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Abstract. Sunda porcupine (*Hystrix javanica* F. Cuvier 1823) is an endemic mammalian fauna from Indonesia which is known as a pest since they damage crops like corn and tubers. Local people hunt the porcupine to decrease the damage, but others hunt for its medicinal benefits. They believe that sunda porcupine has medicinal benefits, yet this ethnomedicine has not been recorded well and has no sufficient scientific report. One of the interesting ethnomedicines is that local people believe the quills of sunda porcupine can treat the toothache. Our research aims to add scientific record of the sunda porcupine's quills as well the acute toxicity effect of sunda porcupine's quills crude extract on male Sprague Dawley rats. The extraction of the active compound of the quills was using ethanol 70%. The determination of acute toxicity effect was using a fixed dose method by oral administration including two steps: sighting study and main study, with four doses: 5 mg/kg; 50 mg/kg; 300 mg/kg; and 2.000 mg/kg. We also observed the histological effect on liver and kidney tissues. The result showed that the oral administration of the sunda porcupine's quills crude extract by method of fixed dose does not cause death in all rats used both after 24 hours (acute effect) and 14 days (delayed effect). Based on the fixed dose method, we can categorize the sunda porcupine's quills crude extract as non-toxic. Although the rats were clinically healthy and showed no symptoms of intoxication during 14 days of observation, histopathological analysis on liver and kidney showed lesions which can not be ignored. Histopathological analysis showed that the higher the dose, the lesions tended to increase the severity on the liver and kidney, but the active substance administered did not kill the rats and the severity was mostly mild. It is in line with the percentage of body weight gain which is a group of placebo > 300 mg/kg > 2000 mg/kg which may occur because the body prioritizes repairing the damages of the tissues due to the exact administration rather than for growth. These findings can be used as a consideration on further toxicity study on Sunda porcupine's quills crude extract, such as chronic and subchronic toxicity tests both to ensure the safety and to complete the toxicity study of the extract.

Keywords: sunda porcupine's quills, acute toxicity, in vivo, histopathology

INTRODUCTION

Sunda porcupine (*Hystrix javanica* F. Cuvier 1823) is a native species from Indonesia which can be found in Java, Bali, Sumbawa, Flores, Lombok, Madura, and Tanah Jampea (South Sulawesi) [1-4]. They are grouped as terrestrial mammals, rodentia order, and *Hystricidae* family. The local people used to hunt them since they are a pest which

damages crops like corn and tubers. However, some local people hunt them for food because they believe the porcupines have benefits to human health.

Porcupine has been reported to have high medicinal value in several countries, including Indonesia, Malaysia, and Singapore [5-6]. The tail meat of sunda porcupine has been reported to have aphrodisiac potency [7]. The porcupine's bezoar, a stone-like mass from indigestible food which can be found in porcupine's gut, usually used in traditional medicine since it is believed has many healing properties [8]. The local people used to kill the porcupine to collect the bezoar, though it can not be found in every individual porcupine. Another body part believed to be used in traditional medicine was quills which can be collected easily without killing the porcupine since they can relinquish the quills periodically. The North American porcupine (*Erethizon dorsatum*) quills reported that have antibiotic properties associated with free fatty acids (but not neutral lipids) coating the quills [9]. Extracts of quill fatty acids strongly inhibited the growth of six strains of gram-positive bacteria. In Indonesia, the quills of sunda porcupine are believed by local people in some regions of Indonesia to be used for treating toothache. However, this ethnomedicine has no scientific report. A series of research should be performed to prove this ethnomedicine scientifically.

Based on limited information about the sunda porcupine's quill as a new medicinal candidate, the safety uses of alternative medicine are important before commercial utilization. Toxicology evaluation is necessary to ensure the safety of the drug candidate, and become the first measurement before conducting efficacy tests in animal models. Several toxicity test procedures were standardized, and currently procedures with minimal animal uses are more common to conduct recently. The Organization for Economic Cooperation and Development (OECD) has already established the standard for several methods. The Procedures require a minimum number of animals to obtain statistical relevance, and also ensure a reduction in animal number to implement animal welfare in research [10]. The OECD already established guidelines that successfully used to evaluate the toxicity of several plant's extract using Guidelines 425 [11-13], Guidelines 423 [10,14] and Guidelines 420 for fixed dose procedure [15,16]. Indonesian National Agency of Drug and Food Control (Badan Pengawas Obat dan Makanan Republik Indonesia-BPOM RI) releases guidelines about in vivo toxicity tests based on OECD Guidelines 420 by The Chairman Regulation Number 7/2014 about Guidelines for In Vivo Testing for Non-Clinical Toxicity Test. This research aimed to provide LD_{50} data and toxicity records of sunda porcupine's quill crude extract on male Sprague Dawley rats using an acute oral toxicity test according to BPOM's Chairman Regulation number 7/2014 and OECD protocols 420. Moreover, we also observed the histopathological effect on liver and kidney as well as the body weight change before and after treatment as supporting data in this study.

MATERIALS AND METHODS

Preparation of Sunda Porcupine's Quills Crude Extract

The sunda porcupine's quills were collected from the remains of physiological research samples which has been approved by The Committee of Ethical Clearance of Animal Use for Research Purposes in Indonesian Institute of Sciences (LIPI) with protocol number B-15897/IPH/KS.02.04/XII/2019. The quills were prepared by cleaning, cut into 2-3 mm in length, and dried at 50 ^{0}C. The dried quills were then ground and sieved with a size of 60 mesh into powder preparation (simplicia). The simplicia was extracted by maceration using ethanol 70% (v/v) with a ratio 1:20. The extraction process was carried out for three days using an orbital shaker at a speed of 120 rpm. After extraction, the filtrate was separated using filter paper and the solvent was evaporated using a rotary evaporator at 40-50 ^{0}C. The crude extract then was stored in a clean and dry container.

Experimental Animals

Twenty male 6-7 weeks Sprague Dawley rats were used in this study. The Rats were obtained from Biopharmaca Research Center (Trop BRC), IPB University, and recorded from the BPOM RI. The experiment was conducted in the Animal Experiment Facility of the Research Center for Pharmaceutical Ingredient and Traditional Medicine, BRIN. The rats were kept in communal cages under room temperature conditions and 12-12 hours light cycle. The rats were given free access to water supply (*ad libitum*) and were fed with rat pellets (18% protein content; produced by IndoFeed, a local feed company Indonesia). The animals were grouped based on their body weight before treatment to ensure each group has no significant difference in the body weight. The animals were acclimated to laboratory conditions for seven days before the treatment was started. Before starting the experiment, rats were weighed individually to calculate the treatment doses. The endpoint of all rats was considered when the rats showed

the terminally ill condition, prognosed as infausta and lost more than 20% body weight. At the end of treatment, before termination, all rats were weighed.

Acute Oral Toxicity Using Fixed Dose Method (OECD Guideline 420)

All procedures conducted on animals were approved by The Committee of Ethical Clearance of Animal Use for Research Purposes in Indonesian Institute of Sciences (LIPI) with protocol number 73/Klirens/VIII/2021. The oral toxicity test was conducted according to OECD test guideline number 420 for Oral Acute Toxicity Test with Fixed Dose and BPOM's Chairman Regulation number 7 2014 with slight modification [17-18]. The method ranks and classifies hazardous properties of a tested substance according to The Globally Harmonized System (GHS). Briefly, the experiment was divided into two parts: Sighting Study and Main Study. The Sighting study was conducted on 5 rats to observe the initial doses from 0 mg/kg (placebo), 5 mg/kg, 50 mg/kg, 300mg/kg and 2000 mg/kg. Meanwhile the main study was conducted after the initial doses were decided. The Main study was conducted on 15 rats, divided into 3 groups with 5 repetitions for each group, according to the decided doses from the sighting study: placebo, 300 mg/kg and 2000 mg/kg. Since there was no previous study on Sunda porcupine's quills crude extract and no death or toxicity signs were observed in the sighting study, we start the doses from 300 mg/kg, followed by one dose higher if no death is recorded in the group. Single oral doses were given for each rat, with sterile aquades for the placebo group. Physical observation was performed during 30 minutes, 1 hours, 4 hours, 8 hours, 12 hours, and for each day until the 14th day. The observation includes physical changes and signs of toxicities, for instance changes in fur, body weight loss, anorexia, lethargy, seizure, tremor, diarrhea and vomit. If death occurred before the 14th day, the rat will be immediately submitted for necropsy. After the 14th day, all the remaining rats were euthanized using lethal doses of Valabarb® (Pentobarbital 300mg/ml) (LD: 200mg/kg bw) via intraperitoneal [19]. Necropsy was performed to collect liver and kidney samples. The organs were preserved and fixed in 10% Neutral Buffered Formalin (NBF).

Histopathology Observations

Histopathology specimens were made from preserved organs. Tissue samples were processed for histological analysis using the paraffin method. The samples were washed with running tap water before being dehydrated with a series of concentrations of ethanol. All samples were embedded in paraffin and sectioned 5-micron using a rotary microtome (Yamato, Japan). Slides then underwent graded xylene washes to remove the paraffin. Following the deparaffinization, all samples were stained with hematoxylin and eosin, cleared by xylene, and then mounted with Entellan® (Merck Millipore). The histopathological condition of samples was examined and micrographs were recorded using compound microscopy (Olympus) connected to a camera and PC with 4x/10x magnification.

Data Analysis

The data was analyzed descriptively by comparing the treatment group with baseline data (placebo). The data of body weight observation was analyzed by SPSS 25 software using ANOVA test with significance level of 5% and the Tukey's test will be performed if the significance score of ANOVA test is less than 0.05.

RESULT AND DISCUSSION

Grouping Animal Model

In the main study of the toxicity test, 15 rats with relatively the same body weight were randomly chosen into 3 groups: placebo, 300 mg/kg, and 2000 mg/kg. The test of normality, homogeneity, and ANOVA with significance level of 0.05 was performed to ensure each group has no significance different based on the body weight. The tests of the animal groups aim to ensure that each group has the same baseline based on the body weight factor, so that the influence of the initial body weight can be ignored toward the result of toxicity test and other related supporting analysis. The result shows that all groups were normally distributed ($p > 0.05$), have a homogen body weight ($p > 0.05$), and have mean with no significantly different ($p > 0.05$) (Table 1). It indicates the group design can be used for the experiment.

TABLE 1. Statistical analysis on grouping animal model (α = 5%)

Group	Mean (g)	Standard Deviation (g)	p-value		
			Normality Test	Homogenity Test	ANOVA Test
Dose 0 mg/kg	231.70	26.08	0.474		
Dose 300 mg/kg	251.22	23.21	0.687	0.594	0.346
Dose 2000 mg/kg	248.38	14.81	0.792		

Toxicity Test and LD_{50} Determination

Oral administration of sunda porcupine's quills crude extract showed no treatment related behavioral and physical alteration during 14 days of observations, both in sighting study and main study. The toxic effects on vital body organs are manifested by clinical signs and symptoms which become indicators for toxicity observation [20]. The observed signs include alteration of behavior, fur, body condition, and clinical signs including vomiting, seizure, tremor, hypersalivation and diarrhea. The signs were not observed in both sighting and main study. There was also no death recorded during 14 days of observation in both sighting and main study. Oral administration is normally used for toxicity study. The method is less painful to the animals, although the absorption may be slower than the parenteral route. As the extracts were administered orally, the animals need to fast at least 4 hours before dosing. The fasting is to minimize the unnecessary effect of the food and other materials in the animal's stomach [12].

The highest dose administration (2000 mg/kg) did not lead to death or produce any toxicity symptom. According to the Globally Harmonized Classification System for Chemical Substances and Mixtures (GHS), the sunda porcupine's quills crude extract (solvent ethanol 70%) was having LD_{50} more than 2000 mg/kg and classified as category 5 or unclassified (2000 mg/kg < LD_{50} < 5000 mg/kg), and considered to be safe as single dose oral medication properties [17]. However, acute toxicity information is still limited for clinical application, since cumulative effects sometimes occurred at very low dosages [14]. The findings of acute toxicity of sunda porcupine's quills crude extract can be used as a consideration for further toxicity research such as subchronic and chronic toxicity studies.

Histopathological Analysis

Histopathological examination was conducted on liver and kidney in all rats from the main study. Although gross anatomy pathological lesions were not observed in all groups, histopathological findings showed mild to moderate inflammation in both organs as shown in Table 2. The higher the dose, tends to produce a more severe lesion compared to the lower one and the placebo. Pathological findings in the placebo group, probably caused by the animals used in this experiment, were non-specific pathogen free (SPF), and probable previous infection had already occurred during our treatment. Although the placebo group already showed pathological lesions, this group still can act as baseline data since the treatment group showed a more severe effect than the placebo. We assumed that although previous infections had already occurred in all of the group, the treatment group assigned more severe lesions compared to the placebo group. It indicates that the treatment, in an unknown mechanism, worsened inflammation conditions which had already occurred in both liver and kidney in the rat model. High doses (2000 mg/kg) of basil extract in the previous study also showed no clinical symptoms, but mild to moderate pathological lesions were also found in histopathology analysis of liver and kidney [15]. The dose higher than 2000 mg/kg of sunda porcupine's crude extract may lead to toxicity effects on the animals.

At a dose of 0 mg/kg it causes mild liver severity in the lesions form of sinusoidal dilation, congestion, the proliferation of bile ducts, degeneration of hepatocyte cytoplasm, infiltration of mononuclear cells in the periportal and peri-acinar areas. The kidneys showed mild severity in 4 rats and moderate severity in 1 rat, with lesions in the form of glomerular hemorrhage, congestion, and mononuclear cell infiltration in the cortex and medulla areas. Lesions in the form of inflammation may have occurred before the animals used in the study (non-SPF mice). A single dose of 300 mg/kg caused mild severity in 2 rats and moderate severity in 3 rats, with lesions in the form of a combination of sinusoidal dilation, congestion, bile duct proliferation, hepatocyte cytoplasmic degeneration, mononuclear cell infiltration in the periportal area. and peri-acinar. The kidneys showed mild lesions in four rats and moderate lesions in two rats, with lesions in the form of glomerular hemorrhage, congestion, and mononuclear cell infiltration in the cortex and medulla areas. The kidneys showed mild severity in 3 rats and moderate lesions in 2 rats, with lesions in the form of glomerular hemorrhage, congestion, and mononuclear cell infiltration in the cortex and medulla areas. A

dose of 2000 mg/kg caused mild severity in 1 rat and moderate severity in 4 rats with lesions in the form of a combination of sinusoidal dilation, congestion, bile duct proliferation, hepatocyte cytoplasmic degeneration, mononuclear cell infiltration in the periportal area. and peri-acinar. The kidneys showed mild lesions in three rats and moderate lesions in two rats, with lesions in the form of glomerular hemorrhage, congestion, and mononuclear cell infiltration in the cortex and medulla areas. The kidneys showed mild severity in 2 rats and moderate severity in 3 rats, with lesions in the form of glomerular hemorrhage, congestion, and mononuclear cell infiltration in the cortex and medulla areas. It was concluded descriptively that the higher the dose of histopathological lesions tended to increase in severity in the liver and kidneys, but the active substances given did not kill rats and the severity was still mild to moderate.

TABLE 2. Pathological findings in Liver and Kidney through histopathological examination.

Organ	Pathological Lesions	Group		
		Placebo	300 mg/kg	2000 mg/kg
Liver	Sinusoidal Dilatation	5/5	5/5	5/5
	Bile duct proliferation	4/5	0/5	2/5
	Mononuclear cell infiltration in periportal area	2/5	1/5	2/5
	Mononuclear cell infiltration in periacinar area	2/5	0/5	1/5
	Hepatocyte cytoplasm degeneration	2/5	3/5	1/5
	Duct Blockages	4/5	2/5	5/5
	Moderate Severity	0/5	3/5	4/5
Kidney	Mononuclear cell infiltration in cortex area	1/5	0/5	0/5
	Duct Blockages	5/5	4/5	2/5
	Glomerular hemorrhage	5/5	5/5	5/5
	Mononuclear cell infiltration in medulla area	1/5	2/5	2/5
	Cytoplasm tubuli degeneration	1/5	2/5	2/5
	Moderate Severity	1/5	2/5	3/5

Notes: The scores 1-5 were given according to the repetition found on each individual rats in each group.

The histopathological analysis on the tissue specimens of liver and kidney are shown on Figure 1. Based on the result, all treatment dosages showed several histological changes both in the liver and kidney. Some factors influence the histological changes. Hepatic sinusoidal dilatation refers to the enlargement of the hepatic capillaries. Almost all these conditions are caused by hepatic venous outflow obstruction, which results in vascular stasis and congestion of hepatic parenchyma [21,22]. Many factors induce this case, including infection. The use of non-SPF rats in this study might trigger this case. Although the use of SPF rats did not guarantee the animals were infection free, it can reduce the possibility of infection. The rat colony of both SPF and non-SFF rats in the US was infected by pinworm (50%), coronavirus or parvovirus (30%), and PVM (20%) [23]. No toxic effect features were found in control and all treatment groups. The most common toxic effect caused by natural products included cholestasis with canalicular cholestasis and scant hepatocyte necrosis and inflammation [24]. While the toxicity caused by drugs includes necrosis, hepatitis with or without cholestasis, acute hepatitis-like with or without massive necrosis, simple cholestasis, reactive hepatitis and steatosis [25]. The histological changes in the current study did not show the effect of toxic substances but might be caused by infections. The histological features proved it in the control group, which also had similar changes to the treatments group. Therefore, the porcupine quills extract had no toxic effect on the liver and kidney.

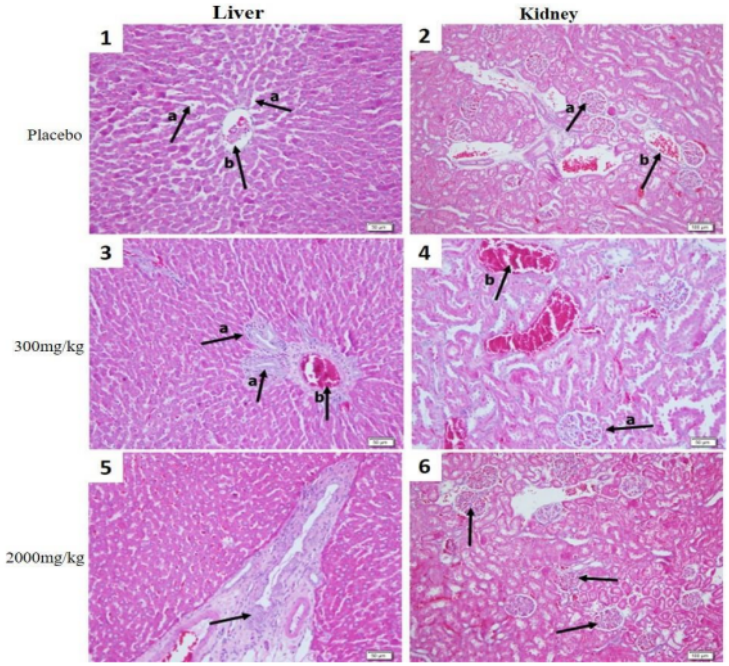
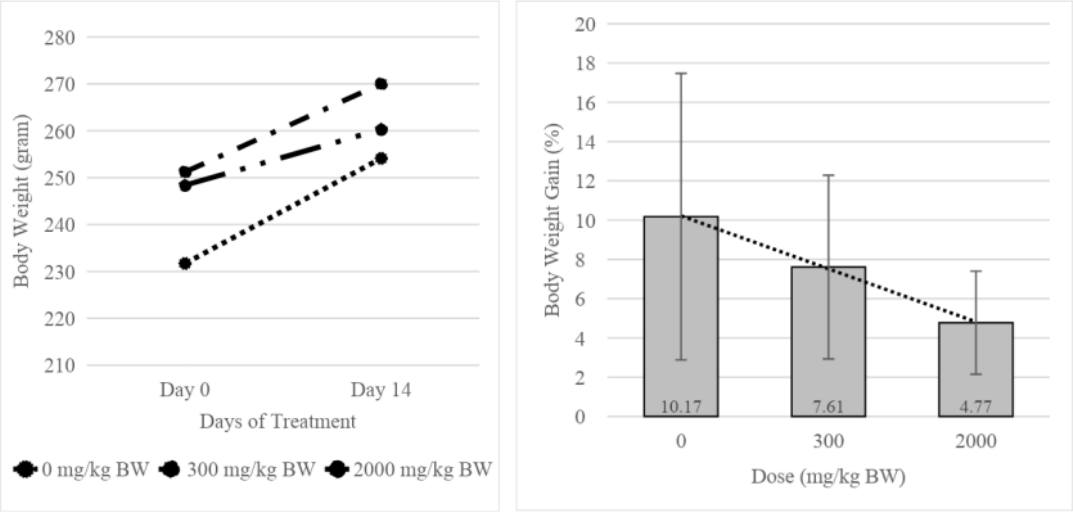


FIGURE 1. Selected histopathology specimens with H&E staining that correspond to each group. 1-2: Rat with 0 mg/kg BW; 1. Liver with sinusoidal dilation (a) and mild congestion of central vein (b); 2. Kidney with glomerular hemorrhage (a) and congestion; 3-4: Rat with 300 mg/kg BW; 3. Liver with bile duct proliferation (a) and mild congestion of central vein (b); 4. Kidney with glomerular hemorrhage (a) and moderate congestion (b); 5-6: Rat with 2000 mg/kg BW; 5. Liver with severe bile duct proliferation; 6. Kidney with glomerular hemorrhage congestion.

Body Weight Observations

We also observed the body weight change during the treatment of the main study of acute oral toxicity test. The body weight change on all groups of animal model and their percentage is presented in Figure 2. We found that the body weight of all groups increased. However, the slope of body weight gain after 14 days of treatment was different for each group, the body weight slope of placebo > 300 mg/kg > 2000 mg/kg (Figure 2(a)). It shows that the high dose is inversely proportional to body weight gain. This correlation is more clearly seen in the percentage of body weight gain (Figure 2(b)). The data shows that the groups of a placebo, 300 mg/kg, and 2000 sequentially have a percentage of body weight gain 10.17% ± 7.30%, 7.61% ± 4.68%, and 4.77% ± 2.62%. Moreover, if we assumed that distances between groups are the same, the decrease in the percentage of body weight gain has a correlation score (R2) of 99.92%, which indicates strong relation between dose and percentage of body weight gain. Furthermore, we performed the statistical analysis, including normality test, homogeneity test, and ANOVA test on treatment groups toward a percentage of body weight gain is presented on Table 3. The result shows that the percentage of body weight in all groups are normally distributed ($p > 0.05$), and all groups are homogenous ($p > 0.05$). However, the ANOVA test shows that the mean of each group is not significantly different ($p > 0.05$), so Tukey's test is unnecessary. Even though the percentage of body weight means of each group seems different, the significance is still not different for $\alpha = 5\%$.



(a) (b)

FIGURE 2. Body weight gain (a); and percentage of body weight gain (b) during the treatment of the main study.

The less body weight gain in the highest dose group, 2000 mg/kg, compared to a group of 300 mg/kg compared to the placebo group may indicate the delay effect of toxicity. The dose treatment may cause toxicity effects systematically signed by the less body weight gain on the increase in dose although the highest doses (2000 mg/kg) in the present study has 0% of mortality and has no symptoms on physical and behavior of the animal model. Although it does not cause death and toxic symptoms, the dose given may damage cells or tissues so that the nutrients that enter the body are prioritized to be used to repair the damage resulting in inhibition of growth signed by the less body weight gain during the treatment. It is in line with the histopathological analysis in the present study that shows the severity of liver and kidney tissue on the group 2000 mg/kg > 300 mg/kg > placebo. Another possibility is that the dose reduces the appetite as one of the toxic symptoms so that the nutrients uptake is reduced and consequently the body weight gain is less increased. Other than that, the mortality and the toxic symptoms may not be found because the dose given in the present study is not more than 2000 mg/kg. The previous study of acute oral toxicity shows the mortality and toxic symptoms for dose more than 2000 mg/kg, including administration of waru leaves (*Hibiscus tiliaceus* L.) extract, mango tree parasite (*Dendrophthoe petandra* L.) extract, and herbal mixture extract [26-28]. Some previous studies also show that a high dose of more than 8000 mg/kg still has no mortality and toxic symptoms, including kirinyuh leaves extract (*Chromolaena odorata* L.) and purple yam (*Dioscorea alata* L.) extract [29-30]. The different results of acute oral toxicity between the present study and previous studies may be caused by the different content of the active compound of the extract. However, the findings of body weight observation in the present study can still be used as consideration for further toxicity study of Sunda porcupine's quills crude extract.

TABLE 3. Statistical analysis on percentage of body weight gain during the treatment of the main study ($\alpha = 5\%$).

Group	Mean (g)	Standard Deviation (g)	p-value		
			Normality Test	Homogenity Test	ANOVA Test
Dose 0 mg/kg	10.17	7.30	0.084		
Dose 300 mg/kg	7.61	4.68	0.156	0.248	0.300
Dose 2000 mg/kg	4.77	2.62	0.622		

CONCLUSION

Although our extract is considered to be Category 5 by the GHS determination and no clinical toxicity signs were observed during 14 days of observation, several pathological findings showed that our extract may worsen the already occurred inflammation in liver and kidney of male Sprague Dawley Rats (severity 2000 mg/kg > 300 mg/kg > placebo). In line with the histopathological analysis, the percentage of body weight gain of placebo > 300 mg/kg > 2000 mg/kg after 14-day single dose oral administration, which may be caused by the systemic control to prioritize the nutrients uptake for repairing the damage of cell or tissue than growth signed by the increase of the body weight. Sunda porcupine quill's crude extract does not cause any terminal acute effect. However, in order to ensure the safety of the extract, further research must be conducted on the safety aspect of the extract including chronic toxicity tests.

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