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Original Research Article

Toxicity Assessment of Ethanol Extract of *Lygodium microphyllum*

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ABSTRACT

Lygodium microphyllum is an invasive plant with various pharmacological activities, but information on its toxicity is limited. Therefore, this study aims to determine the toxic effects of acute oral administration of- ethanol extract of *Lygodium microphyllum* (ELM) in mice. Female mice (n = 30) were divided into six groups, namely; Negative control (KN), ELM 1 (300 mg/kg BW), ELM 2 (625 mg/kg BW), ELM 3 (1250 mg/kg BW), ELM 4 (2500 mg/kg BW), and ELM 5 (5000 mg/kg BW). ELM was administered as a single oral dose. The number of deaths, and signs of toxicity were observed at 30, 60, 120, 180, 240 min, then every 24 h for 14 days. Body weights were measured every 2 days for 14 days. On day 15, the mice were sacrificed and vital organs were harvested for the measurement of organ index. Histological examination of the liver was done according to standard procedure. No death and no sign of toxicity was recorded in the mice within 14 days following ELM administeration. The body weights of the mice did not change significantly ($p > 0.05$) after ELM administeration. There were no significant differences in the relative organ indices of the kidneys, stomach, heart, lungs, brain, intestines, and liver among the various groups. Histological examination of the liver showed mild to moderate changes in liver architecture following treatment with ELM. Overall, ELM may be regarded as practically non-toxic ($LD_{50} > 5000$ mg/kg BW), and relatively safe on acute oral administration.

***Keywords*:** *Lygodium microphyllum*, Krokot herbs, Ethanol extract, Toxicity.

Introduction

Lygodium microphyllum known as Krokot herb is a plant of the genus Lygodium and the division pteridophyta (ferns). It is an invasive plant, considered as a weed, nuisance or harmful plant. Krokot herb is widely distributed in tropical Africa, Southeast Asia including Indonesia, Australia, and some South Pacific islands. Krokot herb can be found creeping or climbing on other plants.[1] The stem of the plant is used by the locales for making rope and baskets, in West Kalimantan, the plant is used in traditional medicine for the treatment of a number of ailments including dysentery, cough, fever, hepatitis, cancer, and malaria.[2] An infusion of Krokot herb is used as a remedy for fever, muscle pain, dysentery, and cancer.[3] The plant is also employed in the treatment of skin diseases,[4] and for digestive disorders such as flatulence.[5]

A number of studies relating to the pharmacological effects of Krokot herb have been conducted. In an *in silico* study by Anggreini *et al*. (2023),[6] *Lygodium microphyllum* phytosterols were docked with Sirtuin 1 (SIRT1) and 5' adenosine monophosphate-activated protein kinase (AMPK), which are important proteins in lipogenesis pathway, two of the phytosterols; β-sitosterol and stigmasterol interacted with the allosteric binding sites of SIRT1 and AMPK. In the same study, an *in vitro* toxicity tests using the brine shrimps lethality test (BSLT), the methanol extract of *Lygodium microphyllum* showed moderate toxicity.[6]

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In another study, the analgesic, antipyretic, anti-inflammatory, antidiarrheal, and anthelminthic properties of the methanol extract of Krokot herb was demonstrated *in vivo*, *in vitro*, and *in silico*.[7] The aqueous extract of *Lygodium microphyllum* has been shown to have strong hepatoprotective effect against CCl_4-induced oxidative stress as well as a good antioxidant effect.[8] The ethyl acetate fraction of *Lygodium microphyllum* has been shown to have antimalarial effect against *Plasmodium falciparum in vitro*.[2] An infusion of Krokot herb was reported to reduce malondialdehyde (MDA) levels in experimental animals.[9] The flavonoid glycosides; quercetin 3-*O*-β-D-glucopyranoside and kaempferol-3-*O*-β-D-glucopyranoside isolated from Krokot herb have been reported to possess antifungal activity against *C. albicans* and *A. niger in vitro*.[1] The sunscreen activity of Krokot herb ethanol extract expecially the ethyl acetate fraction as also been demonstrated.[10]

Despite the numerous pharmacological activities of *Lygodium microphyllum*, only a few studies on the toxicity of the plant have been reported. For example, Anggreini *et al*. (2023)[6] reported moderate toxicity of 70% ethanol extract of *Lygodium microphyllum* against brine shrimp, whereas, the infusion of *Lygodium microphyllum* has been reported to show no toxic effect on brine shrimp.[3] Furthermore, the methanol extract of *Lygodium microphyllum* was said to cause no sign of toxicity or death in swiss albino mice following an acute oral adminisreation.[7] However, the *in vivo* toxicity of the ethanol extract of *Lygodium microphyllum* is yet to be investigated. Therefore, the present study aims to assess the toxicity of ethanol extracts of *Lygodium microphyllum* (Krokot herb) in mice.

Materials and Methods

Collection and identification of plantt material

Approximately 5 kg of *Lygodium microphyllum* (Krokot herb) was collected from Sambutan District in April, 2022. The plant material was identified by Atiek Retnowati, and an herberium specimen was stored at Herbarium Bogoriense, Biology Research Center, Indonesian Institute of Sciences, Indonesia, and authenticated with a voucher specimen number 750IPH101. The fresh plant material was washed,

cut into small pieces, and then air-dried under a shade. The dried plant material was sorted to remove unwanted parts, and debris, after which it was pulverized using a mechanical grinder. The powdered plant sample was weighed and then stored in an air-tight container until ready for use.[11]

Extraction of plant sample
The powdered plant sample was extracted by maceration in 70% ethanol (at a plant sample to solvent ratio of 1:10) at room temperature for 24 h. The extract was filtered and the residue was remacerated three times. The combined filtrate was evaporated using a rotary evaporator at 45℃ to obtain a concentrated ethanol extract of *Lygodium microphyllum* (ELM).[11]

Animals
Thirty (30) DDY strain of mice (6-8 weeks old) weighing between 20-30 g were acclimatized to the laboratory condition (temperature: 22 ± 3℃, relative humidity: 30-70%, lighting: 12 h light 12 h dark cycle) for one week. The animals were allowed access to feed and drinking water *ad libitum*. The experiments were conducted at the Pharmaceutical Research and Development Laboratory, Farmaka Tropis, Faculty of Pharmacy, Mulawarman University in accordance with the Regulation of Food and Drug Administration Number 7 of 2014 of the Republic of Indonesia. The experimental protocol was approved by the Ethics Committee of the Faculty of Pharmacy, Mulawarman University with ethical approval number 00472164722111320230824211.

Acute toxicity test
The acute toxicity test of the plant extract was done following standard procedures.[12,13] Thirty mice were divided into six groups of 5 animals each. The groups consist of a negative control group (KN) which was administered sodium carboxymethyl cellulose (Na CMC) 0.1%, and five test groups which were administered the plant extract as follows; ELM 1 (300 mg/kg BW), ELM 2 (625 mg/kg BW), ELM 3 (1250 mg/kg BW), ELM 4 (2500 mg/kg BW), and ELM 5 (5000 mg/kg BW). The Na CMC and extract were administerd orally as a single dose. The mice were observed for mortality and signs of toxicity including tremors, convulsions, diarrhea, lethargy, coma, and piloerection for the first 30, 60, 120, 180, and 240 minutes of administration. Thereafter, observations were made every 24 h for 14 days. Body weights were monitored on days 0, 2, 4, 6, 8, 10, 12, and 14. Then on day 15, the animals were sacrificed by cervical dislocation and organ indices of kidney, stomach, heart, lung, brain, intestine, and liver were evaluated. Histological examination of the liver was also done.

Mortality and LD_{50} determination
The number of deaths that occurred after the 30th, 60th, 120th, 180th, and 240th minutes of extract administration, and each day for 14 days was recorded. After 14 days, the median lethal dose (LD_{50}) was calculated according to the Miller and Tainter method (probit).[13]

Measurement of body weight
Body weight measurements were taken on days 0, 2, 4, 6, 8, 10, 12, and 14. Body weight were presented in the form of images processed using Microsoft excel application and the data obtained were statistically analyzed using SPSS.[13]

Measurement of organ index
After 14 days, vital organs (kidney, stomach, heart, lung, brain, intestine, and liver) were harvested and their weights determined. Organ index was calculated in form of percentage (%) in relation to the body weight using the formula below.[14,15]

$$\% \text{ Organ index} = \frac{Organ\ weights\ (gram)}{Body\ weights\ (gram)} \times 100 \qquad \text{Equation 1}$$

Observations for signs of toxicity
Observations for signs of toxicity such as tremors, convulsions, diarrhea, lethargy, coma and piloerection were made at the 30th, 60th, 120th, 180th, 240th minute after treatment and then once daily for 14 days. Data were presented in tabular form and then analyzed descriptively.[13]

Histological examination
Histological examination of the liver was performed according to standard procedures.[14,16] The harvested liver were washed sequebtially in 70%, 80%, 90%, 95%, and 100% alcohol. The liver was cleared with absolute xylol followed by embedding in paraffin pastille that has been liquefied at 60℃ (this was done 3 times), the organ was frozen followed by refrigeration. The liver was cut with a microtome into a thickness of 4-6 microns, then transferred into a container of distilled water and warmed in a water bath at 40℃, then placed on a glass slide and dried in an incubator at 40℃. The dried hepatic tissue was stained with Haematoxylin Eosin stain, and observed under a microscope with one field of view at 400x magnification. Histological data obtained were displayed in tabular form in Microsoft excel application and data were analyzed using SPSS. Scoring was done using the Mordue method as follows; score 0: No damage, Score 1: Damage < 25% (mild), Score 2: Damage 25% - 50% (moderate), Score 3: Damage 50% - 75% (severe), Score 4: Damage > 75% (very heavy).[17]

Statistic analysis
Data were analyzed using IBM SPSS Statistics 23. Data were tested for normality using Shapiro-Wilk. Comparison between values was done using Kruskal Wallis non-parametric statistics followed by Pairwise comparison post-hoc test.

Results and Discussion

Mortality (LD_{50})
The mortality data (LD_{50}) in mice administered ELM are shown in Table 1. Median lethal dose (LD_{50}) is the dose that can kill 50% of the test animals. LD_{50} in toxicity testing can be determined by treating experimental animals with varying doses or multiple doses of test sample. The doses used should be at least 3 different doses. The maximum tolerable dose is the highest dose that does not cause death while minimum lethal dose is the lowest dose that can cause death in all the test animals. If up to a dose of 5000 mg/kg does not cause death, then the test dose does not need to be increased further.[12] Acute toxicity studies are conducted to determine the short-term adverse effects of a drug when administered in a single dose, or in multiple doses over a 24-hour period in mammalian (rodent) species. Acute toxicity studies provide information on the potential for acute toxicity in humans, prediction of safe acute doses for humans, target organs with potential toxicity, time course of drug-induced pharmacological effects, appropriate doses for multiple dose toxicity studies, and species differences in toxicity.[18] Acute toxicity testing using the median lethal dose (LD_{50}) is done to determine the effect of administering a single dose of a compound to animals and to assess the acute safety of a drug or substance to be used.[19]
Observations of the number of deaths were made at 30, 60, 120, 180, 240 minutes, and every day for 14 days. This periodic observation aims to see the time it takes for the test sample to have an effect on the test animals. Based on the results of the study as presented in Table 1, ELM did not cause death of the test animals at all doses tested, so the extract do not have the potential to cause harm on acute administration. The maximum dose that does not cause death of the test animals is regarded as the pseudo LD_{50}, while the apparent LD_{50} is the highest dose that can technically still be given to the test animals.[20] Based on the BPOM Regulation (2014) on the guidelines for *in vivo* nonclinical toxicity tests, if a test dose of 5-15 g/kg does not cause death, then the the test substance is practically non-toxic.[12] The results of the present study are in agreement with the study of Alam *et al*. (2021)[7] where up to 3000 mg/kg dose of *Lygodium microphyllum* methanol extract did not cause any sign of toxicity or death in swiss albino mice.

Effect of ELM on body weight
The effect of ELM on the body weight of mice are shown in Figure 1. Determination of the body weight of test animals serves to observe changes in body weight in real time, and it is the most easily visible

indicator of the effect of the treatment used.[21] In addition, body weight can also be used as an early indicator of the toxic effect of the test sample.[22] The test substance or sample has significant adverse effects on an animal if there is a decrease in body weight of more than 10% from the initial weight prior to the administration of the test sample. This may occur as result of a decreased appetite, which causes weight loss in the animal following the treatment with the test substance.[23]

Based on the results of the body weight determination, there was an increase in body weight in the KN (Na CMC 0.1%), ELM 1 (300 mg/kg BW), ELM 3 (1250 mg/kg BW), and ELM 4 (2500 mg/kg BW) groups every day until day 14. While in the ELM 2 (625 mg/kg BW) group, there was a decrease in body weight on days 2 and 12 but the decrease was not up to 10% of the body weight before testing, then increased until day 14. For the ELM 5 (5000 mg/kg BW) group, there was a decrease in body weight on days 2 and 6, but the decrease was less than 10% of the initial body weight prior to testing, then increased until day 14. The results shows that there is no weight loss of more than 10% which indicates that ELM has no toxic effect on the test animals.

Among all the groups, there was no significant changes in the body weight of the mice ($p > 0.05$) which indicates the administration of ELM has no significant effect on the body weight of mice, although body weight tends to increase for the ELM 5 group after the 6th day. This may be attributed to the flavonoids content of ELM,[6] which according to Krisna *et al*. (2022),[24] flavonoids play a role in increasing appetite, resulting in an increase in body weight. The findings from this study is also in agreement with the results of the study by Alam *et al*. (2021)[7] who observed no change in body weight of mice after administration of 3000 mg/kg BW of methanol extract of *Lygodium microphyllum*.

Table 1: Mortality data in mice administered ELM

Group	Number of Animals	Number of Deaths	Log Dose	% Mortality	Probit	Pseudo LD_{50}
KN (p.o)	5	0	0	0	0	> highest dose
ELM 1 (p.o)	5	0	2.477	0	0	5000 mg/kg BW
ELM 2 (p.o)	5	0	2.795	0	0	
ELM 3 (p.o)	5	0	3.097	0	0	
ELM 4 (p.o)	5	0	3.398	0	0	
ELM 5 (p.o)	5	0	3.699	0	0	

ELM: Ethanol extract of *Lygodium microphyllum*

ELM: Ethanol extract of *Lygodium microphyllum*

Effect of ELM on organ index

Figure 2 shows the effect of ELM on the organ index of mice. The organ index is used as an indicator of a toxic effect of a test sample on organs. It only indicates an enlargement or shrinkage of an organ, and cannot be used as a standard in determining organ damage.[25] The major target organ of toxicity test are the kidneys because they have an essential function of collecting, detoxifying and eliminating xenobiotics from the body.[26] In addition to the kidneys, other vital organs like the stomach, intestine, heart, lungs, liver, and brain were also invested in this study. The stomach plays a role in the absorption of xenobiotics into the systemic circulation.[27] The heart may easily be invaded by toxic substances, which may lead to the circulation of blood containing toxic substances throgh the body.[28] The lungs play a role in respiration and also express enzymes involved in xenobiotic metabolism such as cytochrome P450,[29] and toxicity of the lungs may affect oxidative metabolism. Toxic substances may cross the blood-brain barrier and directly act on certain neurons in the brain resulting in neurological damage.[30] The intestine has intestinal microbes that play a role in metabolizing xenobiotics and provide some immunity.[31] The liver plays a role in complex metabolism such as bioactivation and detoxification. The detoxification process in the hepatic cells converts toxic substances to less toxic or non-toxic metabolites.[27]

Statistical analysis showed no significant difference in the average organ index for all the organs in each of the groups ($p > 0.05$).

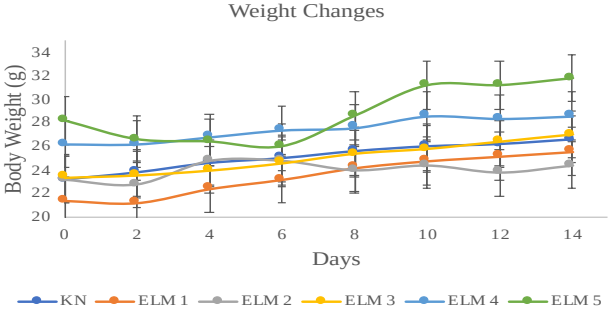

Figure 1: Effect of ELM on body weight of mice. Data represents mean ± SD.

Toxicity signs after ELM administration

Signs of toxicity monitored include; tremors, seizure or convulsions, diarrhea, lethargy, coma, and piloerection. Tremor is an unconscious repetitive vibration-like movement of a body part caused by alternating contractions of opposing muscles.[32] Seizure is a condition in which there is an excessive electrical activity caused by the hyperpolarization of the cell membrane, a decrease in the inhibitory neurotransmitter GABA, or an increase in the excitatory neurotransmitter glutamic and aspartic acids.[33] Diarrhea may occur as a result of toxin-induced damage to intestinal mucosal tissue and may also occur as a mechanism to remove foreign objects that enter the body.[34] Lethargy (somnolence) is the condition where the experimental animal appears drowsy, but can still be awakened by sound or pain stimuli, and easily falls asleep again. Coma is a severe disorder of consciousness and unresponsiveness to pain. Piloerection is a state where the fur becomes erect and hard caused by reflex contractions of small muscles at the base of the hair in the skin. Piloerection is controlled by sympathetic nerves that innervate the arrector pili muscle (APM) and function as a temperature regulator.[35,36]

In the present study, none of the above mentioned signs was observed in the mice in both the control and ELM treated groups, which suggest that ELM no toxic effect on mice on acte oral administration. These observations again corroborate the findings from Alam *et al*. (2021)[7] study where no sign of toxicity was observed in mice following the administration of methanol extract of *Lygodium microphyllum*.

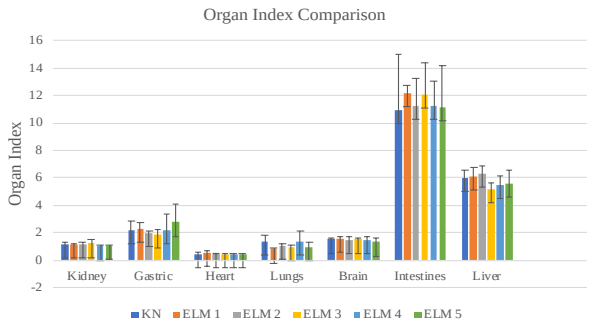

Figure 2: Organ Index of mice administered ELM. Each bar represents mean ± SD (n = 5). ELM: Ethanol extract of *Lygodium microphyllum*

Effect of ELM on liver histology

The histological features of the liver of mice following acute administeration of ELM are shown in Figures 3 and 4. On histological examination, the liver tissues were observed for degeneration, necrosis, and inflammation. From the results obtained, there were different degrees of hepatic damage to the mice administered ELM. Normal hepatocytes are characterized by radial arrangement of cells to the central vein, round and oval cell shapes, and conspicous plates of hepatocytes. Hepatic cells appear to have one nucleus, but some may have more than one nucleus (binucleate) located in the center of the cell.[37]

The KN group (Na CMC 0.1%) had an average score of degeneration 1 (mild), necrosis 1 (mild), and inflammation 1 (mild). The negative control group mice were found to have hepatic damage, presumably already suffering from infection, or other disorders such as malnutrition, hypoxia, and aging before testing.[38] The ELM 1 (300 mg/kg BW) group had an average score of degeneration 2 (moderate), necrosis 1 (mild), and inflammation 1 (mild). ELM 2 (625 mg/kg BW) had an average score of degeneration 2 (moderate), necrosis 1 (mild), and inflammation 2 (moderate). ELM 3 (1250 mg/kg BW) had an average score of degeneration 1 (mild), necrosis 0 (did not occur), and inflammation 1 (mild). ELM 4 (2500 mg/kg BW) had an average score of degeneration 2 (moderate), necrosis 1 (mild), and inflammation 2 (moderate). Lastly, ELM 5 (5000 mg/kg BW) had an average score of degeneration 1 (mild), necrosis 1 (mild), and inflammation 2 (moderate). These observations suggest that ELM may be hepatotoxic. However, statistical analysis showed no significant difference in the hepatic damage among the various groups.

The hepatic damage to the mice following extract treatment or otherwise can occur due to several factors such as environmental factors, food, and other health challenges. In a previous report by Kurniawan *et al*. (2014),[38] it was found that untreated group of mice presented with hepatic damage, which was thought to have occured as a result of infection, or other disorders such as lack of nutrients, hypoxia, as well as aging. Damage may also be caused by secondary metabolites contained in the test sample. ELM was extracted using 70% ethanol and has been shown to contain steroids, phenols, flavonoids, alkaloids, and saponins.[6] Secondary metabolites that are thought to cause hepatocellular damage are alkaloids.[40] Alkaloids are metabolically toxic because they are not readily metabolized and excreted from the body, so the alkaloids will have a longer contact time with hepatic cells resulting in hepatocellular damage.[41]Exposure to high concentrations of alkaloids over a long period can cause hepatocellular enlargement due to lipid accumulation.[42] The toxic effects of alkaloids is debatable because some studies conducted on alkaloids show beneficial effects, while others show that these compounds can damage cells and tissues and even cause death in experimental animals.[42] The observed variation in results obtained from different studies can be related to differences in the geographical location and the stage of maturity of the plants, which may alter the kind and amount of phytoconstituents present.[44]

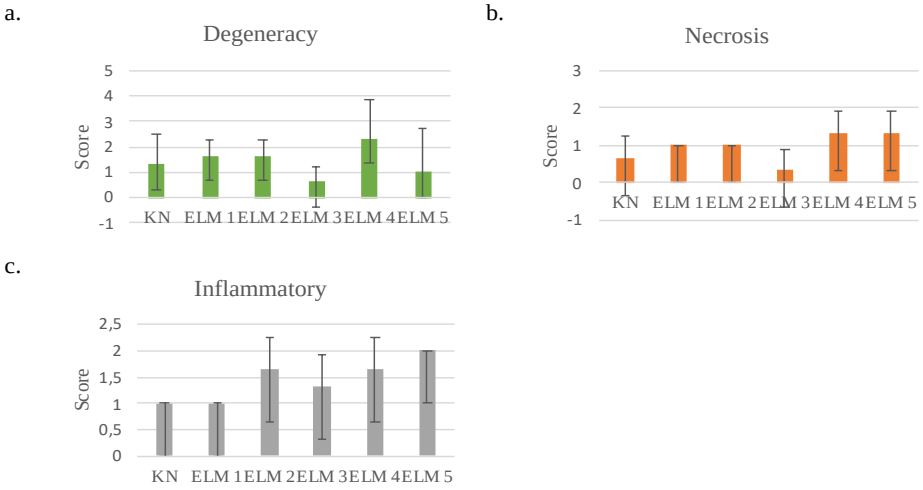

Figure 3: Hepatoxic effect of ELM showing scores for degeneration (a), necrosis (b), and inflammation (c). Each bar represents the mean score based on the Mordue method ± SD (n = 3). ELM: Ethanol extract of *Lygodium microphyllum*

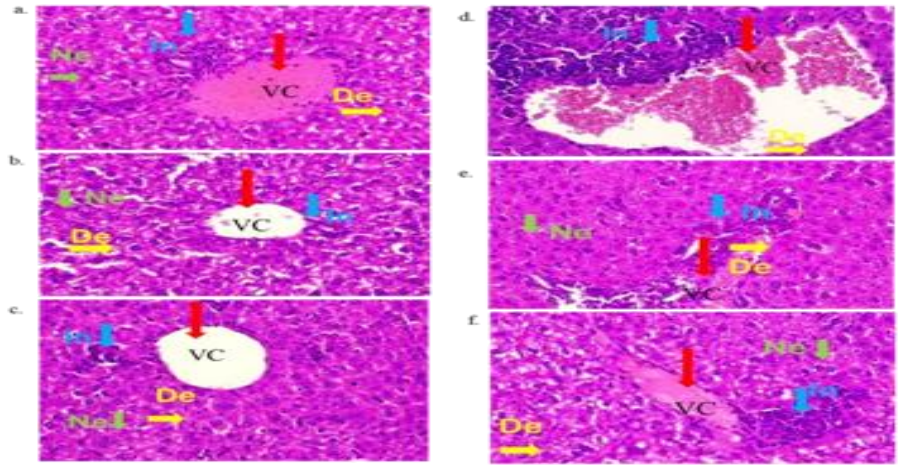

Figure 4: Photomicrograph of hepatic tissue at 400× magnification. (a): KN group, (b): ELM 1, (c): ELM 2, (d): ELM 3, (e): ELM 4, (f): ELM 5. Blue arrows (In): Inflammation, Yellow arrow (De): Degeneration, Green arrow (Ne): Necrosis, Red arrow (VC): Vein Centralis.

Conclusion

Based on the results obtained from this study, it can be concluded that ELM (*Lygodium microphyllum* Ethanol Extract) is not lethal to mice (*Mus musculus*) and is practically non-toxic with LD_{50} > 5000 mg/kg BW. In addition, acute oral administration of ELM is relative safe as there were no significant effects on body weight, organ index, no observable sign of toxicity and no significant changes in hepatic features of mice administered ELM.

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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