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TOXICITY TEST OF IRONWOOD BARK EXTRACT (*Eusideroxylon zwageri*) TOWARD WISTAR RATS KIDNEY (*Rattus norvegicus*)

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ABSTRACT
Background: *The use of herbal medicinal plants by the community is increasing because the side effects are smaller than chemical-based drugs. One of the medicinal plants is ironwood because of its flavonoid and phenolic compounds which have the potential as antioxidants. Ironwood needs to be known for its toxicity, by performing an oral subchronic toxicity test on the kidneys of wistar rats with urea and creatinine parameters.* ***Objective:*** *Proving that the administration of ironwood bark extract at doses of 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL orally for 28 days had no toxic effect on the kidneys of wistar rats with parameters of urea and creatinine.* ***Methods:*** *Pure experimental research with posttest-only with control group design, consisting of 4 groups, each of which contained 4 wistar rats with 1 negative control group and 3 treatment groups given ironwood bark extract at a dose of 524.5 mg/mL, 1151, 5 mg/mL, and 1775.5 mg/mL. It was carried out for 28 days and on the 29th day the rats were taken blood and examined for urea and creatinine levels.* ***Results:*** *In the 3 treatment groups, urea values were 41, 42, and 32.35 mg/dL and creatinine was 0.725, 0.725, and 0.65 mg/dL and the results also showed that there was no significant difference in urea levels (p=0.076) and creatinine (p=0.076). 0.065) in each group.* ***Conclusion:*** *Ironwood bark extract dose 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL was not toxic to the kidneys of wistar rats with parameters of urea and creatinine.*

Keywords: *Creatinine, Ironwood bark extract, Toxicity, Urea.*
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INTRODUCTION

The use of herbal medicinal plants by the Indonesian people has increased because the price is cheap, easier to obtain, and has fewer side effects when compared to drugs with chemical bases. One of the plants used in traditional herbal medicine is ironwood (*Eusideroxylon zwageri*). The Uud Danum Dayak people who live in the area around the Ambalau River, West Kalimantan, use ironwood plants as traditional herbal medicines. Based on the Regulation of the Minister of Environment and Forestry number 106/2018 issued 10 types of forest plants from the attachment to the list of protected plants, one of which is ironwood.[1,2]

Based on the results of phytochemical tests, ironwood bark extract has a higher extract content when compared to other parts, ironwood bark contains compounds at the highest level, namely flavonoids at 30.48 mg CE/g and phenolics at 31.28 mg GAE/g. The content of flavonoids and phenolics is a compound that has the potential as a source of antioxidants found in herbal plants.[3,4] Antioxidants are compounds that can inhibit the oxidation process generated by free radicals and reduce Reactive Oxygen Species (ROS), antioxidants are also useful for accelerating the healing process of oral mucosal wounds.[5,6]

Herbal plants do come from nature, but before using them, the potential for toxicity must be known in order to avoid effects that can be detrimental to body health. Factors that need to be considered in the use of natural materials include the accuracy of dosage, timeliness, and method of use, that it also needs to be tested for safety through toxicity tests.[7]

In vivo toxicity tests include oral acute toxicity test, oral subchronic toxicity test, and oral chronic toxicity test. An oral subchronic toxicity test was carried out by giving the test preparation orally repeatedly to see the toxic effect on test animals from long-term exposure for 28 days.[8] The organ

that often receives negative effects from drug use is the kidney.[7] In general, the kidneys function to excrete waste products such as urea, uric acid, creatinine, and other toxic substances. Evaluation of kidney function can be done using several methods of laboratory examination, one of which is by measuring the waste substances from the body's metabolism that are excreted through the kidneys such as urea and creatinine.[9]

The end products of protein metabolism such as urea and creatinine go through the same process because both of them become toxic when the levels are too high in the body. Indications of kidney function failure can be seen from urea and creatinine levels that are higher than normal levels in the blood, therefore urea and creatinine examinations can be used as parameters to determine the occurrence of impaired kidney function.[9,10] Urea measurement serves to assess kidney function, hydration status, measure nitrogen balance, assess the progress of kidney disease, and assess hemodialysis results. The normal value of rat urea (*Rattus novergicus*) is 10-50 mg/dL. The serum creatinine test has become one of the most widely measured products and is currently used in more than 80% of clinical laboratories worldwide.[11,12] Creatinine is usually produced in equal amounts and excreted in the urine every day, with a normal rat creatinine value of 0.2-0.8 mg/dL, and creatinine is also recommended by The National Kidney Disease Education Program to assess glomerular filtration ability.[9,13,14]

Previous studies have tested the toxicity of ironwood bark extract at concentrations of 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85%, and 95% in vitro against Baby Hamster Kidney-21 (BHK-21) fibroblasts has been conducted. and there is no toxic effect, but there are no studies on in vivo toxicity tests.[15] Based on this, this study aims to prove that the administration of ironwood bark extract (*Eusideroxylon zwageri*) at low concentrations of 25%, medium 55%, and high 95% with doses of 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL orally for 28 days had no toxic effect on the kidneys of Wistar rats (*Rattus norvegicus*) with parameters urea and creatinine levels.

MATERIAL AND METHODS

This research was started by obtaining ethical approval from the Ethics Committee of the Faculty of Dentistry, University of Lambung Mangkurat Number 048/KEPKG-FKGULM/EC/IV/2022. This study used a true experimental design with a posttest-only control group design method. This study used 16 male wistar rats based on the Higgins and Kleinbaum sampling technique formula which were divided into 4 groups with 4 rats in each group. In the first group, as the control group, only aquadest was given and the other 3 groups were given ironwood bark extract with concentrations of 25%, 55%, and 95% at doses of 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL. The population of this research is Wistar Rat (*Rattus norvegicus*).

The materials used in this study were ironwood bark, 96% ethanol, aquadest, WH40 filter paper, potassium dichromate ($K_2Cr_2O_7$), experimental animals in the form of wistar rats (*Rattus novergicus*), Ketamine Xylazine for anesthesia and euthanasia of experimental animals, animal feed test feed BR2, husks for cage bedding, parchment paper, serum creatinine level measurement reagent (*Reiged Diagnostics*) and urea level measurement reagent (Reiged Diagnostics). The tools used in this research are analytical balance (*Precisa*), erlenmeyer flask (*Iwaki*), shaker, hammer mil, mesh screen, autoclave, beaker glass, oven, rotary vacuum evaporator (*Heidolp*), water bath (*SMIC*), dropper pipette for ethanol-free test, UV-Vis spectrophotometry, scalpel, blade, syringe, sterile gloves, vacutainer tube to collect rat blood, and eppendorf. Other tools for test animals such as Wistar rat cage, rat drink bottle, animal scales, gastric probe and 3 ml syringe.

The Making Ironwood Stem Bark Extract

Ironwood bark was taken in Paau Village, Aranio District, Banjar Regency, South Kalimantan. Ironwood bark extract was prepared using the maceration method. Ironwood bark at a height above 50 cm and a diameter of more than 60 cm was taken as much as 1.5 kg using a knife without injuring the cambium. The part of the skin that is taken is the inner skin which is brownish red. The bark of ironwood is cleaned of the outer skin and foreign matter (dirt or moss), then dried using an oven at 40^0C for 4 hours and cut into small pieces with a size of ± 2cm. The pieces of ironwood bark were made into powder using a hammer mill and the results were filtered using a mesh screen. The maceration process was carried out by adding ironwood bark powder into an extractor and adding 96% ethanol as solvent. The extraction process was carried out for 24 hours while stirring with the help of a shaker. The extract obtained was filtered with WH40 filter paper to obtain a clear brownish liquid. Evaporating the solvent using a vacuum rotary evaporator at a temperature of 50-60^0C for 4-6 hours, then heated over a water bath until all of the solvents has evaporated so that a thick brownish extract is obtained. The ethanol-free test was carried out by adding a few drops of potassium dichromate ($K_2Cr_2O_7$) to the ethanol extract sample of ironwood bark. If there is no color change in the sample, then there is no ethanol content in the sample. The ironwood bark extract was diluted into

several concentrations by dissolving several milliliters of the ironwood bark extract with aquadest so that the concentrations of 25%, 55%, and 95% were obtained then the doses obtained were 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL.

Ironwood Bark Extract Toxicity Test

The test animals used were grouped by simple random sampling method, then divided into 4 groups consisting of 4 Wistar rats each and adapted for 7 days in cages away from the noise. Test animals were fed BR2 and aquadest. One group of rats was the negative control group and the other three were the treatment group. Rats were taken at random and given a dose according to the maximum volume of administration for rats weighing 200 g, which is 3 mL. The calculation is based on the regulations set by the Organization for Economic Co-operation and Development (OECD) 423.[16] It was given orally for 28 days and then sacrificed on the 29th day to collect blood serum through the heart. In group 1 (negative control), 4 rats were given aquadest orally 2 times a day every 12 hours for 28 days and the method of administration was using a gastric probe connected to a 3 mL syringe. In the 3 treatment groups, the concentration of ironwood bark extract was given at 25%, 55%, and 95% orally at doses of 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL 2 times a day every 12 hours for 28 days and how to administer it using a gastric probe connected to a 3 mL syringe.

The rats whose blood serum will be taken are anesthetized using ketamine-xylazine at a dose of 0.1 mL/100 g BW then the rats will lose consciousness. Rats were sacrificed using ketamine-xylazine at a dose of 0.4 mL/100 g BW (4 times the anesthetic dose).

The rat's blood was taken through the heart using a syringe as much as ± 3 cc, then put into a vacutainer. Centrifuged for 10 minutes at 4000 rpm. The blood that has been taken is used for examination of urea and creatinine using UV-Vis Single Beam spectrophotometry with the urea *Glutamate dehydrogenase* (GLDH) examination method and the creatinine examination method using the *Jaffe* method.

RESULT

The results of examination of the levels of urea and creatinine of wistar rats after being given ironwood bark extract can be seen in table 1.

Table 1. The Results Of The Examination Of The Levels Of Urea And Creatinine In Wistar Rats After Being Given Ironwood Bark Extract

Groups	Mean ± Std. Deviation (mg/dL)	
	Urea	Creatinine
C-	56 ± 19.44	0.825 ± 0.05
T1	41 ± 5.22	0.725 ± 0.09
T2	42 ± 3.65	0.725 ± 0.09
T3	32.35 ± 10.21	0.65 ± 0.05

Information:
C-: Aquadest negative control group
T1: Treatment group 1 ironwood bark extract with a concentration of 25% with a dose of 524.5 mg/mL
T2: Treatment group 2 ironwood bark extract with a concentration of 55% with a dose of 1151.5 mg/mL
T3: Treatment group 3 ironwood bark extract with a concentration of 95% with a dose of 1775.5 mg/mL

Based on the results of this study, it was found that the dose of ironwood bark extract at 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL were not toxic to the kidneys of wistar rats. Based on table 1, it can be seen that the average value of the highest urea level is in the negative control group (C-) with a value of 56 mg/dL, while the lowest average value of urea level is in the treatment group 3 (T3) with a value of 32.35 mg/dL. Then the highest average creatinine level was in the negative control group (C-) with a value of 0.825. While the average value of creatinine levels was the lowest in treatment group 3 (T3) with a value of 0.65 mg/dL. The following is a diagram of the average urea and creatinine levels in Wistar rats on day 29 in the four treatment groups.

The normality test of the urea test results using the Shapiro Wilk test showed that in the negative control group the value of $p=0.235$ ($p>0.05$), in the treatment group 1 the value of $p=0.256$ ($p>0.05$), in the treatment group 2 the value of $p=0.714$ ($p>0.05$), and the treatment group 3 the value of $p=0.990$ ($p>0.05$) based on these results, it can be concluded that the data is normally distributed ($p>0.05$). The homogeneity test of the data using Levene's test showed the value of $p=0.094$ ($p>0.05$), meaning that the data had homogeneous variations ($p>0.05$). Because the results of the urea examination are normally distributed and homogeneous, the next test is the One-Way ANOVA parametric test which shows the $p=0.076$ ($p>0.05$) meaning that H_0 is accepted. The conclusion there was no significant difference in the urea levels of wistar rats in the negative control

group (C-), treatment group 1 (T1), treatment group 2 (T2), and treatment group 3 (T3). Because the conclusion is that there is no significant difference, the test is not continued with Bonferroni's Post Hoc analysis.

The normality test of the creatinine test data using the Shapiro Wilk test showed that in the negative control group the value of p=0.001 (p<0.05), in the treatment group 1 the value of p=0.272 (p>0.05), in the treatment group 2 the value of p=0.272 (p>0.05), and in treatment group 3 the value of p=0.024 (p<0.05) based on these results, it can be concluded that the data are not normally distributed (p<0.05). Because the data are not normally distributed, the test cannot be continued using One-Way ANOVA, and will continue with the alternative test, namely the Kruskal Wallis test. The results of the Kruskal Wallis test showed the value of p=0.065 (p>0.05) meaning that H_0 was accepted. The conclusion was that there was no significant difference in the creatinine levels of wistar rats in the negative control group (C-), treatment group 1 (T1), treatment group 2 (T2), and treatment group 3 (T3). Because the conclusion is that there is no significant difference, the test is not continued with the Mann Whitney test.

DISCUSSION

Based on the results of this study, it was found that the dose of ironwood bark extract at 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL was not toxic to the kidneys of wistar rats. Because the urea levels are in the normal range (10-50 mg/dL) and the creatinine levels are in the normal range (0.2-0.8 mg/dL), this means that these results are in accordance with the hypothesis in this study.[13,14]

Several factors affect the level of urea in the blood serum of rats, one of which is pathological factors such as acute and chronic kidney damage.[17] In addition, high levels of urea can also be caused by drugs and foods or drinks that have a high protein content. Low urea is not considered abnormal, because it may be caused by low protein in the food consumed.[13,18] Foods high in protein can increase the release of amino acids into the blood and reabsorb in the proximal tubule. Because amino acids and sodium are reabsorbed together from the proximal tubule, increased amino acid reabsorption stimulates sodium reabsorption in the proximal tubule. This reduced sodium delivery to the macula densa then causes a decrease in afferent arteriolar resistance mediated by tubuloglomerular feedback and an increase in renal blood flow and glomerular filtration rate (GFR). This increase in GFR increases the excretion of waste products of protein metabolism such as urea. The increase in urea levels is not only due to a decrease in the glomerular filtration process due to kidney dysfunction.[13]

The National Kidney Disease Education Program recommends serum creatinine to assess glomerular filtration capacity because to determine kidney function creatinine is a more accurate indicator when compared to urea.[9,19] This is because the amount of creatinine produced is more constant. In addition, in the tubules where urea is absorbed, creatinine is not absorbed.[20] Elevated creatinine levels indicate decreased excretion due to renal dysfunction. Creatinine is completely excreted in the urine by glomerular filtration.[13] An increase in creatinine levels can be caused by high free radicals in the body.[21]

Free radicals are one of the causes of increased levels of urea and creatinine. A radical is a molecule that has an unpaired electron in its orbit or a compound that is very unstable in its atomic or molecular structure. As a result, the free radicals present are highly reactive when they try to pair with other atoms and molecules, as well as one electron, to form stable compounds. Free radicals and Reactive Oxygen Species (ROS) induce oxidative stress in the kidney. The increase in free radicals and ROS causes cell death, and the leaked cell contents bind to the fibronectin protein in the tubular lumen. This causes obstruction in the shape of the surrounding cylinder so that as a result there will be impaired excretion of urea and creatinine.[17,21] Free radicals can be stabilized by compounds that are antioxidants such as flavonoids and phenolics.[5]

Ironwood bark extract (*Eusideroxylon zwageri*) contains the highest levels of compounds, namely flavonoids and phenolics.[13] Kidney cell damage caused by toxic substances can be prevented by flavonoid compounds because they have strong antioxidant activity found in ironwood bark extract which can neutralize free radicals.[22] Flavonoids and phenolics are phenolic compounds with the chemical formula C_6H_5OH, phenolic compounds have antioxidant activity which is influenced by the functional group that binds to the main structure, namely the hydroxyl group (-OH) attached to the carbon of the aromatic ring.[21]

Flavonoids have free radical scavenging mechanisms such as hydroxyl radicals, superoxide, and peroxyl. The mechanisms underlying its antioxidant properties are free radical scanning and transition metal ion chelating activity. Due to the reducing activity of phenolic hydroxyl groups, flavonoids are able to donate hydrogen. Along with the delocalization of phenoxy radical products, flavonoids can protect against various disease damage from Reactive Oxygen Species (ROS).[23] In other words, flavonoids stabilize Reactive Oxygen Species (ROS) by reacting with reactive compounds from radicals. Antioxidants can also repair damage to cell membranes using a scanner

mechanism to prevent damage to glomerular cells so that the glomerular filtration process can take place properly and creatinine can be excreted smoothly by the kidneys.[24] In addition, flavonoids also have the ability to inhibit the reabsorption of Na^+, K^+, dan Cl^-, which causes the kidney tubules to experience an increase in electrolytes so that diuresis occurs and binds free radicals caused by exposure to oxalate in the tubular epithelium and inhibits the process of prostaglandin synthesis.[25] The flavonoid and phenolic content of ironwood bark extract is the cause of the results of this study found a decrease in urea and creatinine levels. Based on the results of the research that has been carried out, it can be concluded that there is no toxic effect of giving ironwood bark extract (*Eusideroxylon zwageri*) at a dose of 524.5 mg/mL, 1151.5 mg/mL, and 1775.5 mg/mL orally for 28 days. day to the kidneys of wistar rats (*Rattus norvegicus*) with parameters of urea and creatinine levels given 2 times a day every 12 hours for 28 days.

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