# Potential Use of Breadfruit (Artocarpus altilis) Leaf Extract to Recover Hepatic and Renal Damage in Alloxan-Induced Diabetic Rats

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RESEARCH ARTICLE

# Potential Use of Breadfruit (*Artocarpus altilis*) Leaf Extract to Recover Hepatic and Renal Damage in Alloxan-Induced Diabetic Rats

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***SUMMARY***

*The antihyperglycemic effect of breadfruit leaf (Artocarpus altilis) extract has been demonstrated in a preclinical study using an alloxan-induced diabetic model. This study aimed to examine whether breadfruit leaf extract also ameliorated liver and kidney injury in alloxan-induced diabetic rats. Male Wistar rats (n=35) were used in the study. All other animals except control group (group I, n=5) were injected with alloxan (155 mg/kg body weight). After 3 days, the hyperglycemic rats with blood glucose >200 mg/dl were divided into 4 treatment groups: placebo (alloxan group), Breadfruit Leaf (BL) extract 100 mg/kg, BL extract 200 mg/kg, and BL extract 400 mg/kg. Treatments were administered daily for 14 days, and blood samples were drawn at baseline, after alloxan injection, and following treatments to obtain serum glutamic pyruvic transaminase (SGPT) and creatinine levels. Alloxan was found to cause a significant increase in rat blood glucose, SGPT, and creatinine levels three days after alloxan injection (P<0.01). After treatment, rats that received 200 mg/kg and 400 mg/kg BL extracts had significantly lower SGPT levels compared to those treated with placebo alone (P<0.05). Liver histological damage was also significantly alleviated, especially with the 400 mg/kg dose of BL extract. Although serum creatinine level was restored, alloxan-induced tubular degeneration in renal tissue was still evident. In conclusion, BL extract at a dose of 400 mg/kg improved alloxan-induced liver dysfunction and tissue damage but was less effective at alleviating kidney damage. This result may support the use of breadfruit leaf extract as herbal drug with a hepatoprotective effect.*

***Key Words:*** *Breadfruit leaf, Artocarpus altilis, diabetic rats, alloxan, liver damage, kidney damage*

***Alloxan Nedenli Diyabetik Sıçanlarda Hepatik ve Renal Hasarı İyileştirmek İçin Ekmek Meyvesi (Artocarpus altilis) Yaprak Ekstresinin Potansiyel Kullanımı***

***ÖZ***

*Ekmek meyvesi yaprağı (Artocarpus altilis) ekstresinin antihiperglisemik etkisi, alloksan nedenli diyabet modeli kullanılarak yapılan in vivo bir çalışmada gösterilmiştir. Bu çalışma, ekmek meyvesi yaprak ekstresinin, alloksan nedenli diyabetik sıçanlarda karaciğer ve böbrek hasarını iyileştirip iyileştirmediğini incelemeyi amaçlamıştır. Çalışmada erkek Wistar sıçanları (n=35) kullanılmıştır. Kontrol grubu (grup I, n=5) dışındaki tüm diğer hayvanlara alloksan (155 mg/kg vücut ağırlığı) enjekte edilmiştir. Üç gün sonra, kan şekeri >200 mg/dl olan hiperglisemik sıçanlar 4 tedavi grubuna ayrılmıştır: plasebo (alloksan grubu), ekmek meyvesi yaprak (BL) ekstresi 100 mg/kg, BL ekstresi 200 mg/kg; ve BL ekstresi 400 mg/kg. Tedaviler 14 gün boyunca günlük olarak uygulanmış ve başlangıçta, alloksan enjeksiyonundan sonra ve tedavileri takiben serum glutamik piruvik transaminaz (SGPT) ve kreatinin düzeylerini belirlemek için kan örnekleri alınmıştır. Alloksanın, enjeksiyondan 3 gün sonra sıçan kan şekeri, SGPT ve kreatinin seviyelerinde önemli bir artışa neden olduğu bulunmuştur (P<0.01). Tedaviden sonra, 200 mg/kg ve 400 mg/kg BL ekstreleri uygulanan sıçanların, tek başına plasebo ile tedavi edilenlere kıyasla önemli ölçüde daha düşük SGPT seviyelerine sahip olduğu görülmüştür. (P<0.05). Karaciğer histolojik hasarı da, özellikle 400 mg/kg dozda BL ekstresi ile önemli ölçüde azalmıştır. Serum kreatinin düzeyi eski haline gelmesine rağmen, böbrek dokusunda alloksan nedenli tübüler dejenerasyonun hala belirgin olduğu gözlenmiştir. Sonuç olarak, 400 mg/kg vücut ağırlığı dozunda uygulanan BL ekstresi, alloksan nedenli karaciğer fonksiyon bozukluğunu ve doku hasarını iyileştirmiş, ancak böbrek hasarını iyileştirmede daha az etkili bulunmuştur. Bu sonuç, hepatoprotektif etkili bir bitkisel ilaç olarak ekmek meyvesi yaprağı ekstresinin kullanımını destekleyebilir.*

***Anahtar Kelimeler:*** *Ekmek meyvesi yaprağı, Artocarpus altilis, diyabetik sıçanlar, alloksan, karaciğer hasarı, böbrek hasarı.*

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## INTRODUCTION

The breadfruit plant (*Artocarpus altilis* (Parkinson) Fosberg) is a tropical plant that belongs to the family Moraceae (Akanbi, et al., 2009). The yellowed breadfruit leaves have been used as tea to reduce blood pressure and asthma in the West Indian region. The tea is also known for its therapeutic benefit to control diabetes, which may be derived from its complex organic acid contents (Ragone, 2018). The mechanism of antidiabetic effect of *A. altilis* aqueous extract is believed to be associated with the inhibition of carbohydrate metabolizing enzymes and the stimulation of glucose cellular uptake (Sairam & Urooj, 2012). The aqueous extract of *A. altilis* leaves is safe to use at the dose up to 2000 mg/kg body weight (Sairam & Urooj, 2014).

The therapeutic effects of *A. altilis* leaf ethanolic extract have been studied in diabetic animal models. It has been shown that the aqueous extract of *A. altilis* leaf can reduce blood glucose levels in alloxan-induced diabetic mice and rats (Thubasni, et al., 2012; Djabir, et al., 2021). In addition to the anti-hyperglycemic effect, the ethanolic extract of breadfruit leaves at a dose of 400 mg/kg body weight (BW) has been shown to improve insulin expression in pancreatic beta cells (Indrowati, et al., 2017) and reduce histopathological injury in the pancreatic tissue (Sari, et al., 2020). It is believed that the antioxidant constituents of *A. altilis* significantly contribute to its therapeutic effects on diabetes mellitus since its pathogenesis is predominantly triggered by free radicals and oxidative stress (Ceriello & Motz, 2004).

Many of the chemical compounds of *A. altilis* leaves have been identified years ago. These include tannins, phenols, glycosides, saponins, steroids, terpenoids, and anthraquinones (Graham & De Bravo, 1981). More recently, Sikarwar and co-workers (2014) have listed a range of specific phytochemical constituents of *A. altilis*, including artocarpetin, cycloartinone, cyclogeracommunin, cycloartenyl acetate, cyclocommunol, norartocarpetin, and oxydihydroartocarpesin (Sikarwar, et al., 2014b). These phytochemicals are believed to mediate a range of biological activities, including antioxidant and anti-hyperglycemic activities (Sikarwar, et al., 2014a).

One of the diabetogenic agents that are popularly used to induce diabetes mellitus in animals is alloxan (2,4,5,6-tetraoxypyrimidine). The molecule structure of alloxan that resembles glucose molecules enables its uptake through glucose transport GLUT-2 in beta cells of the pancreas. This facilitates the selective entry of alloxan into beta cells of the pancreas and ultimately damages these insulin-producing cells. As a result, the animals injected with alloxan will experience a decrease in insulin production, leading to hyperglycemia (Ighodaro, et al., 2017).

Although alloxan was initially thought to merely damage the pancreatic beta cells (Gorus, et al., 1982), a number of studies have shown that alloxan injection is not only toxic to pancreatic beta cells but also toxic to other organs expressing GLUT-2, including liver hepatocytes and kidney tubular cells (Gargouri, et al., 2016; Terayama, et al., 2016). Thus, it is often found that alloxan-induced diabetic animals also experience liver and kidney dysfunction. Since alloxan hydrogen toxicity is mediated by reactive oxygen species, including superoxides, peroxides, and radical hydroxyl (Lenzen, 2008), it is believed that plant extract that is rich in antioxidant compounds may reduce the oxidative damage induced by alloxan. Therefore, this study aimed to explore the potential use of *A. altilis* leaf extract to reduce hepatic and renal injury in diabetic rats induced by alloxan.

## MATERIAL AND METHODS

### Chemicals preparation

Alloxan monohydrate (Sigma Aldrich, Singapore) and other chemicals, including 70% ethanol, were purchased through official chemical distributors in Makassar, Indonesia. Reagents for Glutamic-Pyruvic Transaminase (GPT (ASAT) IFCC mod.liquiUV) and Creatinine (creatinine liquicolor) measurements were purchased from HUMAN Diagnostic Worldwide (Germany).

### Breadfruit leaf collection

Breadfruit (*Artocarpus altilis* (Parkinson) Fosberg) leaves were collected at 8-10 am in Timbuseng Village, Patallassang District, Gowa Regency, South Sulawesi in July 2019. The herbarium specimen was stored in Pharmacognosy Laboratory, Faculty of Pharmacy, Hasanuddin University, Indonesia. The plant was authenticated and confirmed by Dr. A. Mu'Nisa from the Laboratory of Biology, State University of Makassar, Indonesia (No. 096/SKAP/LAB.BIOLOGI/VII/2019)

### Breadfruit leaf extract preparation

The collected leaves are thoroughly washed with running water and sorted from foreign materials. The leaves were then washed, dried, and cut into simple 0.5 cm pieces before being macerated with 70% ethanol (1:10 ratio) for five days. Ethanol (70%) was chosen as the maceration solvent to allow optimal extraction of the phenolic compounds of *A. altilis* (Sao Mai, 2015). The maceration process was protected from sunlight. The resulting ethanolic extract was concentrated to dryness using a rotary evaporator (Heidolp®) and stored at room temperature (25°C) in a vacuum desiccator to remove the extra solvent. Prior to administration, the extract was prepared in 1% sodium carboxymethyl cellulose (Na CMC) suspension to facilitate extract administration in animals.

### Animal preparation

Thirty-five 12-week-old (180-300 g) male Wistar rats were obtained from a rodent breeding facility (UD Wistar, Yogyakarta, Indonesia) and transferred to the laboratory where the experiment was conducted. Rats were housed in plastic cages with wood-shaving bedding in the laboratory with 12-hour light and dark cycle. The animals had free access to food and water. Animals were adapted at least 14 days before the start of the experiment. All animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The experiment received ethical approval from the Institutional Animal Ethics Committee of the Faculty of Medicine, Hasanuddin University, with the ethical number UH19050277.

### Experimental protocols

Before starting any treatment, rats were anesthetized by placing the rat one by one in a chamber (2200 $cm^3$) filled with 0.5 ml ether-impregnated cotton balls until the rat was fully anesthetized. Blood samples were drawn from all rats to obtain baseline data. Blood samples (3 ml) were taken from the lateral vein and placed in blood collection tubes (BD vacutainer®) containing EDTA.

Except for healthy controls (n=5, Group I), all other rats (n=30) were injected intraperitoneally (i.p) with alloxan at a dose of 155 mg/kg body weight (BW) to induce diabetes mellitus. Approximately 10 minutes after alloxan injection, 5% glucose solution (2 ml/200 g BW) was administered orally to prevent acute hypoglycemia. Blood glucose level measurements were made after daily injection of alloxan using a digital glucometer (Nesco®). After 72 hours (three days) of alloxan injection, rats were anesthetized via inhalation with ether and 2 ml blood samples were taken to analyze blood glucose levels using Humalyzer 3500 (Human®). Only rats with blood glucose levels >200 mg/dl at 3 days after alloxan injection (n=20) were then assigned to receive one of the following treatments: Na CMC suspension without extract (Group II, n=5), Breadfruit leaf (BL) extract 100 mg/kg BW (Group III, n=5), Breadfruit leaf (BL) 200 mg/kg BW extract (Group IV, n=5), and Breadfruit leaf (BL) 400 mg/kg BW (Group V, n=5). Treatments were administered for 14 days before final blood samples were taken to measure blood glucose, serum glutamic pyruvic transaminase (SGPT), and creatinine levels after treatment. At the end of the experiment, all animals were euthanized by cervical dislocation and the livers and kidneys were collected for histopathological analysis.

### Biomarker analysis

Blood samples were immediately centrifuged at 3000 rpm for 20 minutes to obtain serum and placed in the refrigerator (-20°C) until further analysis. Blood glucose, SGPT, and creatinine levels were analyzed using reagent kits for Humalyzer 3500 (Human®) according to the kits' instructions.

### Histopathological analysis

Rat livers and kidneys were washed with phosphate buffer solution (PBS) before fixing with 10% formaldehyde. Tissue samples were processed in a tissue processor and prepared in paraffin blocks. Tissue blocks were sliced approximately 4-5 µm thick using a microtome, and then tissue sections were stained with standard Hematoxylin and Eosin (HE) staining. The presence of histopathological changes was analyzed under the light microscope by an anatomical pathologist blinded to the treatment groups. Levels of histopathological injuries were determined according to the area and intensity of necrosis, tissue degeneration, and inflammation using methods described in the Gibson-Corley, et al. (2013) study.

### Statistical analysis

Obtained data such as blood glucose, SGPT, and creatinine levels were tested for normal distribution with the Kolmogorov-Smirnov test. To determine the significant difference for group treatments, repeated measured analysis of variance (ANOVA) statistical testing was performed at 95% confidence level, followed by post hoc Tukey's honestly significant difference (HSD) test.

## RESULTS AND DISCUSSION

### Rat blood glucose levels and body weight

Alloxan is a potent diabetogenic agent that can acutely induce hyperglycemia in rats 6 hours after i.p injection. In this study, the initial blood glucose level of rats ranged from 82-110 mg/dl. After three days from alloxan injection, rats' blood glucose levels rose to 261 - 372 mg/dl, which is 2-3 times of the blood glucose level of the healthy controls (Table 1).

There are three mechanisms by which alloxan induces hyperglycemia: 1) it selectively inhibits insulin secretion through glucokinase inhibition, 2) it stops the detection of sugar by beta cells, and 3) it induces the generation of reactive oxygen species (ROS), causing its selective necrosis in beta-pancreatic cells, leading to an insulin-dependent diabetes state (Lenzen, 2008). Two recent studies have reported the presence of inflammation and necrosis in pancreatic tissue of rats treated with alloxan (Sari et al., 2020), as well as shrinkage of pancreatic islets (Djabir et al., 2021). However, induction of hyperglycemia by alloxan has not always been 100% successful. Ten out of 30 animals (33%) did not experience a significant increase in blood glucose levels and were therefore excluded from this study.

Rats with blood glucose levels of >200 mg/dl were then randomly assigned to either receive placebo or BL extract treatments as presented in Table 1. In the alloxan group, which only received placebo, the level of blood glucose was constant above 300 mg/dl after 14 days of treatment. In contrast, a significant reduction of blood glucose level was found (from 372 ± 63.2 to 118 ± 26.1 mg/dl) in rats treated with BL extract at a dose of 400 mg/kg BW ($p<0.05$). Meanwhile, the lower doses (100 and 200 mg/kg BW) were not adequate to alleviate the alloxan-induced hyperglycemia. Hence, the blood glucose levels of those groups remained >200 mg/dl after 14 days of treatment (Table 1).

**Table 1.** Rat blood glucose levels and body weight before injection, after three days from alloxan injection, and following 14 days of treatment.

| Treatment group | Blood glucose level (mg/dl) | | | Body weight (g) | | |
|---|---|---|---|---|---|---|
| | Before injection | After injection | After treatment | Before injection | After injection | After treatment |
| Control (no alloxan) | 104 ± 4.0 | 136 ± 22.5 | 116 ± 10.9 | 196 ± 11.5 | 201 ± 11.7 | 217 ± 16.0 |
| Alloxan | 107 ± 5.2 | 276 ± 26.6* | 273 ± 41.9 | 224 ± 26.2 | 212 ± 26.7 | 204 ± 25.9 |
| Alloxan + BL 100 | 103 ± 6.4 | 292 ± 43.7* | 279 ± 43.2 | 236 ± 15.1 | 220 ± 21.3 | 215 ± 29.4 |
| Alloxan + BL 200 | 107 ± 4.8 | 261 ± 25.3* | 252 ± 69.5 | 234 ± 22.9 | 221 ± 25.5 | 219 ± 18.6 |
| Alloxan + BL 400 | 111 ± 4.5 | 372 ± 63.2* | 118 ± 26.1# | 206 ± 3.3 | 197 ± 7.3 | 205 ± 17.2 |

*$p<0.05$ compared to blood glucose level before injection; #$p<0.05$ compared to blood glucose level after injection

Alongside hyperglycemia, weight loss is also one of the important features of diabetic rats. It is often that diabetic rats lose>10% of their body weight and are accompanied by lethargy and soft stool (Wang-Fischer & Garyantes, 2018). These symptoms were also observed in this study after rats receiving alloxan injection. The baseline body weights of rats ranged from 180-300 g then slightly decreased following alloxan injection. The average of weight loss of alloxan-treated groups was 9 g to 16 g after three days from the injection, while the control group gained weight as much as 5 g on average (Table 1). Indeed, following 14 days of treatments, the rat body weight continued to drop in 80% of rats in the alloxan group. On average the rats in alloxan group had lost 9% of their baseline body weight. Meanwhile, only 40% of rats treated with BL extract 400 mg/kg experienced further weight loss and only lost 0.5% of their body weight. This may indicate an improvement of metabolic function in rats that received BL extract 400 mg/kg.

**Liver function and tissue structure post alloxan injection and following treatments**

The SGPT levels of rats before, after and 14 days after alloxan injection were presented in Figure 1. Alloxan has been shown to significantly increase the level of SGPT after three days of injection. The increase in the mean level of SGPT in alloxan group, alloxan + BL 100, alloxan + BL 200, and alloxan + BL 400 were 62%; 100%; 34%; 113%, respectively. Indeed, the SGPT level of rats in the alloxan group continued to rise to >200 mg/dl after 14 days of treatment, which was five times higher than baseline ($p$<0.05).

The result of this study shows that breadfruit leaf (BL) extract treatment can preserve the liver function of alloxan-injected rats, as the use of BL extract can prevent the increase of SGPT levels (Figure 1). The most effective dose was found to be 200 and 400 mg/kg rat body weight. With the administration of BL extract, the SGPT levels returned to normal despite the initial increase after alloxan injection ($p$< 0.05). The post-treatment SGPT level was also lower in the alloxan + BL extract 100 mg/kg group compared to the alloxan group. Still, the reduction in SGPT was less noticeable with 100 mg/kg BL extract compared with the higher doses.

Increased SGPT level is a specific indicator of liver dysfunction and liver injury, as this enzyme is normally contained in the hepatocytes (Djabir et al., 2020). The presence of liver injury following alloxan injection may be triggered by the direct effect of ROS generation or indirectly by uncontrolled hyperglycemia (Gargouri et al., 2016). Increased ROS production eventually leads to hepatocyte cell degeneration and cellular necrosis (Lucchesi et al., 2015), resulting in increased GPT release from the cytoplasm of hepatocytes into the circulation.

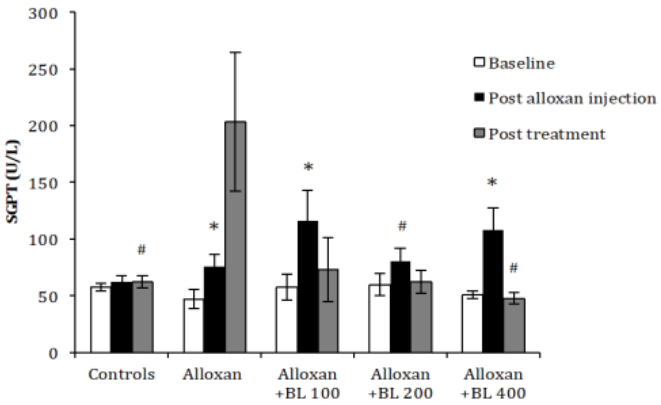


**Figure 1**. SGPT level of treated rats at baseline, post alloxan injection and post 14 days of treatments. *shows $p$<0.05 compared to the baseline level. *shows $p$<0.05 compared to the alloxan group post-treatment.

In this study, liver histology of all rats injected with alloxan was found to show fatty and hydropic degeneration. Still, among all groups, the alloxan group experienced the most extensive necrosis in their livers (Figures 2B and 2C). Oral administration of breadfruit leaf ethanol extract appeared to improve hepatocyte cell structure. At a lower dose (100 mg/kg body weight), rat liver presented hydropic degeneration affecting 50% of liver tissues observed (Figure 2D). As for the 200 mg/kg (Figure 2E) and 400 mg/kg BW (Figure 2F) groups, some hepatocytes still showed signs of hydropic change but had significantly fewer necrotic cells overall compared to the alloxan group. Characteristics of liver injury in rats treated with alloxan can range from inflammation to necrosis (Bilal et al., 2016). The improvement in liver structure seen with BL extract might be enhanced by potent antioxidants and high phenolic content in breadfruit leaves which stabilize the ROS produced by alloxan (Leng et al., 2018).

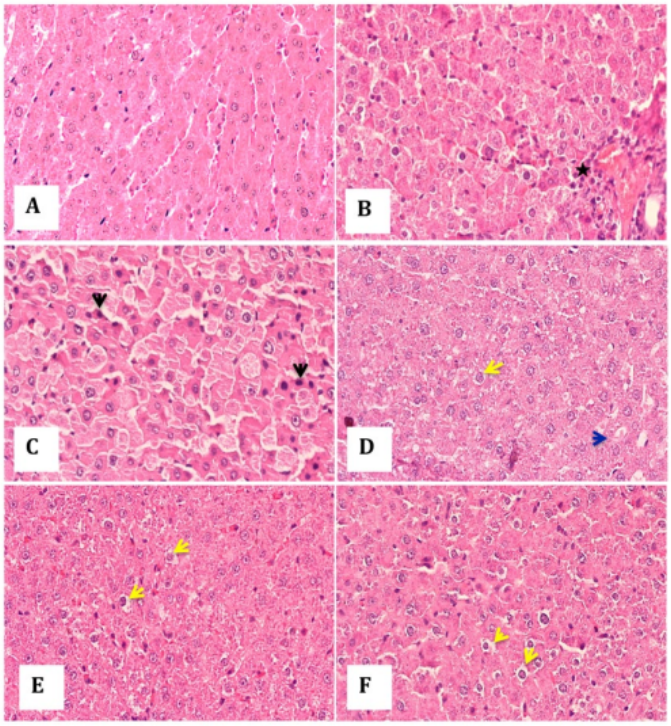


**Figure 2**. The photomicrograph of liver tissues of rats following 14 days of treatments (H&E stained, 400X magnification). **A**) The control group showed a normal architecture of hepatocytes and portal triad. **B**) Alloxan group showed an infiltration of inflammatory cells in liver tissue (*). **C**) Diffuse necrotic area and necrotic cells were found scattered in Alloxan-treated rats (black arrow). **D**) The liver from the rat treated with Alloxan + BL extract 100 mg/kg showed hydropic degeneration (yellow arrow) and dilated sinusoid (blue arrow). **E**) Rat's liver treated with Alloxan + BL extract 200 mg/kg mostly showed hydropic changes (yellow arrow). **F**) Rat's liver treated with Alloxan + BL extract 400 mg/kg showed hydropic degeneration (yellow arrow).

**Renal function and tissue structure post alloxan injection and following treatments**

Figure 3 depicts the serum creatinine levels at baseline and those initially treated with alloxan three days after alloxan injection and 14 days after treatment. After the injection of alloxan, creatinine levels were significantly elevated in all rats injected with alloxan compared to normal controls ($p< 0.05$). Alloxan-treated rats experienced at least a 120% rise

in creatinine on day three after alloxan injection. It is believed that elevated creatinine levels are due to the accumulation of glycogen in the distal tubules of the kidneys during persistent hyperglycemia (Terayama et al., 2016). Regardless of the treatment given, the creatinine level of alloxan-treated rats was simultaneously reduced by 50% (Figure 3). Spontaneous recovery of creatinine levels was also observed by another study after 30 days of alloxan injection in diabetic rabbits (Ahmad et al., 2014).

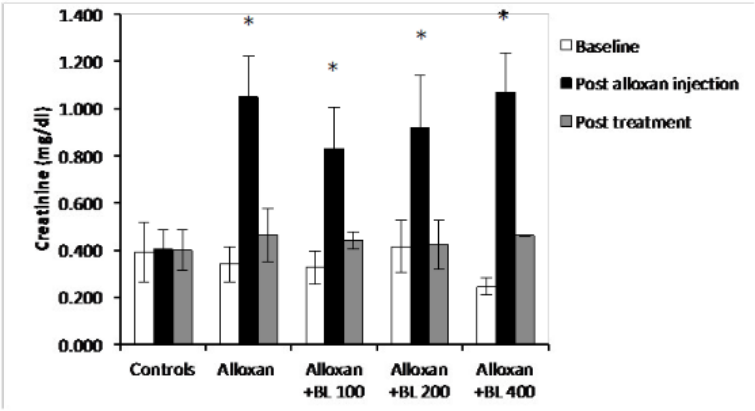


**Figure 3**. Serum creatinine level of rats at baseline, post alloxan injection, and post 14 days of treatments. *shows $p<0.05$ compared to the baseline level.

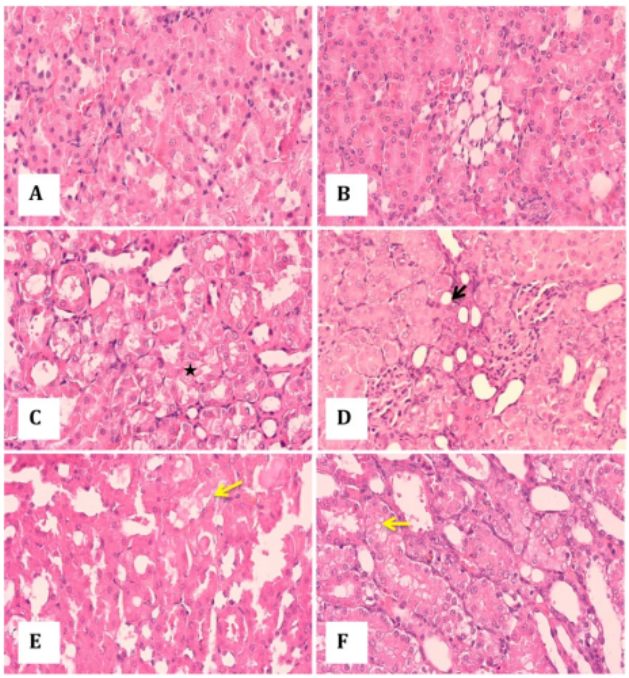


**Figure 4.** The photomicrograph of renal tissues of rats following 14 days of treatments (H&E stained, 400X magnification). **A)** The control group showed normal architecture of renal tubules. **B)** Alloxan group showed substantial lipid degeneration in the tubules. **C)** Alloxan group experienced degeneration in the renal tubule (*). **D)** Rat treated with Alloxan + BL extract 100 mg/kg showed lipid degeneration (black arrow). **E)** Rat treated with Alloxan + BL extract 200 mg/kg showed hydropic degeneration (yellow arrow). **F)** Rat treated with Alloxan + BL extract 400 mg/kg showed hydropic degeneration (yellow arrow).

The increase in creatinine is more likely to reflect acute renal dysfunction rather than permanent kidney damage as it returned to baseline 14 days after injection. Interestingly, renal histological changes were still prominent in placebo-only treated rats (alloxan group, Figures 4B and 4C) (25% to 50% of the field of view at 400X magnification). In this group, both fatty degeneration and hydropic changes were observed in the renal tubules. Application of the BL extract seemed to slightly reduce the severity of kidney injury since the damage was not as intense as in the alloxan group. Although some lipid degeneration was still observed with BL extract treatment at 100 mg/kg (Figure 4D), BL extract 200 mg/kg and 400 mg/kg treatment had less intense injury with scattered hydropic degeneration (Figures 4E and 4F). The discrepancy between the corrected serum creatinine level and the presence of histopathological changes in the renal tubules of alloxan-induced rats suggests that serum creatinine may not be the best biomarker for tubular damage (Chu et al., 2016). Further studies should include other biomarkers to quantify the extent of kidney injuries due to alloxan injection.

## CONCLUSION

The injection of alloxan 155 mg/kg in rats led to hyperglycemia at three days post injection. Liver and kidney injuries were observed in the alloxan-induced diabetes model in rats. The administration of BL extracts at a dose of 200 mg/kg and 400 mg/kg were shown to reduce the SGPT level, indicating its potential role in improving alloxan-induced liver dysfunction. Breadfruit extract, especially at 400 mg/kg dose, significantly alleviated liver tissue injuries, and renal damage to a lesser extent. Due to the spontaneous recovery of serum creatinine level, it was difficult to assess the implication of BL extract in alloxan-induced renal dysfunction.

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## CONFLICT OF INTEREST

The authors declared no conflict of interest

## AUTHOR CONTRIBUTION STATEMENT

HS was responsible for conducting experiment, data acquisition and analysis, as well as writing the manuscript. YY as the project leader was responsible for designing the research protocols and concept, data interpretation, and manuscript revision. HH was responsible for sample extraction, biomarker analysis, and data editing. SL was responsible for data interpretation and revising the manuscript. HC was responsible for histopathological analysis result and interpretation

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