

ACUTE ORAL TOXICITY ASSESSMENT OF THE ETHANOL EXTRACT OF *HOLOTHURIA ATRA* IN MICE

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ABSTRACT

Objective: This study aimed to evaluate the toxicological potential of the ethanol extract of *Holothuria atra* through the acute oral toxicity – acute toxic class method.

Methods: The sample was immersed in ethanol for 72 h at room temperature and repeated 3 times. The extracts were evaporated using a vacuum rotary evaporator. The identification of compounds in the ethanol extract of *H. atra* was carried out using liquid chromatography–mass spectrometry (LCMS) analysis. The acute toxicity test was examined the effects of treating male mice with the ethanol extract of *H. atra* at 300 and 2000 mg/kg by oral administration for 14 days. On the past day of the toxicity test, liver of all experimental animals was taken for histopathological testing.

Results: LCMS analysis showed that the ethanol extract of *H. atra* is contained polar compounds (chlorogenic acid, coumaric acid, a glycosaminoglycan, and holothurin) and non-polar compounds (fatty acids). Acute toxicity study was performed at a dose of 300 and 2000 mg/kg for 14 consecutive days. No deaths or behavioral changes were observed during the administration of both doses. Histopathological test results on the liver showed a few changes at doses of 2000 mg/kg.

Conclusions: The LD₅₀ is equal to 5000 mg/kg and the ethanol extracts of *H. atra* can be classified as practically nontoxic. However, further studies are required to proceed to clinical studies in humans.

Keywords: *Holothuria atra*, Ethanol extract, Safety dose, Acute toxicity, LD₅₀

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INTRODUCTION

Holothuria atra is one of the species of sea cucumbers from the family Holothuriidae and it is found worldwide [1]. *H. atra* is one type of sea cucumber that is relatively inexpensive but rarely consumed due to the presence of saponin compounds that make it taste bitter [2].

H. atra contains a variety of bioactive compounds such as glycoside, lectin, steroidal sapogenin, saponins, triterpenoids, phenols, and alkaloids [3,4]. The previous studies report that *H. atra* has pharmacological activities, including antioxidants [5,6], antibacterial [7], anti-inflammatory [3], and immunomodulatory effects [1,8].

A previous study proved that sea cucumbers (*Holothuria*) also contain a toxic compound called holothurin [9]. *H. atra* is reported to contain saponins (triterpene glycosides) types of holothurin A, holothurin A2, holothurin B, holothurin B1, and holothurin B2 [10,11]. Therefore, we need a toxicity test for the ethanol extract of sea cucumber *H. atra* so that the lethal median dose (LD₅₀; the amount of an ingested substance that kills 50% of a test sample) value is known.

MATERIALS AND METHODS

Sample preparation

Samples of *H. atra* collected from the Mutun Pesawaran Beach, Lampung, Indonesia. The animals were immediately washed under running tap water and cut open, and all visceral organs were removed. The animals were rinsed thoroughly of any internal organs or body fluids and dried at 40–50°C. Then, the dried body walls of the animals were transported to our laboratory in an icebox with dry ice and stored at –20°C until processing [5].

Extraction method

The extraction method is a modification of the previous work [6]. The sample weighed 500 g and cut into pieces (2–3 cm²) and then immersed in ethanol (proAnalyst, MERCK; 1:5; w/v) for 72 h at room temperature and stirring occasionally. The process was repeated 3 times until the filtrate obtained was clear. The filtrate obtained was then filtered using Whatman No.1 filter paper and then evaporated using a rotary vacuum evaporator (IKA RV 10 Basic; 40°C, 90 rpm) with Minichiller 300 Huber. Then, the supernatant residue is stored at –20°C.

Liquid chromatography–mass spectrometry (LCMS) analysis

Chromatographic methods refer to the previous research [12]. Chromatography was performed on a Water Alliance high-performance liquid chromatography 2695 (Waters Associates Inc., Milford, MA, USA) connected to a Water 3100 quadrupole mass spectrometer through an electrospray ionization interface, consisting of an autosampler, a column heater-cooler, a quaternary pump, and a nitrogen gas generator unit (NM30LA-MS Gas Generator from Peak Scientific Instruments Ltd., MA, USA).

Chromatographic separation was carried out on a Symmetry C18 analytical column (50 × 2.1 mm, 3.5 mm particle size, Waters). Mobile phases consisting of water (Solvent A) and MeOH (Solvent B) both with 0.1 mM NH₄Ac and 0.01% HCOOH at different ration and flow rate were tested. The flow rate was 300 μL/min. The sample injection volume was 20 μL. All data were acquired and processed using MassLynx version 4.1 (Waters).

Ethical consideration

Experimental protocols and procedures used in this study were approved by the Brawijaya University, Bioscience Institute, Research

Ethics Committees (Indonesia) (1018-KEP-UB). All the experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals.

Toxicity study

The acute toxicity test was conducted according to the Organization for Economic Cooperation and Development (OECD) Guidebook 423. The acute oral toxicity – acute toxic class method was applied in the present study to determine the median lethal dose (LD₅₀) [13]. The male mice used in the LD₅₀ test were BALB/c strains aged 6–7 w with a body weight of 18–20 g. Animals must adapt to the new environment for 7 days before the study and given standard food and drink *ad libitum*. Three experimental animals used at each step. Starting dose or initial dose to start can be selected from four fixed doses of 5, 50, 300, and 2000 mg/kg BW. Since there is no relevant information on the ethanol extract of *H. atra* that can cause death, the toxicity test will start at a dose of 300 mg/kg [14].

After adjusting to the environment, experimental animals were divided into two groups with three male mice in each group. The first group is the control group, and the second group is the ethanol extract group. The animals in the experimental groups were administered to samples (dissolved in distilled water), and the control group was treated with distilled water. All animals must be fasted before experimentation (but drinking water may be given). Each mouse was marked before the experiment and the initial body weight (fasting for 4 h) was recorded. An ethanol extract of *H. atra* is given in a single dose (one-time administration). The gavage was performed with a volume of 10 ml/kg [15]. Observations were made at 30 min, 1 h, 2 h, and 4 h (given specifically for the first 4 h) after the sample administration, and then, feed with known weight was offered. Further observations were done once a day for 14 days. The test is not continued at the next dose until it is known whether the animal is still alive or dead (OECD, 2001). Meanwhile, the mortality (if any) and abnormality (signs of toxicity) of mice were also recorded [14,16].

Histopathological analysis

A small portion of the liver tissue of each mouse was fixed in 10% formalin, processed, and embedded in paraffin wax to obtain 5 µm thick slices using a microtome. Hematoxylin and eosin stain was applied to the sections. Then, they were examined under a light inverted microscope. Staining was needed to observe the color, shape, and size of hepatocytes. Hematoxylin stained the nucleus blue. Eosin stained the cytoplasm red [17].

Statistical analysis

Data were expressed as mean ± standard deviation. Body weight data of the different groups were determined by analysis of variance (one-way analysis of variance) followed by the Tukey *post hoc* test. $p < 0.05$ was considered statistically significant.

RESULTS

LCMS analysis

The yield of *H. atra* extraction using ethanol was 8.54 g (1.71%). The LCMS analysis revealed that ethanol extract of *H. atra* contains various types of bioactive compounds (Table 1).

The ethanol extract of *H. atra* is known to contain polar compounds such as phenolic (Chlorogenic acid and coumaric acid), glycosaminoglycan, and saponins (holothurin) to non-polar compounds such as fatty acids.

Acute toxicity study

Single doses of 300 mg/kg body weight of the ethanol extract of *H. atra* given to mice during the acute toxicity study did not cause any death or changes in the parameters evaluated when compared to the control group. Then, the dose of ethanol extract was increased at 2000 mg/kg BB also caused no death and no clinical signs of toxicity in any of the treated animals (Table 2). The exposed mice showed no changes in physiological habits during the treatment period.

All animals survived test substance administration gained body weight, appeared active and healthy during the study. There were no signs of gross toxicity, adverse pharmacologic effects, or abnormal behavior. The acute oral lethal dose (LD₅₀) of the ethanol extract of *H. atra* in mice has been estimated to be 5000 mg/kg.

Behavioral sign of toxicity

Mortality is a clear sign of toxicity, but other variables may indicate more subtle adverse effects such as weight loss during the treatment and clinical signs of toxicity [18]. Observations to display signs of toxicity include changes in skin and fur, diarrhea, tremors, convulsions, and mortality [14].

The group mean values of body weights of the mice treated with 300 and 2000 mg/kg body weight of the ethanol extract of *H. atra* did not differ significantly from those of the control groups during the study (Fig. 1). No alterations or adverse effects in growth were observed in animals in any of the dosing groups.

Histopathological analysis

Histological test results of hematoxylin and eosin staining in the liver or liver of mice showed that the liver in mice extract a group of 2000 mg/kg body weight was damaged compared to extract group 300 mg/kg body

Table 1: Chemical composition of the ethanol extract of *Holothuria atra*

No.	Nama Senyawa	Berat Molekul (g/mol)
1.	Chlorogenic acid	354,31
2.	Squalene	410,7
3.	Glycine	75,13
4.	Glutamic acid	147,13
5.	Coumaric acid	164,16
6.	Arginine	174,2
7.	Palmitoleic acid (C16:1)	254,41
8.	Palmitic acid (C16:0)	256,43
9.	Lanosterol	426,7
10.	Chondroitin sulfate	463,37
11.	Heparan sulfate	637,5
12.	Linoleic acid (C18:2)	280,4
13.	Oleic acid (C18:1)	282,5
14.	Stearic acid (C18:0)	284,5
15.	Sulfated glycosaminoglycan	301,27
16.	Eicosapentaenoic acid (C20:5)	302,5
17.	Arachidonic acid (C20:4)	304,5
18.	Glutathione	307,33
19.	Arachidic acid (C20:0)	312,5
20.	Docosahexaenoic acid (C22:6)	328,5
21.	Panaxoside A/Ginsenoside RG1	801
22.	Holothurin B3	866
23.	Holothurin B1	868
24.	Holothurin B/B4	882
25.	Holothurin B2	884
26.	Holothurin A	1221,3

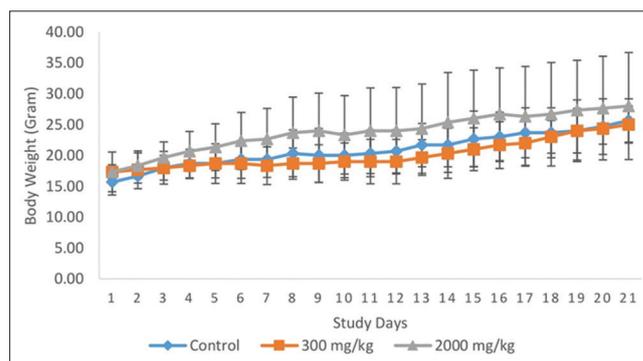


Fig. 1: Body weights of animals

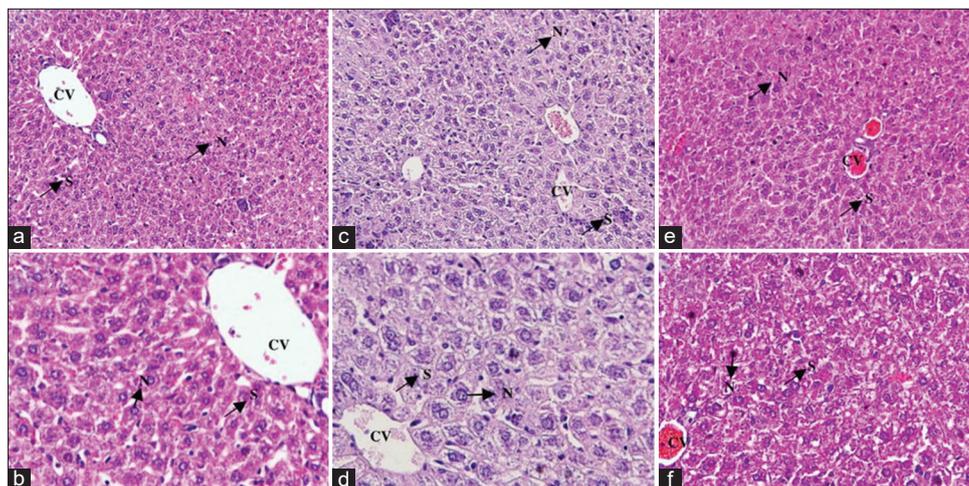


Fig. 2: Histopathological results of livers in mice after oral administration in the acute toxicity (H and E: 200× and 400×), control (a and b); 300 mg/kg ethanol extract (c and d); 2000 mg/kg ethanol extract (e and f), N = nucleus; CV = central vein; S = sinusoid

weight and negative control. This can be seen on the arrows in the picture, which shows that many nucleus cells are damaged or called pyknosis. Observations in the 300 mg/kg BW extract group, the cell nucleus was seen to be unchanged compared with the negative control group. This shows that the extract group of 300 mg/kg body weight and control did not occur in the histological structure of the liver of male mice.

DISCUSSION

The study examined the effects of treating male mice for 14 days with 300 and 2000 mg/kg of body weight of the ethanol extract of *H. atra* by oral administration. Both doses of ethanol extract of *H. atra* did neither cause death and no any obvious toxicity symptom in all mice. Observation of signs of toxicity is carried out individually every 30 min in the first 4 h after administration of the test preparation, and periodically every 4 h for the first 24 h and once a day after that for 14 days. Observations made include the condition of the skin and hair, eyes, convulsions (convulsions), tremors (shaking), diarrhea, and death [14]. The previous study was also reported that phosphate-buffered saline extracts of *H. atra* which was given orally at a dose 2000 mg/kg did not show any signs of toxicity or cause death in mice [5].

The ethanol extract of *H. atra* was used in the acute toxicity test by oral administration that did not show any signs of toxicity or cause death in experimental animals up to a dose of 2000 mg/kg so that the globally harmonized system is classified as category five or unclassified. Therefore, according to the method of acute toxicity classification [19], it can deduce that the LD₅₀ values of ethanol extract of *H. atra* are equal to 5000 mg/kg and regarded as practically nontoxic.

Body weight is summarized in Fig. 1. As shown in Fig. 1, the weight of mice was an increase from day 1 to day 21 in all groups. It was found that there were significant differences in the average body weight of mice each day in each group ($p < 0.05$). Qualitative testing results show that the ethanol extract of *H. atra* contains steroids, which has an effect on metabolic activity so that it can increase appetite [20].

Liver damage can be caused by many exogenous drugs and other chemical substances by a variety of mechanisms, including cellular degeneration and necrosis by interfering directly with various specific biochemical reactions [21]. The liver is the first organ to encounter ingested nutrients, vitamins, metals, and drugs. The liver functions to detoxify toxic substances so that liver damage is an indication of whether a substance is toxic or not.

In this study, control liver tissue indicated the presence of normal hepatocytes which are polyhedral in shape with defined cell lining;

Table 2: Behavioral signs of toxicity effects of the ethanol extract of *H. atra* administered (oral) to mice

Dose of <i>H. atra</i> extract (mg/kg)	Observed mortality (D/T)	Toxic symptoms
0	0/3	None
300	0/3	None
2000	0/3	None

0 mg/kg=control group that was given distilled water through oral; D/T: Dead/treated mice; None: No toxic symptoms were seen during the observation period. *H. atra*: *Holothuria atra*

nuclei are distinctly rounded, with one or two prominent nucleoli [17]. The liver section at doses of 2000 mg/kg indicated the presence of abnormal hepatocytes with an enlarged nucleus (Fig. 2). Damage to the liver is characterized by cell necrosis or changes in the nucleus [21]. The chromatin of the cell clumps into coarse and the nucleus will be shrink, dense, and dark blue when colored by hematoxylin. This process is called pyknosis. The pyknotic nucleus can then be broken down into many small basophilic particles (karyorrhexis) or will be lysis as a result of the action of lysosomal deoxyribonuclease (karyolysis). Pyknosis can be interpreted as the nucleus of cells that have shriveled up, so it looks smaller than normal size, and generally, cells that have pyknosis will look dark in color [17].

Congestion is a term that indicates the excess blood volume in a part of a blood vessel. This can occur because too much blood gets into the arteries or too little goes to the veins. Microscopically congestion can be characterized by dilatation in the artery or capillary wall caused by the large volume of blood in that area [22]. The damage to the central vein can be caused by too much blood being collected, this can cause the accumulation of toxic substances so that the damage becomes even more obvious [23].

The results of the study show that the ethanol extract of *H. atra* detected to contains holothurin A, holothurin B/B4, holothurin B1, holothurin B2, and holothurin B3 (Table 1). Holothurin is a type of saponin that exists in sea cucumbers and generally exists in the form of triterpene glycosides [24]. *H. atra* was reported to contain saponins (triterpene glycosides) of holothurin A, holothurin A2, holothurin B, holothurin B1, and holothurin B2 [10]. The previous studies have shown that holothurin isolated from *Holothuria vagabunda* can have hemolytic effects (damage red blood cells) and hyperemia (increase blood flow to body tissues) which it happens for a long time can cause permanent changes in tissue (atrophy, necrosis, and hypoxia) [9]. The sea cucumber toxin extract can increase the level of hematocrit, hemoglobin, red blood

cells, and white blood cells after 120 min injected intraperitoneally in mice [25]. Holothurin is hygroscopic and soluble in water at unlimited rate. At room temperature it solves in ethanol containing water, but it does not dissolve in ethanol with a concentration of more than 94% as well as in other organic solvents. It's very foamy by stirred, resistant to heat (not decomposed at 100°C), and also have a strong hemolytic effect. The effects of holothurin can be reduced by cholesterol in the blood and hydrolyzed by acids (peptic acid) during the digestive process [9].

The five parameters of the sign of toxicity in the acute toxicity did not show behavioral changes or cause death. It is important to measure the acute clinical signs in an attempt to establish a lethal dose that can cause the death of 50% of the animals tested (LD₅₀) to obtain parameters for further clinical trials of toxicity, specifically subacute, subchronic, and chronic toxicity [26,27]. In this study, therefore, it is assumed that the LD₅₀ is equal to 5000 mg/kg.

CONCLUSIONS

In the present work, the acute toxicity of the ethanol extract of *H. atra* in mice was performed. The result of acute toxicity showed that oral single administration of 300 and 2000 mg/kg of the extract did not cause mortality or sudden death. Therefore, the LD₅₀ of extract for male mice was equal to 5000 mg/kg and regarded as practically nontoxic. However, the ethanol extract of *H. atra* is exhibited toxicities to heart in mice determined by histopathological analysis when treated by oral administration of high doses (2000 mg/kg).

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AUTHORS' CONTRIBUTION

Febriana Untari and Masteria Yunovilsa Putra contributed to the collection of the sample and performed the extraction; Pandu Salim Hanafi contributed to the preparation of the manuscript and carried out the experiments; Aji Sutrisno and Tutik Murniasih has supervised the experimental process; Harijono contributed to article proofreading and revision of the article. The final manuscript was approved by all the authors.

CONFLICTS OF INTEREST

The study was conducted at Marine Natural Product Laboratory, Research Center for Oceanography, Indonesian Institute of Sciences, Jakarta and Food Nutrition Laboratory, Department of Agricultural Product Technology, Faculty of Agricultural Technology, Brawijaya University, Malang, Indonesia. Tutik Murniasih is an employee of the Research Center for Oceanography, Indonesian Institute of Sciences, Jakarta, Indonesia.

REFERENCES

1. Saad DY, Baiomy AA, Mansour AA. Antiseptic effect of sea cucumber (*Holothuria atra*) against multi-organ failure induced by sepsis: Molecular and histopathological study. *Exp Ther Med* 2016;12:222-30.
2. Van Dyck S, Caulier G, Todesco M, Gerbaux P, Fournier I, Wisztorski M, et al. The triterpenoid glycosides of *Holothuria forskali*, usefulness and efficiency as a chemical defense mechanism against predatory fish. *J Exp Biol* 2011;214:1347-56.
3. Bordbar S, Anwar F, Nzamid S. High value components and bioactives from sea cucumbers for functional foods. A review. *J Mar Drugs* 2011;9:1761-805.
4. Murniasih T, Putra MY, Pangestuti R. Antioxidant capacities of

5. *Holothuria* sea cucumbers. *Ann Bogorienses* 2015;19:21-6.
5. Dakrory AI, Fahmy SR, Soliman AM, Mohamed AS, Amer SA. Protective and curative effects of the sea cucumber (*Holothuria atra*) extract against DMBA induced hepatorenal diseases in rats. *BioMed Res Int* 2015;2015:563-652.
6. Pangestuti R, Murniasih T, Putra MY, Rasyid A, Wibowo JT, Ardiansyah A, et al. Free radical scavenging activity of selected sea cucumber species from Mataram-Lombok, Indonesia. *J Teknol Sci Eng* 2016;78:179-85.
7. Dhinakaran DI, Lipton AP. Bioactive compounds from *Holothuria atra* of Indian Ocean. *Springerplus* 2014a;3:673.
8. Dhinakaran DI, Lipton AP. Pharmacological potentials of sea cucumber *Holothuria atra* extracts from the Indian Ocean. *Asian J Biomed Pharm Sci* 2014b;4:36-43.
9. Yamanouchi T. On the poisonous substance contained in holothurians. *Publ Seto Mar Biol Lab* 1995;4:183-203.
10. Dyck SV, Gerbaux P, Patrick F. Qualitative and quantitative saponin contents in five sea cucumbers from the Indian Ocean. *Mar Drugs* 2010;8:173-89.
11. Kalinin VI, Avilov SA, Silchenko AS, Stonik VA. Triterpene glycosides of sea cucumbers (*Holothuroidea*, *Echinodermata*) as taxonomic markers. *Nat Prod Commun* 2015;10:21-6.
12. Salman M, El-Sayed S, Hameed A, Bazzaid SA, Al-Shamranib MG, Mohamed HF. Liquid Chromatography-Mass Spectrometry (LC-MS) method for the determination of sugars in fresh pomegranate fruit juices. *Pharm Chem* 2014;6:320-33.
13. Belhekar SN, Chaudhari PD. Acute and sub-acute oral toxicity assessment of the polyherbal formulation in albino Wister rats. *Int J Pharm Pharm Sci* 2016;8:311-16.
14. Organization for Economic Cooperation and Development. Test Guideline 423 : Acute Oral Toxicity Acute Toxic Class Method. Paris, France: Organization for Economic Cooperation and Development; 2001. p. 1-14.
15. Ajput MA, Zehra T, Ali F, Kumar G. Assessment of analgesic activity of *Nelumbo nucifera* fruit ethanol extract. *Int J Pharm Pharm Sci* 2019;11:1-5.
16. Xiang F, Peng L, Yin Z, Jia R, Hu Z, Li Z, et al. Acute and subchronic toxicity as well as evaluation of safety pharmacology of *Galla chinensis* solution. *J Ethnopharmacol* 2015;162:181-90.
17. Hashim R, Azizan NA, Zamli Z, Zulkipli FH, Mazlan N, Althunibat OY. Toxicity effects of water extracts of *Holothuria atra* jaeger in mice. *Asian Pac J Trop Biomed* 2014;4:614-7.
18. Traesel GM, de Souza JC, de Barros AL, Souza MA, Schmitz WO, Muzzi RM, et al. Acute and subacute (28 Days) oral toxicity assessment of the oil extracted from *Acrocomia aculeata* Pulp in rats. *Food Chem Toxicol* 2014;74:320-5.
19. Duan WL, Liang XM. Technical Guidelines Assembly of Veterinary Medicine Research. Beijing: Chemical Industry Press; 2011.
20. Huberman A. Shrimp endocrinology: A review. *Aquaculture* 2000;191:191-208.
21. Chandrasoma P, Taylor CR. *Concise Pathology*. 3rd ed. Singapore: McGraw-Hill; 1997.
22. Jones TC, Hunt RD, King NW. *Veterinary Pathology*. 6th ed. USA: Lippincott Williams and Wilkins; 1997.
23. Price SA, Wilson LM. *Patofisiologi: Konsep Klinis Proses-Proses Penyakit*. 4th ed., Vol. 4. Jakarta: EGC; 1995.
24. Bahrami Y, Franco CM. Acetylated triterpene glycosides and their biological activity from *Holothuroidea* reported in the past six decades. *Mar Drugs* 2016;14:147.
25. Zaki MA. Effects of the crude toxin of sea cucumbers *Holothuria atra* on some hematological and biochemical parameters in rats. *Egypt J Nat Toxins* 2005;2:71-86.
26. Organization for Economic Cooperation and Development. Guidelines for Testing of Chemical. Acute Oral Toxicity up and Down Procedure. Paris: Organization for Economic Cooperation and Development; 2008.
27. de Lima FF, Traesel GK, Menegati SE, Santos AC, Souza RI, de Oliveira VS, et al. Acute and subacute oral toxicity assessment of the oil extracted from *Attalea phalerata* Mart Ex Spreng. pulp fruit in rats. *Food Res Int* 2017;91:11-7.