In Vivo Cytotoxic Activity and Acute Toxicity Test of Ethanol Extract from *Voacanga foetida* (Blume) Rolfe Leaves

Adriani Susanty [1*], Emrizal[1], Emma Susanti[1], Alfionita BR Saragih[1], Husni Muchtar[2], Dachriyanus[2]

[1] *School of Pharmacy, Sekolah Tinggi Ilmu Farmasi Riau, 28293, Riau, Indonesia*
[2] *Faculty of Pharmacy, Universitas Andalas, 25163, West Sumatera, Indonesia*
[*]*Corresponding author. Email:* adrianisusanty@stifar-riau.ac.id

ABSTRACT

In vivo cytotoxic activity and acute toxicity test of ethanolic extract *Voacanga foetida* leave against male white mice was done. The in vivo cytotoxic activity used Micronucleus Assay Method while the acute toxicity used LD_{50} Test and Delay Toxicity Method. The micronucleus assay of *V. foetida* ethanol extract on the concentration of 100, 200, and 300 mg/kg BW with the observation of the amount of micronuclei cell of femur bone marrow of mice. The micronucleus assay on dose 200 mg/kg BW of the ethanol extract from *V. foetida* leaves was potential as anticarcinogenic activity. The LD_{50} of *V. foetida* ethanol extract for 24 hours of the animal was observed on the dose of 800, 1600, 3200, and 6400 mg/kg BW. The delayed toxicity test results, for the dietary intake, fecal, urine volume, organ weight ratio, and pathophysiologic of the spleen, heart, and liver through the qualitative and quantitatively against that organs. The results showed that the ethanolic extract of *V. foetida* was classified as not toxic with LD_{50} for 24 h > 15,000 mg/kg BW. The delayed toxicity test of *V. foetida* showed that this extract could decrease the daily dietary intake, water consumption, urine volume, fecal, body weight, and organ weight for 14 days of observation. The histopathologic test from dose 1600 mg/kg BW of *V.foetida* extract might be toxic against the heart and kidneys, but not toxic to the liver.

Keywords*: Voacanga foetida, cytotoxic, toxicity*

1. INTRODUCTION

Cytotoxicity is a substance that exhibits a process that results in cell damage or cell death. The prefix "cyto" refers to the cell and "toxic" to poison. This term is used to describe have the chemotherapy drugs with a mechanism of action to kill cancer cells, the cytotoxic also to describe the toxic properties. In our immune system, we have cytotoxic cells like T-cells; that kill bacteria, viruses, and cancer cells [1].

The *Voacanga foetida* (Apocynaceae) plant contains alkaloids such as voacangine, vobtusine, and vobtusine lactone has cytotoxic activity while vobtusine induces apoptosis via the mitochondria pathway [2].

Pharmacological studies have been carried out on *V. foetida*. According to these researchers, we know this plant has several pharmacological activities: analgesic [3], cytotoxic against colon cancer [4], antiproliferation on leukemia, lung, and cervical cancer [5], induces apoptosis, and inhibits the cell cycle in Leukemia cancer [2], and antimicrobial activity [6].

The cytotoxic activity of this extract is assumed by blocking the carcinogenic activity of cyclophosphamide. This research does on the erythrocyte cell of the bone marrow of mice. This research aims to evaluate the delayed toxicity for 14 days by observing the dietary intake, water consumption, urine volume, fecal weight, and heart, heart, and spleen. The histopathological preparation for evaluating the effect of this extract on organs microscopically.

2. MATERIALS AND METHODS

2.1. Materials

Centrifuge (Hettich Zentrifugen EBA 20®), *rotary evaporator*, micropipette, microscope (Griffin®), photo microscope, microtome (American optical®), incubator (Blue M®), and staining plate. Fresh leaves of *V. foetida*, dimethyl sulfoxide (DMSO), NaCMC, *calf bovine serum*, cyclophosphamide, phosphate buffer, May-Gruenwald solution (methylene blue + eosin Y + methanol), Giemsa solution (methylene blue + methylene azure + eosin Y + methanol), xylol, immersion oil, physiologic NaCl, Bouin solution, hematoxylin-eosin, alcohol, 100, 96, 90, 80, 70 and 50%, Mayer's albumin, paraffin, and entelan.

The male white mice (*Mus musculus*) (20-30 g) were divided into five groups, each with three animals of the male sexes. First for a group of negative control; second for positive control; third to fifth for doses 100, 300, and 900 mg/kg BW. The animals were acclimatized for a week before the experiment. Cyclophosphamide (50 mg/kg BW) was used as the standard drug.

2.2. In Vivo Cytotoxic Test

The sample and Calf Bovine Serum were prepared according to Micronucleus Assay Test [7, 8], with slight modification. Samples at dose 100 mg/kg of body weight were suspended with Na CMC 1%, and added volume to 10 mL. The same procedure was done for doses 200 and 300 mg/kg BW).

Group I (negative control) of animals were fed with NaCMC 1% for two days orally. Group II (positive control) on the second day, fed with cyclophosphamide at dose 50 mg/kg BW intraperitoneally. Meanwhile, III, IV, and V groups were fed with extract at various doses (100, 200, and 300 mg/kg BW) orally. On the second day fed with cyclophosphamide (50 mg/kg BW) intraperitoneally. After 30 hours, the animal was killed by neck dislocation, and its bone marrow was collected.

The micronucleated cells are indicated by dark-colored cells, while normal cells are light-colored. The number of micronucleated cells was counted as 200 cells. Each mouse was made one bone marrow smear and each smear was made 2 times observations to reduce errors in calculations. Observations were made using a microscope with a magnification of 10 × 100 with the help of immersion oil.

2.3. Toxicity Analysis

2.3.1 Toxicity Acute Test

The method was prepared according to the LD_{50} (median lethal dose) test [9, 10]. Animals (20-30 g) were divided into six groups, each with three animals of the male sex. The first group for negative only feed with NaCMC 1%; second to fifth for extract doses 800, 1600, 3200, 6400, and 15000 mg/kg BW. The extract samples were fed orally and volume of administration according to this formula:

$$V\ (mL) = \frac{\text{Dose (mg/kg of BW) x Body Weight (kg)}}{\text{Concentration (mg/mL)}}$$

Where;

V = volume of drugs administration

2.3.2 Evaluation of Delayed Toxicity for 14 Days

This evaluation was carried out with the Paraffin Method [11, 12]. This method was accounted for the evaluation of the body weight, water consumption, urine volume, the weight of heart, spleen, and lever ratio of the animal testing.

2.3.3 Evaluation of Heart, Liver, and Kidneys

This evaluation is known as the Paraffin method [13,14], including the evaluation of histological preparation microscopically and counting for destroying of vena centralized and glomerulus of kidneys of animal testing.

2.4. Data Analysis

The micronucleus assay test data were analyzed using the SPSS one-way ANOVA followed by the Post Hoc Tukey test. LD_{50} calculation is done by the Probit Analysis Method. Parameter data of body weight, drinking water consumption, food consumption, and urine volume were performed using two-way ANOVA, while relative organ weights were performed statistically with one-way ANOVA and Tukey's follow-up test.

3. RESULTS AND DISCUSSION

In vivo cytotoxic test gave the percentage of micronuclei cell of positive and negative controls are 66 % and 18.5 % respectively, and animal test at doses 100, 200 and 300 mg/kg BW are 31.7; 23.6 and 20.5% respectively (**Table 1.**) The micronucleus assay test aims to compare the number of micronuclear cells with the number of normal cells, as an indicator of the ability of the test compound to protect the carcinogenic effect of cyclophosphamide. This study began by taking the femur bone marrow of mice and then making smear preparations using May-Gruenwald dye and Giemsa's solution, under a microscope the difference between micronuclear cells and normal cells was seen. The result of this research is that the test compound has the potential as an anticancer at a dose of 200 mg/kg BW which inhibits the carcinogenic effect of cyclophosphamide to normal conditions $p<0.05$.

Acute toxicity is an effect that occurs after exposure to the extract in a single dose within 24 hours given orally. The extract with doses 800, 1600, 3200, and 6400 mg/kg BW showed no lethality for 24 hours of treatment. The LD_{50} test is the dose of the test substance

that causes death in 50% of test animals, this estimates the potential side effects of the test extract on humans.

The acute toxicity test of *V.foetida* extract result is the LD_{50} > 15,000 mg/kg, the classification is relatively harmless. The substance with LD_{50} below 5 mg/kg is classified to be highly toxic while substances with LD_{50} >15,000 mg/kg are termed relatively harmless [11]. The LD_{50} as a result of the acute toxicity test is used as a dose determination in long-term toxicity testing in test animals [11]. The LD_{50} value as the result of the acute toxicity test was used to conclude the toxicity calcification of the test extract.

Although the main variable observed was mortality, acute non-lethal effects may occur as a sign of toxicity depending on the dose and content of the active compounds in the extract. The acute toxicity test aims to assess the potential acute toxicity of the extract as a basis for determining the side effects of the use of the extract in the short term (24 hours)[15]. The *V.foetida* extract with this concentration showed the side effects such as decrease of activity, decrease water consumption, deep breath, decrease dietary intake, sleepness, and at a dose of 6400 mg/kg BW exerted red eyes, defecation, and darkness of fecal, and then feet paralyzed.

A delayed toxicity test was performed by observing changes in diet, drinking, amount of stool, amount of urine, body weight, and relative organ weight of the heart, liver, and kidneys over 14 days. Histological preparations were made using hematoxylin-eosin dye so that information was obtained on how much the test compound damaged the liver, kidneys, and heart tissue microscopically. The test compound did not cause death within 24 hours of observation but caused toxicity to the kidneys, liver, and heart after the test compound was in the body for 14 days at very high doses, starting at a dose of 1600 mg/kg BW up to a dose of 6400 mg/kg BW.

The changing of body weight of animal test gave until 14 days result significantly $p<0.05$. It was proven by there is an interaction between dose and time with $p<0.05$. The histogram in **Figure 1**, showed the difference of body weight at dose 6400 mg/kg BW with positive control. Decrease of body weight significant statistically $p<0.05$, that depending on the dietary intake. From this following histogram image, it can be seen that a dose of 6400 mg/kg BW showed a very significant difference when compared to the control group.

Observation of food consumption in control and treatment showed a decrease in food consumption after administration of ethanol extract depending on doses **(Figure 2)** with a p-value <0.05. This result is in line with the observation that the bodyweight of experimental animals decreased depending on the dose as well. The stool weight of experimental mice was significantly affected by dose and time at $p<0.05$. Changes in average fecal weight due to the administration of ethanolic extract of *V. foetida* leaves at doses of 800 and 1600 mg/kg BW were not significantly different from the control, while the weight of fecal gave $p<0.05$, only at dose 3200 dan 6400 mg/kg of body weight compared to a positive control (**Figure 5)**. Drinking water consumption was significantly influenced by dosage and time of preparation with a p-value <0.05, there was a decrease in drinking volume with increasing dose. This result showed in **Figure 3**. The urine volume of experimental mice was dose and time-dependent significantly where $p<0.05$. This observation is in line with the observation of drinking water volume where the decrease in urine volume is influenced by the decrease in the drinking volume of experimental animals (**Figure 4).**

Evaluation of liver, heart and kidneys organ relative ratio is shown in **Figure 6**. The ratio of relative liver weight was significantly affected by extract administration, $p<0.05$. Liver weight ratio decreased with increasing dose of extract. The ratio of the relative weight of the heart was significantly affected by the extract, $p<0.05$. The ratio of relative organ weight of the heart is due to the administration of the extract. The relative weight ratio of the kidney was not significantly affected by the administration of the extract, $p>0.05$. The ratio of kidneys organ weight in the control group was greater when compared to the treatment.

In the observation of the liver, heart, and kidneys organs morphology, it was seen that the color of the organ did not show a significant difference between the control mice and the treated mice that were given with extract. Microscope observation of the liver was shown in **Figure 7a-7e**. The positive control, treated with extract of *V. foetida*. Vena cell wall clearly with endothelial cell in normal arrangement and complete, hepatocyte and sinusoid in normal form and nucleus are rounded **Figure 7a**. While, in the animal test, treated with extract at dose 800, 1600, 3200, and 6400 mg/kg of BW, respectively at **Figure 7b-7e,** administration of ethanol extract at a dose of 800 mg/kg BW, it was seen that the hepatocytes still regularly surrounded the central vein, but at doses of 1600, 3200, and 6400 mg/kg BW, the hepatocyte arrangement began to be irregular. Observation of the nucleus of the control mice and the mice that were given the ethanolic extract did not show a clear difference and the nucleus looked round.

The cytoplasm in the liver tissue of control mice was bright and clear. From the observation of the cytoplasm treated at a dose of 800 mg/kg BW, there was no difference with the control, while at doses of 1600, 3200, and 6400 mg/kg BW, the cytoplasm began to undergo lysis. Sinusoids in the liver tissue of control mice were intact with a flat shape and were located between the hepatocyte plates. Observations on the liver at doses of 1600, 3200, and 6400 mg/kg BW showed that the sinusoids were slightly enlarged when compared to the

liver of control mice. And the treatment with the dose did not change when compared to the control.

Histopathologic of heart mice **Figure 8.** Observations on the control showed that the endothelial cells around the veins and arterioles were completely circular and arranged in an orderly manner **(Figure 8a).** Animal tests **(Figure 8b-8e)**, treated with various doses of extract did not show any significant difference when compared to the heart tissue of control mice. In the heart tissue of control mice, it was seen that the cardiac muscle cell walls were arranged lengthwise with a round nucleus located in the middle of each cell. Observation of the heart tissue of mice given with the ethanolic extract at various doses did not show any difference in the shape of the myocardium when compared to the control group. However, at doses of 1600, 3200, and 6400 mg/kg BW, the myocardium began to thicken.

Observation of kidneys' histopathology characterization with Glomerulus, Bowman's space, proximal tubule, distal tubule conditions **(Figure 9).** In the kidneys tissue of control mice, it was seen that the glomerulus was surrounded by Bowman's capsule to form the urinary space **(Figure 9a)**. In conditions of glomerular damage, it is characterized by the condition of the Bowman's capsule gap (urinary space) being narrow. In this study, at doses of 800 and 1600 mg/kg BW, it can be said that Bowman's space has not narrowed **(Figure 9b and 9c)**, but at doses of 3200 and 6400 mg/kg BW, the Bowman's capsule gap and the urinary space are already narrow, the parietal and visceral layers are fused **(Figure 9d and 9e)**.

The proximal tubule is lined by simple cuboidal or columnar epithelial cells. These tubules have a lumen and are surrounded by peritubular capillaries. From microscopic observations, it was seen that there were no changes in the proximal tubules of the kidneys tissue of the mice in the treatment group that was given a dose variation when compared to the mice in the control group. The distal tubule has a larger lumen than the proximal tubule. The cells are smaller and cabbage-shaped. From microscopic observations, it was seen that there were no changes in the distal tubule of the kidneys tissue of the mice in the treatment group that was given a dose variation when compared to the mice in the control group.

4. CONCLUSION

Ethanolic extract of the *V. foetida* leaves at a dose of 200 mg/kg BW is effective in inhibiting carcinogenesis by the reduction of the number of micronuclear cells (have an anticarcinogenic effect) and did not cause toxicity, because the results of the delayed toxicity test at a dose of 800 mg/kg BW did not cause significant damage to the kidneys, liver, and hearts. The ethanol extract of *V. foetida* leaves has the LD_{50} > 15,000 mg/kg BW and long-term use at high doses >1600 mg/kg BW have side effects on the liver, heart, and kidneys.

5. FIGURES AND TABLES

Figure 1 Histogram of average body weight of male white mice at several doses of ethanolic extract of *V. foetida*.

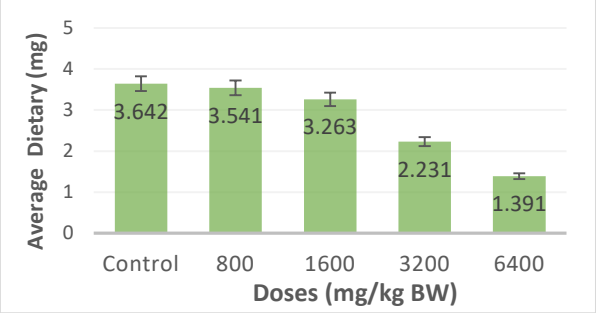

Figure 2 Histogram of an average dietary intake of male white mice at several doses of ethanolic extract of *V. foetida*.

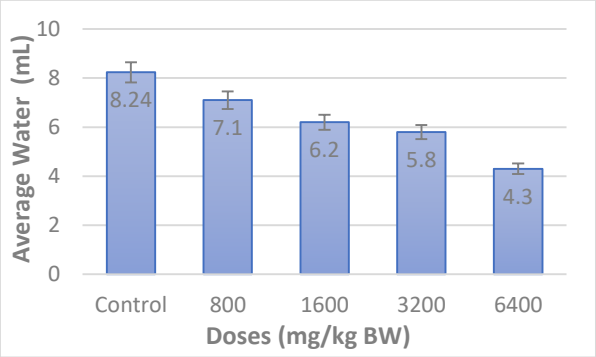

Figure 3 Histogram of water consumption of male white mice at several doses of ethanolic extract of *V. foetida*.

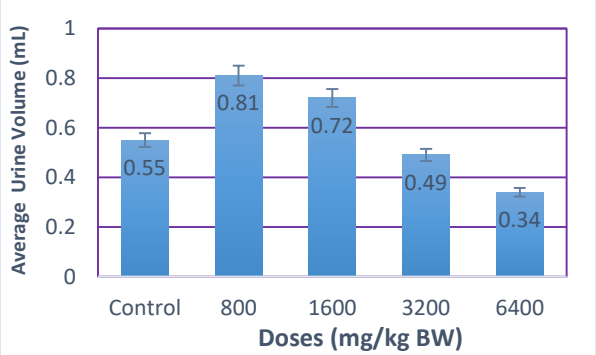

Figure 4 Histogram of the water volume of male white mice at several doses of ethanolic extract of *V. foetida*.

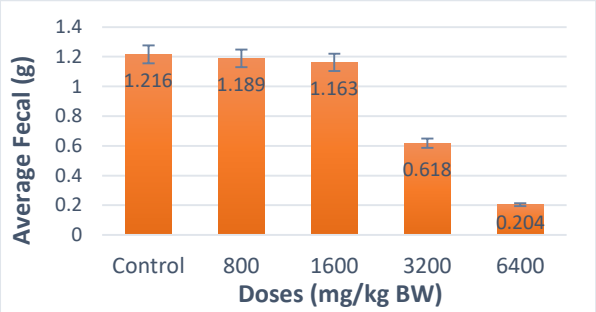

Figure 5 Histogram of fecal weighing of male white mice at several doses of ethanolic extract of *V. foetida*.

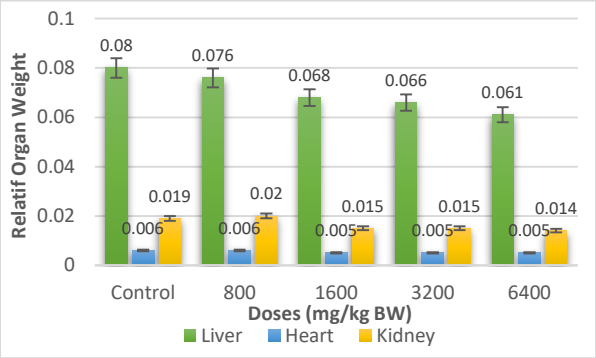

Figure 6 Histogram of the ratio of the liver, heart, and kidneys weight of male white mice at several doses of ethanolic extract of *V. foetida*.

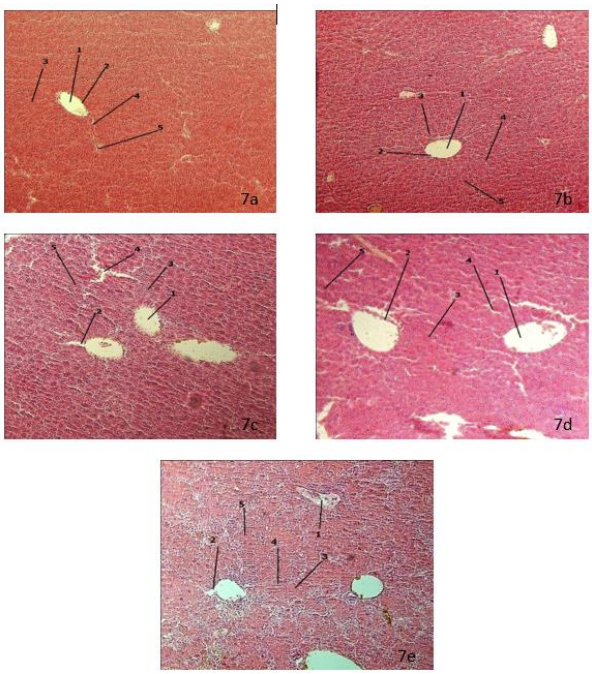

Figure 7 Histopatology of liver mouse (100 X). 1. Vena centralized 2. endothelium, 3. hepatocyte, 4. Sinusoid, 5. nucleus. Control Observation of intact central vein walls, with tightly arranged endothelial cells, regular hepatocytes forming plates, normal sinusoids, and round cell nuclei, the results of this study showed that there was no difference between positive controls and treatment at a dose of 800 mg/kg BW (**Figure 7a and b**), but at doses of 1600, 3200 and 6400 mg/kg BW, the walls of the central veins began to be incomplete, with endothelial cells lysis, hepatocytes irregularly forming plates, enlarged sinusoids, and round cells nuclei (**Figure 7c-e**) and different conditions with positive control.

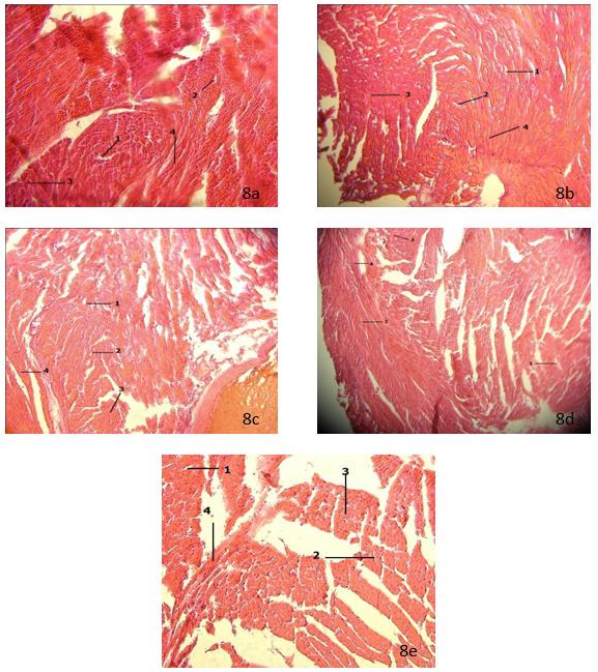

Figure 8 Histopatology of heart mouse (100 X). 1. Vena, 2. Arterial, 3. Longitudinal muscular cell wall, 4. Transversal muscular cell wall. **(8a)** The positive control, treated with ethanolic extract of *V. foetida*. Endothelial cell rounded by vena and arterial, muscular cell wall was seen clearly, in normal arrangement and complete. **(8b-8e)** Animal test, treated with ethanolic extract of *V. foetida* at dose 800, 1600, 3200, and 6400 mg/kg BW, respectively. All endothelial cells rounded by vena and arterial, muscular cell wall was seen, in normal arrangement, and completed.

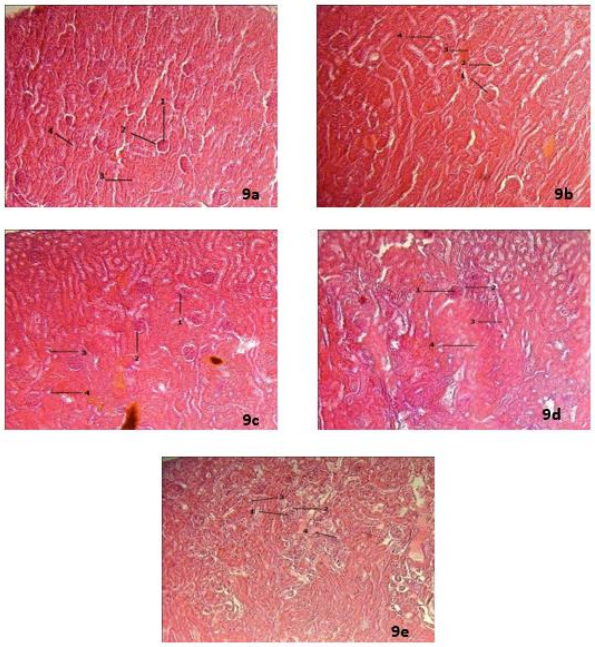

Figure 9 Kidneys histopathology of male white mice due to the administration of ethanolic extract of *V. foetida* leaves at doses of 800, 1600, 3200, and 6400 mg/kg BW (H-E staining, 100X magnification). 1. Glomerulus, 2. Bowman's space, 3. Proximal tubule, 4. Distal tubule. Control **(9a)** and sample at doses of 800 **(9b)** and 1600 mg/kg BW **(9c)** the glomerulus is surrounded by Bowman's capsule which forms Bowman's space, the proximal and distal tubules are visible, but at doses of 3200 **(9d)** and 6400 mg/kg BW **(9e)** normally the Bowman's space has begun to lyse so that the glomerulus has almost no Bowman's space.

Table 1. Micronuclear cell percentage data treated with various doses of ethanol extract of the leaves of the plant *V. foetida*

Samples	Micronuclei Percentage (%)
Negative Control	18.5
Positive Control	66
100 mg/kg BW	31.7
200 mg/kg BW	23.6
300 mg/kg BW	20.5

Tukey Test, $p < 0.05$

AUTHORS' CONTRIBUTIONS

Adriani Susanty, toxicology test, Prof. Dachriyanus, and Husni Muchtar are my supervisors, while Alfionita is my student with Emma Susanti contribution at the micronucleus assay test, and Emrizal contributed to extract preparation.

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