Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 20/10/2024;
Date Revised: 05/01/2025;
Available Online: 22/03/2025;

Author's Affiliation:
1. Division of Veterinary Clinic, Department of Health and Life Sciences, Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga, Banyuwangi – Indonesia
2. Division of Veterinary Clinic, Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya – Indonesia
3. Faculty of Medicine, Universitas Jember – Indonesia

***Corresponding Author:**
Tridiganita Intan Solikhah
Email:
tridiganita-intan-s@fkh.unair.ac.id

How to Cite:
Putra YAS, Solikhah TI, Setiawan B, Miftakhurrozaq RK (2025). Effects of Sapodilla Leaf Extract (*Manilkara zapota* L.) Administration on the Histopathological Presentation of Kidney Tubules and Glomeruli in Alloxan-Induced Mice. Adv. Life Sci. 12(2): 317-323.

Keywords:
Alloxan; Diabetes; Kidney Histopathology; Sapodilla leaf extract

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Effects of Sapodilla Leaf Extract (*Manilkara zapota* L.) Administration on the Histopathological Presentation of Kidney Tubules and Glomeruli in Alloxan-Induced Mice

Yan Arengga Syah Putra[1], Tridiganita Intan Solikhah[1]*, Boedi Setiawan[2], Ragil Kusnandar Miftakhurrozaq[3]

Abstract

Background: Diabetes mellitus is a metabolic disorder characterized by high blood glucose levels or insulin deficiency. Sapodilla leaves (*Manilkara zapota* L.) are known to be potentially utilized as an alternative remedy for diabetes. The objective of this research is to investigate the effect of Sapodilla leaf extract on the improvement of histopathological presentation of kidneys in alloxan-induced diabetic mice.

Methods: This research used male mice 3 months old, weighing 25-35g. A total of 25 mice were used, with 5 mice per each group. The treatments employed included P1 (Sapodilla leaf extract at a dosage of 100 mg/kg BW), P2 (Sapodilla leaf extract at a dosage of 300 mg/kg BW), K+ (Pioglitazone at a dosage of 2 mg/kg BW), K0 (No treatment administered), and K- (Diabetic control). Kidney sample collection was conducted on the 14th day after therapy administration. Histopathological samples were examined using a trinocular microscope. Data analysis was conducted using the Kruskal-Wallis method, followed by the Mann-Whitney test.

Results: The results of the Kruskal-Wallis test with the parameter of necrosis indicate a p-value of 0.001. The degeneration of cells shows a p-value of 0.001, while congestion shows a p-value of 0.001 as well. The Mann-Whitney test was conducted on the parameters of necrosis, cell degeneration, and congestion. The results indicated that the treatment group P1 did not show a significant difference from the treatment P2 and K+ groups but showed a significant difference from the K- and K0 groups.

Conclusion: The study concludes the data analysis revealed that the administration of sapodilla leaf extract can ameliorate kidney damage in diabetic mice induced with alloxan.

Introduction

Diabetes mellitus is a condition that leads to the most common and prevalent abnormalities across various age groups. This occurrence is associated with the disrupted metabolism of carbohydrates, fats, and proteins within the body. Individuals with diabetes mellitus lose the ability to metabolize blood glucose, resulting in elevated blood glucose levels (hyperglycaemia) [1-3]

One of these changes is attributed to free radical stress. Free radicals can react with cells and induce cellular damage [4]. Free radicals also play a role in the progression of hyperglycaemia resulting from decreased insulin secretion and action [5]. One of the conditions caused by these free radicals is diabetic nephropathy. Diabetic nephropathy is a structural abnormality in the glomeruli and renal tubular elements of the kidney, characterized by hypertrophy, increased thickness of the glomerular basement membrane, formation of nodular glomerulosclerosis, accumulation of extracellular matrix components, elevated *Glomerular Filtration Rate* (GFR) with intraglomerular hypertension, proteinuria, systemic hypertension, and kidney function loss [6-8].

Diabetic nephropathy is the leading cause of end-stage kidney disease worldwide and is associated with an increased risk of cardiovascular disease [9-10]. Diabetic nephropathy is a major contributor to mortality among diabetes-related complications and the leading cause of death due to cardiovascular complications [11,12]. Cell damage caused by free radicals can be mitigated through antioxidant supplementation.

The kidney is a vital organ that contributes to maintaining environmental stability, regulating water and electrolyte balance, and producing metabolic waste products, which are bodily metabolic byproducts. In addition to its role in regulation and excretion, the kidney generates a group of endocrine compounds known as prostaglandins [13]. Failure in the vital function of the kidney leads to the condition of uraemia. Impaired kidney function gives rise to a clinical and laboratory syndrome that affects various organs, known as uraemia [14].

The treatment of diabetes mellitus aims to maintain glycaemic control within the normal standards and prevent the emergence of complications [15]. Efforts to prevent complications involve stabilizing blood glucose levels, which is achieved through regular and continuous treatment. The medication commonly used for the treatment of diabetes mellitus is pioglitazone, which operates by reducing the levels of vascular proinflammatory molecules [16]. Some of the benefits of pioglitazone include insulin resistance recovery, ß-cell improvement, glycaemic control, enhancement of metabolic syndrome, as well as liver and kidney function enhancement [17]. Nonetheless, continuous use of pioglitazone can lead to side effects such as weight gain, edema, gastrointestinal disturbances, and even heart failure. This has prompted efforts to explore alternative options, such as the use of herbal remedies with minimal side effects.

Indonesia possesses a high level of biodiversity, allowing for the utilization of various medicinal plants within the field of health [18-20]. sapodilla is one such medicinal plant that can be harnessed for this purpose. Plants belonging to the Sapotaceae family have been extensively researched and proven to be used as traditional medicine. Phytochemical identification of sapodilla leaf extract (*Manilkara zapota* L.) indicates the presence of alkaloids, flavonoids, saponins, polyphenols, tannins, quinone, and steroids [21]. Several studies have stated that flavonoid compounds can be used for antidiabetic, anti-ulcer, anti-spasmolytic, anti-allergic, anti-diarrhoea, and antioxidant activity purposes [22,23].

The research conducted by Prihanti et al [24] indicated that black garlic extract at doses of 0,15, 0,3, and 0,6 (ml/200g BW/day) on the decrease of hydropic degeneration in the kidney tubular cells. Research conducted by Al-Malki and ElRabey [25] demonstrated the effectiveness of flavonoids and antioxidants in restoring kidney and pancreatic damage histologically. Paliwal et al [26] stated that antioxidants can support nephroprotective activity in mice with kidney disorders. Nephroprotective compounds possess the ability to safeguard kidneys from renal disturbances caused by free radicals.

Based on several previous studies and considering the side effects associated with the drug pioglitazone, the researcher intends to conduct research on the effectiveness of the antidiabetic properties of sapodilla leaf extract (*Manilkara zapota* L.) on the histopathological presentation of kidneys in mice induced with alloxan.

Methods

Research Sample

The population in this research consisted of male mice (*Mus musculus*) approximately 3 months old. The mice utilized had not been previously used in other research, were healthy, devoid of anatomical defects, and weighed between 25-35 grams. The samples employed in this research were kidneys from male mice (*Mus musculus*). The total number of test animals employed in this research was 25 male mice (*Mus musculus*), with 5 mice allocated to each treatment group.

Materials and Research Methodology

Research Materials

This research employed high quality sapodilla leaves obtained from Puri Subdistrict, Mojokerto Regency, East Java Province. The selection of sapodilla leaves was purposive, which means only one location was chosen with no comparative analysis of sapodilla leaves from other regions to ensure uniformity in the plant material used throughout the study also to minimize the variability that could occur due to geographical differences. Male mice (*Mus musculus*), feed, sawdust, pioglitazone, 10% formalin, 96% ethanol, 80%, 90%, and 95% alcohol, xylene, liquid paraffin, 0.9% NaCl solution, alloxan, and Carboxymethyl Cellulose (CMC) were used in this research.

Lesions	Score	Note
Necrosis	0	No necrosis occurred
	1	Necrosis occurred in <25% of the entire field of view
	2	Necrosis occurred in 25-50% of the entire field of view
	3	Necrosis occurred in >50% of the entire field of view
Degeneration	0	No degeneration occurred
	1	Degenerative changes occurred in <25% of the entire field of view
	2	Degenerative changes occurred in 25-50% of the entire field of view
	3	Degenerative changes occurred >50% of the entire field of view
Congestion	0	No congestion occurs
	1	Congestion present in <25% of the entire field of view
	2	Congestion present in 25-50% of the entire field of view
	3	Congestion present in >50% of the entire field of view

Table 1: Histopathological scoring system for histopathological lesions in kidney tissue, including necrosis, degeneration, and congestion. Each lesion type was assessed under light microscopy and scored based on the percentage of the affected field of view.

Research Materials

The instruments utilized encompass the glucometer (ACCU-CHEK Instant), glucose strips, yellow-capped vacutainer, glass beaker, aluminium foil, syringe with needle, probe, plastic cage, surgical equipment, water bottle, scissors, pins or needles, light microscope, surgical board, and feeding container.

Medical Ethics Test

An ethical test was conducted to ensure that all actions and treatments provided to the experimental animals complied with the *Standard Operating Procedures* implemented at the Faculty of Veterinary Medicine, Universitas Airlangga, Campus C, Surabaya, under the reference number 2.KEH.068.06.2022.

Preparation of Experimental Animals

The mice were placed in plastic cages measuring (50 cm x 50 cm x 40 cm). The cages were lined with a 1 cm thick layer of sawdust, which was changed daily. The cages containing the mice were kept at room temperature with a 12-hour light-dark cycle. The mice were provided with *ad libitum* access to food and water. The mice were adapted for a period of seven days [27].

Extraction of Sapodilla Leaves (*Manilkara zapota* L.)

Sapodilla leaves are washed with clean water and oven-dried at a temperature of 60ºC, then pulverized and sieved to obtain sapodilla leaf powder. The sapodilla leaf powder is macerated three times for 3x24 hours with a ratio of 1:3, comprising 500 g of sapodilla leaf powder in 1500 mL of 96% ethanol. The second and third macerations are carried out with a ratio of 1:2, involving 500 g of sapodilla leaf powder in 1000 mL of 96% ethanol each time. Subsequently, the resulting maceration filtrates are concentrated using a rotary evaporator.

The concentrated extract is placed into a glass beaker and covered with aluminum foil, then stored in a *refrigerator* to prevent the extract from deteriorating. A 0.5% concentration of (CMC) is used as the extract solvent to achieve the desired concentration.

Diabetes Induction

According to Solikhah (2021) (27), induction of type 1 diabetes mellitus was conducted through an injection of alloxan at a dose of 150 mg/kg BW dissolved in 0.9% NaCl solution. Blood glucose levels of the mice were assessed five days after the injection by collecting blood from the tail vein following a 12-hour fasting period. Mice with blood glucose levels ⩾ 200 mg/dL were categorized as having diabetes mellitus and were subsequently subjected to extract and drug therapies [21].

Antidiabetic Test

This research utilized 30 male mice, randomly divided into 5 treatment groups with 5 replications each, as detailed below:

K0 : Non-diabetic mice (without treatment).

K- : Diabetic mice treated with 1.5 ml of 0.9% NaCl solution.

K+ : Diabetic mice treated with Pioglitazone at a dose of 2 mg/kg BW.

P1: Diabetic mice treated with 100 mg/kg BW dosage of sapodilla leaf extract.

P2: Diabetic mice treated with 300 mg/kg BW dosage of sapodilla leaf extract.

Technique and Data Collection Instruments

Sampling Procedure

The collection of mice kidneys was carried out on the 14th day after administration of the drug and extract. Euthanasia of the mice was performed through intraperitoneal injection of xylazine and ketamine at doses of 16 mg/kg BW and 80 mg/kg BW, respectively. The *onset of the effect* of ketamine itself ranges from 2 to 40 minutes after injection. A paracostal incision

surgery technique was employed to obtain kidney organ samples. Subsequently, the kidney organs were cleaned using a 0.9% NaCl solution and preserved in a 10% formalin solution.

Histopathological Slide Preparation

The kidneys were fixed in a series of alcohol solutions in ascending concentrations (dehydration), starting from 70%, 80%, 90%, 95%, and reaching 100%. Subsequently, they were clarified in xylene *(clearing)* before being *embedding* in paraffin. Tissue within the paraffin blocks was sectioned at a thickness of 5-6 µm using a microtome, then placed onto glass slides, and stored in an incubator at 40°C for 24 hours. The tissue sections were stained using the standard *Hematoxylin Eosin (HE)* staining technique. The staining process began with tissue deparaffinization using xylene and gradual rehydration with alcohol, followed by a 24-hour clearing step in xylene for clarification. Subsequently, the tissue was retrieved and appropriately mounted before being covered with a cover glass. Microscopic images of the kidney were observed using a Nikon Eclipse E200 light microscope at a magnification of 400x.

Data Analysis

The acquired data was analyzed using the *Kruskal-Wallis* test, followed by the *Mann-Whitney* test, utilizing SPSS 24.

Results

The observation results of mice kidney histopathological specimens were conducted using a trinocular microscope at a magnification of 400x. The assessment of organ damage *scoring* in kidney was performed on five different fields of view using the H&E staining technique, with parameters including necrosis, degeneration, and congestion. Data analysis was carried out using the *Kruskal-Wallis* test, followed by the *Mann-Whitney* test to determine significant differences in each treatment group.

Groups	X±SD Necrosis Scoring	X±SD *Cell Degeneration Scoring*	X±SD *Congestion Scoring*
K-	2,72 ± 0,17^{a}	2,72 ± 0,17^{a}	2,6 ± 0,14^{a}
K+	2,0 ± 0,14^{b}	1,96 ± 0,08^{bd}	2,0 ± 0,14^{b}
P1	1,56 ± 0,35^{c}	1,48 ± 0,1^{cd}	1,76 ± 0,21^{bc}
P2	1,2 ± 0,2^{c}	1,6 ± 0,54^{d}	1,48 ± 0,3^{c}
K0	0,8 ± 0,2^{d}	0,52 ± 0,22^{e}	0,76^{d} ± 0,26^{d}

Table 2: Mean *scoring* of necrosis, cell degeneration, and congestion in the histopathological image of treated mice kidney. Note: Different superscripts indicate significant differences ($p < 0.05$).

The results of the Kruskal-Wallis test with the parameter of necrosis indicate a p-value of 0.001. The degeneration of cells shows a p-value of 0.001, while congestion shows a p-value of 0.001 as well. Subsequently, the analysis continued with the *Mann-Whitney* test to determine significant differences between treatment groups. The *Mann-Whitney* test was conducted on the parameters of necrosis, cell degeneration, and congestion. The results indicated that the treatment group P1 (sapodilla leaf extract at a dosage of 100 mg/kg BW) did not show a significant difference from the treatment group P2 (sapodilla leaf extract at a dosage of 300 mg/kg BW) and the K+ *control* group (pioglitazone at a dosage of 2 mg/kg BW). However, it did show a significant difference from the K- (diabetes control) and K0 (normal control) groups.

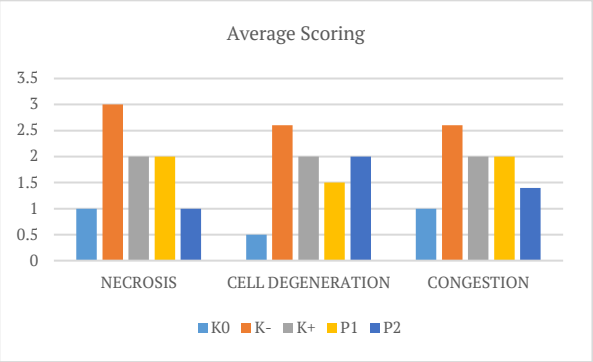

Figure 1: Graph of average histopathological scoring of necrosis, cell degeneration, and congestion in kidney tissue. Low scores indicate less tissue damage.

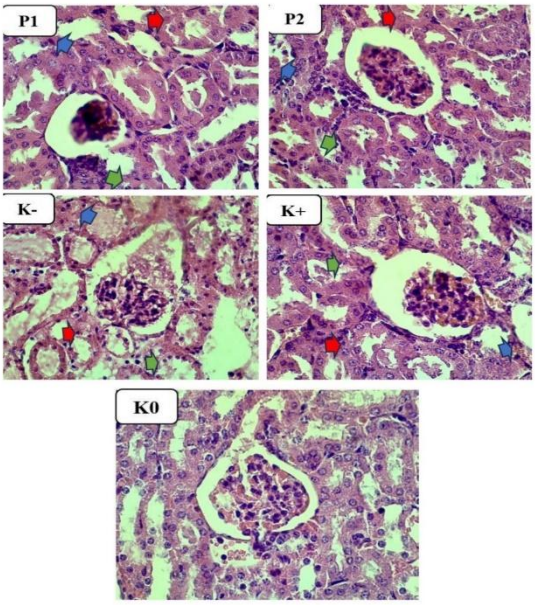

Figure 2: Microscopic View of Kidneys for Each Treatment with Hematoxylin and Eosin Staining, Magnification 400x.

The results obtained from the test indicate that P1 can be considered sufficient to improve the histopathological appearance of kidney organs in mice. However, in the case of P2, it demonstrates superior outcomes compared to P1, suggesting that the optimal

dosage for improving the histopathological appearance of kidney organs in mice, based on the parameters of cell degeneration and congestion is P2.

Discussion

The histology of the mice kidneys in a normal state displays intact renal tubules and glomeruli, with a characteristic appearance of normal kidney tubules and glomeruli. The epithelial cells of the tubules have cuboidal shapes with homogeneously pink cytoplasm and intact basement membranes. The histological depiction of the K0 group (normal control) kidneys appears normal, showing minimal necrosis in both glomeruli and tubules. In contrast, the K- group (diabetes control) experienced renal cell necrosis. Alloxan leads to necrosis and degeneration, with reports indicating that 40-50% experience necrosis. Damage to the proximal tubules occurs due to elevated blood glucose levels and oxidative stress in diabetes [28]. During hyperglycemic conditions, glucose uptake by proximal tubular cells becomes independent of insulin, making these cells highly susceptible to damage caused by hyperglycemia. The period of hyperglycemia results in an increased workload for proximal tubular cells in reabsorbing glucose, which subsequently induces hypertrophy of these tubular cells [29]. Hyperglycemia also triggers oxidative stress in diabetes, characterized by an accumulation of free radicals in the tissues. Hyperglycemia leads to an elevated production of peroxynitrite within the proximal tubular cells [30]. Peroxynitrite enhances the activity of caspase 3, 8, and 9. The activities of caspase 3, 8, and 9 lead to DNA fragmentation, ultimately resulting in proximal tubular cell damage in the form of necrosis, karyorrhexis, and karyolysis [31]. Cell degeneration and congestion occur throughout most of the field of view. Alloxan influences the histopathological changes in the kidneys of mice, as observed through lesions of degeneration, congestion, and necrosis. This statement is consistent with the research by Widiastuti et al [32], which asserts that alloxan administration affects the histopathological changes in the kidneys of white rats, evident through congestion lesions and necrosis.

The administration of sapodilla leaf extract therapy can improve the histopathological appearance of kidney organs. The results of data analysis reveal that treatment groups P1 (sapodilla leaf extract at a dosage of 100 mg/kg BW) and P2 (sapodilla leaf extract at a dosage of 300 mg/kg BW) significantly differ from the K- group (diabetes control). Data analysis based on the necrosis parameter indicates that the groups receiving extract therapy do not exhibit a significant difference compared to the normal group.

Administering a dose of an extract that yields an effect not significantly different from the normal control demonstrates that the bioactive compounds contained reach an effective concentration. Both administered extract doses also do not exhibit significant differences across the three utilized *scoring* parameters. An increase in drug dosage should ideally correspond to an escalated improvement response, yet the higher extract dose (300 mg/kg BW) does not show a substantial difference compared to the dose of 100 mg/kg BW. Medications made from natural ingredients often undergo this phenomenon because the component compounds they contain are not singular but consist of various compounds that work together to produce an effect. This results in detrimental interactions that cause effects to not significantly differ between doses [21]. This is also related to receptor saturation and interactions with chemical compounds within the sapodilla leaf. Saturated receptors lead to doses being unable to achieve a maximal effect.

Sapodilla leaf extract is known to contain antioxidant compounds such as flavonoids, alkaloids, and tannins. The function of flavonoids in managing diabetes is by enhancing the antioxidant status within tissues. Flavonoids capture or neutralize free radicals (such as ROS or RNS) [27]. Flavonoids regulate the activity and expression of enzymes involved in carbohydrate metabolism pathways, which act to supply insulin by influencing the insulin signaling mechanism [33]. Flavonoids play a role in repairing pancreatic tissue damage caused by DNA alkylation resulting from alloxan induction, leading to increased insulin secretion in the blood and a reduction in blood glucose levels. In the absence of glucose accumulation in the bloodstream, kidney nephrons will not be suppressed by glucose, thereby mitigating kidney damage and restoring kidney function to its proper operation.

The research conducted by [34] demonstrated that the flavonoid content found in pletekan (Ruellia tuberosa) can enhance tissue repair in the glomerulus. Flavonoids are among the antioxidants that serve as *scavengers* for free radicals (superoxide anion and hydroxyl radicals) and play a role in inhibiting lipid peroxidation. The antioxidant capability of flavonoids is attributed to their function as *scavengers* for free radicals. The data analysis indicates that the treatment groups P1, P2, and K+ (pioglitazone at a dosage of 2 mg/kg body weight) did not show significant differences; however, all three demonstrated an influence in ameliorating diabetic kidney damage induced by alloxan. Pioglitazone employs a therapeutic mechanism in diabetes by inhibiting oxidative stress and reactive oxygen species (ROS) production through a mechanism mediated by PPAR-γ [35]. The increase in total antioxidants can improve the histopathological

structure of affected organs. This medication can be combined with other glucose-lowering drugs, including GLP-1RA and SGLT2 inhibitors. Some benefits of pioglitazone include restoring insulin resistance, improving β-cells, managing glycemic control, and kidney function [36]. *Thiazolidinedione-class* drugs primarily function to synthesize insulin by activating PPARγ [37].

Based on the results of this research, the data analysis revealed that the administration of sapodilla leaf extract (*Manilkara zapota* L.) can ameliorate kidney damage in diabetic mice induced with alloxan. The administration of sapodilla leaf extract at a dosage of 300 mg/kg body weight exhibited superior outcomes compared to the administration of sapodilla leaf extract at a dosage of 100 mg/kg body weight and pioglitazone at a dosage of 2 mg/kg body weight.

Competing Interest

The authors declare that there is no conflict of interest.

Authors contributions

All authors were responsible for the study design, data gathering, data analysis, manuscript preparation, and editing of the manuscript.

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