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TOXICITY TEST OF *Eusideroxylon zwageri* BARK EXTRACT ON KIDNEY HISTOPATHOLOGY GLOMERULAR HYPERTROPHY AND HYDROPIC DEGENERATION

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ABSTRACT
Background*: Ironwood bark extract (Eusideroxylon zwageri) has antioxidant properties such as tannins, phenolics, flavonoids, saponins and alkonoids. Most compounds in the ironwood bark extract were phenolics (31.28 mg GAE/g), flavonoids (30.48 mg CE/g), and proanthocyanidins (183.30 mg PE/g). These can be used as alternative herbal medicines, but also has toxic effects, so a toxicity test is necessary. Toxicity tests can be seen through histopathological parameters based on glomerular hypertrophy and hydropic degeneration.* ***Purpose****: To determine the toxic effect of ironwood bark extract administration at doses of 1,250 mg/kg, 2,750 mg/kg, and 4,750 mg/kg orally on the kidneys of Wistar rats based on histopathological appearance of glomerular hypertrophy and hydropic degeneration.* ***Methods****: Pure experimental study with a posttest-only with control design, consisting of 4 groups with 3 treatment groups given ironwood bark extract at doses of 1,250 mg/kg, 2,750 mg/kg, and 4,750 mg/kg, and 1 control group was given distilled water 2x1 ml every 24 hours orally for 14 days.* ***Results****: In administration of ironwood bark extract at doses of 2,750 mg/kgBW and 4,750 mg/kgBW, glomerular diameter was found increased. The histopathological hydropic degeneration showed a score of 1 in all dose groups. The research data were analyzed using the One Way Anova and Kruskal Wallis tests. Both tests showed no significant differences between groups.* ***Conclusion****: There was no toxic effect of ironwood bark extract at doses of 1,250 mg/kg, 2,750 mg/kg, and 4,750 mg/kg on the kidneys of Wistar rats based on histopathological appearance of glomerular hypertrophy and hydropic degeneration.*

Keywords: *Glomerular hypertrophy, Histopathology, Hydropic degeneration, Ironwood bark extract, Toxicity*
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INTRODUCTION

Basic Health Research (Riskesdas) 2018 shows that around 48% of Indonesia's population consumes traditional medicines. [1] One of the plants used in traditional medicine is ironwood (*Eusideroxylon zwageri*), which is one of the typical plants of Kalimantan. The ironwood plant has long been used by the community as a traditional medicine for several diseases such as diarrhea, toothache, and swelling. Based on the results of phytochemical tests, ironwood bark contains several secondary metabolites, namely tannins, phenolics, flavonoids, proanthocyanidins, saponins, terpenoids, and alkaloids. Most of the compounds in the extract of ironwood bark are phenolic (31.28 mg GAE/g), flavonoids (30.48 mg CE/g), and proanthocyanidins (183.30 mg PE/g).[2,3]

Phenols and flavonoids have antioxidant effects by donating hydrogen atoms to radical

molecules and forming phenoxyl flavonoid radicals (FI-O•).[3] However, in several studies, phenolics and flavonoids also show toxic potential because they can act as pro-oxidants at high concentrations and the presence of transition metal ions in the body. Phenoxyl flavonoid radicals can react with oxygen to produce quinones and superoxide anions. The interaction with metal ions has the potential to break down superoxide anions into more reactive hydroxyl radicals, leading to cell damage.[4]

One of the cell damage occurs in the kidney as an excretory organ for removing foreign or toxic compounds from the body.[5] Parts of the kidney that are easily damaged are the glomeruli and tubules. The interaction of toxic compounds and blood vessels results in vasodilation of blood vessels which leads to the increasing size of the glomerular cell or called glomerular hypertrophy. Nephrotoxic substances can also cause damage to mitochondria causing decreased ATP production which will make the tubules swelling due to interference with sodium and potassium pumps.[6] This process causes reversible damage known as hydropic degeneration. Alteration in kidney cell structure can be observed with histopathological parameters through a test called the toxicity test.[7]

A toxicity test is needed to provide knowledge on the degree of danger of the test preparation before it is used by humans. Toxicity tests can be carried out in vitro or in vivo.[6,7] In vitro study conducted by Salwa et al (2021) stated that ironwood stem bark extract (*Eusideroxylon zwageri*) had no toxic effect on Baby Hamster Kidney-21 (BHK-21) fibroblast cells at concentrations of 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85%, and 95%. In vivo studies can be divided into acute, subchronic, and chronic toxicity tests.[3] Acute toxicity test was carried out by administering a single dose or repeated test preparation within 24 hours, after that, the samples were observed for 14 days.[7] For this reason, this study aims to determine the toxic effects of ironwood bark extract (*Eusideroxylon zwageri*) administration at doses 1,250 mg/kgBW, 2,750 mg/kgBW, and 4,750 mg/kgBW orally on the kidneys of Wistar rats based on histopathological features of glomerular hypertrophy and hydropic degeneration.

METHODS

This research has received ethical approval from the Ethics Commission of the Faculty of Dentistry, University of Lambung Mangkurat No. 023/KEPKG-FKGULM/EC/II/2023. The method used in this research is purely experimental with a posttest-only control design. In this study, 16 male Wistar rats were used and then divided into 4 groups with 4 rats in each group. The control group (K) was given distilled water, treatment group 1 (P1) was given ironwood bark extract at a dose of 1,250 mg/kg, treatment group 2 (P2) was given ironwood bark extract at a dose of 2,750 mg/kg, and treatment group 3 (P3) was given ironwood bark extract dose of 4750 mg/kgBB.

Fourteen kilograms of ironwood bark was taken from Paau Village, Aranio District, Banjar Regency, South Kalimantan. The ironwood bark is cleaned, planed using a wooden planer machine, dried in an oven at 40°C for 4 hours, and blended to make simplicia powder. Extraction was carried out using the maceration method using 96% ethanol for 3x24 hours. The extract was filtered using filter paper to obtain a clear brownish liquid. The macerate is evaporated using a vacuum rotary evaporator at a temperature of 50-60°C for 4-6 hours, then heated over a water bath until a thick brownish extract is obtained. The ethanol-free test was carried out with potassium dichromate ($K_2CR_2O_7$). The ironwood stem bark extract was diluted to concentrations of 25%, 55%, and 95% by dissolving a few milliliters of ironwood stem bark extract with distilled water which was then converted into doses of 1,250 mg/kg, 2,750 mg/kg, and 4,750 mg/kg. Ironwood bark extract was administered orally 2x1 ml every 24 hours for 14 days using a gastric tube.

On the 15th day, all rats were sacrificed to have their kidneys removed with euthanasia dose via intraperitoneal injection using 91 mg/kg of ketamine and 0.6 ml of 9.1 mg/kg xylazine. Kidneys were washed with NaCl and fixated with Neutral Buffered Formalin 10% solution, and preparations were made with Hematoxylin Eosin (HE) staining.

Histopathological features, glomerular hypertrophy and hydropic degeneration were seen and observed in five visual fields using a light microscope with 400x magnification. Histopathological observation of glomerular hypertrophy was carried out using ImageJ software version 1.53 by calculating the average diameter of each glomerulus from five visual fields. The diameter of one glomerulus is the average of the diameters of the longest and shortest glomeruli.[8] Histopathological observation of hydropic degeneration was carried out by calculating the percentage of cell damage in one field of view by dividing the number of kidney cells which experienced hydropic degeneration by the total normal cells, then multiplying by 100%. The percentage of lesion area in the five visual fields was summed and divided by five, then the score was given based on the average percentage of

histopathological features of hydropic degeneration. Score histopathological picture of hydropic degeneration as follows, score 0 (normal) if no kidney damage is found, score 1 (mild) if kidney damage is <25%, score 2 (moderate) if kidney damage is 25-50%, score 3 (severe) if kidney damage is >50%.[5]

RESULTS

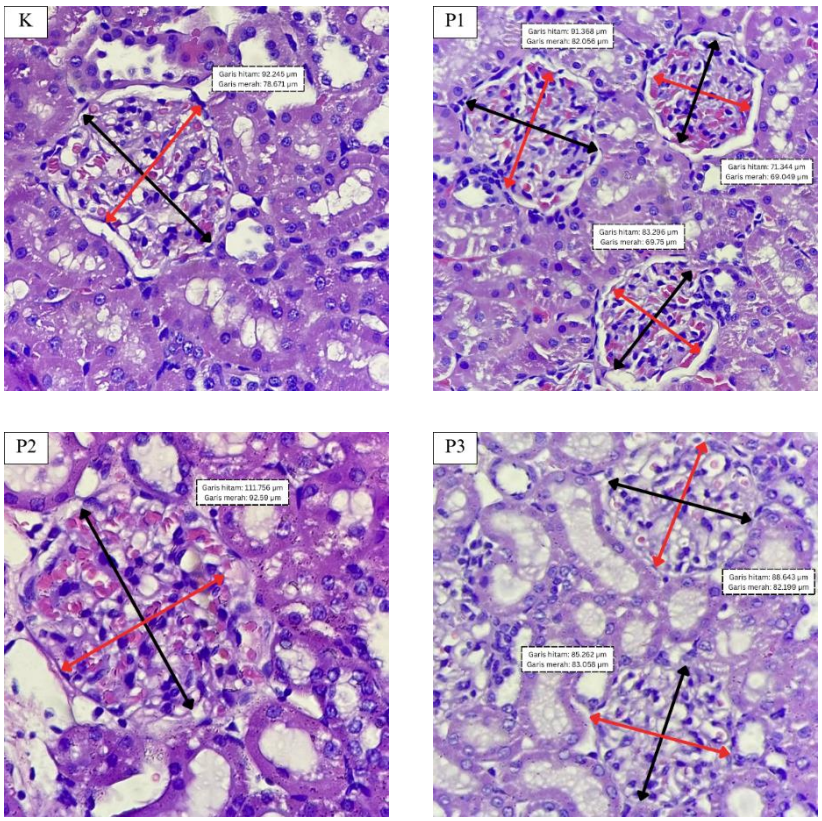

Figure 1. Histopathological Overview of Wistar Rat Kidney Glomerular Hypertrophy. (K) control; (P1) ironwood bark extract dose of 1,250 mg/kg BW; (P2) ironwood bark extract dose of 2,750 mg/kg BW; and (P3) ironwood bark extract at a dose of 4,750 mg/kg BW. *Olympus CX43* (400x, HE).

Note : ↔ Shortest diameter
↔ Longest diameter

Table 1. Kidney Glomerular Diameter Mean of Wistar Rats in Groups K, P1, P2, and P3.

Kelompok	Jumlah Data (n)	Rata-Rata ± SD Hipertrofi Glomerulus (µm)
K	4	84,45 ± 2,54
P1	4	85,93 ± 6,88
P2	4	90,27 ± 8,67
P3	4	91,23 ± 13,38

Based on table 1, it can be seen that the highest glomerular diameter mean was found in group P3, which was 91.23 ± 13.38 µm and the lowest was found in group K, which was 84.45 ± 2.54 µm. The normality test for the glomerular diameter mean showed that the control group had a value of p=0.827, group P1 had a value of p=0.310, group P2 had a value of p=0.802, and group P3 had a value of p=0.502, so that the data was declared normally distributed (p>0.05). The data homogeneity test showed a value of p=0.228 (p>0.05), meaning that the data has a homogeneous variation. Data analysis was continued using the One Way Anova test which showed a value of 0.651 (p>0.05) meaning that there was no significant difference between groups.

Groups K and P1 in Figure 1 shows a normal glomerular illustration characterized by Bowman's capsule covering the whole part with the pars visceralis epithelium and pars parietalis epithelium still clearly visible and in between there is a capsular space. In the P2 and P3 groups, there was an increase in glomerular size due to the vasodilation of blood vessels which caused the capsular space to narrow.

Damage in the form of hydropic generation was found in Groups K and P1 in Figure 2 which is indicated by the yellow arrows with a large

number of normal tubular cells which is indicated by the black arrows. The P2 and P3 groups showed extensive damage in the form of hydropic degeneration which was characterized by enlarged tubular epithelial cells, pale cytoplasm, and clear empty spaces (vacuoles) in the cytoplasm.

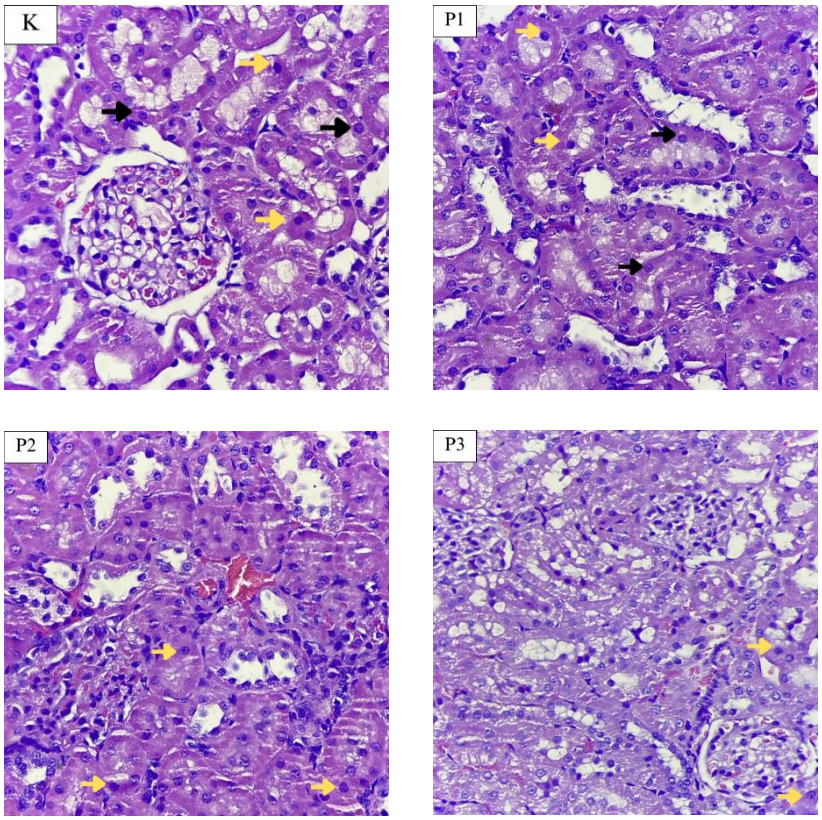

Figure 2. Histopathological Features of Wistar Rat Kidney Hydropic Degeneration. (K) control; (P1) ironwood bark extract dose of 1,250 mg/kg BW; (P2) ironwood bark extract dose of 2,750 mg/kg BW; and (P3) ironwood bark extract at a dose of 4,750 mg/kg BW. *Olympus CX43* (400x, HE).

Note : ➡ Normal tubules
➡ Hydropic generation tubules

Table 2. Percentage Mean Histopathological Features of Wistar Rat Kidney Hydropic Degeneration

Kelompok	Jumlah Data (n)	Rata-Rata ± SD Degenerasi Hidropik (%)	Skor
K	4	0,60 ± 0,43	1
P1	4	0,50 ± 0,20	1
P2	4	0,65 ± 0,34	1
P3	4	0,80 ± 0,16	1

Based on table 2, it can be seen that the histopathological picture of hydropic degeneration was experienced by all groups with the highest percentage mean value for the histopathological picture of hydropic degeneration in group P3, which was 0.80 ± 0.16, and the lowest percentage value was found in group P1, which was 0.50 ± 0.20. The percentage mean histopathological features of hydropic degeneration was scored 1 for all groups, namely groups K, P1, P2, and P3.

The data normality test for the percentage mean histopathological features of hydropic degeneration showed that in the control group the value was p = 0.577, the P1 group had a p value = 0.001, the P2 group had a p value = 0.850, and the P3 group had a p value = 0.683, so that the data was not normally distributed ($p<0.05$). Data analysis continued with the Kruskal-Wallis test. The results of the Kruskal-Wallis statistical tests showed a value of $p=0.448$ ($p>0.05$), which means that there was no significant difference between groups.

DISCUSSION

The results showed that glomerular diameter in all groups, both in the control and treatment groups, doses of 1,250 mg/kgBB (P1), doses of 2,750 mg/kgBB (P2) and doses of 4,750

mg/kgBB (P3) were still within the normal range of glomerular diameters. The group of rats that were given distilled water (K) had the smallest glomerular diameter mean, which was 84.45 ± 2.54 µm. This glomerular diameter is still relatively normal, supported by research by Rohman et al (2021) which stated that the glomerular diameter mean of normal adult white rats is 77-151 µm.[9] This result is in line with research by Fahriyansyah et al (2021) which showed that normal rats that were not given neem leaf ethanol extract had the smallest glomerular diameter among the other groups.[10] In group K, a normal glomerulus was obtained, marked by the presence of clearly visible capsular space on all sides of the glomerulus. This is supported by the research of Agi et al (2021) stating that a normal glomerulus is characterized by a Bowman's capsule which covers it as a whole with a normal glomerulus having a polyhedral shape and consisting of a parietal layer and a visceral layer that has a Bowman's capsule space in between.[11]

In this study, the group of rats given ironwood bark extract at a dose of 1,250 mg/kg BW (P1) obtained a diameter mean 85.93 ± 6.88 µm. Rohman et al (2021) stated that the normal glomerular diameter mean in adult white rats is 77-151 µm so the results obtained in the P1 group are still within the normal range.[9] The results of this study are also in line with the study of Septiva et al (2019) which found that the glomerular diameter mean in the treatment group given flavonoid compounds was still relatively normal and did not affect the overall increase in glomerular diameter. This is because flavonoid compounds act as nephroprotectors which can improve the histological structure of the kidney and prevent damage to the glomerulus of the rat kidney.[12]

In the rat group given ironwood bark extract at a dose of 2,750 mg/kgBW (P2) and a dose of 4,750 mg/kgBW (P3), each had a diameter mean 90.27 ± 8.67 µm and 91.23 ± 13.38 µm. In this study, it was found that the histology of the kidneys in groups P2 and P3 experienced an increase in glomerular size as seen from the Bowman's capsule space which was narrowed due to the merging of the parietal and visceral layers. This is in line with research by Septiva et al (2019) which states that changes in glomerular cells due to the administration of medicinal plant extracts can occur as the dose given increases.[12] Increasing the concentration of certain compounds will cause pressure and flow in the glomerular capillaries to increase which can affect the size of the glomerular diameter due to increased filtration load.[13] This structural change in glomerular diameter is a form of cell adaptation to regulate metabolic functions, which is a normal pattern of changes in tissues. Significant changes occur when cells are unable to adapt to environmental changes.[12]

Alterations in the structure of the kidney tissue, especially the glomerulus, are caused because the tissue is the first component of the nephron to be exposed to toxic substances.[14] Glomerular cells, matrix, and mesangial cells are susceptible to exposure to toxic substances through several mechanisms such as direct injury to cellular components and formation of free radicals originating from O_2 formation.[14] Under normal conditions, reactive oxygen species (ROS) play a role in physiological processes such as the body's defense system, cellular signaling, and hormone biosynthesis. Excessive ROS levels can cause cell damage. Hemodynamic changes are mediated by increased production of angiotensin II which stimulates and increases the production of podocyte-derived Vascular Endothelial Growth Factor (VEGF), suppresses nephrin expression, and induces Transforming Growth Factor Beta (TGF-β).[15] Increasing VEGF expression will activate intra and intercellular signals, especially proliferation signals in cells that express Vascular Endothelial Growth Factor Receptors (VEGFR-1) and VEGFR-2. These cells include podocytes, mesangial cells, and endothelial cells.[15,16] The proliferation of these cells causes the size of the glomerulus to increase. This increase will cause changes in the histological picture of the kidney in the form of glomerular hypertrophy.[16]

Based on the results of the study, in the control group (K) which was only given distilled water, the percentage mean histopathological features of hydropic degeneration was 0.60 ± 0.43 and a score of 1 was found, meaning that hydropic degeneration was found in an area of <25% of the entire visual field. The results of this study are in line with research conducted by Wahyuningsih et al (2016) and Rahman et al (2020) who also found hydropic degeneration in the control group that was given aquades with damage in the mild category and which was reversible.[17,18]

In the treatment group at a dose of 1,250 mg/kg BW or in group P1, the histopathological features of hydropic degeneration were shown with a percentage mean 0.50 ± 0.20. The results of this study based on the histopathological level of hydropic degeneration obtained a score of 1, which means that hydropic degeneration was found in an area of <25%. The histopathological picture of hydropic degeneration in this study is still in the mild category and does not have a toxic effect on the kidneys. The percentage mean histopathological features of hydropic degeneration in group P1 decreased compared to group K. This is in line with a study conducted by

Fahrimal et al (2016) which stated that the use of flavonoids at the right dose could improve the picture of rat kidney hydropic degeneration.[19]

The ironwood stem bark extract is known to contain 31.28 mg GAE/g of phenolic compounds, 30.48 mg CE/g of flavonoids, and 183.30 mg PE/g of proanthocyanidins.[2] Antioxidant activity of flavonoids, phenolic and tannin compounds can prevent the initiation of lipid peroxidation which can damage the structure and function of mitochondria because these three compounds are phenolic compounds that can donate one hydrogen atom from the hydroxyphenolic group when reacting with free radicals.[20,21] Rosyida et al (2022) stated that the decreased picture of hydropic degeneration is due to the presence of secondary metabolites which act as antioxidants that can prevent fluid retention and reduce capillary permeability. Capillary permeability that returns to normal causes fluid does not return to the capillaries so swelling of the tubules does not occur.[22] Chikrista et al (2020) mentioned, flavonoids and phenolics act as antioxidants by increasing the activity of the enzymes superoxide dismutase, glutathione peroxidase and catalase which are endogenous antioxidant enzymes so they can prevent injury due to oxidative stress.[21]

The percentage mean hydropic degeneration histopathology was found to be 0.65 ± 0.34 and 0.80 ± 0.16 respectively in the group of mice with ironwood stem bark extract dose of 2,750 mg/kg BW (P2) and a dose of 4,750 mg/d kgBB (P3). Based on the level of the histopathological picture of hydropic degeneration, a score of 1 was found, namely there was hydropic degeneration of <25%. According to Intan et al (2018) in her research it was stated that the percentage of hydropic degeneration covering an area of <25% is still categorized as normal.[6] In this study, the percentage mean histopathological features of hydropic degeneration in the P3 group increased compared to the other groups. This is in line with research by Ernawati et al (2018) which stated that there was an increase in kidney tubular cell damage with increasing doses of plant extracts.[23] According to Kristian (2022) excessive flavonoids can cause accumulation in kidneys so that they will interfere with the function of the kidney tubules.[20] The nature of phenolic compounds and flavonoids can be antioxidants or pro-oxidants depending on the administration concentration. According to Sotler et al (2019) antioxidants can act as pro-oxidants because an increase in antioxidants can affect the rate of oxidation which can trigger the formation of ROS.[24]

Formed ROS will react with the fatty acid of the cell membrane to cause a chain reaction called lipid peroxidation. Continuous lipid peroxidation will lead to damage to mitochondria and make the organelles produce adenosine triphosphate (ATP).[18] Cells need ATP to activate the sodium and potassium pumps so when cells in lack ATP they will push sodium ions into the cells and potassium ions out of the cells. This causes an increase in sodium levels in the cell and the influx of water into the cell so the cytoplasm will swell due to the accumulation of fluid between the cell matrix and the rough endoplasmic reticulum.[6]

The results of the statistical analysis stated that there was no significant difference between the treatment groups at the doses of 1,250 mg/kg, 2,750 mg/kg, and 4,750 mg/kg, with the control group so that the administration of ironwood bark extract in groups P1, P2, and P3 was not toxic based on features of glomerular hypertrophy and hydropic degeneration. This is supported by Makiyah et al (2021) which show that statistically there is no significant difference, it can be stated that it is not toxic to the kidneys.[25] In this study it can be concluded that the administration of ironwood stem bark extract (*Eusideroxylon zwageri*) at a dose of 1,250 mg/day kgBB, 2750 mg/kgBW, and 4750 mg/kgBW via oral did not cause acute toxicity to the kidneys of Wistar rats based on histopathological features of glomerular hypertrophy and hydropic degeneration.

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