

Acute toxicity test of *Rhizophora apiculata* bark extract on rat liver and kidney histology using fixed dose method

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ABSTRACT

Background: The therapeutic properties of the ethanol extract derived from the stem bark of *Rhizophora apiculata* have been investigated. Nevertheless, there is a lack of studies regarding its acute toxicity.

Objective: This research aims to evaluate the toxicity of ethanolic extract of *R. apiculata* bark by examining histological changes in the liver and kidney of rats.

Method: The ethanol extract of *R. apiculata* bark was administered using a fixed-dose approach in preliminary and primary tests, focusing on the kidneys and liver of male *Sprague Dawley* rats. Four treatment groups received doses of 5, 50, 300, and 2000 mg/kg BW, while one group served as the control. An uninterrupted observation period of 14 days was conducted to determine any indications of acute toxicity in these animals. On the fifteenth day, the rats were terminated.

Results: This study indicated the absence of any toxic manifestations, such as tremors, excessive salivation, convulsions, coma, and mortality. Nevertheless, it caused histological damage to the rats' liver and kidney when administered at doses 300 and 2000 mg/kg BW.

Conclusion: While the administration of *R. apiculata* extracts at doses of 300 and 2000 mg/kg BW resulted in histological damage to the kidneys and livers of the rats, it did not induce any immediate symptoms.

Keywords: acute toxicity, fixed dose method, kidney histology, liver histology, *Rhizophora apiculata*

Introduction

The mangrove plant *Rhizophora apiculata* is found in Indonesia's coastline. Mangrove plants are known for their natural antioxidants and other bioactive substances essential for health. Some Indonesians use the plant's fruits, flowers, and leaves in traditional medicine, particularly for treating heartburn and stomachaches [1]. Additionally, it has been used in India to treat amoebiasis, nausea, diarrhea, and vomiting [2].

Previous studies have shown that the bark extract of *R. apiculata* possesses antioxidant and anti-inflammatory properties [3-5]. Administering the extract can protect the pancreas [6], coronary artery [7], and testicles [8] of rats from damage

caused by exposure to cigarette smoke. The extract contains active components such as tannin [9] and pyroligneous acid [10], which act as antioxidants and free radical scavengers, primarily found in the bark. Furthermore, studies indicated that ethanol extract of the bark exhibits anti-tumor [11], anti-diabetic [12], and anti-microbial [9] effect. However, a subacute toxicity test of *R. apiculata* at a dose of 114 mg/kg body weight (BW) showed signs of liver toxicity in rats, while a dose of 228 mg/kg BW resulted in pancreatic toxicity [13].

Given the numerous benefits and uses of this extract, it is essential to conduct a toxicity test using an animal model. The study aims to assess the safety of this extract in an animal model.



Figure 1. Image of the *R. apiculata* plant. This plant grows on the coast, has distinctive roots reaching a height of 5 meters, and sometimes has aerial roots that come out of the branches. The bark is gray.

The research investigates the acute toxicity of the ethanolic extract of *R. apiculata* on the liver and kidney histology of Sprague Dawley rats, providing insights into its potential toxic effects on human and the relative toxicity of the substance [14].

Methods

Extract preparation

The maceration method was used to extract *Rhizophora apiculata* bark obtained from KPHL Gunung Balak on the shoreline of the Pasir Sakti sub-district, East Lampung (Figure 1). First, contaminants were removed from the barks, and it was sundried. Then, 1500 grams of dried bark were washed, chopped, and ground into powder using a grinder machine. The bark powder was macerated in 4 liters of ethanol 80% and blended homogeneously for 24 hours. Next, the mixture was filtered using filter paper to obtain the filtrate. The filtrate was then evaporated using a rotatory

evaporator at 50 °C to produce a solvent-free thick extract [6-8].

Animals

An acute toxicity study of the ethanol extract from the bark of *R. apiculata* was performed in healthy male rats using the fixed dose method. The study was approved by the Ethical Commission of Medical Faculty, Universitas Lampung (1346/UN26.18/PP.05.02.00/2023).

The sample in this study consisted of 30 male Sprague Dawley rats, aged 8-10 weeks, and weighing 200-250 gram. Acclimatization and administration of the extract were carried out in the pet house of the Faculty of Medicine, University of Lampung. The rats were kept in a room with controlled conditions: temperature of 20~25°C, relative humidity of 50-70%, and 12 hours of artificial lighting time from 8:00 am to 8:00 pm. The rats were fed 20-25 grams per day, equivalent to 10% of their body weight, with feedings at 07.00 am and 4 pm. Drinking water was provided ad libitum. The cages cleaned by replacing the husks every three days.

Acclimatization lasted for seven days. After the acclimatization, rats were randomly divided into five groups and placed into five cages, each containing six rats. The cages measured 40 x 30 x 20 cm and were made from plastic [13,14]. Mangrove extract was administered to the rats on the first day, and the rats were observed for 14 days. On the 15th day of the experiment, termination was carried out.

Administration of the extract

After acclimatization, the rats were randomly divided into five groups. The KK group served as the negative control (healthy group) and was only given standard food and drink. Group KP1-4 were assigned single-dose oral administration of the extract at doses 5, 50, 300, and 2000 mg/kg BW, respectively [13, 14]. The rats were terminated on 15th day, and their organs were collected for histological preparations [13-15].

Table 1. Histological damage score of rat liver and kidney due to exposure to toxic substances

Score	Liver description	Kidney description
0	No degeneration of hepatocytes and necrosis in one microscope field of view observed	Normal
1	About 1-20% degeneration of hepatocytes and necrosis found in one microscope field of view (mild damage)	Mild (change less than 30%)
2	About 21-50% degeneration of hepatocytes and necrosis found in one microscope field of view observed (moderate damage)	Moderate (change less than 50%)
3	About 51-75% degeneration of hepatocytes and necrosis found in one microscope field of view (severe damage)	Severe (change more than 50%)
4	More than 75% degeneration of hepatocytes and necrosis found in one microscope field of view (very severe damage).	

Histological preparation and examination

Six steps were used to construct the histological preparations for the liver and kidney. Organs were sectioned, infiltrated, fixed, dehydrated, and cleared. The process began with 48 hours of fixation in a 10% buffer-neutral formalin solution. After trimming to a thickness of less than 0.5 cm, the organ samples were placed in a tissue cassette for use in the automatic tissue processor.

The samples were immersed in graded alcohol solution (75%, 95%, and absolute alcohol) for dehydration, with each immersion lasting two hours. Clearing was done in two phases using xylol I and xylol II. Histoplast paraffin was used for impregnation. The embedding process was completed using a tissue embedding console. A rotary microtome Spencer was used for sectioning, resulting in pieces 4-5 mm thick. The sections were transferred to glass slide and kept in an incubator at 37°C for 24 hours.

Before staining, the histological preparations were deparaffinized using xylol (I and II) solutions for two minutes. The preparations were then rehydrated by submerging them in graded alcohol (absolute alcohol, 95% alcohol, and 80% alcohol) for one minute each. The samples were then rinsed under running tap water for one minute and stained with Mayer's Hematoxylin dye [6-8].

Histological examination was performed under a 400x microscope to determine the number

of necrotic and degradation cells in the liver. Additionally, tubular degeneration and inflammatory cells in renal histology were assessed. The severity of liver or kidney damage due to exposure to toxic substances was categorized according to the scoring system in Table 1 [6,15].

Statistical analysis

SPSS statistical analysis software was used for statistical analysis. The Shapiro-Wilk test was used to assess normality. Additionally, Kruskal-Wallis and Post Hoc Mann-Whitney tests were conducted.

Results

The results showed that there were no deaths or toxic symptoms in any of the treatment groups. During the 14-day observation period, there was no significant change in the weight of the mice in either the standard group or the group dosed up to 2000 mg/kg BW.

Histological examination revealed no significant adverse effect of the extract at doses of 5 and 50 mg/kg BW. The liver histology showed typical hepatocyte cells arranged radially, with no apparent swelling or abnormalities in the hepatic sinusoids (Figure 2). However, cell degeneration and necrosis were observed at doses of 300 mg/kg BW (KP3) and 2000 mg/kg BW (KP4).

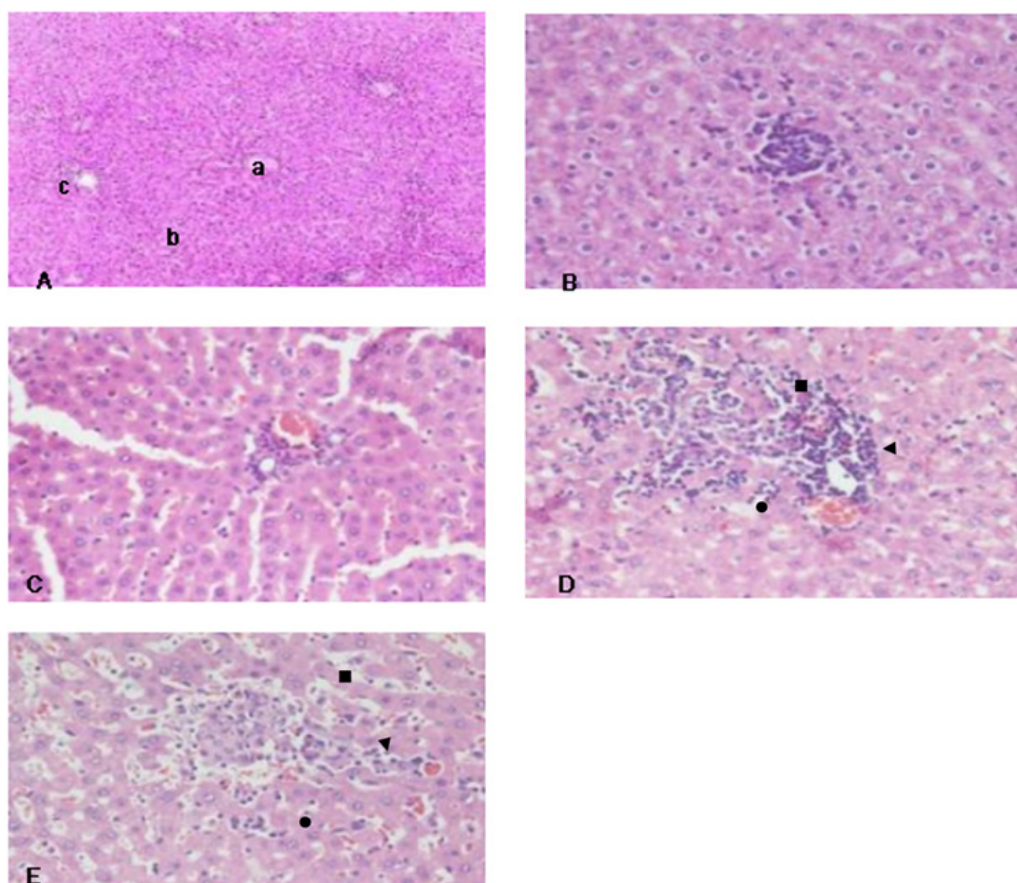


Figure 2. Histological examination of rat liver of 400x magnification after treatment with *R. apiculata* bark extract. A. Key: a: vena porta hepatica, b: normal sinusoid, c: normal hepatocytes, \blacktriangleright : hepatocyte necrosis, \bullet : clowdy swelling, \blacksquare : sinusoidal dilation. **B.** 5 mg/kg BW extract group, **C.** 50 mg/kg BW extract group, **D.** 300 mg/kg BW extract group, **E.** 2000 mg/kg BW extract group

Table 3. The rats' histopathological liver damage evaluation (*significant)

Groups	Average of histological liver damage score	Mann-Whitney Post Hoc Test (liver)
KK (Control)	0	-
KP1 (5 mg/kg BW the extract)	0.8	0,071
KP2 (50 mg/kg BW the extract)	0.8	0,071
KP3 (300 mg/kg BW the extract)	1.04	0,036*
KP4 (2000 mg/kg BW the extract)	1.08	0.036*

Table 4. The rats' histological kidney damage evaluation (*significant)

Groups	Average of histological kidney damage score	Mann-Whitney Post Hoc Test (Kidney)
KK (Control)	0	-
KP1 (5 mg/kg BW the extract)	0.4	0,134
KP2 (50 mg/kg BW the extract)	0.6	0.050
KP3 (300 mg/kg BW the extract)	1.4	0,005*
KP4 (2000 mg/kg BW the extract)	2.4	0,005*

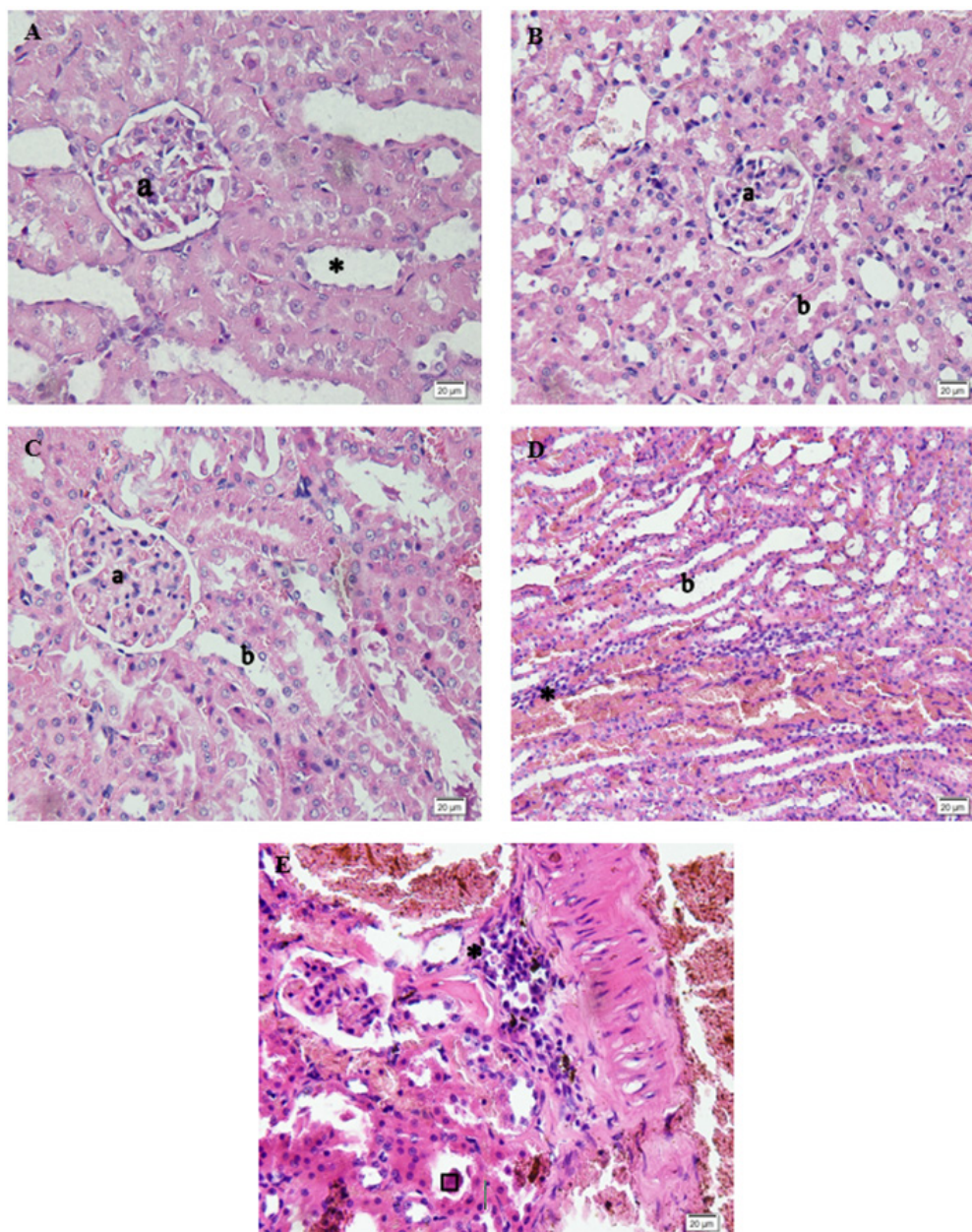


Figure 3. Histological examination of rat kidneys at 400x magnification after treatment *R. apiculata* bark extract. A. Healthy control group, **B.** 5 mg/kg BW extract group, **C.** 50 mg/kg BW extract group, **D.** 300 mg/kg BW extract group, **E.** 2000 mg/kg BW extract group. Key: a: glomerulus (in A, B,C), b: tubulus. *: inflammatory cells, □: tubular thyroidization (in E)

In the kidney histology, tubular degeneration and inflammatory cells were found in the groups treated with 300 mg/kg BW and 2000 mg/kg BW (Figure 3). The extract exhibited toxicities to the liver and kidneys at doses over 300 mg/kg BW, with significant differences ($p < 0.05$) between the control group and the groups exposed to these higher dose (Tables 3 and 4).

The main histological changes in the rats' liver induced by the extract included cell degeneration

and necrosis, dilation of the sinusoid, focal congestion, and vacuolar degeneration. These changes indicate metabolic disturbances caused by the extract. A dose of 300 mg/kg BW caused mild kidney damage, with approximately less than 30% of the tissue containing inflammatory cells. The 2000 mg/kg BW caused moderate kidney damage (Figure 2). In contrast, doses of 5 and 50 mg/kg BW did not cause inflammation of the kidney tissues.

Discussion

Based on ethnographic exploration of traditional medicine, further *in vitro* and *in vivo* research is needed to reveal *R. apiculata* extract as a potential raw material for medicine. Previous studies have reported that this extract has antioxidant, anti-inflammatory, anti-tumor, and other pharmacological effects [5-7,11]. However, before can be further developed into a medicinal product, it is necessary to study the potential side effects and toxic effects associated with its consumption [14,15].

In this study, rats were used as a model due to their similar sensitivity and metabolic systems to humans, as well as their rapid growth and ease of handling during experiments [14,15]. The liver and kidney were the primary organs observed in histological toxicity tests. The liver is a principal organ for metabolic activities, including the detoxification of endogenous and exogenous substances such as xenobiotics [16]. Some xenobiotics induce liver injury and damage, characterized by hepatocyte swelling, inflammation, and necrosis. These xenobiotics generate free radicals indirectly, leading to oxidative stress, which in turn causes pro-inflammatory cytokine expression and local inflammation [17,18].

The present study revealed toxic effect of *R. apiculata* extract on the liver and kidneys of rats, likely due to the presence of tannins and pyroligneous acid in the extract. These substances can cause cell damage and apoptosis [19,20]. Tannins, widely distributed in the plant kingdom, may inhibit microorganism growth and support plant tissues defense against infection. However, high doses of tannin can irritate mucous membranes. The phenolic group in tannins has a high affinity for protein, forming complexes that can bind with metal ions, leading to their absence. Metal ions are essential for certain enzymes to bind their active sites, and their lack can render cells inactive [19,21,22]. Previous findings indicate that acacia tannin extracts cause changes in cell internal structures and membranes, leading to organelle destruction and membrane rupture of microorganisms [23].

Inflammatory cells were observed in the kidneys when toxic doses were administered acutely. Inflammation is a protective response involving blood vessels, cells, proteins, and other mediators, aimed at eliminating the cause of cell injury, removing necrotic cells and tissue, and initiating tissue recovery. Poisons are diluted, destroyed, or neutralized by inflammation as a protective measure [24]. At a dose of 2000 mg/kg BW, tubular thyroidization was observed, with some of the tubules appearing smaller and others dilated with colloidal mass content covered by squamous epithelium.

Conclusion

The study indicates that the safe and effective dose of *R. apiculata* bark extract for rats is 300 mg/kg BW or below. Administration of *R. apiculata* extracts at acute doses of 300 and 2000 mg/kg BW resulted in mild to moderate histological damage to the kidneys and livers of the rats. However, no immediate symptoms were observed.

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Declaration of interest

The authors declare that none of them has any conflict of interest.

Author contributions

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