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Subacute Toxicity Test of Ethanol Extract of Sungkai Leaves (*Peronema canescens* Jack.) on Renal Histology of Male Wistar Rats

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ABSTRACT

The Sungkai plant (*Peronema canescens* Jack) is widely used as an immunostimulant. This study determined the toxicity of the ethanol extract of Sungkai leaves in Wistar rats by histological evaluation of their kidneys. The study used 36 male rats administered ethanol extract of Sungkai leaves for 7, 14 and 21 days at three doses of 140, 280 and 560 mg/kg bw, respectively. In each group, three rats were taken to be sacrificed on days 8, 15 and 22, and their kidneys were exercised for further histological evaluation. The degree of kidney tissue damage and the ratio of kidney organs were noted. The descriptive results showed that the highest kidney damage was due to an extract dose of 560 mg/kg bw with a score of 2.2 in the mild damage category, which occurred at 21 days of administration. The observation of the ratio of kidney organs showed that there was a significant effect of variations in dose and duration of administration on the ratio of kidney organs (p<0.05). However, there was no significant effect on the interaction between dose and duration of administration on the ratio of kidney organs (p>0.05).

Keywords: *Peronema canescens* Jack., Histology, Renal, Sungkai, Subacute toxicity

Introduction

The sungkai plant (*Peronema canescens* Jack) is a well-known herb for the treatment of various health problems by the people of Indonesia for centuries. The people of Lampung and Sumatra use sungkai as an antimalarial and for fever. The Dayak tribe in East Kalimantan uses sungkai as a medicine for worms, fever, colds and toothache. The Serawai tribe also uses Sungkai to treat fever, while the Lembak tribe use it to maintain immunity.[1,2]

The use of traditional medicine has been known long before the existence of formal health services by considering its benefits empirically. Nowadays, herbs are used as a complement to primary treatment.[3] In the last decade, many have turned to traditional medicine products and practices with the assumption that natural means safe, which is not necessarily true. All effective drugs can have adverse reactions, including herbal medicines. For this reason, in the use of herbal medicine, it is important to consider the dose, time of use, method of use, and selection of drugs for the disease.[4]

The immune system can be divided into two types: the nonspecific immune system (innate immunity) and the specific immune system (adaptive immunity). The nonspecific immune system has faster activity because it does not involve the memory cells. Several components that are involved in the nonspecific immune system are macrophage cells (phagocytic white blood cells) and natural killer cells, which protect the body from pathogen attacks so that the body eventually builds its defence system. The body's defence system can be activated by providing compounds that can increase the body's immune response.

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Many of these compounds are found in plants that can stimulate these immune response functions, which are called immunomodulators.[5]

To ensure the safety of traditional medicine, it is necessary to conduct toxicity tests. A toxicity test is a test conducted to detect the toxic effect of a substance on biological systems and to obtain dose-response data that is typical of the test preparation used. The data obtained can be used to provide information about the degree of danger of the test preparation in the event of human exposure so that a safe dose can be determined for its use in humans. One test used to evaluate the toxicity of phytochemicals is the subacute toxicity test. The subacute toxicity test is part of a long-term toxicity test that aims to obtain data on the poisoning of drugs or (chemical) substances used intentionally or unintentionally that enter the body repeatedly and over a long period.[6]

Although often described as safe, it has been noted that many medicinal plants have the intrinsic potential to be toxic or to interact with other traditional medicines or with conventional drugs.[7]

Histopathology has been used to identify changes in morphology associated with *in vivo* diagnosis, evaluation of response to therapy, and nonclinical safety assessment. There is often a strong correlation between specific histopathologic findings and clinical signs or some clinical chemistry parameters.[8]

In this study, a subacute toxicity test of sungkai leaf extract (*Peronema canescens* Jack) was conducted using male Wistar rats (*Rattus norvegicus*) by observing the kidneys. Parameters observed in kidney histology are the degree of kidney tissue damage and the ratio of kidney organs.

Material and Methods

Chemicals

Aquadest (Andeska laboratory), 70% ethanol (Andalas laboratory), Hematoxylin and Eosin dye (Pupick Med), physiological NaCl (PT Rajawali Nusindo), Na CMC 1%, formalin buffer (Leica), 80% Ethanol (Brata Med), 95% Ethanol (Brata Med), absolute ethanol (Brata Med), 80% xylol (Merck), 95% xylol (Merck), 100% xylol (Merck), paraffin (Merck), Canadian balsam (DPX mountant).

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Animals
Healthy male Wistar rats (36) weighing 200 - 300 grams were used. The animals were acclimatised for one week and provided standard animal food and water *ad libitum*. Ethical approval was obtained from the Faculty of Medicine Ethics Committee of Universitas Andalas, with approval number 109/UN.16.2/KEP-FK/2023.

Plant Materials and Collection
Samples of sungkai (*Peronema canescens* Jack.) were obtained in June 2022 from UPTD Rumah Potong Hewan Aia Pacah, Padang City, West Sumatera. The plant was identified and validated by Dr Nurainas of Andalas University Herbarium (ANDA), Department of Biology, Faculty of Mathematics and Natural Science, Universitas Andalas, Padang City, West Sumatera. A voucher specimen number 259/K-ID/ANDA/V/2022 was assigned.

Preparation of the Extract
About 4 kg of fresh plant sample was dried and ground to a fine powder (650 g). This sample was subsequently macerated with 70% ethanol at a ratio of 1:10, sample-to-solvent, in a dark-coloured glass container for 6 hours. The filtrate was dried using a rotary evaporator at 45 ℃ under reduced pressure.[10]

Characterisation of the Extract
The characterisation of the extract included nonspecific, specific, and chemical testing as follows: a) nonspecific testing measured the loss of weight on drying, total ash content, acid insoluble ash content, and water-soluble ash content; b) specific testing included organoleptic tests (i.e. shape, colour, taste, odour), parameter identify (i.e. names identified and compounds contained); and c) chemical testing determined the phytochemical components (e.g. alkaloids, saponins, phenols, flavonoids, steroids, and terpenoids), including thin-layer chromatography, and total flavonoid content determination.[9]

Sub-acute Toxicity Testing
The 36 male Wistar rats were weighed and divided into four groups: Group I (the control group) received NaCMC (0.5%). Groups II, III, and IV received oral doses of sungkai (*Peronema canescens* Jack) extract at 140, 280, and 560 mg/kg BW, respectively. The extract was administered orally once daily for 7, 14, and 21 days at 10.00 h. The animals were sacrificed on the 8^{th}, 15^{th}, and 22^{nd} days respectively.
The percentage ratio of organs to body weight of each experimental animal was calculated with the following formula:

$$RO = \frac{BO}{BB}$$

Description:
RO = Ratio of rat organs
BO = weight of rat organs (g)
BB = Rat body weight (g)

Preparation of Kidney for Histological examination
Rats were sacrificed, and kidneys were removed. Then, the organ was rinsed with physiological NaCL and fixed in a formalin buffer solution.
Each kidney was dehydrated with 80%, 95% and absolute alcohol for 1 hour.
Transfer into absolute alcohol: xylol (1:1), xylol 1 and xylol 2 for 1 hour for clarification.
The dehydrated kidneys were placed into an infiltration solution in an incubator. Xylol: paraffin, paraffin I, II, III (for half an hour each).
The cut kidney tissues were embedded in a metal mould/paper box filled with liquid paraffin, heated in an incubator, and allowed to cool and freeze.
The embedded tissues were mounted on a paraffin block holder and then thinly sliced with a microtome knife (3 μm).
The incision tissues were placed on glass slides, rubbed with Mayers albumin and placed on a hot plate until the incision expanded, then attached to the glass slide.
The tissues were stained with Erlich's hematoxylin and eosin. Xylol I, xylol II (15 minutes each), absolute alcohol I, absolute alcohol II, 80%, 95% and 100% (3 minutes each), Ehrlich's hematoxylin for 10 minutes, then washed with running water.
The slides were counterstained with eosin for 5 minutes, absolute alcohol III, absolute IV (7 minutes each), xylol III, and xylol IV (15 minutes each) and washed with running water.
Canadian balsam was applied to stained tissues and covered with a slide glass cover.
The labelled glass slides were examined under the microscope.
Examination of Kidney Histopathology Preparations
Histopathological preparations of the kidneys were examined microscopically with a magnification of 400x.

Statistical Analysis
The data were analysed by two-way ANOVA between the time (duration of administration) and doses. Subsequently, significant results were analysed by Duncan's multiple range test (p<0.05) using IBM SPSS Statistics V24.

Results and Discussion

Phytochemical screening was conducted to identify the secondary metabolites contained in sungkai leaves. The results showed that the leaf extract contained flavonoids, phenolics, saponins, alkaloids and triterpenoids (Table 1). A subacute toxicity test of ethanol extract of the leaves was conducted by observing the kidney histopathology of the treated animals. Histopathology has been used in identifying changes in morphology associated with *in vivo* diagnosis, evaluation of response to therapy and nonclinical safety assessment.[8] The reason for using the kidney in this study is because the kidney is the main filtration and excretion organ and is very important in removing toxic metabolic products and other substances that enter the body.[5] Based on the results obtained on the histology of the kidneys of animals treated with the ethanol leaf extract of sungkai after 7 days post-administration, the control group showed kidneys with cortex containing tubuli and glomeruli arranged regularly without any signs of inflammation, degeneration and necrosis of tubular epithelium, or bleeding (Figure 1a), with a score of 0 (Table 2). The administration of a dose of 140 mg/kg bw showed minimal changes in kidney tissue where there were no significant changes in the glomerulus, with a score of 1 (<10%) (Figure 1b). Dosing with 560 mg/kg bw showed focal changes in kidney tissue in several places in the form of degeneration and necrosis of tubular epithelial cells characterised by cloudy cytoplasm and lysed nuclei, decreased loss of tubular structure. Dilated blood vessels were hypaeremic and haemorrhagic, glomerulus with mild hypaeremic capillaries. The 280 mg/kg bw dose showed the same changes as the 560 mg/kg bw dose but milder (Figure 1d). Changes observed in the histopathology of rat kidneys were degeneration and necrosis. Degeneration is an event of changes in cell morphology due to injury characterised by enlarged cells, round nuclei in the middle of small vacuoles that appear white around the nucleus and pale cytoplasm.[14] While necrosis is cells that undergo pathological changes that lead to cell death, caused by toxic substances that enter the kidneys with blood flow to the kidneys. According to Suhita[15], necrosis begins with changes in the morphology of the nucleus, which loses the chromatin picture, becomes wrinkled, and the nucleus is denser and dark in colour.

Table 1: Results of Phytochemical Screening

Phytochemical Test	Result
Alkaloids	+
Flavonoid	+
Phenolic	+
Saponin	+
Steroid	+
Terpenoid	+

(+) = contains a secondary metabolite
(–) = does not contain a secondary metabolite

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Table 2: Effect of sungkai leaf ethanol extract dose and time on the mean value of rat kidney organ ratio

Dosis	Average ± SD			Average ± SD
	Day-7	Day-14	Day-21	
Control	0.010 ± 0.0002	0.009 ± 0.0005	0.009 ± 0.0008	0.0093 ± 0.0005
Dose 1	0.008 ± 0.0006	0.008 ± 0.0006	0.007 ± 0.0007	0.0077 ± 0.0006
Dose 2	0.009 ± 0.0007	0.008 ± 0.0011	0.008 ± 0.0009	0.0083 ± 0.0009
Dose 3	0.009 ± 0.0003	0.007 ± 0.00012	0.007 ± 0.0006	0.0077 ± 0.0003
Average ± SD	0.007 ± 0.0005	0.007 ± 0.0006	0.007 ± 0.0008	

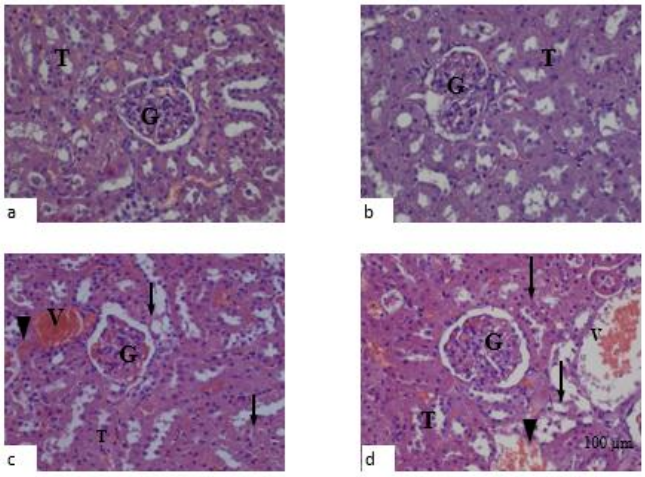

Figure 1: Histology of Rat Kidney after 7 days administration of Sungkai Leaf Ethanol Extract.
Description: a = control; b = dose of 140 mg/kg bw; c = dose of 280 mg/kg bw; d = dose of 560 mg/kg bw; T = Renal cortex with tubules; G = Glomeruli; V = blood vessels; (↓) = loss of tubular structure; (▼) = hyperemic and hemorrhagic dilated blood vessels.

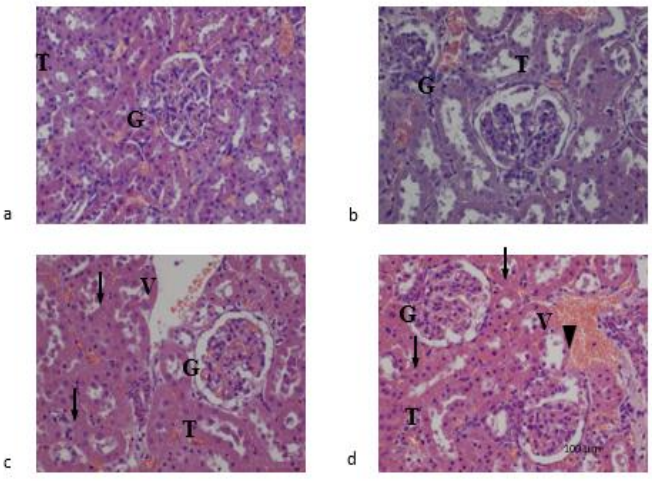

Figure 2: Histology of Male Rat Kidney after 14 days administration of Sungkai Leaf Ethanol Extract
Description: a = control; b = dose of 140 mg/kg bw; c = dose of 280 mg/kg bw; d = dose of 560 mg/kg bw; T = Renal cortex with tubules; G = Glomeruli; V = blood vessels; (↓) = loss of tubular structure; (▼) = hypaeremic and haemorrhagic dilated blood vessels.

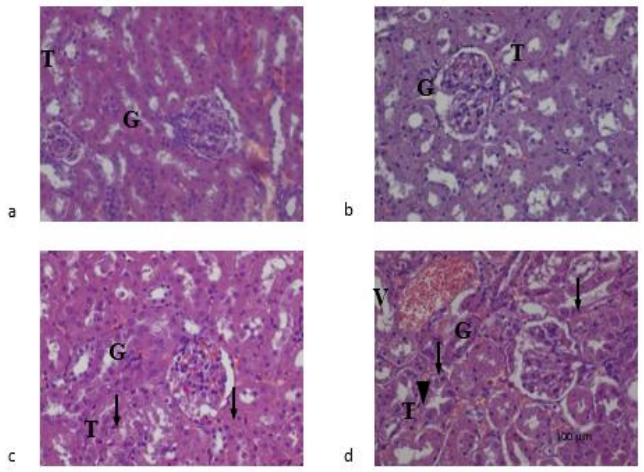

Figure 3: Histology of Male Rat Kidney after 21 days administration of Sungkai Leaf Ethanol Extract.
Description: a = control; b = dose of 140 mg/kg bw; c = dose of 280 mg/kg bw; d = dose of 560 mg/kg bw; T = Renal cortex with tubules; G = Glomeruli; V = blood vessels; (↓) = loss of tubular structure; (▼) = hyperemic and hemorrhagic dilated blood vessels.

Necrosis is the death of tissue cells due to injury while the organism is still alive. Microscopically, there were changes in the nucleus, including the loss of chromatin, a wrinkled nucleus, undefined vascular tissue, and a denser dark black (pyknosis) coloured nucleus, with fragmentation and tear (karyorrhexis).[15]
In addition, at a dose of 560 mg/kg bw, changes such as hyperaemia and haemorrhage also occur. Hyperaemia is a condition where blood volume increases in blood vessels accompanied by widening of blood vessels. One of the causes of hyperaemia in the kidneys is the presence of toxic substances that enter the kidneys and can affect the walls of blood vessels. Continued hyperaemia will cause bleeding in the tissue. Hyperaemia is the first pathological symptom of tissue damage that shows a red colour change, depending on the degree of blood oxygenation. The next change is haemorrhage (bleeding), which is the discharge of blood from blood vessels, usually identified as the presence of red blood cells outside the blood vessels or in the tissue.[14]
Another feature seen was oedema in the glomeruli due to fluid retention arising from damage to the tubules. Inflammation in the glomeruli can occur due to an increase in capillary permeability so that the glomerular capillaries become permeable to proteins. This increase in glomerular permeability leads to an increase in glomerular filtration load and, eventually, oedema. One sign of glomerular oedema is atrophy or shrinkage of glomerular capillaries so that Bowman's space appears enlarged.[15]
A sensitive indicator that can be used to observe the toxicity effect of a material or compound is seen in changes in the weight of the organs in experimental animals.[16] The observation of the kidney-to-body weight ratio showed no significant effect of the interaction between dose and duration of administration (p>0.05). However, there was a significant effect on the dose variation of the extract of sungkai leaves on the ratio of kidney organs (p<0.05). The administration of the extract of sungkai leaves to the experimental animals caused a decrease in the

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value of the kidney-to-body weight ratio. The ethanol extract of sungkai leaves caused minimal to mild damage to the kidneys of the male Wistar rats.

Conclusion

There was an effect of dose variation and duration of administration of ethanol extract of sungkai leaves on the histology of kidneys, with increased kidney histology damage. The highest increase occurred at 21 days of administration at a dose of 560 mg/kg bw. The study concluded that there is a need for caution in the prolonged use of sungkai leaf extract, especially at a dose of 560 mg/kg bw and beyond.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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