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TOXICITY TEST OF *Eusideroxylon zwageri* BARK EXTRACT BASED ON LIVER HISTOPATHOLOGY HYDROPIC DEGENERATION AND NECROSIS

Dhiya Salma Azminida[1)], I Wayan Arya Krishnawan Firdaus[2)], Juliyatin Putri Utami[3)], Beta Widya Oktiani[4)], Erida Wydiamala[5)]
[1)]Preclinical Dentistry Program, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin
[2)]Department of Oral Biology, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin
[3)]Department of Biomedicine, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin
[4)]Department of Periodontology, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin
[5)]Department of Parasitology, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin

ABSTRACT
Background: *Ironwood bark extract contains antioxidant properties such as flavonoids, phenolics, and proanthocyanidins, which can stabilize ROS in the body and help accelerate wound healing. The liver plays a role in nutrient metabolism, detoxification, and ROS production. The lack of antioxidants to neutralize excess ROS makes the liver vulnerable to damage.* ***Purpose****: This study aimed to determine that there was no toxic effect of giving ironwood bark extract (Eusideroxylon zwageri) doses of 1.250 mg/kg, 2.750 mg/kg, and 4.750 mg/kg on the liver of Wistar rats based on histopathological features of hydropic degeneration and necrosis.* ***Methods:*** *Pure laboratory experimental quantitative (true experimental) with a post-test-only research design with control group design and One Way Anova statistical test. Treatment was given to 4 groups, namely group (K) was given aquadest control, group (T1) was given ironwood bark extract at a dose of 1.250 mg/kgBW, group (T2) was given ironwood bark extract at a dose of 2.750 mg/kgBW, and group (T3) given ironwood bark extract at a dose of 4.750 mg/kgBW.* ***Results:*** *The results showed no significant difference between the four treatment groups based on the average percentage of hydropic degeneration and necrosis.* ***Conclusion:*** *There was no toxic effect of giving ironwood bark extract doses of 1.250 mg/kgBW, 2.750 mg/kgBW, and 4.750 mg/kgBW on the liver of Wistar rats based on histopathological appearance of hydropic degeneration and necrosis for 14 days.*

Keywords: *Eusideroxlon zwageri, Hydropic degeneration, Liver, Necrosis, Toxicity.*
Correspondence: Dhiya Salma Azminida; Dentistry Study Program, Faculty of Dentistry, Lambung Mangkurat University, Veteran Street 128B, Banjarmasin, South Kalimantan, Indonesia; E-mail corresponding author: dhiyasalmaazminida@gmail.com

INTRODUCTION

Dental and oral health problems are common issues that Indonesians often suffer from.[1,2] As reported by Riskesdas in 2018, around 57.6% of the Indonesian population have oral and dental problems.[1] One way to treat this condition is by getting a tooth extraction.[1,3] Tooth extraction causes a wound in the dental socket, which has a healing process.[3] Inflammatory cells will release Reactive Oxygen Species (ROS) during the healing process.[4] If excessive amounts of ROS occur in the cellular detoxification system, this can cause oxidative stress and may affect wound healing.[3,4] Furthermore, antioxidants are required to neutralize the effects of such high levels of ROS.[4]

South Kalimantan is a diverse region rich in medicinal plants, based on the 2018 Riskesdas data that recorded the use of Yankestrad (Traditional Health Treatment) at 54.1% in the province.[1] One of the medicinal plants native to South Kalimantan is the ironwood tree (*Eusideroxylon zwageri*), which has been used as an alternative treatment for diarrhea, jaundice (liver), and has anti-inflammatory and antioxidant effects.[5,6] The part of the ironwood tree that contains secondary metabolic compounds useful as antioxidants are highest in the bark.[6] Phytochemical tests show that ironwood bark extract contains secondary metabolic compounds such as flavonoids (30.48 mgCE/g), phenolics (31.28 mgGAE/g), and proanthocyanidins (183.30 mgPE/g) that are antioxidants.[7–8]

Flavonoids can rapidly provide hydrogen atoms to lipid radicals and convert them into more stable forms.[8] Meanwhile, phenolics and proanthocyanidins as antioxidants can provide one electron to free radicals that have unpaired electrons, thereby stabilizing the aroxyl radicals formed through resonance.[8] In addition, phenolic antioxidants can reduce cholesterol levels and

increase the formation of nitric oxide (NO), which can inhibit atherosclerosis (anti-atherosclerosis) and cause blood vessel dilatation (vasodilator).[8] However, if the concentration of these secondary metabolites is at an excessive level, it can cause an increase in the toxic ROS and disrupt mitochondrial function in metabolic organs.[9]

The functions of the liver are the metabolism of nutrients and the detoxification of substances.[10] The liver also produces ROS, making it an organ susceptible to damage when the amount of antioxidants is insufficient to neutralize excessive ROS.[11] High levels of ROS can cause oxidative stress in the liver and trigger the development of various liver diseases such as hepatitis, alcoholic liver disease, and non-alcoholic steatohepatitis.[11] Oxidative stress will disrupt homeostasis and cause lipid peroxidation, protein and DNA damage in the liver.[11] This can cause cells to swell or rupture due to the activation of cytochrome p-450, which forms covalent bonds between chemicals and intracellular proteins.[10] Chemicals can also bind to proteins and trigger a cytotoxic T-cell response, leading to antibody formation and intracellular dysfunction.[10] Symptoms of liver damage can be seen through histological changes.[10] Histopathological examination of the liver can reveal hydropic degeneration as reversible and necrosis as irreversible.[10] Hydropic degeneration occurs due to the inability of hepatocyte cells to maintain ion and electrolyte balance.[10] If the damage is severe and persistent, hepatocyte cells may undergo necrosis.[10]

According to a prior study, ironwood bark extract has the ability to eliminate *Aggregatibacter actinomycetemcomitans* at a concentration of 20%.[12] Further in vitro toxicity testing revealed that ironwood bark extract is non-toxic to Baby Hamster Kidney-21 (BHK-21) fibroblast cells at concentrations ranging from 5% to 95%.[13] However, the potential impact of ironwood bark extract on the liver histopathology of Wistar rats has not yet been explored. To establish ironwood bark extract as a phytopharmaceutical, an in vivo toxicity test is necessary to gauge its safety. This study uses previous research to determine the oral doses of 1.250 mg/kgBB, 2.750 mg/kgBB, and 4.750 mg/kgBB for extract concentrations of 25%, 55%, and 95%, respectively, and observes the rats over a period of 14 days.[12] The purpose of this study is to establish that ironwood bark extract does not pose any harm to the liver, as indicated by the absence of hydropic degeneration and necrosis in the histopathological analysis.

METHODS

The research conducted in this study was ethically approved by the Health Ethics Commission of the Faculty of Dentistry at Lambung Mangkurat University with the number 022/KEPKG-FKGULM/EC/II/2023. The research design utilized a pure laboratory experimental quantitative research (true experimental) approach with a post-test only and control group design. The purpose of the study was to analyze the toxic effect of orally administered doses of ironwood bark extract (*Eusideroxylon zwageri*) at 1.250 mg/kgBB, 2.750 mg/kgBB, and 4.750 mg/kgBB on the liver of Wistar rats based on the histopathological picture of hydropic degeneration and necrosis.

For this study, a simple random sampling technique was used to select the sample. The sample size was calculated using the unpaired numerical comparative analytic equation, resulting in a total of 16 rats divided into 4 test groups, each consisting of 4 rats. The control group (C) received aquadest, while treatment group 1 (T1) was given ironwood bark extract at a dose of 1.250 mg/kgBB, treatment group 2 (T2) received ironwood bark extract at a dose of 2.750 mg/kgBB, and treatment group 3 (T3) received ironwood bark extract at a dose of 4.750 mg/kgBB.

In Paau Village, Aranio Subdistrict, Banjar District, South Borneo, ironwood bark measuring over 50 cm in height and 60 cm in diameter was carefully harvested without damaging the cambium. A total of 14 kg of the red-brown inner bark was collected using a knife. The harvested bark was then cleaned and tapped to remove any foreign objects and the outer bark. Then, Ironwood bark is pulverized with a wood-planer machine.

The simplicial powder was obtained by drying ironwood bark in an oven at 40°C for 4 hours, pulverizing it with a blender, and filtering it with a mesh screen, resulting in 2,100 g of powder. To extract the ironwood bark, a maceration method was used, in which the powder was soaked in 96% ethanol for 3 x 24 hours while stirring it using a shaker. The resulting extract was filtered using WH 40 filter paper. The macerate was evaporated with a rotary vacuum evaporator at 60°C for 4-6 hours and heated with a waterbath until a thick brown extract of 102 g was formed after complete evaporation. To test for ethanol content, a few drops of potassium dichromate ($K_2Cr_2O_7$) were added, and a color change from orange to green indicated the absence of ethanol in the ironwood bark extract. Dilution using aquadest was carried out to prepare ironwood bark extract with concentrations of 25%, 55%, and 95%, which were converted into doses of 1.250 mg/kgBB, 2.750 mg/kgBB, and 4.750 mg/kgBB, respectively.

Wistar rats were acclimatized for seven days in the laboratory in a 35 cm × 29 cm × 13 cage with rice husk bedding that was changed twice a week. The laboratory temperature was maintained between 25-28°C and not humid. The rats were given standard BR2 feed at 20% of body weights (40 g/200 gBB) and sterile aquadest in a drink bottle *ed Libitum*. Rats were gently used to interact with handlers to minimize handling stressors and facilitate response to the stimulus.

The rats were fastened for ± 6 hours so that the ironwood bark extract was absorbed without being

affected by food in the rat gastric. The maximum volume of the rat gastric is 5 ml. Oral intake of the section was done with a gastric probe connected to a 3 ml syringe slowly to avoid reflux of vomitus. Aquadest and ironwood bark extracts were administered 2 x 1 ml daily for 14 days.

All rats were fasting meal for ± 8-12 hours before being euthanasia with a combination of ketamine-xylazine as much as three times the anesthesia dose (0.6 ml/200 gBB). The liver was taken using 1 set of minor surgical instruments. After organ excision, the animals were cleaned, wrapped in fabric, and buried with a minimum depth of 75 cm to be protected from wild animals. Liver organs were fixation using 10% BNF solution in a labeled specimen container. Then the liver organs were made into slides and stained with *Hematoxylin-eosin*.

Observation of the preparations using an Olympus CX43 microscope in 5 fields of view with 100x and 400x magnification in each field of view by the researcher, three other researchers, and confirmed by an anatomical pathology specialist. The mean percentage of damage to hepatocyte cells was calculated by dividing the number of cells that underwent hydropic degeneration or necrosis by the total number of cells in one field of view and multiplied by 100%. The mean percentage of damage from 5 fields of view was calculated. The calculation determination was determined by each group's mean rate of hydropic degeneration or necrosis.

Table 1. Assessment of Histopathologic Features of Liver Hydropic Degeneration.[11]

Scores	Categories	Descriptions
0	Normal	Hydropic degeneration around the centrilobular or central vein (<25%)
1	Mild	Hydropic degeneration that extends to the midzone or central zone (25-50%)
2	Moderate	Hydropic degeneration that extends to the periportal or perilobular zone (50-75%)
3	Severe	Hydropic degeneration that extends to the periportal or perilobular zone (>75%)

Table 2. Assessment of Histopathologic Features of Liver Necrosis by Suzuki Scoring.[14]

Scores	Categories	Descriptions
0	Normal	No necrosis found
1	Minimal	A single cell with necrosis is found.
2	Mild	Necrosis <30%
3	Moderate	Necrosis 30-60%
4	Severe	Necrosis >60%

RESULTS

Excessive fluid buildup in cells, known as hydropic degeneration, causes cytoplasmic swelling resulting in a puffy appearance with clear vacuolization and a centered nucleus. All test groups displayed normal hepatocyte histopathology with evenly distributed granules and clear sinusoid boundaries. The hydropic degeneration observed in all test groups was mostly concentrated in the centrolubular area (central vein). The data regarding the mean percentage of hydropic degeneration in both control and treatment groups can be found in Table 3.

Hydropic degeneration was observed in all test groups. The results of Table 3 show that the average percentage of hydropic degeneration for group C was 0.55 ± 0.19, group T1 was 1.05 ± 0.19, group T2 was 1.15 ± 0.52, and group T3 was 1.75 ± 1.01. Across all study groups, the average percentage of hydropic degeneration was less than 25% in the centrolobular (central vein) region, indicating a damage score of 0 (normal). The data on the mean percentage of hydropic degeneration with the One Way ANOVA test yielded a value of p=0.084 (p>0.05), suggesting that there were no significant differences between the test groups.

Table 3. Mean Percentage Data of Histopathological Features of Hydropic Degeneration

Treatment Group	Repetition	Mean (%) ± SD	Scores
		Hydropic Degeneration	
C	4	0,55±0,19	0
T1	4	1,05±0,19	0
T2	4	1,15±0,52	0
T3	4	1,75±1,01	0

Descriptions:
C: The control group was given the aquadest;
T1: The group has been given ironwood bark extract at a dose of 1.250 mg/kg BW;
T2: The group has been given ironwood bark extract at a dose of 2.750 mg/kg BW;
T3: The group has been given ironwood bark extract at a dose of 4.750 mg/kg BW.

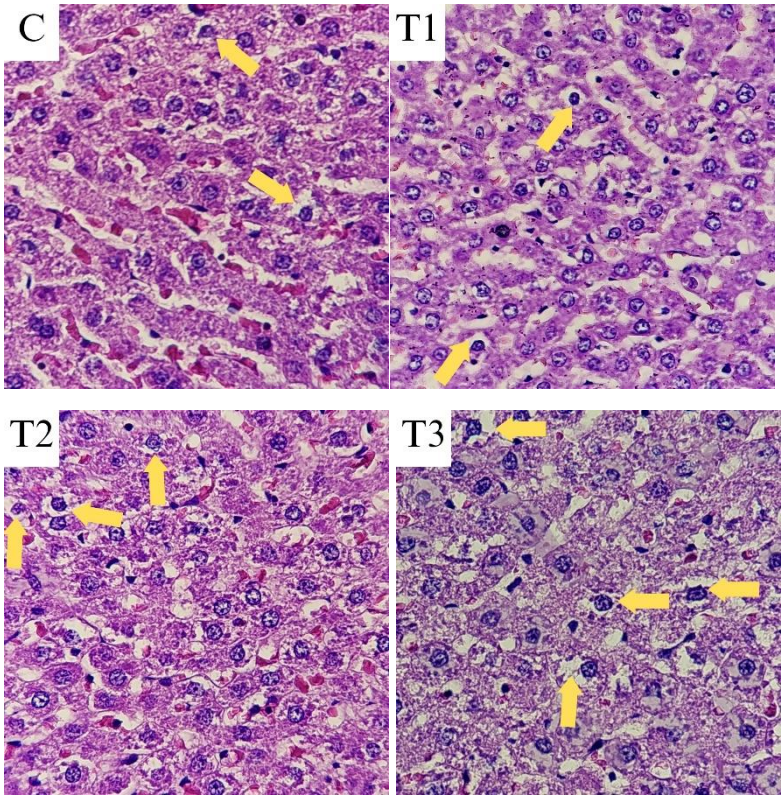

Figure 1. Histopathology of Hydropic Degeneration of Wistar Rat Liver Tissue in Group C, Group T1, Group T2, and Group T3 using *Olympus CX43* Microscope (400x, HE).
Descriptions:
(➡): Overview of hepatocytes with hydropic degeneration;
C: The control group was given the aquadest;
T1: The group has been given ironwood bark extract at a dose of 1.250 mg/kg BW;
T2: The group has been given ironwood bark extract at a dose of 2.750 mg/kg BW;
T3: The group has been given ironwood bark extract at a dose of 4.750 mg/kg BW.

The liver tissue histopathology findings in Figure 2 indicated that all test groups had normal arrangement of hepatocyte cells with clear sinusoid boundaries. Necrosis was characterized by lysed cell membranes, smaller cytoplasmic diameter compared to surrounding normal cells, red cytoplasmic color, and changes in the nucleus. Pyknotic patterns, which are bony nuclei, an increase in basophilia, fragmented cell nuclei known as karyorrhexis, and cells without clear boundaries and lost cell nuclei due to faded chromatin basophilia known as karyolysis were observed in all test groups.

In Table 4, the mean percentage of histopathological appearance of liver tissue necrosis in Wistar rats is presented. The group with the lowest average necrosis rate was C at 0.55 ± 0.50, while the highest was T3 at 0.90 ± 0.26. All research groups exhibited histopathological necrosis with a mean percentage of <30%, which falls under the category of mild damage (score = 2). The One Way Anova statistical test for the mean percentage of necrosis data revealed a significance value of 0.780 ($p>0.05$), indicating no significant difference between the groups.

Table 4. Mean Percentage Data of Histopathological Features of Necrosis

Treatment Group	Repetition	Mean (%) ± SD	Scores
		Necrosis	
K	4	0,55±0,50	2
T1	4	0,60±0,59	2
T2	4	0,75±0,66	2
T3	4	0,90±0,26	2

Descriptions:
C: The control group was given the aquadest;
T1: The group has been given ironwood bark extract at a dose of 1.250 mg/kg BW;
T2: The group has been given ironwood bark extract at a dose of 2.750 mg/kg BW;
T3: The group has been given ironwood bark extract at a dose of 4.750 mg/kg BW.

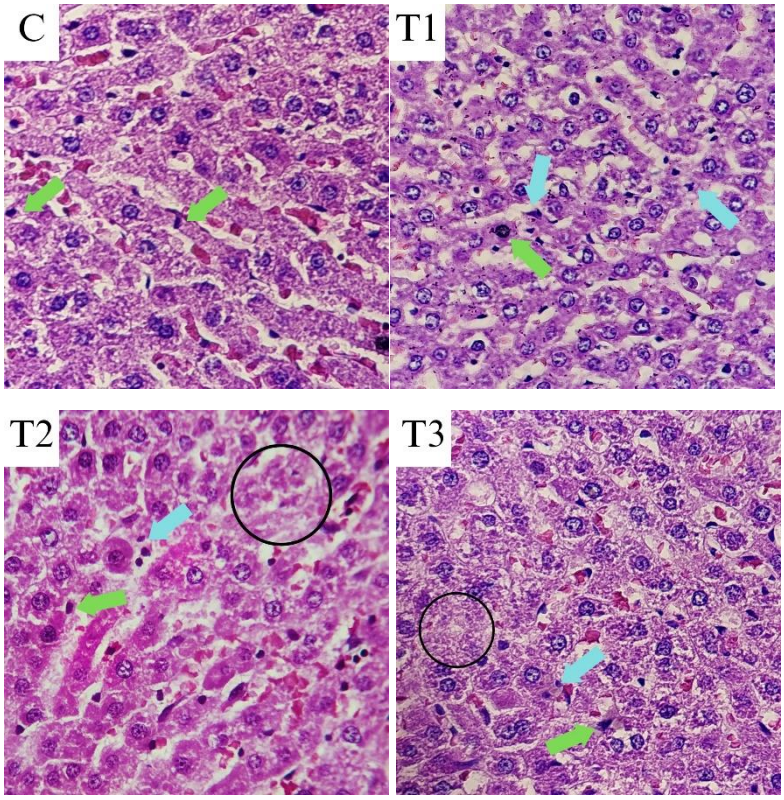

Figure 2. Histopathology of Necrosis of Wistar Rat Liver Tissue in Group C, Group T1, Group T2, and Group T3 using *Olympus CX43* Microscope (400x, HE).
Descriptions:
(➡): Overview of hepatocytes undergoing necrosis with the nucleus being pyknotic;
(➡): Hepatocyte necrosis with karyorrhexis of the nucleus;
(O): Overview of necrotizing hepatocytes with the nucleus undergoing karyolysis;
C: The control group was given the aquadest;
T1: The group has been given ironwood bark extract at a dose of 1.250 mg/kg BW;
T2: The group has been given ironwood bark extract at a dose of 2.750 mg/kg BW;
T3: The group has been given ironwood bark extract at a dose of 4.750 mg/kg BW.

DISCUSSION

During the study, hydropic degeneration was observed around the centrilobular (central vein) in all five visual fields of each group. However, none of the groups had an average percentage of more than 25%, which meant that they fell within the normal category and scored 0.[11] The group given aquadest, Group C, had the lowest mean percentage value at 0.55 ± 0.19. Similar research conducted by Fitmawati et al. (2018) also found that the control group given aquadest had the least percentage of damage at 14.89 ± 2.77, and was included in the normal category with a score of 0.[11]

The appearance of normal hepatocytes generally does not show degeneration and necrosis.[11] However, hepatocyte cells in normal conditions experience periodic turnover and can undergo degeneration processes by various causes, such as cell aging, but degenerative cells found in normal conditions are usually <3%.[15] Hydropic degeneration is a reversible change in hepatocyte cells.[10] If the injury can be compensated by the hepatocytes and the stimulus that caused the injury subsides, the cells can return to normal.[10] Research by Parapasan, et al (2023) also found hydropic degeneration in the control group given aquadest with a mean percentage of liver cell damage of 1.08 ± 0.11.[16]

The hydropic degeneration found is thought to be related to the role of the liver as an organ of metabolism and detoxification of drugs and toxins in the body.[10] The highest content of ironwood bark extract is proanthocyanidin (183.30 mgPE/g).[9] Proanthocyanidin can stabilize aroxyl radicals, increase the amount of reduced glutathione (GSH), and increase the activity of endogenous enzymes.[17] However, in high concentrations, flavonoids, phenolics, and proanthocyanidin can form hydroxyl radicals that are prooxidants so that they can increase ROS.[13] An excessive increase in ROS will disrupt the sodium-potassium pump in the hepatocyte cell membrane, increase plasma membrane permeability, and cause hypernatremia in the cell so that the cell can swell into hydropic degeneration.[10] A study conducted by Amer, et al (2022) also stated that there was hydropic

degeneration in the histopathology of rat liver given extracts with proanthocyanidins.[18]

The hydropic degeneration found in all treatment groups given ironwood bark extract with different dose levels was stated to have a percentage of <25%, so it had a hepatocyte damage score of 0 or was still in normal condition based on the description of hydropic degeneration.[11] However, every increase in the dose of ironwood bark extract given, there was an increase in the average percentage of hydropic degeneration as well. Research conducted by Muhartono et al. (2019) also stated that the average hydropic degeneration shown in liver histopathology increased along with the increasing dose of treatment given.[20]

In a study conducted by Makiyah et al. (2021) on hepatocyte cells, it was found that the results of statistical tests on the average percentage of hydropic degeneration had significant differences, so the extract used was considered toxic to the liver.[20] Meanwhile, the results of statistical tests with One Way Anova in this study showed that there were no significant differences in the average percentage of hydropic degeneration in the treatment groups with ironwood bark extract doses of 1.250 mg/kg BW, 2.750 mg/kg BW, and 4.750 mg/kg BW against the control group with aquadest for 14 days. The histopathological analysis indicates that there is no toxic effect on the liver of Wistar rats, as the hydropic degeneration observed is within normal limits.[11,15]

In the study of Wistar rat liver histopathology, necrosis was observed in all groups. he cytoplasm and cell nucleus changes indicated the presence of necrosis in liver cells.[10] The percentage of necrosis found varied among the groups, with Group C having the smallest mean percentage value of 0,55 ± 0,50 and Group T3 having the largest mean percentage of 0,90 ± 0,26. The histopathological picture of necrosis observed showed lobular hepatocyte cells towards the central vein with clear sinusoid boundaries, which is still considered normal.[11]

The necrosis in the control group with aquadest was classified as mild (score 2) since the average percentage was less than 30%.[14] This is consistent with Prasetyo et al.'s research, which found mild (focal) necrosis in the control group using aquadest.[21] Necrosis occurs as a natural process of replacing old and damaged cells, which can lead to liver organ dysfunction.[22] In this study, the necrosis that occurred was passive cell death due to extracellular events, which are not programmed.[22]

The necrosis found in the treatment group increased with increasing doses, but it remained in the mild category (score 2) since the average percentage was less than 30%[14] However, the study found that the average percentage of necrosis increased as the dose increased. Research conducted by Makiyah et al. (2021) also stated that an increase in the average percentage of necrosis in each treatment group occurred as the dose increased.[20] Necrosis that occurred in this study was found in the form of pyknotic, karyorexis, and karyolysis. Studies conducted by Budianto (2022) also stated that the three patterns of necrosis in the mild category were found in all test groups and did not have a toxic effect on the liver.[23]

According to research, the presence of necrosis in the liver may be linked to ironwood bark extract, specifically proanthocyanidin (183.30 mgPE/g).[9] Research conducted by Hemant et al. (2019) stated that mild necrosis was found in the liver histopathology group of Wistar rats given grape seed proanthocyanidin 200 mg/kgBB for 14 days.[24] Proanthocyanidin can act as a prooxidant and increase ROS production.[9] The higher amount of ROS than antioxidants in the liver can produce reactive lipid peroxidation products, such as malondialdehyde (MDA) that damage cell membranes, mitochondrial swelling, and plasma membrane rupture, which trigger necrosis.[19,25]

The mean percentage of necrosis is stated to have no significant difference, so the administration of stem bark extract was found to have mild necrosis but not toxic. This is supported by the research of Makiyah et al. (2021); if the average percentage of necrosis provides a significant difference, then the extract used in the study has a toxic effect on the liver organs of Wistar rats.[20] Based on the research that has been done, it can be concluded that there is no toxic effect of giving ironwood bark extract (Eusideroxylon zwageri) doses of 1.250 mg/kg BW, 2.750 mg/kg BW, and 4.750 mg/kg BW orally to the liver of Wistar rats based on the histopathological appearance of hydropic degeneration and necrosis for 14 days. It is recommended that research be continued on other vital organs and toxicity testing to subchronic or chronic levels to obtain further information on the safety of ironwood bark extract.

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