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TOXICITY TEST OF KELAKAI LEAF EXTRACT (*Stenochlaena palustris*) TOWARD WISTAR RAT KIDNEY (*Rattus norvegicus)*

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ABSTRACT
Background: *Excessive free radicals cause oxidative stress which is dangerous for the body. The number of free radicals in the body can be controlled with the presence of antioxidants. Kelakai leaves (Stenochlaena palustris.) are known to have activity as an antioxidant which has the potential to become an ingredient in herbal medicine.* ***Purpose:*** *To prove whether there is a toxic effect of administering kelakai leaf extract on the kidneys of Wistar rats based on analysis of blood urea and creatinine levels.* ***Method:*** *A pure experimental study with a posttest-only with control group design method, there were 4 groups consisting of 1 negative control group and 3 treatment groups who were given kelakai leaf extract orally for 28 days and then analyzed the blood urea and creatinine levels of the Wistar rats.* ***Results:*** *The average values of urea and creatinine levels in the three treatment groups were still within the normal range. There was a significant difference in the urea levels of all groups ($p<0.05$), and there was no significant difference in the creatinine levels of treatment groups 2 and 3 ($p>0.05$).* ***Conclusion:*** *Kelakai leaf extract given orally for 28 days did not cause toxic effects on the kidneys of Wistar rats based on analysis of blood urea and creatinine levels.*

Keywords*: Creatinine, Kelakai Leaf Extrac,T Toxicity, Urea.*
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INTRODUCTION

Free radicals or ROS (Reactive Oxygen Species) are by-products produced by the body due to energy generation. The amount of free radicals in certain situations can increase so that oxidative stress can occur which causes cellular damage to carbohydrates, lipids, proteins, and DNA structures.[1] The negative effects caused by these free radicals can be suppressed by the presence of antioxidants. Antioxidants are needed to prevent the harmful effects caused by excessive free radicals. Antioxidants can be produced by the body itself, but antioxidants can be reduced or depleted quickly due to infections caused by bacteria or viruses, chronic inflammation and the aging process so that antioxidant sources from outside the body or exogenous antioxidants are needed. Exogenous antioxidants can be obtained naturally or artificially. Natural sources of antioxidants can be found in various plants, one of which is kelakai leaves (*Stenochlaena palustris.*).[2]

The kelakai plant is a fern that grows in peatland areas, the potential of kelakai leaves as herbal medicine is currently still being researched. Based on phytochemical tests, kelakai leaves contain secondary metabolites such as flavonoids, phenolic, alkaloids, and terpenoids. The main content in mature kelakai leaves is in flavonoids with a concentration of 503.56 mg QE/g, while the phenolic content is around 252.32 mg GAE/g.[3,4] The flavonoids and phenolic content in kelakai leaves have the potential to become antioxidants or prooxidants depending on the dose received by the body. Flavonoids and phenolics work as

antioxidants by capturing ROS through the process of hydrogen atom donation, the capture of ROS will react with the second radical which will produce a more stable compound. Meanwhile, flavonoids work as prooxidants by generating ROS through autooxidation and redox-cycling.[5] The mechanism of flavonoids and phenolics being antioxidants in kelakai leaves is what makes kelakai leaves potential as herbal medicine.

The content of secondary metabolites from plants is a substance from outside and in certain doses the substance cannot be fully accepted by the body.[6] The substances contained in a plant that will be used as herbal medicine should not have toxic properties and harm the body.[7] To test the toxicity of a substance, it is necessary to conduct pre-clinical tests in vitro and in vivo to determine a safe dose before clinical trials are carried out.[8]

Previously, the toxicity test of kelakai leaves (*Stenochlaena palustris.*) was carried out in vitro and in vivo. In vitro toxicity tests were conducted on BHK-21 fibroblast cells. Based on the results of this study, the results obtained at concentrations of 40%, 50%, 60%, 70%, 80%, and 90% did not show any toxic effects on BHK-21 fibroblast cells.[9] Based on the results of this study, it is necessary to conduct further in-depth research, namely the subchronic in vivo toxicity test on the kidneys which is carried out for at least 28 days to fulfill the requirements for kelakai leaf extract to be used as herbal medicine.

Examination of subchronic toxicity tests on the kidneys according to WHO can use main parameters such as urea and creatinine levels in blood serum.[10] Urea comes from amino acid catabolism and creatinine comes from creatine phosphate metabolism, both of these substances are removed by the kidneys from the body through urine. The kidney as an excretory organ functions to filter out harmful substances and remove these substances from the body, but its ability will be further reduced if the kidney is exposed to toxic substances. Toxic substances will damage important parts of the kidney such as the glomerulus so that the Glomerular Filtration Rate (GFR) will decrease, then metabolic products such as urea and creatinine which should be removed from the body instead re-enter the blood circulation and there will be an increase in blood urea and creatinine levels.[11] A substance is said to be non-toxic to the kidneys if urea and creatinine levels are still at the normal threshold after being given the substance, normal urea levels in Wistar rats are around 10-50 mg/dL while normal creatinine in rats ranges from 0.578-1.128 mg/dL.[12,13]

Based on the background, the purpose of this study was to determine whether there is a toxic effect in the subchronic toxicity test of kelakai leaf extract on the kidneys of Wistar rats at doses of 2,000 mg / kg BW, 2,500 mg / kg BW, and 3,000 mg / kg BW, namely concentration conversions of 40%, 50%, and 60% for 28 days.

METHODS

Ethical approval has been obtained from the Ethics Committee of the Faculty of Dentistry, Lambung Mangkurat University Number 092/KEPKG-FKGULM/EC/VIII/2023. The design used in this study was true experimental design with posttest only control group design method. The number of Wistar male white rats used was 16 rats aged 8-12 weeks with a body weight of 200-250 grams. Rats were grouped based on the formula for simple random sampling technique which was then grouped into 4 groups, with each group totaling 4 rats. The group was divided into a control group given distilled water and 3 other groups were given kelakai leaf extract at a dose of 2,000 mg, 2,500 and 3,000 mg/kg BW.

The materials used for extraction are mature kelakai leaves, 96% ethanol, WH40 filter paper, potassium dichromate (K2Cr2O7), beaker glass, oven, erlenmeyer flask (*Iwaki*), blender, shaker, mesh screen, rotary vacuum evaporator (*Eyela*), waterbath (*SMIC*), and drop pipette. As for the treatment of experimental animals, wistar rats (*Rattus norvegicus*), analytical balance (*Precisa*), distilled water, extracts that have been diluted according to the dose, wistar rat cages, husks for cage mats, rat drinking bottles, gastric syringes, and 3 ml syringes are needed. For blood collection and examination of urea and creatinine levels, Ketamine Xylazine is needed for anesthesia, BR2 for animal feed, scalpel, razor, syringe, sterile gloves, vacutainer tube as a place for rat blood, Eppendorf, reagents for measuring serum creatinine levels (*Reiged Diagnostics*), reagents for measuring urea levels (*Reiged Diagnostics*) and UV-Vis spectrophotometry (*PG Instrument*).

Preparation of Kelakai Leaf Extract

Kelakai leaves were collected in the Anjir area, Barito Kuala Regency, South Kalimantan Province. Preparation of kelakai leaf extract was carried out by maceration method. Kelakai leaves were taken as many as 20 kg of old leaves with criteria measuring more than 10 cm in length and more than 1-2 cm in width. Kelakai leaves are cleaned from foreign objects, then oven at 40°C to get dry leaves for 4 hours. Dry kelakai leaves are made into powder using a blender and then filtered to get simplisia powder.

The extraction process is carried out by dissolving kelakai leaf powder into 96% ethanol solvent. The solvent is replaced every 1x24 hours until the solution is clear white. To get clean

extraction results, the extract was filtered using WH40 paper. Solvent evaporation is carried out using a rotary vacuum evaporator for 4-6 hours at a temperature of 50-60 ° C, then heated in a waterbath until all the solvent evaporates so that 250 grams of thick blackish extract is obtained. Potassium dichromate (K2Cr2O7) is added to the kelakai leaf extract sample to determine if there is any solvent remaining. Samples that do not change color mean that they do not contain ethanol. Pure kelakai leaf extract is diluted using distilled water so that a concentration of 40%, 50%, and 60% of kelakai leaf extract is obtained, the doses obtained are 2,000, 2,500 and 3,000 mg/kg BW.

Toxicity Test of Kelakai Leaf Extract

Test animals were selected by simple random sampling method. Rats were grouped into 4 groups, each consisting of 4 wistar rats. grouped rats were put into cages and adapted for 7 days. Rats were given BR2 feed and distilled water. The first group (negative control), rats were given distilled water twice a day using a 3 mL syringe with a gastric probe tip for 28 days orally. In 3 treatment groups, kelakai leaf extract was given orally using a 3 mL syringe with a gastric probe tip at a dose of 2,000, 2,500 and 3,000 mg/kg BW twice a day for 28 days.

On day 29, rats were sacrificed for blood serum and examined. Rats that will be taken blood serum are anesthetized using ketamine-xylazine at a dose of 0.1 mL for every 100g BW of rats using a syringe. When the rat was unconscious, the rat was dissected using a blade and the rat's blood was taken through the heart while still beating. ± 3 cc of blood was taken using a syringe and then put into a vacutainer. Rat blood was centrifuged at a rotation speed of 4000 rpm for 10 minutes. The blood serum obtained was then examined for ureum and creatinine using the Glutamate dehydrogenase (GLDH) ureum examination method and the Jaffe method for creatinine examination using a Single Beam UV-Vis spectrophotometric instrument.

RESULTS

The results of the examination of urea and creatinine levels of Wistar rats after being given kelakai leaf extract can be seen in Table 1.

Table 1. Results of Examination of Urea and Creatinine Levels of Wistar Rats After Being Given Kelakai Leaf Extract.

Groups	Mean ± Std. Deviation (mg/dL)	
	Urea	Creatinine
C	30,372±0,414	1,031±0,063
T1	25,414±0,238	0,884±0,063
T2	23,864±0,207	0,719±0,063
T3	20,661±0,337	0,656±0,063

Description:
C: Group 1 Negative Control
T1: Group 2 (Treatment 1 dose of 2,000 mg/kg WB)
T2: Group 3 (Treatment 2 dose of 2,500 mg/kg WB)
T3: Group 4 (Treatment 3 dose of 3,000 mg/kg WB)

Based on the normality test, it is known that the data for the results of urea and creatinine levels have a value $p<0.05$ so that the data is not normally distributed. Based on this, data that are not normally distributed are continued using the Kruskal Wallis non-parametric test to see if there are significant differences. The results of data testing using Kruskal Wallis show that H0 is rejected ($p<0.05$), which means that there were significant differences in the urea and creatinine groups. Then proceed with the Mann-Whitney test to see which group has a significant difference between groups.

Table 2. Mann-Whitney Test Analysis Values for Urea Levels of Wistar Rats

Groups	C	T1	T2	T3
C	-	0,017*	0,015*	0,017*
T1	0,017*	-	0,017*	0,019*
T2	0,015*	0,017*	-	0,017*
T3	0,017*	0,019*	0,017*	-

Description:
(*): There is a significant difference ($p<0.05$)

Based on the table above, it is known that there are significant differences between all groups.

Table 3. Mann-Whitney Test Analysis Values for Creatinine Levels of Wistar Rats

Groups	C	T1	T2	T3
C	-	0,015*	0,015*	0,015*
T1	0,015*	-	0,04*	0,022*
T2	0,015*	0,04*	-	0,186
T3	0,015*	0,022*	0,186	-

Description:
(*): There is a significant difference ($p<0.05$)

Based on the table above, it is known that in treatment group 2 with treatment group 3 there is no significant difference.

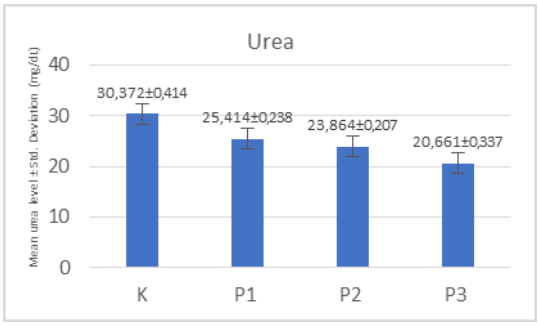

Figure 1. Graph of Average Urea Level of Wistar Rats

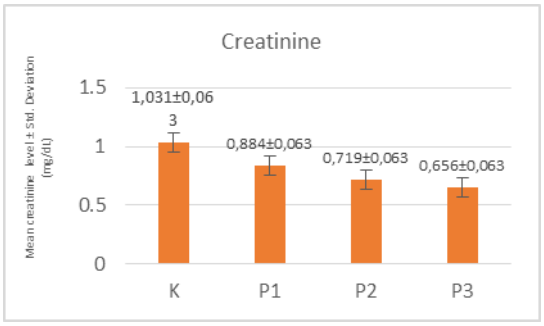

Figure 2. Graph of Average Creatinine Level of Wistar Rats

Based on Figure 1 and Figure 2, it is known that there is a decrease in the average blood urea and creatinine levels of Wistar rats in each group as the dose of kelakai leaf extract given increases.

DISCUSSION

Based on the results obtained, the urea levels of treatment groups 1, 2 and 3 were lower than the negative control group and there was a decrease in the average as the dose of kelakai leaf extract increased. The difference in urea levels in each group also shows that there are statistically significant differences. The highest urea concentration was in the control group which was 30.992 mg/dL, in treatment group 3, the lowest urea concentration was obtained, which was 20.248 mg/dL. The value of the average urea level of rats in each group is still in the range of 10-50 mg/dL which means it is still within normal limits. The results showed that kelakai leaf extract was not toxic to rat kidneys based on the parameters of blood urea levels.[12]

Based on the average creatinine levels of each group, it also shows a decrease from the control group and treatment group along with an increase in the dose of kelakai leaf extract. The highest creatinine level was 1.031 mg/dL in the control group, while the lowest creatinine level was 0.625 mg/dL in treatment group 3.The average creatinine level of rats in each group is still in the range of 0.578-1.128 mg/dL which means it is still within normal limits. The results showed that kelakai extract is not toxic to rat kidneys based on the parameters of blood creatinine levels.[13]

Urea and creatinine levels in rats can be affected by damage to the kidneys due to exposure to free radicals.[14] Free radicals or ROS have one or more unbound electrons such as superoxide (O^{2-}).[15] The unbound electrons possessed by ROS are unstable and highly reactive, because of this condition ROS requires electrons from other molecules to be more stable. The molecule whose electrons are taken by ROS will become the second radical and will likely take electrons from nearby molecules resulting in a chain reaction. ROS can cause oxidative stress which results in impaired vascular function, cell damage and nucleic acid (DNA) damage until cell death.[14,16] Increased ROS in rat kidneys will cause cell death so that the contents of dead cells will come out and bind to fibronectin protein so that it will form a blockage in the form of a cylinder in the tubular lumen. These factors can affect GFR so that the process of excretion of urea and creatinine can be disrupted.[17,18] Disruption to excrete urea and creatinine due to free radicals results in the accumulation of urea and creatinine substances in the blood. The problems caused by ROS can be reduced by the presence of antioxidants as ROS stabilizers such as flavonoids and phenolics.

Kelakai leaf extract (*Stenochlaena palustris.*) has the main content of flavonoids and phenolics that can suppress the negative effects of ROS. It is proven in this study that the treatment group has an average rat urea and creatinine levels that are significantly lower than the control group, and there is a decrease in the average urea and creatinine levels in each treatment group as the dose of kelakai leaf extract given to rats increases. The ability of flavonoids and phenolics of kelakai leaves to protect cells from ROS can make it a renoprotective agent, so that damage to kidney cells can be avoided and the process of excretion of urea and creatinine can also run smoothly.[19]

Flavonoids and phenolics in kelakai leaves suppress ROS by increasing the activity of endogenous antioxidants such as superoxide dismutase (SOD), catalase enzyme (CAT), glutathione peroxidase (GPX).[20] Flavonoids also work by binding Fe^{2+} and Cu^{+} ions and breaking the chain reaction of free radicals so that they inhibit the enzyme that forms superoxide anion (O^{2-}). O^{2-} will be converted into hydrogen peroxide (H_2O_2) by the enzyme SOD.[21] H_2O_2 will be converted into water (H_2O) by CAT and GPX.

H_2O_2 that is not converted into water will be transformed into hydroxyl radicals (OH) through the catalytic reaction of Fe^{2+} or Cu^{+} groups. OH gets a hydrogen ion contribution from flavonoids, so that ROS can be reduced and become unreactive.[20,22] The flavonoids and phenolic content in kelakai leaves protect cells to prevent the harmful effects by ROS and the enzyme system in cells can more easily control metabolic processes. so that kelakai leaf extract can be a source of natural antioxidants that are not toxic to the kidneys.[23]

Apart from working as an antioxidant, the ability of flavonoids and phenolic substances in kelakai can also be a diuretic agent. This mechanism takes place by increasing electrolytes in the renal tubules which causes diuresis through inhibition of Na^{+}, K^{+} and Cl^{-} reabsorption. This process will increase the volume of urine production and increase GFR so that the excretion of solutes such as urea and creatinine also increases.[24,25] This increase in excretion will cause urea and creatinine in the blood to decrease.[25]

In conclusion, this study proved that kelakai leaf extract doses of 2,000, 2,500 and 3,000 mg/kg BW given orally for 28 days are not toxic to the kidneys of Wistar rats and can be a source of natural antioxidants that can suppress negative effects by excess ROS and have a diuresis effect so that it can reduce blood urea and creatinine levels.

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