



SUBACUTE TOXICITY TEST OF *Rhizophora apiculata* BARK EXTRACT ON LIVER AND PANCREAS HISTOPATHOLOGY OF RATS

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ABSTRACT

Background: *Rhizophora apiculata*, one of the mangrove plant widely spread in Indonesia, can be developed as a medicinal plant. The extract of the bark has been found to have antioxidant and anti-inflammatory. However, the toxicity of *Rhizophora apiculata* has not been established yet.

Objective: This research aims to evaluate the toxicity of ethanolic extract of *Rhizophora apiculata* bark on histopathological changes in rat's liver and pancreas.

Methods: Subacute toxicity study of the ethanol extract of *Rhizophora apiculata* bark was performed in healthy male rats by administering the extract at doses of 57, 114, 228, 456, and 918 mg/kg of body weight daily for 28 days. The subacute toxicity in rats was determined by histological analyses.

Results: No significant adverse effect of the extract at dose 57 mg/kg was found. However at and over 114 mg/kg dose of the extract exhibited toxicities to the rats' liver. In addition, the toxic effect appeared in rats' pancreas at and over 228 mg/kg dose.

Conclusions: *Rhizophora apiculata* bark extract showed no toxicity at or below 57 mg/kg. The ethanol extract from bark of *Rhizophora apiculata* showed toxicity at 114 mg/kg by subchronic toxicity.

Keywords : rat liver histopathology, rat pancreas histopathology, *Rhizophora apiculata* bark extract, subchronic toxicity.

Received Aug 31, 2020 ; Revised Dec 22, 2020 ; Accepted Dec 30, 2020

INTRODUCTION

Rhizophora apiculata is a mangrove plant that grows along the coastline in Indonesia. The plants produce numerous bioactive substances that play important role in health, such as source of natural antioxidants. Fruits, flowers, and leaves of the plant are used by some Indonesian for traditional medicine especially for treating heartburn and stomach aches.[1] Furthermore, it was also utilized for treating nausea, vomiting, diarrhea, and amoebiasis in India.[2]

Previous studies indicated that the bark extract of *Rhizophora apiculata* performed as antioxidant activities and anti-inflammatory effects.[3-5] The administration of the extract is able to protect pancreas[6], coronary artery[7], and testicles[8] of rats from damage caused by exposure to cigarette smoke. Tannin[9] and pyroligneous acid[10] are active components that represent as antioxidants and free radical scavengers in the extract which mostly can be found in its bark. The studies also indicated that the ethanol extract of the bark has an effect of anti-tumor[11], anti-diabetic[12], and anti-microbial[9]. However, there is no report regarding toxicity testing of the extract. Furthermore, our previous study of the extract's acute toxicity test has not been published. We found that the dose of this extract that caused acute toxicity in the form of liver histopathological damage was 300 mg (unpublished data).

By considering many benefits and uses of this extract, it is required to conduct a toxicity test using animal model. The study aims to evaluate the safety of this extract in animal model. The results of this test will provide an indication of relative toxicity and to identify toxic effects of humans.[13] Therefore, we explored the subacute toxicity of ethanolic

extract of *Rhizophora apiculata* on liver and pancreas histopathology Sprague Dawley rats.

MATERIAL AND METHODS

Subacute toxicity study of the ethanol extract from bark of *Rhizophora apiculata* was performed in healthy male rats. It was enrolled in February-May 2020 at Universitas Lampung. The study was approved by Ethical Commission of Medical Faculty Universitas Lampung (3885/UN26.18/PP.05.02.00/2019).

Animals

The sample in this study was 30 male, 3-month age, 200-300 gram Sprague Dawley rats. Acclimatization and administration the extract was carried out in the pet house of the Faculty of Medicine, University of Lampung. Rats were held in a room with controlled conditions (temperature sustained at 20~25°C, relative humidity of 50~70%, 12 h of artificial lighting time from 8:00 am to 8:00 pm). Rats were fed according to 10% body weight, that is about 20-25 grams/head/day. Feed was given in the morning at 07.00 am and in the afternoon at 4 pm. Drinking water was given ad libitum. The cleanliness of the cage was undertaken by replacing the husks every 3 days. The acclimatization has been done for 7 days. After the acclimatization was completed, rats were randomly divided into 6 groups and placed into 6 cages. Each of them contained of 5 rats. The cages size was 40 x 30 x 20 cm which was made from plastic.[13,14]

Extract preparation

The maceration method was used for extracting *Rhizophora apiculata* bark obtained from KPHL Gunung Balak, in shoreline of Pasir Sakti sub-district, East

Lampung. The contaminants were removed from the barks and they were sundried frequently. Then, 1600 grams of dried bark were washed, chopped then turned into powder by using grinder machine. The bark powder was macerated in 2.6 liters of ethanol 95% and they were blended homogenously for 24 hours. Next, the mixture were filtered by using filter paper to obtain the filtrate. The filtrate then was evaporated by using rotatory evaporator at 50°C to obtain solvent-free thick extract. [6-8]

Administration of the extract

After the acclimatization, the study was undertaken by dividing the rats into 6 groups randomly A, B, C, D, E, and F group. The A group was a negative control (healthy group), which was only given standard food and drink. Group B to F were given daily oral administration of the extract at doses of 57, 114, 228, 456, and 918 mg/kg every morning for 28 days.[13, 14] The lowest dose was based on our previous research which states the dose which had an anti-oxidant and anti-inflammatory effect was 57 mg/kg.[6, 7, 8] Rats were terminated on 29th day. The organs were used as sample for histopathological preparations.[13, 14]

Histological preparation and examination

The hepatic and pancreatic histopathological preparations were constructed in 6 step procedures. Organs were fixed, dehydrated cleared, infiltrated, embedded, and sectioned. First, fixation was carried out using 10% buffer neutral formalin solution for 48 hours. The organ samples were then trimmed with a thickness of ± 0.5 cm then put in the tissue cassette for inclusion in the automatic tissue processor. Second, dehydration process was carried out by immersing the

sample in an alcohol solution with graded concentrations (75%, 95%, and absolute alcohol). The immersion process at each alcohol concentration was carried out for 2 hours. Third, clearing was carried out in two stages using xylol I and xylol II. Fourth, impregnation was carried out using histoplast paraffin. Fifth, the embedding process was carried out by using a tissue embedding console. Finally, sectioning was carried out using a rotary microtome spencer which produced pieces with a thickness of 4-5 μ m. The preparation was then placed in a glass slide and stored in an incubator at 37°C for 24 hours. Next, the preparation needed to be stained for examination. [6-8]

Table 1. Histopathological damage score of rat liver due to exposure to toxic substances

Score	Description
0	There was no degeneration of hepatocytes and necrosis in one microscope field of view observed part
1	There was found about 1-20% degeneration of hepatocytes and necrosis in one microscope field of view observed part (mild damage).
2	There was found about 20-50% degeneration of hepatocytes and necrosis in one microscope field of view observed part (moderate damage).
3	There was found about 50-75% degeneration of hepatocytes and necrosis in one microscope field of view observed part (severe damage).
4	There was found more than 75% degeneration of hepatocytes and necrosis in one microscope field of view observed part (very severe damage).

Before staining, the histopathological preparations were deparaffinized with xylol (I and II) solutions for two minutes. Then the rehydration process was carried out by immersing the preparation in graded alcohol (absolute alcohol, 95% alcohol,

and 80% alcohol) for 1 minute at each alcohol grade. Then the preparation was washed under running water (tap water) for 1 minute finally stained with Mayer's Hematoxylin dye.[6-8]

Histopathological observations under a microscope with 400x magnification in five fields of view were conducted to calculate the number of degradation cells and necrotic cells in the rats' liver. In addition, it looked for signs of inflammation in the rats' pancreas for obtaining data. In the histopathological examination of the liver due to exposure to toxic substances, we divided the rat liver damage according to the scores in Table 1. While, on the pancreas, the score 0 (zero) indicated that there was no inflammation cell. On the other hand, score 1 indicated that inflammation cell was found.[6,14]

Statistical analysis

SPSS statistical analysis software was employed for statistical analysis. It used the Shapiro-Wilk normality test. One Way ANOVA test and Chi-square test were involved.

RESULTS

The result showed that there was no significant adverse effect of the extract at dose 57 mg/kg. Non-significant results ($p > 0.05$) were found between the control group and 57 mg/kg group in liver (Table 2) and pancreas (Table 3) histopathology. There was no cell degeneration and necrosis in the observed part of liver histopathology. Hepatocyte cells appeared normal and radially in composition. There was no visible swelling of the hepatocytes. The hepatic sinusoids also appeared normally. There were no enlarged cells and fatty liver. In addition, they were constructed as radially pattern (Figure 1).

Related to pancreas, there was no inflammation in observed part of pancreas histopathology in control group and 57 mg/kg group. The pancreas appeared normally. We also found that there were no dilated blood vessels and no invasions of inflammatory cells in parts of the pancreas (figure 2).

Moreover, at an over 114 mg/kg dose, the extract exhibited toxicities to the rats' liver. There were significant ($p < 0.05$) differences between the control group and other groups that exposure by extract at and over 114 mg/kg dose (Table 2). The main histopathological changes induced by the extract in rats' liver were cell degeneration and necrosis. Cell degeneration was influenced by the extract in the liver included dilation of sinusoid, focal congestion, and vacuolar degeneration. It indicated that there was metabolic disturbance upon the exposure of the extract. A dose of 114 mg/Kg caused mild liver damage in rats, approximately 1-20% of cells became degraded and necrosis. Even, at an over 456 mg/kg dose it caused moderate to severe rats' liver damage (Figure 1).

Table 2. The rats' histopathological liver damage evaluation (*significant)

Groups	Average of histopathological liver damage score	p-Value to control group (ANOVA)
A (Control)	0.12	1
B (57 mg/kg the extract)	0.16	0.365
C (114 mg/kg the extract)	1.40	0.007*
D (228 mg/kg the extract)	1.52	0.007*
E (456 mg/kg the extract)	2.36	0.000*
F (912 mg/kg the extract)	2.60	0.000*

On other hand, the toxic effect appeared in rats' pancreas at and over 228 mg/kg dose. The doses 57 and 114 mg/kg did not cause inflammation of the pancreatic tissues (Table 3). However, in higher doses groups, the extract administration caused significant dilatation of inflammatory cell infiltration compared to the control group. The administration of stem bark extract *Rhizophora apiculata* at dose of 228, 456, and 912 mg/kg had a toxic effect on the histopathology of pancreas of white Sprague Dawley rats. It was evidenced by the inflammatory cells in the pancreatic parenchyma. In those groups, there were more dilatations of blood vessels and inflammatory cell

infiltrations compared to the control group (Figure 2).

Table 3. The rats' histopathological pancreas inflammation evaluation (*significant)

Groups	Average of histopathological pancreas inflammation score	p-Value to control group (Chi Square)
A (Control)	0	1
B (57 mg/kg the extract)	0	1
C (114 mg/kg the extract)	0.12	0.235
D (228 mg/kg the extract)	0.4	0.001*
E (456 mg/kg the extract)	0.72	0.000*
F (912 mg/kg the extract)	0.92	0.000*

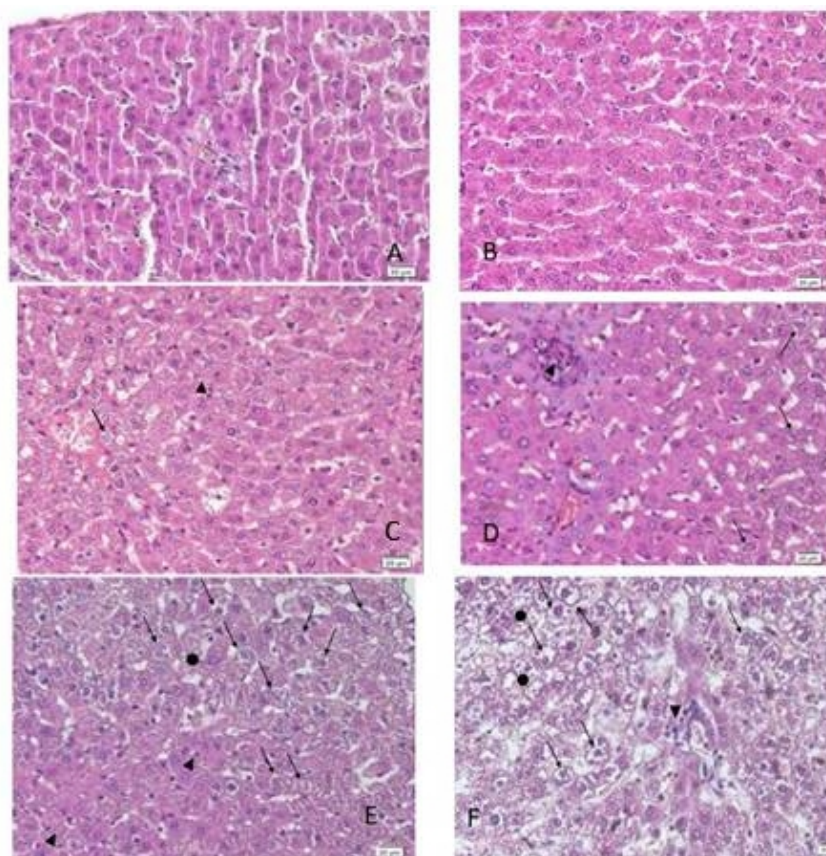


Figure 1. Histopathological of rats' liver of 400x magnification in the treatment of *R. apiculata* bark extract.

Black arrows (→) indicate cell degenerations, black pointing triangles (▼) indicate inflammatory cell infiltrations, Black circles (●) indicate cells necrosis. (A : Healthy control group; B: 57 mg/kg extract ; C: 114 mg/kg extract group; D: 228 mg/kg extract group; E: 456 mg/kg extract group; F: 912 mg/kg extract group.

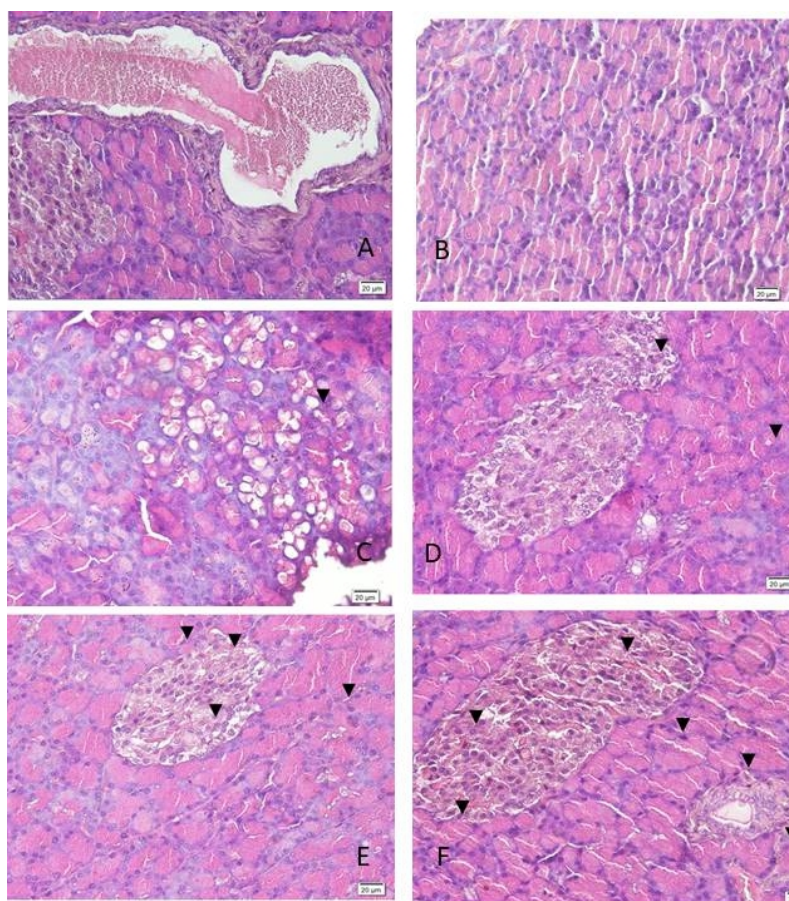


Figure 2. Histopathological of rats' pancreas of 400x magnification in the treatment of *R. apiculata* bark extract. Black pointing triangles (▼) indicate inflammatory cell infiltrations. (A: Healthy control group; B: 57 mg/kg extract; C: 114 mg/kg extract group; D: 228 mg/kg extract group; E: 456 mg/kg extract group; F: 912 mg/kg extract group)

DISCUSSION

Studies including ethnographic research on traditional medicine, in vitro research, in vivo research using various cells and experimental animals reported *Rhizophora apiculata* extract as potential medicine.[1,3-5] This extract has antioxidant, anti-inflammatory, antitumor, and certain effects.[5-7,11] However, it is necessary to study the possible side effects caused by the consumption of this extract by humans to be developed as a medicine. Studies of subacute toxicity for 2–4 weeks are undertaken to assess a new drug's potential adverse effects. They are performed as range-finding studies in order to select dose levels to be used in

further research of toxicity. Furthermore, it may reinforce initial clinical trials when the duration of treatment may be up to 4 weeks. They are constructed to evaluate the increasing and declining of drug-induced lesions.[13,14]

In this study, rats were used as a model because they had similar sensitivity and metabolic systems for the test preparation to humans. In addition, they grow quickly and easily to be handle during experiments.[13,14]

The organ commonly observed in histopathological examination in subacute toxicity test is liver. Besides, to see the inflammatory effect of exposure to this extract, we also examined the

histopathology of the pancreas. The most principal organ for metabolic utility involving detoxification of endogenous and exogenous challenges such as xenobiotics is liver.[15] Related to this case, some xenobiotics induce liver injury and damages which are characterized by hepatocyte swelling, inflammation, and necrosis. Xenobiotics generates free radicals indirectly, which in turn to lead oxidative stress in organism. Free radicals caused the expression of pro-inflammatory cytokine, which can lead a local inflammation.[16] Some studies showed that xenobiotics can be toxic for human organs, especially on the pancreatic tissue. Cell surface receptors on the exocrine pancreas, including nicotinic acetylcholine receptor, are activated by xenobiotics, and then they mediate pancreatitis.[17]

In the present study, the toxic effect appeared in rats' liver and pancreas. It is most likely due to the content of the two active substances in the extract, namely tannins and pyroligneous acid. Both of these substances have the potential to cause cell damage and apoptosis.[18,19] Tannins are widely distributed in the plant kingdom. Tannins may inhibit microorganism growth and support the defense plant tissues from infection. In high doses of tannin, it may irritate the mucous membranes. Phenolic group in tannins have high affinity to the protein to form complexes. Tannins are also able to bind with metal ions, lead to the absence of these ions. On the other hand, the enzymes in cells require metal ions for binding to the active sites. The lack of metal ions for some important enzymes may cause the cells become inactive.[18,20,21] Furthermore, previous finding indicated that acacia tannin extracts cause changes in cell internal structures and membrane lead to organelle

destruction and membrane rupture of microorganisms.[22]

Pyroligneous acid contains phenolics. There are two signaling events that are stimulated by the phenolic compounds in the mammalian cell lines. They are called mitogen-activated protein kinases (MAPKs) and ICE/Ced-3 proteases (caspases). The MPPKs regulate cell function such as gene expression, differentiation, proliferation, cell survival, mitosis, and apoptosis. The ICE/Ced-3 proteases play a key role in apoptotic cell death. Phenolics activated the MAPKs at low concentrations, lead to homeostasis response. On the contrary, the ICE/Ced-3 proteases pathway is activated by increasing phenolics concentrations. This condition is potential cytotoxicity because leading to apoptosis.[19]

CONCLUSION

It can be concluded that the safe dose for use of *Rhizophora apiculata* bark extract was at and below 57 mg/kg. The ethanol extract from bark of *Rhizophora apiculata* showed toxicity at 114 mg/kg by sub chronic toxicity. We suggest to explore further toxic dose of *Rhizophora apiculata* bark extract and to administer carcinogenicity test.

Acknowledgment

The authors would like to thank Penelitian Dasar Universitas Lampung 2020 for funding this research, and KPHL Gunung Balak for providing *Rhizophora apiculata* bark.

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