

The effect of lemon extract (*Citrus limon*) on the blood sugar levels and pancreatic beta cell regeneration in alloxan-induced hyperglycemic mice

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ABSTRACT

Background: High blood sugar levels that exceed normal limits or commonly referred to as hyperglycemia, is an early symptom of diabetes mellitus.

Objectives: This study explored the effect of lemon (*Citrus limon*) extract on blood sugar levels and pancreatic β cell regeneration in alloxan-induced hyperglycemic mice (*Mus musculus*).

Method: This research is an experimental study using a post-test group. The sampling was carried out using a randomization method. By administering 125 mg/kg BW of alloxan, the sample was conditioned for hyperglycemia. The samples were divided into five groups: normal control, alloxan-induced control, and three treatment groups that received lemon extract with dosages of 100 mg/kg BW, 300 mg/kg BW and 500 mg/kg BW, respectively. Semiquantitative analysis was used to evaluate pancreatic damage.

Results: The results showed that lemon extract can decrease blood sugar levels. Histopathological imaging revealed a significant improvement in β cell distribution and decreased vacuolization in the Langerhans islets of mice administered lemon extract. No significant differences were observed among different dosages of lemon extract ($p > 0.05$).

Conclusion: Our findings underscore the potential therapeutic benefits of lemon extract in managing blood sugar levels and promoting pancreatic β cell regeneration in alloxan-induced mice.

Keywords: diabetes mellitus, hyperglycemia, lemon, *Mus musculus*

Introduction

High blood sugar levels exceeding normal limits, a condition known as hyperglycemia, is an early symptom of diabetes mellitus (DM). Numerous epidemiological studies have shown an increasing incidence and prevalence of DM across the globe, making it a growing global epidemic. According to the International Diabetes Federation (IDF), Indonesia ranks sixth worldwide with the highest prevalence of DM, affecting 19.3 million people in 2021 [1].

Although this chronic disease cannot be cured, it can be managed or potentially prevented through regular dieting [2]. Therefore, understanding diabetes risk factors is crucial for its prevention and management. Unmodifiable risk factors include gender, age, family history of DM, race, and ethnicity. On the other hand, modifiable risk factors refer to lifestyle choices that promote unhealthy living, such as lack of physical activity, an unbalanced diet, habitual smoking, hypertension, dyslipidemia, and obesity [3]. Given these risk

factors, controlling the modifiable ones through lifestyle changes, such as changes in physical activity and dietary patterns, becomes an important strategy.

DM management involves using insulin therapy and oral anti-diabetic drugs, such as metformin, sulfonylurea, and glinides [4]. However, these antidiabetic medications can cause several side effects, potentially reducing the patient's quality of life. For instance, metformin may lead to lactic acidosis and is therefore not recommended for patients with renal impairment [5,6]. Meanwhile, antioxidants show promising antidiabetic effects due to their ability to reduce blood sugar levels. These antioxidants play a vital role in preventing and controlling various diseases. However, most drugs leveraging antioxidant activity are still in clinical trials [7].

Given the potential side effects of these medications, alternative ways to control blood sugar levels are required. Diet modifications, for instance, can be beneficial. People with diabetes are advised to consume low-calorie, low-fat, and nutrient-rich foods, such as vegetables and fruits. Low-sugar fruits such as lemons are recommended. Lemons contain citric acid, ascorbic acid, flavonoids, alkaloids, tannins, saponins, and steroids [8,9]. These elements could help reduce blood glucose levels through two primary mechanisms. Firstly, flavonoids as antioxidants can reduce oxidative stress, preventing pancreatic damage. Secondly, ascorbic acid can inhibit the binding of glucose to hemoglobin, thereby reducing high levels of glycated hemoglobin.

Several studies have illustrated the positive effects of lemon. For example, the fraction of lemon water can reduce glucose levels in aged mice on high-fat diets, presumably due to the flavonoids in lemons [10]. Studies by Neovita *et al.* (2020) and Kadhum *et al.* (2020) using lemon peel extract and lemon extract, respectively, demonstrated a decrease in blood sugar levels in hyperglycemic mice [11, 12]. However, the effects of lemon extract on the pancreas have not been thoroughly studied. Therefore, this study aims to

evaluate the potential for pancreas regeneration in diabetic mice, examining the histopathological changes in the pancreas following the administration of lemon extract.

Methods

The research was conducted at the Laboratory of the Faculty of Medicine and Veterinary Medicine, Universitas Nusa Cendana, Kupang, with preparations performed at Prof. Dr. W.Z. Johannes Kupang Hospital. The study utilized a true experimental design with a post-test control group.

Sample

The sample size was calculated to be five mice (*Mus musculus*) for each group using Federer's formula. An additional mouse was added to each group to account for potential loss, resulting in six male mice per group. Therefore, the total sample required was 30 male mice aged between 10 and 16 weeks and weighing between 20 and 40 grams. The mice were sourced from Solo, Central Java, Indonesia. The pancreas samples were stored in a 10% formaldehyde solution until hematoxylin-eosin slides were prepared. This study received ethical clearance from the Research Ethics Committee, Faculty of Veterinary, Universitas Nusa Cendana, with number: 025/KEH/SK/II/2022.

Treatment

Each group consisted of six mice that met the inclusion and exclusion requirements. The five groups comprised a normal control group, an untreated alloxan-induced group, single-dose treatment of 100 mg/kg body weight (BW) (P1), single dose treatment of 2 doses of 300 mg/kg BW (P2), and single-dose treatment of 3 doses of 500 mg/kg BW (P3). Blood sugar levels in the mice were monitored to analyze changes before (pre-test) and after (post-test) lemon extract treatment. A post-test histopathological examination of pancreatic β cells was performed on mice given lemon extract at the specified dosages.

Alloxan induction

The mice were fasted for 8-12 hours before alloxan monohydrate was administered at 125 mg/kg BW to 24 mice via an intraperitoneal injection using a 1 mm syringe. The injection was carried out on the lower left quadrant of the abdomen of the mice. After the alloxan injection, glucose 1 g/kg BW was administered orally to prevent hypoglycemic shock.

Blood glucose test

Blood glucose testing was carried out three times during the experiment: one day before alloxan injection (day 0), the 5th day after alloxan induction (day 5), and the 28th day after lemon extract treatment (day 28). The test used fasting blood glucose from mice that had fasted for 8 hours. Blood was drawn from the tail vein of each mouse, which had been disinfected with an alcohol swab. A 2- 3 mm distal segment of the tail was cut using a sterile scissor, and a single drop of blood was placed on a strip of blood glucose test meter (Accu Chek Instant®).

On day 0, an examination was conducted to ensure that none of the mice were diabetic before the alloxan induction. On the fifth day, a blood glucose test was administered following the alloxan induction to monitor any increase in blood glucose levels. For mice injected with alloxan, their fasting blood glucose must exceed 126 mg/dL to be classified as diabetic. The normal group of mice must maintain a blood glucose level between 90-110 mg/dL. On the 28th day, another blood glucose test was performed to evaluate the effect of the lemon extract on the diabetic mice by comparing the results with those from the diabetic mouse groups treated with and without lemon extract.

Lemon extract treatment

The lemon extract was derived from fresh imported lemons purchased from a local market. The extraction composition consisted of 99% lemon juice and 1% distilled water (aquades). The lemon extract was administered for 14 days using oral

gavage with 100 mg/kg BW, 300 mg/kg BW, and 500 mg/kg BW.

Histopathological scoring

Data were obtained from the scores of changes in the histopathology of the Langerhans islet of the pancreas and the extent of lesions of these islets. The examination was based on infiltration, inflammation, congestion, vacuolization, necrosis, and fibrous. Each Langerhans islet underwent a histopathological assessment and scored as follows: 0 = no damage (lesions < 25%), 1 = mild (lesions 25-50%), 2 = moderate (51-75%), and 3 = severe (lesions >75%) [13]. The final score of each sample was obtained by averaging the scores observed on each islet.

Data analysis

Data were analyzed using the Saphiro-Wilk test and a One-Way ANOVA test to determine any significant differences. If significant differences were identified, the analysis proceeded with the LSD test to compare values between groups [14].

Results

Blood glucose level

Blood samples were drawn from the mice to monitor the effects of alloxan administration. The blood was collected from the tip of the mice's tails and tested in a glucometer strip. Measurements of fasting blood glucose were taken the day before alloxan induction (day 0), the fifth day after alloxan induction (day 5), and immediately prior to the termination of the experiment (day 28).

The administration of 125 mg/kg BW alloxan resulted in an increase in fasting blood sugar levels in mice on day 5, showing an increase in blood sugar levels. The untreated control group showed an increase of 64.2 mg/dL, while treatment groups P1, P2, and P3 also exhibited increases of 28.2 mg/dL, 55.2 mg/dL, and 48.8 mg/dL, respectively. As the normal group was not administered alloxan, their fasting sugar levels remained stable (Figure 1).

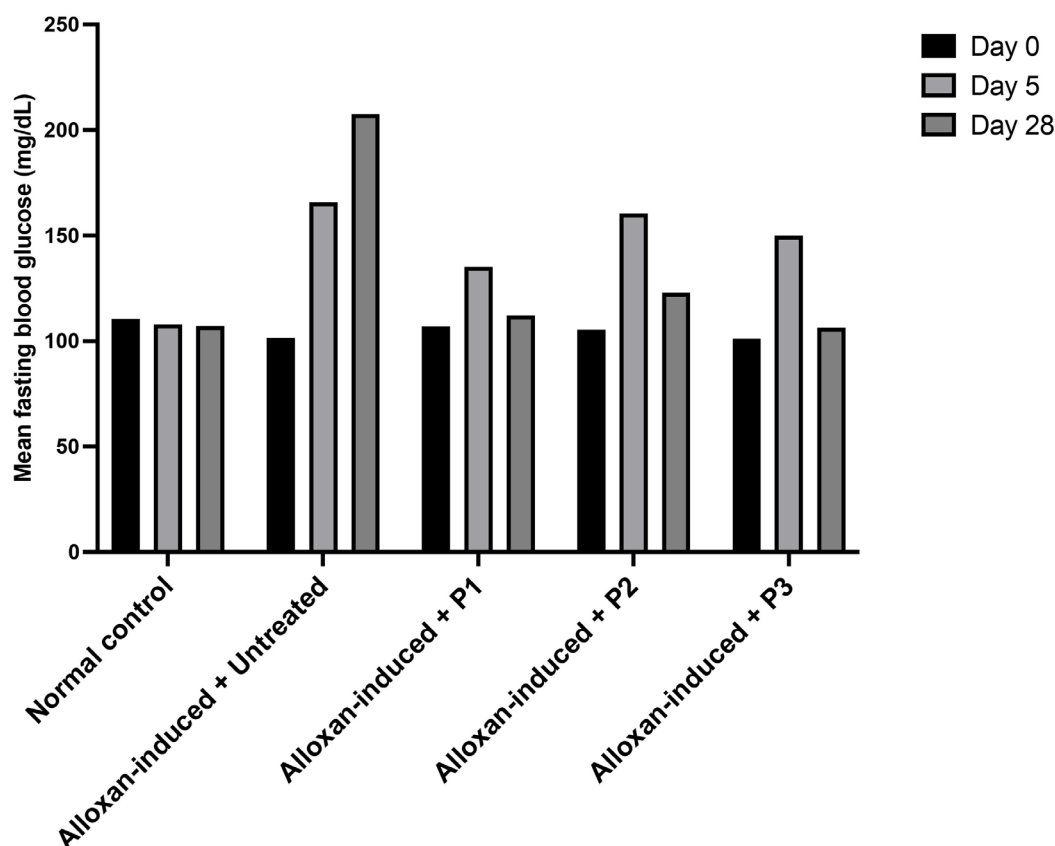


Figure 1. Fasting blood glucose levels. The average blood glucose levels on day 0, 5, and 28 in alloxan-induced mice. The groups represented include untreated mice, as well as those treated with lemon extract 100 mg/kg BW (P1), 300 mg/kg BW (P2), 500 mg/kg BW (P3)

The lemon extract was administered to mice for two weeks after 14 days of alloxan induction. The normal group, which did not receive the lemon extract and alloxan induction, showed a decrease of 0.84 mg/dL. Alloxan-induced mice without lemon extract treatment experienced an increase in fasting blood glucose levels up 41.95%. Treatment groups P1, P2, and P3 showed decreases in fasting blood glucose levels by 23 mg/dL, 37.6 mg/dL, and 43.6 mg/dL, respectively.

Histopathological results

Histopathological assessments of the pancreas were conducted, focusing on the islets of Langerhans, which regulate blood sugar levels. These assessments were semi-quantitative. We found that the normal control group had the lowest score, close to 0 (indicating normal conditions), signifying that the average damage to the Langerhans islet was less than 25%. Mice induced with alloxan scored close

to 3 (indicating severe lesions), showing that the average damage to the Langerhans islets was over 75%. The treatment group showed similar results with a score of 1 (indicating mild lesions), with moderate damage to the Langerhans islets (Figure 3).

Under normal conditions, β cells still filled the Langerhans islet, and no noticeable cell necrosis. There was also no visible formation of fibrous tissue inside the Langerhans islets (Figure 3b). The histopathological image of the pancreas from alloxan-induced mice without extract treatment showed evidence of β cell necrosis. As a result, the β cells were sparse compared to normal ones. Vacuolization was also observed on the Langerhans islets (Figure 3a).

In the P1 treatment (dosed at 100 mg/kg BW), cells showed improvement, with β cells undergoing regeneration and decreased vacuolization (Figure 3c). Similar improvements were seen in the P2 group (dosed at 300 mg/kg BW) and P3 group (dosed

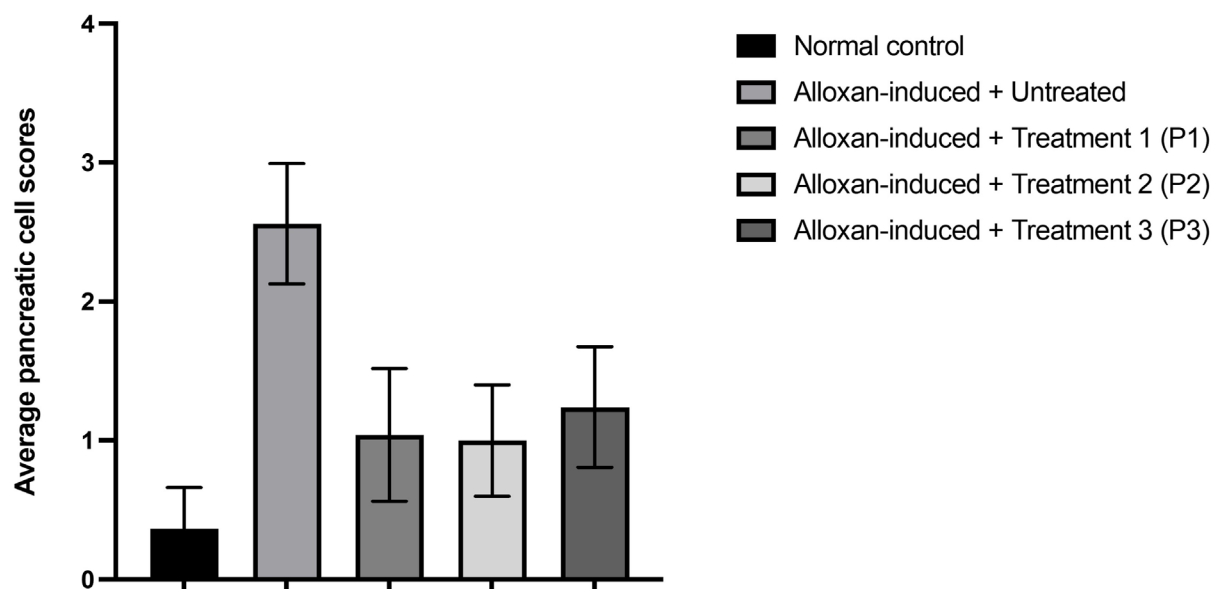


Figure 2. Pancreatic cell scores. Average damage to the islets of Langerhans, based on observations across five field of view at 400x magnification. One-way ANOVA test reveals a significant relationship between administration lemon extract and histopathological appearance of islet of Langerhans ($p < 0.05$)

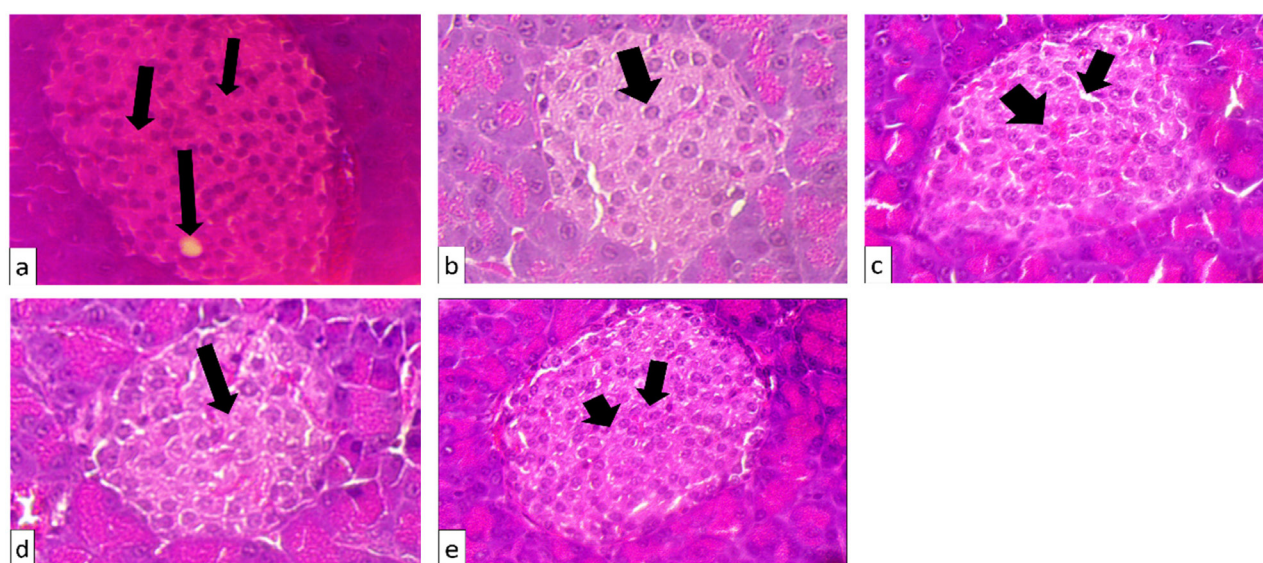


Figure 3. Histopathology of mouse pancreas. a. Pancreatic histopathology in alloxan-induced mice, b. Pancreatic histopathology of the normal control group, c. Pancreatic histopathology of the following lemon extract treatment 1 (100 mg/kg BW), d. Pancreatic histopathology of the following lemon extract treatment 2 (300 mg/kg BW), e. Pancreatic histopathology of the following lemon extract treatment 3 (500 mg/kg BW)

at 500 mg/kg BW), where regenerated β cells and decreased vacuolization were noted (Figure 3d, 3e).

During the experiment, four out of the 24 mice died following alloxan induction, leaving 26 mice at the end of the experiment. The main cause of death was not determined, but it is hypothesized that the deaths resulted from alloxan induction or stress due to injection.

Discussion

Alloxan, employed as a selective cytotoxic agent against β cells, induces insulin level alterations by increasing reactive oxygen species (ROS) production and damaging the antioxidant defense system. These factors primarily contribute to oxidative stress, playing a significant role in the pathogenesis of type 1 diabetes. Alloxan begins its effects by

being absorbed by the β cells of the pancreas. Glutathione-reducing agents then interact with alloxan by binding to the -SH group, producing dialuric acid. This acid is later oxidized back to alloxan, which results in the formation of ROS and superoxide radicals. These radicals can undergo dismutation, leading to the production of hydrogen peroxide (H_2O_2). Furthermore, alloxan can increase Ca^{2+} in the cytosol by depolarizing pancreatic β cells and opening calcium channels. The combination of high levels of Ca^{2+} ions and ROS formation leads to pancreatic β cell damage [15].

Elevated blood sugar levels can trigger protein glycosylation. This reaction occurs due to the interaction between proteins and glucose at higher concentrations. The reaction's by-products, known as advanced glycation end products (AGEs), signal the occurrence of oxidative stress. Oxidative stress in subsequent stages can aggravate pancreatic β cell damage, leading to cell apoptosis.

This study demonstrates that lemon extract can help lower blood sugar levels in mice due to its content, such as ascorbic acid (vitamin C) and flavonoid. Vitamin C, structurally similar to glucose, acts as a glucose competitor for cellular entry, thereby reducing protein glycosylation within the body [16]. This action can decrease glycated hemoglobin levels, lower high blood sugar, and reduce oxidative stress produced by this process. Additionally, flavonoids are crucial in reducing oxidative stress and ROS, aiding in β cell regeneration. Previous studies have shown similar results regarding the role of lemon extract in reducing blood sugar levels in older mice [10]. Notably, the groups administered lemon extract for 14 days showed a significant decrease in blood sugar levels compared to the alloxan-induced untreated group, which experienced an increase in fasting blood glucose levels.

Histological imaging revealed β cell regeneration in diabetic mice administered lemon extract, in contrast to the alloxan-induced group. In the alloxan-induced group, we observed β cell destruction in the Langerhans islets caused by oxidative stress following alloxan induction. Unlike the distribution

of β cells in human islets of Langerhans, β cells in mice are centrally located within the islets [17,18]. The apoptosis of β cells is marked by empty space (fibrous tissue) in the central islets and the development of insulitis, indicated by islet inflammation (Figure 3a) [19].

Transient or irreversible cytoplasmic vacuolization in mammalian cells is typically observed during exposure to inducers. Known inducers of transient vacuolization include lipophilic compounds that contain weakly basic amines. In neutral extracellular fluids, lipophilic bases are uncharged and can be transported across the plasma membrane through passive diffusion or active transport. However, upon entering acidic endosomal-lysosomal organelles and Golgi cisterns, they become positively charged and cannot diffuse back to the cytoplasm through the organelle membrane. The accumulation of these charged forms of weak bases increases intra-organelle osmotic pressure, leading to vacuole formation (Figure 3a) [20].

The group administered lemon extract showed β cell regeneration, reduced empty space in the Langerhans islets, and decreased inflammation and vacuolization in the Langerhans islets (Figure 3c, 3d, and 3e). The lemon extract helps β cell regeneration by reducing oxidative stress and preventing cell apoptosis. Flavanoids play a crucial role as anti-inflammatories during β cell regeneration, as shown in histopathological imaging of diabetic mice treated with lemon extract (Figure 3c, 3d, and 3e) [21,22]. No significant differences in the histopathological imaging results were observed between 100 mg/kg BW (P1), 300 mg/kg BW (P2), and 500 mg/kg BW (P3) of lemon extract. The LSD test showed a p-value greater than 0.05, indicating no significant difference among the groups given different doses of lemon extract, as per histopathological imaging.

Conclusion

The study concludes that the administration of lemon extract has a significant impact on blood sugar levels and pancreatic β cell regeneration in alloxan-induced diabetic mice. Histopathological

imaging revealed substantial improvements in β cell distribution and reduced inflammation and vacuolization in the Langerhans islets of mice administered lemon extract.

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Author contributions

PM collected research data, wrote the manuscript. EMBD, ELSS, PDP supervised study, contributed to design, and helped the completion of the manuscript.

Declaration of interest

The authors declare no conflict of interest.

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