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A Subacute Toxicity Test of Rhizophora apiculata Stem Bark Ethanol Extract on the Number, Motility, and Morphology of Male Rattus Norvegicus Spermatozoa

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Abstract

Indonesia has the largest mangrove ecosystem in the world. Rhizophora apiculata is a mangrove often used as traditional medicine by Indonesians. It has previously been researched that the ethanol extract of Rhizophora apiculata stem bark has benefits as a source of natural antioxidants. However, there has yet to be much research on the toxicity of this extract. Therefore, researchers want to know the subacute toxic dose of Rhizophora apiculata bark extract on fertility parameters. The type of research is experimental research with a completely randomized research design. The sample consisted of 30 male rats, which were divided into 5 groups, namely the control group, which received no treatment, and the P1-P4 treatment group, which was given Rhizophora apiculata bark extract every day with 4 dose levels starting from 114 mg/kg BW to a dose of 912 mg/kg BW for 28 days. On day 30, the rats were euthanized, and the fertility parameters of the rat spermatozoa were examined using a microscope. Kruskal-Wallis analysis and One-Way ANOVA showed $p<0.05$ for the spermatozoa's number, motility, and morphology. The toxic dose that can reduce the number of spermatozoa is found at 912 mg/kg BW; the toxic dose that reduces spermatozoa motility is 228 mg/kg BW; and the toxic dose that affects spermatozoa morphology is 456 mg/kg BW. This research concludes that the subacute toxic dose of the ethanol extract of Rhizophora apiculata stem bark against the spermatozoa of the male Rattus norvegicus Sprague Dawley strain in the subchronic toxicity test was 228 mg/kg BW.

Keywords: Ethanolic extract, Mangrove, Rhizophora apiculata Bark, Spermatozoa, Subacute toxic dose

1. Introduction

Indonesia has the largest mangrove ecosystem in the world, with an area of more than 42,550 km² and more than 45 species. Mangrove species found in Indonesia include Rhizophora apiculata, Rhizophora mucronata, and Rhizophora stylosa. Rhizophora apiculata is one type of mangrove plant that is most often found and used as medicine. Almost all parts of Rhizophora apiculata can be used as medicine because they contain useful active ingredients. In general, mangroves are classified into the families Rhizoporaceae, Avicenniaceae, Sonneratiaceae, and Ceriops. The Rhizoporaceae type, especially Rhizopora apiculata, is one of the mangrove plants that is most commonly found in coastal areas.[1,2]

Mangrove plants are widely used as medicinal plants. Based on previous research, the compounds contained in the Avicennia alba mangrove plant are known to be used as pregnancy control or contraception. Rhizophora apiculate contains many antioxidants.[3] Ingredients that can be found in the Rhizophora apiculata plant include alkaloids, flavonoids, triterpenoids, steroids, saponins, and tannins. The antioxidants contained in Rhizophora apiculata bark extract have the ability to inhibit oxidative stress caused by free radicals. This happens because the bark of Rhizophora apiculata contains tannin compounds, which are found in high quantities.[4]

The bioactive compounds contained in Rhizophora apiculata are found in the fruit, leaves, stems, and roots. Rhizophora apiculata bioactive compounds can be used as antioxidants, antifungal, antiviral, antibacterial, antitumor, antidiarrheal, anti-inflammatory, and anticancer.[5]

Based on previous research, Rhizophora apiculata is known to act as an antioxidant that inhibits free radicals, thereby preventing oxidative stress. Based on other research, Rhizopora apiculata is known to be able to protect organs such as the coronary arteries, testicles,[6] liver,[7] kidney[8] and pancreas[9] in male white rats (Rattus novergicus) of the Sprague-Dawley strain that were exposed to cigarette smoke.

The toxic effects of a biological substance can be determined through a toxicity test. A toxicity test is a test carried out to determine toxic or dangerous substances contained in a preparation to obtain dose-response data from the preparation. Based on the length of exposure given, toxicity tests can be divided into oral acute toxicity tests, oral subchronic toxicity tests, and oral chronic toxicity tests. Toxicity tests with test animals aim to see biochemical, physiological, and pathological reactions in humans to a preparation. Test results cannot absolutely be used to prove the safety of a substance in humans, but they can provide an indication of the dangerous substances in the preparation being tested.[10] Organs examined histopathologically in the subchronic toxicity test include the brain, pituitary, lungs, heart, liver, kidneys, spleen, adrenals, pancreas, testes, seminal vesicles, bladder, uterus, epididymis, intestines, lymph nodes, nerve edge, stomach, sternum, femur, spinal cord, or at least five main organs, namely the liver, spleen, heart, kidneys, lungs, and additional specifically known target organs. Apart from histopathological examinations, other examinations such as hematological, macropathological, and clinical biochemical examinations are also carried out.[11]

Our previous research found that the sub-acute toxic dose of this ethanol extract for the liver and pancreas was 128 mg/kg BW.[12] Therefore, we were interested in examining the sub-acute toxic dose of this extract on fertility parameters.

2. Methods

This type of research is true experimental quantitative analytical This research was experimental research using a post-test-only control group design. This study used samples of 30 male white rats (Rattus norvegicus) of the Sprague Dawley strain aged 8¬12 weeks. Rats were randomly selected and divided into 5 groups; these groups were considered the same before being given treatment. Data collection in the form of fertility parameters was carried out only at the end of the study by comparing the results in the control group and the treatment group.[12]

The research was carried out in October 2020. This research was approved by the Health Research Ethics Committee, Faculty of Medicine, University of Lampung, with letter number 1432/UN26.18/PP.05.02.00/2020. The research was carried out in the animal house of the Faculty of Medicine, University of Lampung, the Biochemistry and Molecular Biology Laboratory, Faculty of Medicine, Unila, and extraction in the Organic Chemistry Laboratory, Faculty of Mathematics and Natural Sciences, University of Lampung. The samples for this research were white rats (Rattus norvegicus) of the Sprague Dawley strain, male, aged 8 - 10 weeks, with a body weight of 200 - 250 grams obtained from the Bogor Agricultural Institute.

The sample size used in this study was 30 male white rats (Rattus norvegicus) of the Sprague Dawley strain. White rats were divided into 5 groups, with 6 male white rats in each group. Control group (K) was only given

standard feed and drink. Treatment group 1 (P1) was given Rhizophora apiculata bark extract at 114 mg/kg BW. Treatment group 2 (P2) was given Rhizophora apiculata at 228 mg/kg BW. Treatment group 3 (P3) was given Rhizophora apiculata bark extract at a 452 mg/kg BW dose. Treatment group 4 (P4) was given Rhizophora apiculata bark extract at a 904 mg/kg BW dose. The extract was given every day for 28 days.[12]

On day 30, rats were euthanized using ketamine at a dose of 100 mg/Kg BW and xylazine at a dose of 10 mg/Kg BW which were injected intraperitoneally. Then, after the rat was confirmed dead, specimens of rat spermatozoa were taken, and observations were made. The spermatozoa suspension that has been obtained was homogenized first. Counting the number of spermatozoa was done through dilution using 0.9% NaCl. Next, 10 µl of the sample was taken, put into improved Neubauer hemocytometer boxes, and covered with a cover glass. A hemocytometer was placed under a light microscope with 40x magnification, and the number of spermatozoa in the counting chamber box was counted. The results of calculating the number of spermatozoa were then entered into the following formula for determining the number of spermatozoa: Number of Spermatozoa = n x dilution x 200,000 (million/ml).[13]

Spermatozoa motility was calculated by calculating the percentage of spermatozoa under a light microscope at 40x magnification. Based on the motility of moving or not rat spermatozoa. Observations were made in four to six fields of view to obtain one hundred spermatozoa in sequence, then classified to produce a percentage for each motility category. Total % Motility by dividing the number of progressive spermatozoa by the number of all observed spermatozoa multiplied by 100%.[14]

Spermatozoa morphology test was carried out by taking spermatozoa from the cauda epididymis, then making a smear using a glass object, then drying it. Then, it was fixed by administering methyl alcohol for 5 minutes, then dried and treated with Giemsa dye for 5 minutes. After that, rinse with distilled water and then dry again. Then, it was observed and counted in one field of view with 100x magnification under a light microscope. The normal interpretation of spermatozoa morphology is obtained when >30%. The number of morphological % was calculated by dividing the number of normal-shaped spermatozoa by the number of all observed spermatozoa and multiplying by 100%.[13.14]

The collected research data was entered into data processing software, and then univariate and bivariate analysis was carried out. Bivariate analysis was carried out using One-way ANOVA followed by Post Hoc. The alternative Mann-Whitney test was also performed if necessary.

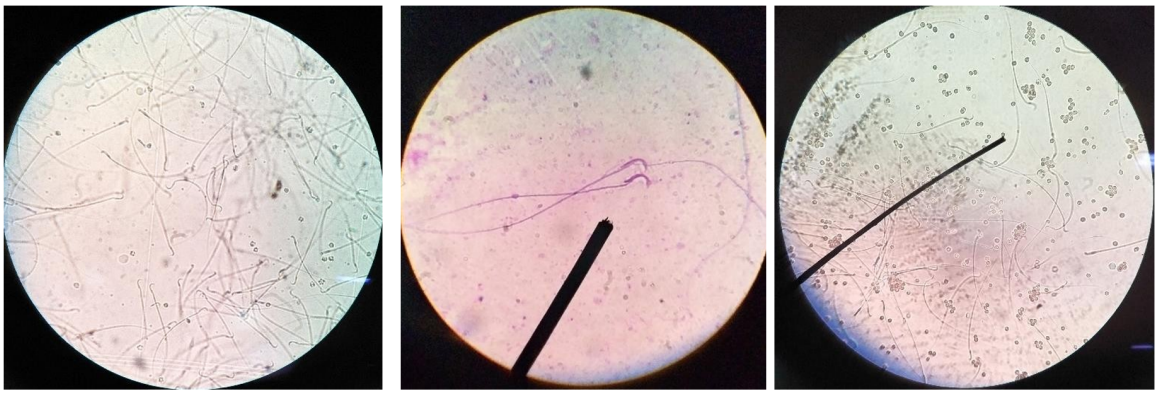
3. Results

Observations of sperm preparations from test rats have been carried out. Microscopic observations on sperm smear preparations included the number, motility, and morphology of spermatozoa seen using a light microscope with 40x magnification (figure 1).

The results showed that the mean number of spermatozoa obtained the highest value in group P1; in the control group, the mean number was slightly smaller than group P1, and the mean number of spermatozoa decreased in group P2 but was not much different from the value of groups P1 and K. The P value decreased significantly compared to group K in groups P3 and P4 (see Table 1).

When examining spermatozoa motility, the median spermatozoa motility value for the control group was 81.50. The median value of spermatozoa motility in group P1 was found to

be lower than the control group, namely 55.00, and in group P2, the median value was also lower, namely 23.00. In group P3 and group P4, the median spermatozoa motility value greatly decreased below the control group to 11.50 and 8.50 (see Table 2).



A B C

Figure 1. Process of assessing spermatozoa fertility parameters. A Calculates the number of spermatozoa; B calculates spermatozoa motility; C calculates the morphology of spermatozoa.

The results of the spermatozoa morphology assessment showed that the mean morphology of spermatozoa in group K was higher than all treatment groups, namely 65.50%. The mean morphology of spermatozoa decreased in group P1 but was not much different from group K, namely 59.50%. In group P2, the mean morphology of spermatozoa was lower than in group P1, namely 52.33%. The mean morphology of spermatozoa decreased greatly in groups P3 and P4, namely to 24.17% and 17.50% (see Table 1).

Table 1. Mean and Standard Deviation of Number and Morphology of Spermatozoa

Variabel	Average	Standard deviation
Number of Spermatozoa (million)		
K	197.27 /ml	8.641
P1	198.55 /ml	17.681
P2	183.43 /ml	6.109
P3	87.10 /ml	8.545
P4	12.16 /ml	4.461
Spermatozoa morphology		
K	65.50 %	13.62
P1	59.50 %	15.50
P2	52.33 %	12.22
P3	24.17 %	11.03
P4	17.50 %	7.58

Next, appropriate data analysis was carried out on the number, motility, and morphology of spermatozoa based on the data distribution. So, for the number and morphology of spermatozoa, a parametric one-way-ANOVA test was carried out, and for

spermatozoa motility, a non-parametric test, namely Kruskal Wallis, was carried out. In the One-Way ANOVA test, a p-value of 0.001 was obtained for the number of spermatozoa and a p-value of 0.000 for spermatozoa morphology, which means the p-value was <0.005, so it was known that there was a significant difference. In the Kruskal Wallis Test for spermatozoa motility, a p-value of 0.000 was obtained, so it was known that the data on spermatozoa motility was also significant.

Tabel 2. Median and Minimal-maximal Spermatozoa motility

Spermatozoa motility	Percentages	Min-max
K	81.50	(45-95)
P1	46.00	(15-88)
P2	23.00	(12-49)
P3	11.50	(11-28)
P4	8.50	(5-16)

In Table 3, it can be seen that only in groups P4 with K, P1, P2, and P3 were the results p<0.005, which means there is a significant difference. Meanwhile, the other groups got a p-value> 0.005, which means there was no significant difference except for group P4. It means that the toxic dose of ethanol extract of Rhizophora apiculate, which can reduce the number of spermatozoa, was 904 mg/kg BW.

Table 3. *Post Hoc Bonferroni* test the Number of Spermatozoa (* significantly different)

Groups	K	P1	P2	P3	P4
K	-	1.000	1.000	0.196	0.003*
P1	1.000	-	1.000	0.183	0.003*
P2	1.000	1.000	-	0.388	0.007*
P3	0.196	0.183	0.388	-	1.000
P4	0.003*	0.003*	0.007*	1.000	-

Tabel 4. *Post Hoc Mann-Whitney* tests the motility of spermatozoa (* significantly different)

Groups	K	P1	P2	P3	P4
K	-	0.077	0.006*	0.004*	0.004*
P1	0.077	-	0.150	0.006*	0.006*
P2	0.006*	0.150	-	0.045*	0.008*
P3	0.004*	0.006*	0.045*	-	0.261
P4	0.004*	0.006*	0.008*	0.261	-

Tabel 5. *Post Hoc Bonferroni test the* Morphology of Spermatozoa (* significantly different)

Groups	K	P1	P2	P3	P4
K	-	1.000	0.752	0.000*	0.000*
P1	1.000	-	1.000	0.000*	0.000*
P2	0.752	1.000	-	0.005*	0.000*
P3	0.000*	0.000*	0.005*	-	1.000
P4	0.000*	0.000*	0.000*	1.000	-

In Table 4, it can be seen that there is a significant difference in spermatozoa motility in group K with P2, P3, and P4 because the p-value was <0.05. There is no significant difference between K and P1 because p>0.05. it means that the toxic dose of Rhizophora apiculate ethanol extract, which can reduce spermatozoa motility, was 228 mg/kg BW.

In Table 5, it can be seen that there are significant differences in spermatozoa morphology in groups K with P3, K and P4, P1 with P3 and P4, P2 with P3 and P4, P3 with K, P1, and P2, and P4 with K, P1, and P2 because it was found that p<0.05. it means that the toxic dose of ethanol extract of Rhizophora apiculata, which can reduce the normal morphology of spermatozoa, was 452 mg/kg BW.

4. Discussion

In this study, Male rats given a high dose of ethanolic extract from Rhizophora apiculata bark saw a notable reduction in sperm count, movement, and shape. The decrease occurred as the dose concentration of the extract supplied increased, in comparison to the control group. The decline in sperm viability corresponded with a decrease in progressive sperm motility, as stationary sperm were identified as dead due to their uptake of the Eosin/Nigrosin stain during examination of the smear. This outcome could be attributed to the impact of the concentrated extract's high dosage on the epididymis, where it functions as a spermatotoxic agent affecting developing or fully developed sperm cells.[15] The rise in sperm head abnormalities in exposed rats, correlating with the dosage increase, indicates

that Rhizophora apiculate bark extract may have harmed the pre-meiotic stages of spermatogenesis. DNA synthesis takes place before the pre-meiotic phase in spermatogenesis, with no additional DNA synthesis occurring during the cell cycle.[16]

Various causes have been identified to support the rise in the incidence of sperm head defects in organisms exposed to certain substances. Sperm cell damage can result from physiological, cytotoxic, or genetic reasons. High concentrations of the extracts may have caused impacts on the pituitary, hypothalamus, or sex hormones, impacting sperm production. Alternatively, exposure could have led to anomalies in seminal fluid, resulting in impaired sperm function or structure.[17]

Research has demonstrated that elevated oxidative stress, as well as decreased levels of enzymatic and non-enzymatic antioxidants in Leydig cells, are associated with poor spermatogenesis and a notable decrease in epididymal sperm count. Abnormal sperm head shape and changes in sperm DNA content are frequently determined by genetics. These abnormalities are caused by chromosomal aberrations during genetic material packing in the sperm head or by point mutations in testicular DNA. It can also occur due to normal errors in the differentiation process of sperm cells during spermatogenesis.[18]

The study found that a concentrated ethanolic extract of Rhizophpora apiculata exhibited a mutagenic effect by dramatically increasing the occurrence of errors during spermatogenesis, similar to other chemical mutagens. The high-dose extracts had a deleterious effect on sperm differentiation, leading to sperm abnormalities. The high-dose extracts could change spermatogenesis and decrease or eliminate the viability of sperm cells. This indicates that the extracts contain components that can create damaged sperm cells, perhaps leading to infertility by hindering fertilization or causing mutations in zygotes, while also decreasing the number of healthy sperm cells.

Our previous studies regarding the subacute toxic dose of the extract showed that no toxicity was seen at doses equal to or below 57 mg/kg. The ethanol extract from Rhizophora apiculata bark exhibited hepatotoxicity in rats' livers at a dosage of 114 mg/kg during sub-chronic toxicity testing.[12] The results of this study strengthen the sub-acute toxicity test of the ethanol extract of Rhizophora apiculata bark by confirming that at a dose of 228 mg/kg BW, the extract can disrupt fertility parameters in male rats. With increasingly detailed preclinical research data regarding the ethanol extract of Rhizophora apiculata stem bark, this will increase the opportunity for this extract to be studied further in human clinical trials.

5. Conclusion

The subacute toxicity test determined that the hazardous dosage of the ethanol extract from Rhizophora apiculata stem bark against the spermatozoa of male Rattus norvegicus Sprague Dawley strain was 228 mg/kg BW.

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