioWorld), PBS solution (Phosphate Buffer Saline), 10% trichloroacetic acid (TCA), PBS-Tween, PMSF (Poly Methyl Sulfonil Fluoride), buffer Tris-HCl pH 6.5, phenolphthalein indicator, starch, DNS reagent, tyrosine, casein. The in vivo study used male rats (Rattus norvegicus), Wistar strain, purchased from Institute of Biosains, Brawijaya University, Malang. The study has obtained a certificate of ethics from the Research Committee of Universitas Brawijaya No: 873-KEP-UB. The plant simplisia of R. tuberosa L. were obtained from Materia Medica, East Java. The plant was identified and authenticated by a plant taxonomist of the herbarium unit, UPT Materia Medica, Batu. Instruments used were UV-Vis spectrophotometer (1601, Shimadzu).

2.2. Extract Preparation and Characterization
Extract preparation was reported previously [10]. The identification and characterization of the extract was published in our preceding paper [3].

2.3. In Vivo Study
Rats were divided into five groups: (1) control group; (2) diabetic group; (3) treatment group 1; (4) treatment group 2; and (5) treatment group 3. Rats were kept in the animal house, in Biosains Laboratory, Brawijaya University. Streptozotocin in a dose of 20 mg/kg of body weight was injected intraperitoneally to rats in groups 2 to 5, for 5 days consecutively. Rats in groups 3, 4, and 5 were treated with R. tuberosa L. extracts in doses of 250; 375; and 500 mg/kg body weights per day, respectively, for 21 days. Changes in blood glucose levels were monitored per week during the treatment. At the end of the experiment (day 21), rats were sacrificed, and the blood serum was collected and stored at 4 °C for subsequent process.

2.4. Measurement of Serum Enzyme Activity
2.4.1. Enzyme Isolation. PBS-Tween:PMSF (9:1) solution was added to serum, 5× sample volume. The mixture was mixed, then centrifuged for 10 min at 6000 rpm (4 °C). The supernatant was separated, ethanol solution was added to the supernatant with a ratio of 1:1 and left overnight until the precipitate formed. The precipitate then re-centrifuged for 15 min at a speed of 10,000 rpm. The precipitate was dried until all solvent evaporated. The Tris-HCl buffer solution pH 6.5 was added to the precipitate, in 1:1 ratio of volume.

2.4.2. Enzyme Activity Determination. For amylase serum activity, 1% starch was used as enzyme substrate. A 1% starch (0.5 mL) was mixed with serum (0.05 mL), free-reductant water (0.5 mL), and then incubated for 10 min at 42 °C. The DNS reagent was added to the mixture, and boiled for 5 min. The absorbance of the final solution was determined using UV-Vis spectrophotometer at λ 490 nm. The amylase activity was calculated using this equation:

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\text{enzyme activity} \left( \frac{\text{mg}}{\text{mL} \cdot \text{min}} \right) = \frac{[\text{glucose}]}{\text{mol weight of glucose} \times \frac{v}{p \times q} \times \text{dilution factor}}
\]
with: $v$ is sample total volume (mL); $p$ is enzyme volume (mL); and $q$ is incubation time (min).

For lipase activity, olive oil was used as substrate. A 2.5 mL of olive oil was transferred into a Erlenmeyer flask. Mixture of 22.5 mg of Arabic gum, 15 mL of CaCl$_2$, 10 mL of 3 M NaCl, and phosphate buffer, pH 7. From this mixture, a 2.5 mL of solution mixture was mixed with 0.5 mL sample, and shaken for 15 min. The mixture was incubated for 6 min, at 30°C, and boiled for 1 min. The solution was titrated with 0.05 M NaOH, with phenolphthalein indicator. The lipase activity was calculated using this equation:

$$\text{enzyme activity (}\mu\text{g mL}^{-1}\text{min}^{-1}) = \frac{(V_s - V_b) \text{NaOH} \times M \text{NaOH} \times 1000}{\text{enzyme volume} \times \text{min}}$$

with: $V_s$ is volume of sample titration; and $V_b$ is volume of blank titration.

For protease activity, casein was used as substrate. A 500 ppm of casein was mixed with phosphate buffer and sample solution, and incubated for 60 min at 37°C. A 4% of TCA solution was added and incubated for 30 min at room temperature. The solution mixture was centrifuged at 4000 rpm, for 10 min, at room temperature. Supernatant was separated and diluted 3× sample volume with phosphate buffer, and the sample absorbance was measured at $\lambda$ 275 nm.

$$\text{enzyme activity (}\mu\text{g mL}^{-1}\text{min}^{-1}) = \frac{[\text{tyrosine}]}{\text{mol weight of tyrosine}} \times \frac{v}{p \times q} \times \text{dilution factor}$$

with: $v$ is sample total volume (mL); $p$ is enzyme volume (mL); and $q$ is incubation time (min).

### 3. Results and Discussion

One unit of amylase activity is defined as $\mu$mol glucose that resulted from hydrolysis of glycoside bond in starch, due to amylase action. Enzyme activity is determined by converting samples absorbance to glucose concentration. Based on Table 1, on diabetic rats (group 2), amylase activity increased 229.46%, with the average of amylase activity was 2.142±0.027 $\mu$mol/mL.min. In treated groups, 3 to 5, amylase activity has decreased. In group 3, treated group with 250 mg/kg body weight, has shown the highest decreasing effect, to 3.466±0.047 $\mu$mol/mL.min (51.16%). At doses of 375 and 500 mg/kg body weights, amylase activity decreased to 4.477±0.042 (36.65%) and 5.579±0.030 (20.94%) $\mu$mol/mL.min, respectively.

**Table 1. Amylase activity on serum of rats in groups 1 to 5, after treatment with *R. tuberosa* L. root extracts.**

| No | Group                        | Amylase activity* (\(\mu\text{mol/mL min}\)) |
|----|------------------------------|-----------------------------------------------|
| 1  | 1 (healthy rats)             | 2.142 ± 0.027                                 |
| 2  | 2 (diabetic rats)            | 7.057±0.046                                  |
| 3  | 3 (treated rats, 250 mg/kg body weight) | 3.466 ± 0.047                             |
| 4  | 4 (treated rats, 250 mg/kg body weight) | 4.477±0.042                                |
| 5  | 5 (treated rats, 250 mg/kg body weight) | 5.579±0.030                                 |

*different letters (a-e) indicate significant statistical effects in each group, at $p<0.05$

Lipase activity on rats from groups 1-5 is presented in Table 2. The lipase activity in diabetic rats group increased up to 381.4%, with the average of 43.125±1.2 $\mu$mol/mL.min. In the groups 3, 4, and 5 or treatment groups, there were decline in the lipase activity. Again, at a dose of 250 mg/kg body weight of *R. tuberosa* L. extract, resulted in the highest decrease of lipase activity. At doses of 375 and 500 mg/kg body weights, also had effects in lowering lipase activity, at 41.55% and 15.46%, to 25.208±1.423 and 36.458±2.295 $\mu$mol/mL min, respectively.
Table 2. Lipase activity on serum of rats in groups 1 to 5, after treatment with *R. tuberosa* L. root extracts.

| No | Group | Lipase activity* (μmol/mL min) |
|----|-------|-------------------------------|
| 1  | 1 (healthy rats) | 8.958 ± 1.8a |
| 2  | 2 (diabetic rats) | 43.125 ± 1.2b |
| 3  | 3 (treated rats, 250 mg/kg body weight) | 16.042 ± 1.5c |
| 4  | 4 (treated rats, 250 mg/kg body weight) | 25.028 ± 2.2d |
| 5  | 5 (treated rats, 250 mg/kg body weight) | 36.048 ± 2.1e |

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

Table 3 lists protease activity on serum of rats in groups 1 to 5. Similar trends in protease serum activity with those in amylase and lipase activity have been obtained. In the diabetic conditions, as in group 2, protease activity increased up to %, with the average of 0.068±0.007 μmol/mL min. Treatments with *R. tuberosa* L. root extracts had effects in decreasing protease activity. These have been shown in the protease activity in rats group 3, 4, and 5. Dose of 250 mg/kg body weight of *R tuberosa* L extract, resulted in the highest decrease of protease activity, at 0.029±0.002 μmol/mL min or decreased to 52.83%. Doses of 375 and 500 mg/kg body weights, also affected to the protease activity. These doses had decreased protease activity to 36.23% and 20.16%, or to 0.041±0.004 μmol/mL and 0.054±0.001 μmol/mL, respectively.

Table 3. Protease activity on serum of rats in groups 1 to 5, after treatment with *R. tuberosa* L. root extracts.

| No | Group | Protease activity (μmol/mL min) |
|----|-------|-------------------------------|
| 1  | 1 (healthy rats) | 0.019 ± 0.008a |
| 2  | 2 (diabetic rats) | 0.068 ± 0.007b |
| 3  | 3 (treated rats, 250 mg/kg body weight) | 0.029 ± 0.002c |
| 4  | 4 (treated rats, 250 mg/kg body weight) | 0.041 ± 0.004d |
| 5  | 5 (treated rats, 250 mg/kg body weight) | 0.054 ± 0.001e |

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

In these results, all digestive serum enzymes activity increased dramatically in diabetic conditions, and decreased after rats treated with root extracts of *R. tuberosa* L. Pancreatic β-cell damage after streptozotocin induction caused decrease insulin production. It is known that insulin as a hormone that facilitates glucose uptake to the cells. In addition, this hormone is also important for the liver, since it helps the liver to absorb glucose and store glucose in the form of glycogen. In the case of glucose deficiency, this induces acinar cells to secrete the amylase enzyme, which catalyzes the hydrolysis of starch into glucose, thus the glucose requirement in the body is still adequately fulfilled [11]. As a result, amylase activity in diabetic rats without any treatments (group 2) increased and had the highest amylase activity among others. Other digestive enzymes, lipase and protease, also have shown the highest activity in diabetic rats. In the diabetic states, lack of insulin secretion activates lipase to hydrolyze triglycerides into fatty acids and glycerol, hence, they produce energy. In addition, increasing blood glucose levels triggers the formation of advanced glycosylation products (AGEs), which are formed in the process of non-enzymatic glycosylation [12]. In AGEs pathway, neutrophils are activated as an indication of inflammation. The formation of AGEs in various biological pathways also lead to the synthesis of cytokines and free radicals. Protease activity is a sign of increased inflammation in cells.
Decreases in enzyme serum activity after treatment with root extracts of *R. tuberosa* L, can be related to the secondary metabolites compounds that contained in the extracts. Extracts of *R. tuberosa* L. contained mostly phytosterol and flavonoids compounds [3, 10]. The phytosterol compounds were including stigmasterol, campesterol, and β-stigmasterol [3]; whereas the flavonoids in the extracts were sorbifolin, cirsimarin, cirsimarin dan cirsiol 4-glucoside [10].

Phytosterol and flavonoid compounds are suggested act as free radical scavengers. In the molecular structure of flavonoids, the hydroxyl (-OH) groups can release H atoms and bind to free radicals, forming R-H bond, subsequently produce phenoxy flavonoid radicals (FIO*). These flavonoid radicals are stabilized by C=C double bonds in the flavonoid rings [3]. The proposed mechanism of action of flavonoids compound acting as free radical scavenger is illustrated in Figure 1. In addition, phytosterol compounds (stigmasterol, β-sitosterol, and campesterol) also contributed to the anti-diabetic activity. Stigmasterol improves GLUT 4 translocation which causes an increase in glucose uptake, therefore, glucose levels are reduced and insulin increases [13]. β-sitosterol is also useful in stimulating insulin secretion from the pancreatic-β cells.

![Figure 1. Proposed mechanism of actions of flavonoid as free radical scavengers.](image)

4. Conclusion
The current work has investigated effects of *R. tuberosa* L. root extracts in serum enzymes activity. The root extracts contained steroids, flavonoids, ascorbic acids, phytosterol, and flavonoid compounds. The root extracts of *R. tuberosa* L. have reduced enzymes (lipase, protease, and amylase) activity on the serum of diabetic rats. In summary, root extracts of *R. tuberosa* L. have the potential to be used as a natural cure for diabetes.

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