ASIAN JOURNAL OF HEALTH AND APPLIED SCIENCES
2023, VOL. 2, NO. 2, 7 – 13
https://doi.org/10.53402/ajhas.v2i2.280

The Effect of Garlic Decoction (*Allium sativum L.*) on The Histological Features of The Liver of Alloxan-induced Mice (*Mus musculus*)

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ABSTRACT

Giving alloxan can cause disruption of insulin production resulting in hyperglycemia. Hyperglycemia causes Reactive Oxygen Species (ROS) resulting in oxidative stress and liver cell damage. The use of natural ingredients, one of which is garlic (*Allium sativum L*) in the form of boiled water, because it contains flavonoid and organosulfur compounds. Therefore, the purpose of this study was to determine the histology of the liver of mice (Mus musculus) induced by alloxan after being given a decoction of garlic (*Allium sativum L*). This study used experimental research with a completely randomized design method on mice consisting of 6 groups with 4 replications each. The groups in this study consisted of a negative control group (without treatment), a positive control group (alloxan injection) a comparison control group (alloxan injection + glibenclamide) treatment groups at doses I, II and III (alloxan-induced + garlic boiled water (*Allium sativum L*) respectively 20 gr, 40 gr and 60 gr). Histology of the liver of mice (*Mus musculus*) was made by paraffin method with Hematoxylin Eosin (HE) staining. The result showed that there was a significant difference due to the value (p<0,05) but for a KP and P3 there was no significant difference with the value (p>0,05) on hydropic degeneration. Based on the results of the study, it can be concluded that the decoction of garlic (*Allium sativum L*) has an effect on the alloxan-induced liver histology of mice (Mus musculus).

KEYWORDS

Garlic; Mice liver; Alloxan; Hyperglicemia; Antioxidant

Received: February 22, 2023
Accepted: May 31, 2023
Published: August 2, 2023

Introduction

Diabetes mellitus is an increasing number of degenerative diseases and one of the leading causes of death in the world. Since 2019 IDF indicates the number of diabetes is indicates that the number of diabetes raised by 463 million adults, by 2021 there were 537 million adults, and estimated 700 million people worldwide in 2045. With a percentage of 80% of sufferers from developing countries to the poor, including Indonesia with the highest number of diabetics (Sun *et al.*,2022). Increased blood glucose (hyperglycemia) is one of the specific signs of Diabetes mellitus. Hyperglycemia causes the accumulation of Reactive Oxygen Species (ROS) in pancreatic β cells. The accumulation of ROS can cause cell damage. Excessive ROS level causes oxidative stress. Conditions of oxidative stress will increase the concentration of free radicals in the human body. An increase in free radicals will cause lipid peroxidation also DNA and protein oxidation, causing degeneration of liver cells and causing cell death (Subandrate, 2016).

Alloxan is used intraperitoneally, intravenously, and subcutaneously as a causative agent for diabetes mellitus (diabetogenic). Diabetes induction methods are widely used in experimental animals including mice (*Mus musculus*) because they have a similar structure to glucose, the structural integrity of the cytoskeleton, lysosomes, DNA, and mitochondria will be lost and then the cells will be destroyed causing a lack of insulin production when cells induced by alloxan (Cheekati *et al.*,2017). Therefore, it requires therapy or medication to repair the damaged or destroyed cells.

Garlic (*Allium sativum L.*) has antioxidant compounds such as phenolic and organosulphur. These compounds have the main benefit of avoiding damage to cells and organs as a result of the oxidation stage. Phenolic compounds contained in Garlic (*Allium sativum L.*) contain groups that gather with the number of one to more hydrogen proton donor agents to neutralise free radicals and the effect of Reactive Oxygen Species (ROS) so that they can repair liver damage due to oxidative stress originating from alloxan induction (Prasonto *et al.*, 2017).

Based on some of these explanations, the researchers carried out research on the effect of garlic (*Allium sativum L.*) decoction on the histological features of the liver of alloxan-induced mice (*Mus musculus*).

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Method

This study used an experimental research method with a completely randomized design. Using 24 mice (*Mus musculus*) swiss webster strain was divided into 6 groups. Sampling used a simple randomization technique with the criteria of male mice, aged 8 – 10 weeks, weighing 20 – 30 grams, healthy, and having normal activities. The primary data analyzed in this study were the results of the degree of liver damage scores from all treatment groups.

Animal preparation

The experimental animals used were swiss webster strain white mice with the aforementioned criteria. For one week the mice were left for adaptation in order to reduce the effects of stress on the mice which could affect the mice's body metabolism. Mice must be in good health with these criteria: normal fur, clear eyes, normal activities, and no abnormalities in the body. At the time of the one-week adaptation, the mice were only given food and drink.

Animal treatment

This study was conducted in vivo on 24 male mice (*Mus musculus*) which were divided into 6 treatment groups, each group consisting of 4 male mice (*Mus musculus*).

Table 1. Grouping and Treatment of Mice (*Mus musculus*)

Group	Number of mice	Day of Treatment			
		1-7	8	10-25	26
Negative Control (KN)	4	Adaptation	Given normal feed and distilled water	Given normal feed and distilled water	The liver was removed surgically and put into a 10% NBF solution for making histological preparations
Positive Control (KF)	4	Adaptation	Given normal feed, distilled water, and induced by alloxan	Given normal feed and distilled water	
Comparison Control (KP)	4	Adaptation	Given normal feed, distilled water, and induced by alloxan at a dose of 130 mg/20 gram body weight with a volume of 1 ml/20 gram body weight	Given normal feed, distilled water, and induced by glibenclamide at a dose of 0,013 mg/20 gram body weight with a volume of 1 ml/20 gram body weight	
Dose I (P1)	4	Adaptation	Given normal feed, distilled water, and induced by alloxan at a dose of 130 mg/20 gram body weight with a volume of 1 ml/20 gram body weight	Given normal feed, distilled water and Garlic decoction 0.52 ml/kg body weight at a dose of 20 grams	
Dose II (P2)	4	Adaptation	Given normal feed, distilled water, and induced by alloxan at a dose of 130 mg/20 gram body weight with a volume of 1 ml/20 gram body weight	Given normal feed, distilled water and Garlic decoction 0.52 ml/kg body weight at a dose of 40 grams	
Dose III (P3)	4	Adaptation	Given normal feed, distilled water, and induced by alloxan at a dose of 130 mg/20 gram body weight with a volume of 1 ml/20 gram body weight	Given normal feed, distilled water and Garlic decoction 0.52 ml/kg body weight at a dose of 60 grams	

Preparation of Alloxan Solution

260 mg of alloxan dissolved in 0.9% NaCl to a 50 ml total volume, then it's homogenized using a hot plate magnetic stirrer mixer. Alloxan solution is used for injection (1 ml/20 gram body weight) with the volume taken according to the body weight of the mice at a dose of 130 mg/20 gram body weight.

Preparation of Glibenclamide Solution

Glibenclamide as much as 1.3 mg is dissolved with distilled water until the volume is 100 ml, then homogenized using a hot plate magnetic stirrer mixer. The glibenclamide solution is used for injection (1 ml/20 gram body weight) with the volume taken according to the body weight of the mice used at a dose of 0.013 mg/kg body weight.

Preparation of Garlic Decoction

Garlic is separated from the skin and then washed using running water until clean, Dose I uses 20 grams of garlic, Dose II uses 40 grams of garlic, and Dose III uses 60 grams of garlic, then chop the garlic coarsely. Prepare 400 ml of hot water to boil the garlic, then add the chopped garlic for about 15 minutes at 90^0C until the cooking water has reduced by 200 ml. Strain the stew using a filter then wait until it cools down and put the garlic decoction into the bottle.

Dosing of Alloxan in Mice

The group mice were divided into 2, namely the Negative Control Group (KN) or non-diabetic and the Positive Control Group (KF) or diabetic mice. Diabetic mice were induced with alloxan at a dose of 130 mg/kg body weight with a volume of 1 ml/20 gram body weight. The solution was given by one-time injection, given for 14 consecutive days.

Dosing of Glibenclamide in Diabetic Mice

The group mice was divided into 2, namely the Negative Control Group (KN) or non-diabetic and the Positive Control Group (KF) or diabetic mice. Diabetic mice were induced with alloxan

Dosing of Garlic Decoction

Mice with diabetes mellitus were then given garlic decoction to prevent or repair liver cells and organs as a result of oxidative stress. The diabetic mice group consisted of the Positive Control Group (KF), Comparative Control Group (KP), Dose I Treatment Group (P1), Dose II Treatment Group (P2), and Dose III Treatment Group (P3). Garlic decoction was given to P1, P2, and P3 with different concentrations and the volume given was 0.52 ml/20 grams orally (sonde).

Mice Organ Taking

Hold the tail of the mice with a right hand and let the mice's body stretch, then hold the neck and inject ketamine into the thigh intravenously. After injection, keep the mice until it killed. Take the killed mice into a container and incinerated them. After that, do surgery on the abdomen and remove the liver. Place the liver in a container that has been labelled and filled with 10% NBF solution.

Tissue Cutting

Cutting the liver tissue using a sharp knife with a 1.5 cm × 1 cm × 0.5 cm size. The cut tissue is inserted into the cassette using tweezers. Name labelled the cassette according to the treatment group, then the cassette is closed. Insert the cassette into a 10% NBF solution.

Fixation

The tissue cassette is inserted into 10% NBF. There are two stages, namely: I: 10% NBF for 30 minutes, II: 10% NBF for 60 minutes.

Dehydration

At this stage, the tissue cassette is inserted in I: 70% alcohol for 30 minutes, II: 80% alcohol for 60 minutes, III: 90% alcohol for 60 minutes, and IV: 96 alcohol for 30 minutes.

Clearing

After the tissue has gone through the stages of dehydration, then the tissue cassette is put into the xylol solution. Xylol I for 30 minutes, Xylol II for 60 minutes, and Xylol II for 60 minutes.

Infiltration

The tissue cassette is put into the liquid paraffin in an oven with a 40^{o} – 60^{o} C temperature. Paraffin I for 60 minutes and Paraffin II for 12 hours.

Blocking

The process of tissue embedding is carried out on a paraffin block that has been thawed and allowed to stand until it hardens.

Tissue Sectioning and Offxing

The tissue blocks that have been cut using a microtome produce tissue bands with a thickness of 4 – 10µm, then it taken carefully and placed in a water bath filled with 40 – 60 ^{0}C water.

Staining

The glass object is heated on the hotplate for 15 – 20 minutes or until the paraffin melts at 40^o – 60^{o}C temperature. Soak the tissue preparation into Xylol, Xylol I for 5 minutes and Xylol II for 5 minutes; the process is called deparaffinization. After that, the tissue preparation is soaked in alcohol to pass the rehydration step, the tissue preparation is soaked in Alcohol 96%, 90%, 80%, and 70% for 2 minutes in each concentration. Then the tissue preparation is put into distilled water for 1 minute. Then put the tissue preparation into a hematoxylin stain for 3 – 5 minutes. After that, soak it in water for 2 – 5 minutes. Dip into 0.5% acid alcohol solution for 3 dips. Then soak in water for 1 minute. Then put into 0.5% lithium carbonate for 3 dips to maintain the blue colour. Place the tissue preparation in water for 1 minute. Then the tissue preparation is put into the eosin solution for 3 – 5 minutes. Place the tissue preparation in water for 1 minute. Then the tissue preparation is put into alcohol for the dehydration step (70%,80%,90%, 96%) for 3 minutes in each concentration. After that, the tissue preparation is put into xylol to standardize the red and blue colours (Xylol I and Xylol II) for 2 minutes in each step.

Mounting

All tissue preparations were added by Stellan and covered with a covered glass to prevent air bubbles from forming.

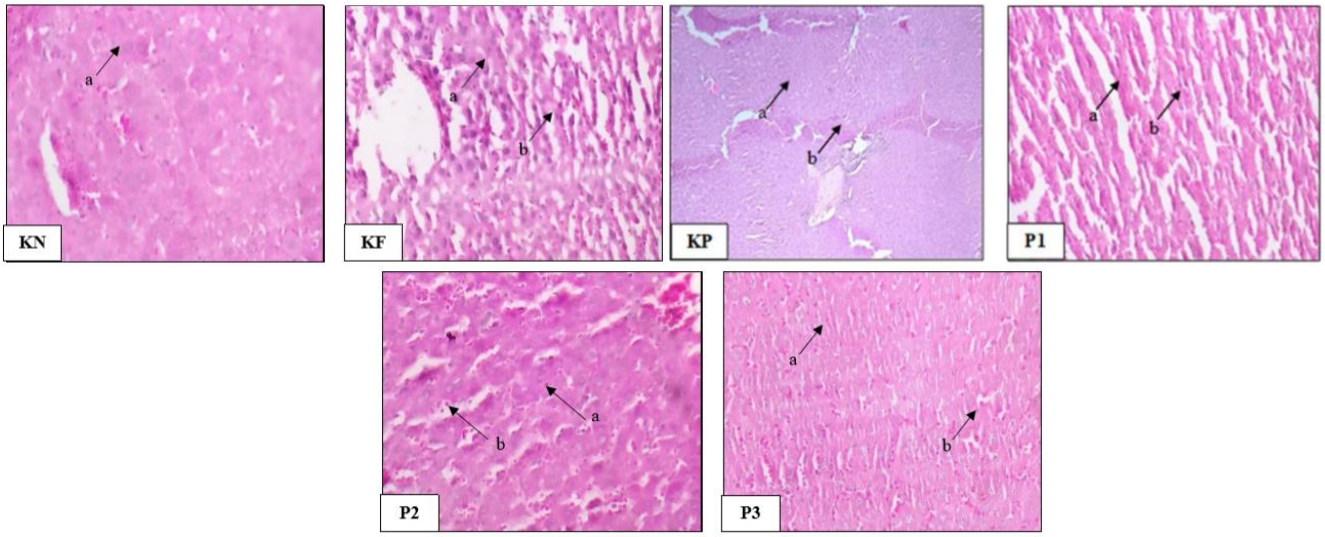
Labelling

The tissue preparation is then labelled with a description. Then observed under a microscope with 400 times magnification.

Examination of Liver Histopathology Preparations

Examination of liver histology preparations using a microscope with 400 times magnification. Assessment criteria to determine how severe the histological changes in the liver of mice (*Mus musculus*). Then the average liver histological damage score was calculated from 4 preparations for each mouse using the Mitchel method (Gufron, 2001).

Results



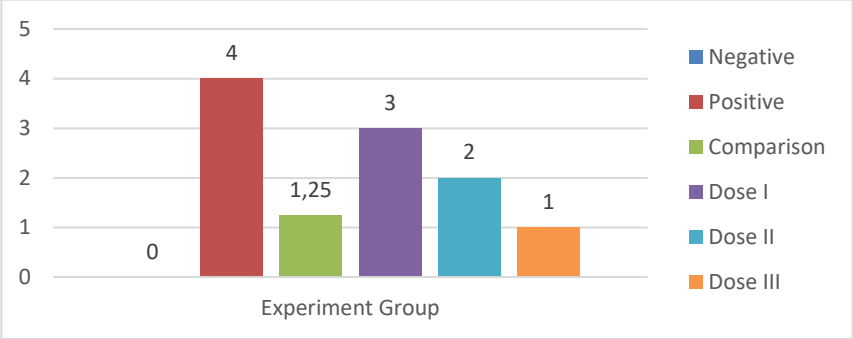
Picture 1. Histology of The Liver of Mice

Picture 1. Histology of the liver of mice (*Mus musculus*) induced by alloxan, glibenclamide, and garlic (*Allium sativum L.*) decoction which was then stained with Hematoxylin Eosin with 400× magnification. Description: a. Normal hepatocyte b. Hydropic degeneration.

Table 2. Degree of Damage Mitchel's Method

Mice	Negative	Positive	Comparison	Dose I	Dose II	Dose III
1	0	4	1	3	2	1
2	0	4	1	3	2	1
3	0	4	2	3	2	1
4	0	4	1	3	2	1
Average	0	4	1.25	3	2	1

Hows the Degree of Damage to the Mitchel Method (Gufron, 2001) with the description 0 no liver cell damage occurs, 1 liver cell damage up to 0.1 – 5%, 2 liver cell damage up to 6 – 25%, 3 liver cell damage up to 26 – 50%, 4 liver cell damage reaches 50%.



Picture 2. Bar Chart of Mean Score Degree of Liver Cell Damage

Table 3. Result of Shapiro Wilk Normality Test

Group	P value
Negative Control (KN)	0.000
Positive Control (KF)	0.000
Comparison Control (KP)	0.001
Dose I (P1)	0.000
Dose II (P2)	0.000
Dose III (P3)	0.000

Table 3. The Results obtained were that the six treatment groups had a p-value <0.005. This shows that the data for all treatment groups are not normally distributed. So then a non-parametric statistical test was carried out, namely the Kruskal Wallis test.

Table 4. Results of Non-Parametric Analysis of The Kruskal Wallis Test

Group	P value
Negative Control (KN)	0.000
Positive Control (KF)	0.000
Comparison Control (KP)	0.000
Dose I (P1)	0.000
Dose II (P2)	0.000
Dose III (P3)	0.000

Table 4. The results showed that the six treatments group had a p-value <0.05. This shows that if the treatment groups have differences with the provisions of the p-value <0.05 to see a real difference from each treatment group then it will continue to use the Mann-Whitney test.

Table 5. Results of Mann Whitney Test

Main Group	Group	P value
Negative Control (KN)	KF	0008*
	KP	0.011*
	P1	0.008*
	P2	0.008*
	P3	0.008*
Positive Control (KF)	KP	0.011*
	P1	0.008*
	P2	0.008*
	P3	0.008*
Comparison Control (KP)	P1	0,011*
	P2	0.040*
	P3	0.317
Dose I (P1)	P2	0,008*
	P3	0.008*
Dose II (P2)	P3	0,008*

Explanation: * there were significant differences in the treatment group

Table 5. The results show that there is a significant difference in the score of liver cell damage between the treatment groups because the p-value is <0.05. But, in the comparison control (KP) with dose III (P3) there was no significant difference because the p-value>0.05.

Discussion

The results of observations in the negative control treatment group did not occur liver cell damage. There are normal sinusoid and normal hepatocytes with round or oval cell morphology, and hepatocyte plate with a condensed circular nucleus in the middle (Rarangsari, 2015). This happened because mice (*Mus musculus*) were only induced by food and distilled water. The positive control group shows hydropic degeneration with an average value of 4 according to the degree of damage by Mitchel's method (Gufron, 2001). This happened because alloxan-induced mice experience damage to vital components in pancreatic cells resulting in a lack of insulin-carrying granules in the pancreatic cells while also causing toxic effects on the liver (Bilal *et al*., 2016).

In the positive control group, hydropic degeneration of liver cells was damaged with an average damage value of 4. Degenerated liver cells were characterized by picnotic and sinusoids that appeared to be regular, experiencing dilation or dilatation which could occur due to the presence of toxic substances that can put pressure on the walls of the sinusoids and caused this (Rarangsari, 2015). This happened because mice (*Mus musculus*) are induced by alloxan. In accordance with the mechanism of action of alloxan, namely by involving its activation so that it can inhibit insulin secretion in a way that damages pancreatic cells and selects intermediaries from GLUT 2 intermediaries and also induced the formation of Reactive Oxygen Species (ROS), which can cause liver cells to degenerate to become necrosis. Due to the role of the liver in the metabolism of semi-biotic substances, liver cells are the main tissues that are the target of free radicals (Millati *et al*., 2019).

In the comparison control group which was treated with glibenclamide, it was shown that there was an improvement in hydropic and sinusoid degeneration of liver cells which had improved close to that of the negative control (KN) treatment group with a mean value of 1.25. in accordance with the way of action of glibenclamide which involves the production of the hormone insulin from pancreatic cell granules. The membranes of these cells undergo depolarization as a result of their interaction with ATP-sensitive K channels and this opens Ca channels. When the channel opens, Ca^{2+} ions flow into the cell thereby activating the insulin-containing granules and increasing insulin production. This results in considerable repair of hydropic degeneration in liver cells (Utami, *et al*., 2019).

In the group treated with garlic (*Allium sativum L.*) decoction, there was mild hydropic degeneration and sinusoidal damage was evident and regular compared to the positive control (KF) treatment group with mean values of damage at P1 = 3, P2 = 2, P3 = 1. Because one way to neutralise oxidative stress is to consume foods that are antioxidants, one of the natural antioxidants is garlic (*Allium sativum L.*). The protective effect of garlic (*Allium sativum L.*) is related to its antioxidant properties as in (Rachman, 2003) which can accelerate the regeneration capacity of hepatocytes which causes hepatocyte cell membrane stabilization and can ultimately protect liver cells against damaging agents and toxic-free radical damage. This is also in line with research (Oyebadejo, 2014) proving that garlic (*Allium sativum L.*) has antioxidant and hepatoprotective activity. Garlic (*Allium sativum L.*) contains flavonoids and organosulphur compounds such as *diallyl thiosulfinate* (Allisin) which function as an antioxidant agent so that they can neutralize cell damage including cells in the liver. Antioxidant content also functions as a repair agent or can reduce oxidative damage in diabetics. This is consistent with the anti-diabetic benefits of garlic (*Allium sativum L.*) where the main bioactive components of organosulphur compounds such as *diallyl thiosulfinate* (Allisin) and *S–allyl–cysteine sulfoxide* (Allin) are able to stimulate the pancreas to be able to secrete more insulin into β cells. Pancreas of mice (*Mus musculus*) diabetes due to alloxan-induced (Hernawan & Setyawan, 2003).

Conclusion

Based on the research that has been done, giving garlic (*Allium sativum L*) boiled water with a volume of 0.52 ml/kg BW and different dose concentrations of 20 grams, 40 grams, and 60 grams has the potential to repair liver cell damage hydropic degeneration due to attacks Reactive Oxygen Species (ROS) which occurs due to hyperglycemia due to alloxan induction in mice (Mus musculus). The high dose concentration of garlic (*Allium sativum L.*) will show improvement changes that occur, as evidenced by the average dose III (P3) treatment group with an average value of 1. This is a reference for the field of medicinal plants and has the opportunity to develop research.

Acknowledgement

The authors are grateful to all parties in Medical Laboratory Technology, Faculty of Health, Institut Kesehatan Rajawali

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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