Effects of soursop leaf extract and physical training on decreasing oxidative stress and pancreatic histopathology in diabetic rat models

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Abstract

Context: Diabetes mellitus is a degenerative disease characterized by chronic hyperglycemia. Hyperglycemia is the cause of MDA levels increases due to damage to pancreatic beta cells. Regular physical exercise can improve insulin sensitivity. Soursop leaves are considered to have antioxidant effects. Aims: Here we aimed to prove the effectiveness of the soursop leaf extract and physical exercise could reduce blood glucose and MDA levels in diabetic rats. Settings and Design: The research method was experimental using the pretest–posttest design with a control group in a completely randomized design. Methods and Material: Ammout 28 diabetic rats using Alloxan were divided into five groups: negative control, positive control, and treatment groups: Physical exercise, soursop leaves with the dose of 150 mg kg−1, and a combination of both. The test was done by measuring MDA levels. Statistical analysis used: Data analysis of MDA levels was performed using one way ANOVA (α = 0.05) and description of pancreatic histology. Results: This study found that soursop leaf extract and moderate physical exercise of 20 m min−1 were able to reduce the MDA levels better than the combination treatment of both and positive control. The pancreatic histological picture shows inhibition of pancreatic beta–cell damage. Conclusions: In general, the result indicates that the administration of soursop leaf extract with the dose of 150 mg kg−1 BW−1 and physical exercise have a better antioxidant effect and can inhibit pancreatic damage in diabetic rat models.

Keywords: Annona muricata L., diabetes, oxidative stress, soursop leaf

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**Introduction**

Diabetes Mellitus (DM) is a degenerative disease caused by metabolic syndrome with hyperglycemia caused by decreased insulin secretion or progressive insulin receptor resistance. Chronic hyperglycemia conditions will lead to a rise in the oxidative stress. In 2009, International Diabetes Federation (IDF) also predicted an increase in the number of people with DM from 7,000,000 in 2009 to 12,000,000 in 2030. This data shows an increase in the number of DM patients as much as two times to three times\(^1\).

Oxidative stress is a condition of imbalance between free radical (ROS) production and antioxidants\(^2\). Hyperglycemic conditions in DM are caused by damage to the pancreatic beta cell lipid membrane due to the process of lipid peroxidation, so that the structure and physiological processes in the pancreatic beta cell membrane become disrupted\(^3\). Malondialdehyde is formed through lipid peroxidation in cell membranes, namely free radical reactions with poly unsaturated fatty acids (PUFA). Increased MDA serum and pancreatic tissue has a large potential for complications, both micro and macrovascular in DM\(^3\). Regular physical exercise as a therapy method can improve glucose homeostasis, reduce glucose/insulin ratios, and improve insulin sensitivity\(^4\). Physical training causes glucose uptake to increase seven times to 20 times\(^5\). Regular and measurable physical exercise can maintain endogenous antioxidants and reduce the number of end products from lipid peroxidase reactions, so that free radicals in the body will decrease\(^5\).

As an effort to reduce the side effects of antidiabetic drugs and control blood sugar levels and also reduce medical costs, the public and researchers began to focus on the use of herbal/traditional medicinal plants such as soursop leaves, that have been much in demand as antidiabetic agents and antioxidants. Soursop leaves are thought to have the potential as antioxidants because of its flavonoid content. Flavonoids are a group of polyphenol compounds which have high antioxidant activity and the potential to prevent the formation of free radicals so that they can prevent tissue damage. Treatment with soursop leaves can increase β cell regeneration in the islets of Langerhans in diabetic pancreas, which in turn will increase the secretion of the insulin hormone\(^6\)\(^7\). Therefore, a scientific test is needed so that soursop leaves can be further developed as antioxidant drugs.

**Materials and Methods**

**Preparation of experimental animals**

Ethical clearance in research this was approved by the medical research ethics commission of the Faculty of Medicine UPN Veteran Jakarta with letter number: B/1950/5/2019/KEPK. The experimental animals used were male Wistar Strain of rats (Rattus norvegicus) aged 11 wk to 12 wk with a body weight of 200 g to 250 g, which were then divided into five groups, namely group one as the negative control (K1): Rats not given alloxan neither treatment, group two as the positive control (K2): Rats given alloxan with dose of 125 mg kg\(^{-1}\) BW\(^{-1}\) intraperitoneal and metformin, group three as treatment 1 (P1): Rats given alloxan with dose of 125 mg kg\(^{-1}\) BW\(^{-1}\) intraperitoneal and regular physical exercise which was then measured,
group four as treatment 2 (P2): Rats given alloxan with dose of 125 mg kg\(^{-1}\) BW\(^{-1}\) intraperitoneal and soursop leaf extract with dose of 150 mg kg\(^{-1}\) BW\(^{-1}\) d, and group five as treatment 3 (P3): Rats given alloxan with dose of 125 mg kg\(^{-1}\) BW\(^{-1}\) intraperitoneal and a combination of soursop leaf extract with dose of 150 mg kg\(^{-1}\) BW\(^{-1}\) day and physical exercise.

Making and determining the extraction dose of soursop leaves
Samples in the form of soursop leaf (*Annona muricata* L.) originated from the Manako plantation, Lembang Bandung, Indonesia weighed as much as 1 kg were washed, then aerated in indirect sunlight for 5 d. After they dried, they were weighed and mashed with a blender. Afterwards, they were soaked using 95 % ethanol solution for 24 h. The results were then filtered. The solution was distilled off, then evaporated for 6 h to 8 h so that a pure thick 100 % soursop extract was obtained\(^{[8]}\). The dose of 150 mg kg\(^{-1}\) BW\(^{-1}\) was based on the study by Moghadamtousi\(^{[6]}\). By converting a human dose to a 200 kg BW\(^{-1}\) rat dose by using the conversion factor of 0.018, the soursop leaf extract needed for one use of sonde per 1 kg BW\(^{-1}\) rat was obtained, which was 13.5 mg kg\(^{-1}\) BW\(^{-1}\) rat.

Phytochemical screening
Phytochemical screening of soursop leaves was carried out in the laboratory of *Balai Penelitian Tanaman Obat dan Aromatik* (Research Institute for Spice and Medicinal Crops) Bogor, Indonesia by using a tube test to determine the content of secondary metabolites in soursop leaves, namely flavonoids, phenolics, saponins, terpenoids, steroids, tannins, alkaloids and glycosides.

Determination of alloxan dosage
The dose of alloxan used was 125 mg kg\(^{-1}\) based on a research conducted by Gumelar, Ekowati et al.\(^{[9]}\). By converting a human dose to a 200 kg BW\(^{-1}\) rat dose by using the conversion factor of 0.018, the alloxan dose was obtained, which was 2.25 mg 200 g\(^{-1}\) rat, or 2.25 mg × 1000 200 g\(^{-1}\) = 11.35 mg kg\(^{-1}\) BW\(^{-1}\) rat.

Determination of metformin dosage
The dose of metformin in humans weighing 70 kg is 500 mg / administration\(^{[11]}\). By converting a human dose to a 200 kg BW\(^{-1}\) rat dose by using the conversion factor of 0.018, the metformin dose was obtained, which was 9 mg 200 g\(^{-1}\) rat, or 9 mg × 1000 200 g\(^{-1}\) = 45 mg kg\(^{-1}\) BW\(^{-1}\) rat.

Treatment of physical training
The intensity of physical training was based on a research conducted by Moraes-Silva et al.\(^{[10]}\). The physical exercise given was classified as moderate at 20 m min\(^{-1}\) using a treadmill and carried out for 3 wk. The first week, the rats were given an adaptation period of physical exercise at the speed of 5 m min\(^{-1}\) for 15 min, followed by 2 wk of multilevel exercise speeds ranging from 5 m min\(^{-1}\) to 20 m min\(^{-1}\) in stages, again followed by three d exercise with the speed of 5 m min\(^{-1}\) for 15 min, then for each day afterwards the speeds were 10 m min\(^{-1}\) for 30 min, 11 m min\(^{-1}\) for 30 min, 12 m min\(^{-1}\) for 30 min, 20 m min\(^{-1}\) for 30 min and finally at a speed of 20 m min\(^{-1}\) for 45 min.
**Treatment of experimental animals**

For 7 d the rats were acclimatized and given standard diet. During the adaptation process, the body weight and animal activity were continued to be monitored. Later from 8 d to 43 d, the rats were given high–fat diet, and on 44 d an induction of alloxan 125 mg kg$^{-1}$ BW$^{-1}$ was carried out intraperitoneally. Amount 2 d later, the blood sugar levels were examined to make sure that hyperglycemia had occurred in the rats. From 47 d to 54 d, treatment was given to each treatment group. After completion of the treatment on 54 d, termination of the test animals was carried out.

**The termination process of experimental animals**

Termination of test animals was done by the cervical dislocation method, then positioning the rats on a surgical board using pins so as to facilitate the surgical stage. The rat's hair on the abdomen was then shaved and the rest of the hair was then cleaned using cotton soaked in water. Surgery started from the abdomen using crooked scissors. The pancreas was then taken by straight scissors, and cleaned from the fat that was still attached. Afterwards, the pancreas was washed with 0.9 % NaCl several times quickly and carefully, drained on filter paper, and weighed. Then the pancreas was cut into several parts. For histopathology examination, the organ was inserted in a pot containing 10 % formalin buffer. Then a paraffin method preparation was made, starting from material selection, fixation, dehydration, purification, paraffin infiltration, embedding, cutting, attachment, deparaffination, Hematoxylin–Eosin (HE) staining, closing and labeling. After that, the observation of pancreatic histology was carried out.

**Examination of MDA levels**

A piece of pancreas weighing 1 g was taken then crushed with a mortar until smooth. Tissue homogenate was then piped into a 1.5 mL microtube as much as 50 µL then diluted with 350 µL of distilled water. 200 µL of TCA 20 % was added into the solution and vortexed until it looked cloudy. The mixture was centrifuged at the speed of 5 000 rpm for 10 min. The result of the supernatant was mixed with 400 µL of TBA 0.67 % to produce a pink compound then heated over a 96 °C water heater for 10 min. After the heating process, the solution was centrifuged again at the speed of 5 000 rpm for 5 min and the absorbance was read with a spectrometer with a wavelength of 530 nm$^{[11]}$.

**Analysis of data**

Data analysis was carried out using Shapiro–Wilk data normality test ($p > 0.05$) and data homogeneity test (Levene's test) ($p > 0.05$) to determine the effect of soursop leaf extract on the reduction of MDA levels using one–way analysis of variance (ANOVA) test ($p < 0.05$).

**Result**

The result of phytochemical test in Balitro’s laboratorium, Bogor, Indonesia from soursop leaf extract has been identified to contain alkaloid, saponin, tannin, phenolic, flavonoid, triterpenoid, steroid, and glycoside compound. It showed that flavonoid was suspected to have antioxidant effects, evident from the difference of MDA levels in each group.
The results of the study showed that the negative control rats group (C1) had the lowest MDA absorbance value of pancreatic tissue at 0.110, while the treatment rats group given a combination of physical exercise and soursop leaf extract with dose of 150 mg kg⁻¹ BW⁻¹ (T3) had the highest mean MDA absorbance of pancreatic tissue at 7.216 compared to the physical exercise treatment group (T1)’s MDA levels of 0.579, the treatment group given soursop leaf extract with dose of 150 mg kg⁻¹ BW⁻¹ (T2)’s of 1.627 and the positive control group (C2)’s of 1.983. The MDA absorbance value of P1 group was lower than the MDA absorbance number of group C2, T2 and T3 (Table 1).

Table 1. Average number of pancreatic MDA levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Pancreatic MDA levels (nmol mL⁻¹)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.110 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>1.983 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Physical Exercise (PE)</td>
<td>0.579 ± 0.04</td>
<td>0.000</td>
</tr>
<tr>
<td>Soursop Leaf Extract (SLE) 150 mg kg⁻¹ BW⁻¹</td>
<td>1.627 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>PE and SLE 150 mg kg⁻¹ BW⁻¹</td>
<td>7.216 ± 0.23</td>
<td></td>
</tr>
</tbody>
</table>

Note: the results are significant if the p-value ≤ 0.05 and have the same significant letter with the control.

MDA levels were obtained by measuring absorbance homogenate number which was diluted in 530 nm wave length. Afterwards, standard curve formula was input, which was $y = 0.0662x + 0.0559$ with $R^2 = 0.9988$, $x$ as the concentration of MDA levels in pancreatic samples. In this formula, nmol mL⁻¹ was used as the unit. (fig. 1)

![Fig. 1 Average number of MDA levels in each treatment groups](image)

Data processing and analysis of MDA levels of pancreatic tissue was shown in the form of standard deviation average (mean±SD). After that, the normality and
variance of the data were tested using Shapiro–Wilk parametric statistic test and ANOVA test ($p < 0.05$). The result of Shapiro–Wilk statistic test showed ($p < 0.05$) which meant data wasn’t distributed normally. Then, transformation data test was done with logarithm. From transformation data test, homogeneity variance test with Levene test yielded sig. number of 0.367, which was more than 0.05, thus the variance of the data was homogeneous. Requirement of ANOVA test was fulfilled, due to the data being distributed normally and variance of the data being homogeneous (Fig. 2)

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>Df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>2.370</td>
<td>4</td>
<td>.593</td>
<td>10.732</td>
<td>.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>1.104</td>
<td>20</td>
<td>.055</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3.474</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 ANOVA test of average number of MDA levels in each treatment groups

The results showed that A. muricata extract affected rat pancreatic cells (Rattus norvegicus) that had been given Alloxan, which induced hyperglycemia as one of the manifestations of DM. The Langerhans islets consisting of four different types of cells, alpha, beta, delta, and F cells, could not be differentiated using H&E (Hematoxylin and Eosin) staining, therefore this research only focused on pancreatic cells in general. The histology of the cells can be seen in figure 3.

Fig. 3 Result of HE staining staining (400×) in pancreatic organ histology of rat model diabetic

Annotation ➔ : necrotic ➔ : normal

Discussion

In this study, the rats were conditioned to have the characteristics of type 2 DM by being given a high–fat diet for 5 wk and alloxan, thus resulting in hyperglycemia. Hyperlipidemia affects the increase of free radicals resulting in an increase of peroxidation products, which causes the body cells to experience oxidative stress\cite{12}. Hyperglycemia can also cause the formation of reactive oxygen species (ROS) through glucose autooxidation to produce $\alpha$–hydroxy–aldehyde, which takes part in
the oxidation of reactive oxygen species (ROS). Free radicals formed by these two inductions in rats will cause interactions with lipid bilayers, resulting in lipid peroxidation\[^{13}][^{14}][^{15}\]. This is in accordance with the healthy group of rats group C1 having normal blood sugar levels and lower MDA level of 0.110 nmol mL\(^{-1}\), compared to the group C2 and the treatment groups with diabetes all having higher MDA levels. This shows the formation of ROS in the pancreas. High–fat diet and alloxan induction could cause interference in the process of insulin secretion or damage to pancreatic beta cell membranes, resulting in decreased sensitivity of insulin receptors. The combination of these two was in accordance with the etiological characteristics of non–genetic type 2 DM. In type 2 DM there is a significant increase in MDA levels because it is not balanced with an increase of body endogenous antioxidant\[^{13}][^{14}][^{15}\]. In the treatment group T1 the MDA level was 0.579 nmol mL\(^{-1}\), which was lower than the other treatment groups. Physical exercise has been shown to increase insulin sensitivity, and muscle contractility that is carried out regularly is capable of increasing glucose uptake by muscles so that blood sugar levels decrease\[^{5}\]. In group T2, the MDA level was 1.627 nmol mL\(^{-1}\), which was lower than group C2 and T1. This also proved that flavonoid contained in soursop leaves had a strong affinity for Fe ions. Fe is known to be able to catalyze processes that cause free radical formation. The work of flavonoid as antiperoxidative agents by means of the Fe chelation process will cause an inert ion complex which will then inhibit the initial process of lipid peroxidation\[^{16}\].

However, the combination treatment showed that the MDA level of group T3 was 7.216 nmol mL\(^{-1}\), which was higher than the treatment groups T1 and T2, and still higher than group C2. This is presumably because at the time the soursop leaf extract was administered, there had been a decrease in blood glucose levels. And the treatment of physical exercise led to the possibility of rats experiencing stress. According to research conducted by Kregel et al.\[^{17}\], it was stated that exercise in experimental animals resulted in vulnerability to stress. It was assumed that this was due to first, the repeated exercise time was not the same every day even though its rhythms had been measured. Second, the adequacy of eating and drinking activity during physical exercise was not considered, while physical exercise requires enough calories as energy fuel to be used. Therefore, if there is a lack of calories, it will cause a build–up of ketone objects due to the burning of excess fat and lactic acid build–up. The three stages of the intensity of physical exercise were not quite appropriate, causing rats to experience fatigue. This could be seen from the performance of rats such as some rats not running, rats hitting electric shock devices on the treadmill four times in a minute, increased temperature and pulse, and increased lactic acid levels. According to Cooper et al.\[^{18}\], physical fatigue caused by large oxygen consumption in active tissues will increase the formation of free radicals.

The pancreatic histology can be described by assessing the morphology of pancreatic beta cells that have a round and large shape, then assessing necrosis which is a type of cell death in the form seen as swollen cells then proceeding to the
level of shrinking cell nucleus (pyknosis), where the cell nucleus is destroyed and chromatin material fragments are scattered in the cells (karyorrhexis), and the final level is dead cells and cannot be tinted anymore (karyolytic)\(^{[19]}\).

Based on Figure 1 above, it is known that the negative control group (C1) without being treated with alloxan showed normal pancreatic cell condition, the cell arrangement was regularly spread on the islets of Langerhans and had uniform cell forms. In the group C2 that had been induced by alloxan there was a cell change, it appeared that the cells spreaded irregularly in the islets of Langerhans and in empty spaces (with less number of beta cells) in the Langerhans islet area. In empty spaces, cells that experienced necrosis could be seen with only collagen fibers present. The small number of beta cells showed an interference of insulin metabolism, causing hyperglycemia and beta cell damage characterized by a higher number of pancreatic MDA levels than in negative control group. Group T1 showed improvement with many round cells with nucleus, where the cells were scattered in the islets of Langerhans, and the cell arrangement was seen to begin organization, while group T2 showed better conditions than group T1 with rounded cells scattered regularly, uniformly, and fewer empty spaces due to cell loss as seen in the group C2. In the group T3, normal pancreatic beta cells were seen, but many cells experienced necrosis and there were empty spaces due to cell loss as it happened in C2. Histologically, in cells that do not get alloxan/STZ induction, Langerhans islet is said to be normal if the composition of endocrine cells is spread regularly with uniform cell shape, round shape, clear cell nucleus and no edema cells found\(^{[20]}\).

According to Andri\(^{[21]}\), bioactive compounds present in plants such as tannin and flavonoid have been shown to have antioxidant activity that is able to neutralize free radicals, which are the cause of pancreatic beta cell damage, thus the remaining beta cells can still function normally. If the cell damage lasts long or is heavy enough, it can cause cell death (necrosis) and the cell cannot carry out its metabolic functions, thus will cause changes in the size of the pancreatic islets, and cells with pyknotic nucleus and eosinophilic cytoplasm appear. Free radicals damage cell membranes which cause cytosolic calcium concentration to increase, resulting in Langerhans beta cell deconstruction and beta cell sensitivity to insulin decrease due to influx of calcium into cells, causing depolarization of pancreatic beta cells\(^{[22]}\).

**Conclusion**

Based on the result of this study, Group treated with soursop leaf extract had pancreatic MDA level of 1.627 nmol mL\(^{-1}\), Group treated with moderate exercise (20 m min\(^{-1}\)) had pancreatic MDA level of 0.579 nmol mL\(^{-1}\), and Group treated with physical exercise and soursop leaf extract with dosage of 150 mg kg\(^{-1}\) BW\(^{-1}\) showed improved pancreatic Langerhans islet cells compared to positive controls.
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