https://doi.org/10.31925/farmacia.2023.2.12 **ORIGINAL ARTICLE**

SUBCHRONIC TOXICITY OF *HOLOTHURIA ATRA* ETHANOL EXTRACT FOR 90 DAYS IN HISTOPATHOLOGY OF THE LIVER AND KIDNEY OF RATS

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Abstract

This study aimed to determine the effects of sea cucumber extract *Holothuria atra* on the histopathology of the liver and kidney of Sprague Dawley rats after 90 days of oral administration. The ethanol extract of *H. atra* was used. The rats were divided into six groups according to the dose of extract given, namely Group A (control), Group B (dose of 25 mg/kg BW), Group C (dose of 50 mg/kg BW), Group D (dose 100 mg/kg BW), Group E (dose of 200 mg/kg BW), and Group F (dose of 400 mg/kg BW). The extract was administered orally to the rats for 90 days, after which liver and kidney histopathological observations were carried out. The study showed that the administration of *H. atra* extract to rats caused liver and kidney histopathological changes, especially at 200 mg (group E) and 400 mg (group F). Based on the histopathological description, *H. atra* extract was toxic to the liver and kidney at 200 mg/kg BW and 400 mg/kg BW. The safe doses of *H. atra* extract in the liver and kidney were 25 - 100 mg/kg BW. The results also showed that the extract with doses of 50 and 100 mg/kg BW positively affected the liver. *Holothuria atra* extract was toxic to the liver and kidney at 200 mg/kg BW and 400 mg/kg BW; thus, the recommended safe doses are 25 - 100 mg/kg BW.

Rezumat

Prezentul studiu a avut ca scop evaluarea efectelor extractului etanolic de castravete de mare (*Holothuria atra)* asupra modificărilor histopatologice la nivelul ficatului și rinichilor de șobolani Sprague Dawley, după 90 de zile de administrare orală. Șobolanii au fost împărțiți în șase grupuri în funcție de doza de extract administrată, și anume grupul A (martor), grupul B (doza de 25 mg/kg corp), grupul C (doza de 50 mg/kg corp), grupul D (doza de 100 mg/kg corp), grupul E (doza de 200 mg/kg corp) și grupul F (doza de 400 mg/kg corp). După 90 de zile de administrare orală a extractului, s-a efectuat analiza histopatologică a ficatului și rinichilor. Pe baza analizei histopatologice, extractul de *H. atra* s-a dovedit toxic pentru ficat și rinichi la 200 mg/kg corp și 400 mg/kg corp, în timp ce dozele sigure recomandate au fost de 25 - 100 mg/kg corp.

Keywords: subchronic toxicity, liver, kidney, sea cucumber, *Holothuria atra*

Introduction

The Holothuroids, commonly known as sea cucumbers, are marine invertebrates in the echinoderms phylum. These animals live along coastal waters in tropical and subtropical regions; however, the tropical Indo-Pacific region has higher diversity and abundance than other regions [1-3].

Sea cucumbers have been long consumed by people in Asia and the Middle East as food and traditional medicine. Sea cucumbers have become popular because they contain high-quality nutrients that consist of protein, amino acids, vitamins, minerals and fatty acids [4-5]. In addition, sea cucumbers contain various bioactive compounds in high amounts, such as peptides, polyunsaturated fatty acids, triterpene glycosides, and chondroitin sulphate. The potential of sea cucumbers as anti-asthma, anticancer, anti-inflammatory, anti-oxidant, immunomodulatory and anti-coagulant has been reported [6-7].

One of the sea cucumbers that have the potential as a source of anticancer medicine is *Holothuria atra*. This sea cucumber had the highest cytotoxicity among 15 species of sea cucumbers collected from Gorontalo, Indonesia [8]. In addition, ethanolic extract of *H. atra* has potential cytotoxicity against several cancer cell lines, namely T47D, HeLa, WiDr [9], HepG2 [10], SP-C1 [11], A549, and B16F10 [12]. Ethanol extract of *H. atra* could induce apoptosis and activate caspase-3 in T47D cells [13]. *Holothuria atra* extract also ameliorated methotrexate damages and showed protective potential against testicular cytotoxicity; therefore, it has the potential as an immunosuppressant [14]. *Holothuria atra* extract contains triterpene glycosides, such as holothurin A, holothurin A5,

echinoside A and 24-dehydroechinoside A [15]. These compounds were reported to be responsible for various biological activities. Furthermore, Shahinozzaman *et al*. [12] found that desulfated echinoside B from *H. atra* acted as an inhibitor of PAK1 (p21-activated kinase 1). Based on the results of these studies, *H. atra* has the potential to be used as a source of natural medicine. Therefore the safety information of *H. atra* extract is needed.

Hashim *et al*. [16] and Hanafi *et al*. [17] have reported the acute toxicity (14 days) of *H. atra* water extract and ethanol extract, respectively, in the liver of mice. However, so far, the sub-chronic toxicity evaluation of ethanol extract of *H. atra* has not been reported, specifically for the kidney and liver. This study aimed to investigate the effect of ethanol extract *H. atra* for 90 days on the liver and kidney through histopathological examination.

Materials and Methods

Experimental animals

Adult Sprague Dawley male rats (220 - 250 g) were obtained from The National Agency of Drug and Food Control Republic of Indonesia. The experiment was conducted at the Faculty of Veterinary Medicine, IPB University, Bogor. The rats were housed in cages 39 x 30 x 11 cm (two animals/cage) in dimension. Animals were acclimatized for two weeks before the experiment and fed with a standard rodent pellets diet and Pureit™ *ad libitum*. The investigational procedures adopted in this experiment followed the requirements of the Experimentation Ethics Committee on Animal Use of the Faculty of Veterinary Medicine, IPB, Bogor, Indonesia.

Sub-chronic toxicity study

The sub-chronic oral toxicity was carried out according to OECD-408 guidelines [18]. Rats were weighed to determine the dose of *H. atra* extract. The animals were grouped divided into six groups, where each group consisted of three animals. The extract doses used in this study were 25 mg/kg rat BW (body weight) (group B), 50 mg/kg rat BW (group C), 100 mg/kg rat BW (group D), 200 mg/kg rat BW (group E), and 400 mg/kg rat BW (group F). The control groups (group A) were treated with the same volume of distilled water. About 1 mL of extract was administered daily to each rat by oral gavage for 90 days. At the end of the treatment period, all animals were anesthetized with ketamine 10% (100 mg/kg body weight) and xylazine 2% (10 mg/kg body weight) by intraperitoneal injection. After euthanasia, the rats were sacrificed, and organs were removed by necropsy, and prepared for histopathological examination.

Histological examination

The liver and kidney were carefully cut and preserved in Buffered Neutral Formalin 10%. The tissue's small piece marked (0.5 cm x 1 cm x 1 cm) was then dehydrated with ethanol, followed by xylol, and embedded in paraffin. The paraffin blocks were cut using microtomes with a thickness of 3 - 5 μm and then stained with Haematoxylin-Eosin (HE). The stained sections were examined under a light microscope.

Data analysis

Cell observations of the liver and kidney were carried out quantitatively by counting the number of normal cells, apoptosis, necrosis, fatty degeneration, hydropic degeneration and perivascular inflammatory cells on the area of 1280 x 1024 μm^2 micrographic photo. For the kidney, the part observed was the tubules and glomerulus in the renal cortex. The calculation of these variables was done by using ImageJ software. The observations were statistically analysed using the one-way ANOVA using SPSS 16.0 software.

Results and Discussion

Liver histopathology

The histopathology of rat liver in the portal triad and central vein in all treatment groups is presented in Table I. In contrast, the histopathology of normal cells, apoptosis, necrosis, and lipid degeneration is shown in Figure 1. In general, the administration of sea cucumber extract at 200 mg (group E) and 400 mg (group F) caused significant changes in liver histopathology. These changes were characterized by increased hydrophic degenerative and inflammatory cells in the periphery of the portal triad and central vein. Moreover, a decreasing number of normal cells was also observed in the same area. The number of normal cells, hydrophilic degeneration, and inflammation in the central veins of groups E and F were significantly different ($p < 0.05$) compared to those of the other groups.

Normal hepatocyte cells have a wide homogeneous pinkish cytoplasm and a large and transparent nucleus, so the nucleolus and chromatin can be seen clearly (Figure 1A). Hepatocyte cells undergoing apoptosis were shown in Figure 1B; the cell nucleus was covered by a clear transparent zone, indicating that the cell nucleus's size began to shrink due to apoptosis. The highest number of hydrophic degeneration cells was found at 200 mg/kg BW and 400 mg/kg BW ($p < 0.05$), while the lowest was observed at 100 mg/kg BW. The morphology of hepatocyte cells that underwent lipid degeneration was shown in the cytoplasm containing microvesicles or white vacuoles (Figure 1D), while hydropic degeneration was characterized by thick cytoplasm (Figure 1E). The lowest number of necrotic cells was found at a dose of 100 mg/kg BW (group D). The morphology of necrotic cells was characterized by the expanded cytoplasm due to the nucleus shrinking (karyopyknotic) and hyperchromatic.

Table I

The average number of normal cells, apoptosis, necrosis, lipid degeneration, hydrophic degeneration, and inflammation cells in the portal triangle and central vein of rat liver treated with *H. atra* extract for 90 days

Portal Triangle						
Groups	**Normal**	**Apoptosis**	**Necrosis**	**Lipid Degeneration**	**Hydrophic Degeneration**	**Inflammation cells**
A (control)	13.2 ± 3.3^{b}	5.1 ± 2.6^{b}	27.6 ± 3.9^{b}	2.1 ± 1.1^{b}	23.4 ± 13.8^{b}	11.8 ± 10.7^{b}
B (25 mg/kg)	20.6 ± 10.1^{c}	3.8 ± 5.1^{ab}	22.7 ± 9.6^{bc}	1.2 ± 2.7^{ab}	20.7 ± 10.9^{ab}	10.7 ± 17.6^{b}
C (50 mg/kg)	22.4 ± 6.8^{cd}	2.8 ± 1.8^{a}	20.3 ± 8.3^{ab}	0.9 ± 3.1^{ab}	16.2 ± 5.5^{ab}	4.6 ± 6.8^{a}
D (100 mg/kg)	25.6 ± 5.5^{d}	2.3 ± 1.9^{a}	8.4 ± 8.3^{c}	0.2 ± 0.4^{a}	13.7 ± 7.2^{a}	2.4 ± 2.7^{a}
E (200 mg/kg)	5.6 ± 4.9^{a}	2.7 ± 2.3^{a}	17.6 ± 4.6^{b}	1.1 ± 1.7^{ab}	40.6 ± 9.6^{c}	12.2 ± 5.5^{b}
F (400 mg/kg)	4.8 ± 5.6^{a}	3.6 ± 1.2^{ab}	20.4 ± 5.6^{b}	1.0 ± 1.4^{ab}	46.6 ± 9.7^{c}	14.2 ± 9.6^{c}
Central Vein						
Groups	**Normal**	**Apoptosis**	**Necrosis**	**Lipid Degeneration**	**Hydrophic Degeneration**	**Inflammation cells**
A (control)	21.4 ± 18.2^{b}	3.6 ± 3.5^{b}	23.2 ± 6.2^{b}	1.7 ± 1.4^{ab}	28.8 ± 8.8^{b}	2.5 ± 1.8^{a}
B (25 mg/kg)	23.7 ± 19.2^{b}	3.6 ± 4.5^{b}	19.0 ± 7.7^{ab}	1.6 ± 2.06^{ab}	23.8 ± 4.6^{ab}	2.0 ± 1.1^{a}
C (50 mg/kg)	28.8 ± 18.2^{b}	2.4 ± 3.0^{ab}	17.8 ± 6.1^{a}	1.4 ± 1.3^{ab}	22.8 ± 9.8^{ab}	1.93 ± 1.7^{a}
D (100 mg/kg)	30.1 ± 7.6^{b}	0.9 ± 1.0^{a}	16.0 ± 4.2^{a}	0.6 ± 0.8^{a}	19.4 ± 10.6^{a}	1.6 ± 1.5^{a}
E (200 mg/kg)	8.0 ± 4.1^{a}	2.3 ± 2.9^{ab}	18.4 ± 4.2^{a}	1.6 ± 1.3^{ab}	42.0 ± 5.6^{c}	6.7 ± 3.5^{b}
F (400 mg/kg)	0.0 0.0	2.8 ± 2.2^{ab}	23.0 ± 3.4^{b}	2.3 ± 0.9^{b}	46.4 ± 7.9^{c}	7.4 ± 3.5^{b}

Data are expressed as mean ± standard deviation. Treatments not sharing the same letters in the same column are significantly different by ANOVA ($p < 0.05$).

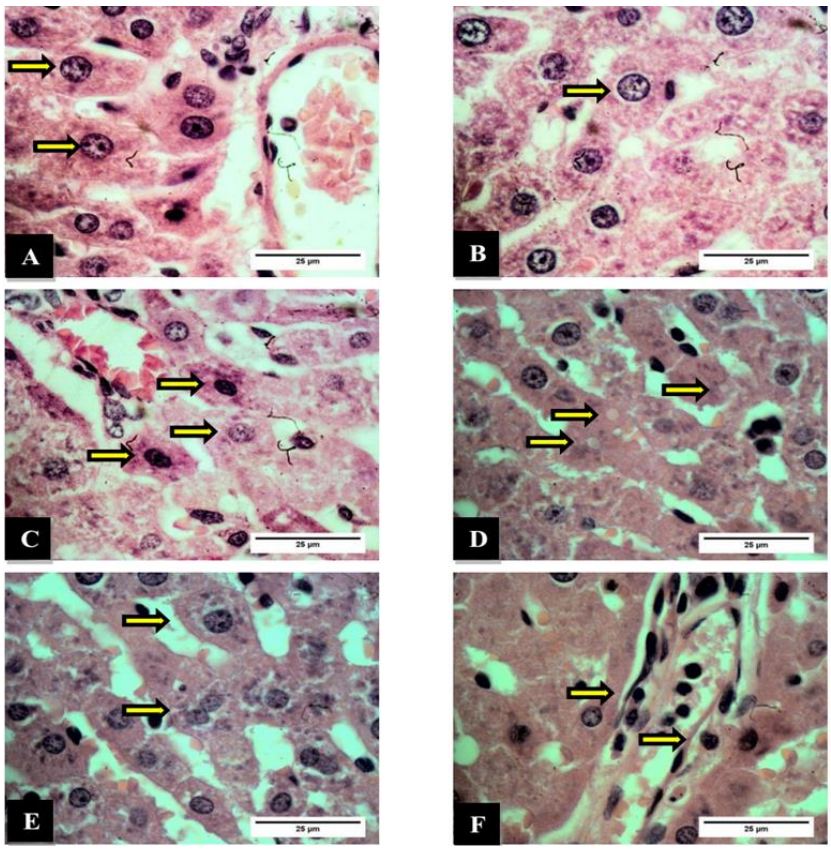

Figure 1.

Microphotography of rat hepatocytes (100 x); A: normal cells B: apoptosis; C: necrotic; D: fat degeneration; E: hydropic degeneration; F: perivascular inflammatory cells. Bars = 50 µm

Furthermore, some of the hepatocytes had ruptured (karyorrhexis) and lysis (karyolysis). The highest number of perivascular inflammatory cells was found at 200 mg/kg BW and 400 mg/kg BW, while the lowest was observed at 100 mg/kg BW. These inflamed cells can be observed in the portal triad and the central vein. The hepatocyte structure was irregular at 200 mg/kg BW and 400 mg/kg BW. The extract at a 100 mg/kg BW dose gave the best results compared to the other groups. The administration of the extract

at this dose resulted in the highest number of normal cells. Conversely, apoptotic, necrosis, fat degeneration, hydrophytic degeneration and perivascular inflammatory cells were small in number.

Kidney histopathology

Table I and Table II summarize the mean number of normal, degeneration, and necrotic cells of the glomerulus and tubules. The histopathologic features of degenerated and necrotic cells are presented in Figure 2. Degenerated cells have vacuoles in their cytoplasm, while necrotic cells are morphologically characterized by a compact, round, dark nucleus. The administration of the extract at doses of 25 - 100 mg/kg BW did not show significant damage to the glomerulus. However, at higher doses (200 and 400 mg/kg BW), there was an increase in the number of degenerated and necrotic cells ($p < 0.05$) (Table II). By increasing the dose given, more cell damage was observed. The same pattern was observed in tubules, with the highest degeneration and necrosis cells found in groups E and F ($p < 0.05$).

Normal glomerulus cells have less extensive than Bowman's capsule. The administration of the extract at 25 - 100 mg/kg BW relatively did not affect the Bowman's capsule area, similar to that of the control group (Figures 2A, 2B, 2C and 2D). In contrast, groups E and F were more expansive due to the occurrence of necrotic cells (Figure 3E and Figure 3F). The increase in the area of Bowman's capsule indicated a shrinkage of the glomerulus due to reduced glomerular-forming cells caused by necrosis. Necrosis is further damaged by irreversible cell degeneration; cell death will occur if the degenerated cells cannot return to their normal state [19]. Cell death can be in the form of shrinkage, round, and dark cells called pycnosis. The shrinking of cells and their number decreases, causing Bowman's space to expand [20].

The presence of protein in the tubule lumen indicated damage in the form of necroes in tubular epithelial cells, as shown in Figure 3E and Figure 3F (doses of 200 and 400 mg/kg BW). The arrangement of the tubules was varied, and there was an expanding tubule lumen, which indicated that the tubule epithelium had atrophied due to degeneration that led to necrosis.

Damage in the tubules is a sign of degeneration characterized by swelling of the tubule epithelial cells causing the tubular lumen to narrow. Degeneration is cell damage that occurs in the early phase before cell death and is reversible, characterized by direct damage to cell structure and function [19].

In addition to degeneration of the tubules, it also undergoes dilatation. Cell necrosis is characterized by the expanded lumen, the thinning of the epithelial cells covering the tubules, and the darkening colour of cell nuclei. Necrosis can occur due to exposure to a toxic metabolite secreted by the glomerulus from the blood. Necrotizing cells in the tubules were found in rats treated with extracts at 200 and 400 mg/kg BW. Although the degenerated and necrotic cells in the kidneys were found, no extracellular inflammatory cells leading to kidney failure or interstitial inflammation were observed.

Table II

The average number of normal cells, degeneration, and necrosis in the glomerulus and kidney tubules of rats treated with *H. atra* extract for 90 days

Glomerulus			
Groups	**Normal cells**	**Degeneration cells**	**Necrosis cells**
A (control)	15.62 ± 1.74^{a}	1.00 ± 1.07^{c}	1.60 ± 0.50^{a}
B (25 mg/kg)	16.46 ± 6.02^{a}	1.46 ± 1.50^{c}	1.40 ± 0.50^{a}
C (50 mg/kg)	14.33 ± 3.65^{a}	1.86 ± 1.12^{c}	1.46 ± 0.63^{a}
D (100 mg/kg)	15.46 ± 1.84^{a}	1.14 ± 1.53^{c}	1.28 ± 0.46^{a}
E (200 mg/kg)	8.53 ± 1.55^{b}	5.46 ± 1.35^{b}	2.06 ± 0.77^{b}
F (400 mg/kg)	4.93 ± 1.66^{c}	10.60 ± 3.11^{a}	2.73 ± 0.76^{c}
Tubules			
Groups	**Normal cells**	**Degeneration cells**	**Necrosis cells**
A (control)	64.40 ± 2.89^{ab}	12.33 ± 2.55^{c}	1.93 ± 1.79^{c}
B (25 mg/kg)	66.86 ± 4.60^{a}	12.20 ± 0.67^{c}	2.20 ± 0.86^{c}
C (50 mg/kg)	66.60 ± 5.60^{a}	12.80 ± 1.52^{c}	2.06 ± 1.86^{c}
D (100 mg/kg)	63.73 ± 2.18^{b}	10.20 ± 1.42^{d}	2.20 ± 1.12^{c}
E (200 mg/kg)	51.73 ± 2.05^{c}	22.26 ± 1.38^{b}	4.13 ± 0.99^{b}
F (400 mg/kg)	39.86 ± 1.84^{d}	36.66 ± 1.11^{a}	6.93 ± 1.53^{a}

Data are expressed as mean ± standard deviation. Treatments not sharing the same letters in the same column are significantly different by ANOVA ($p < 0.05$).

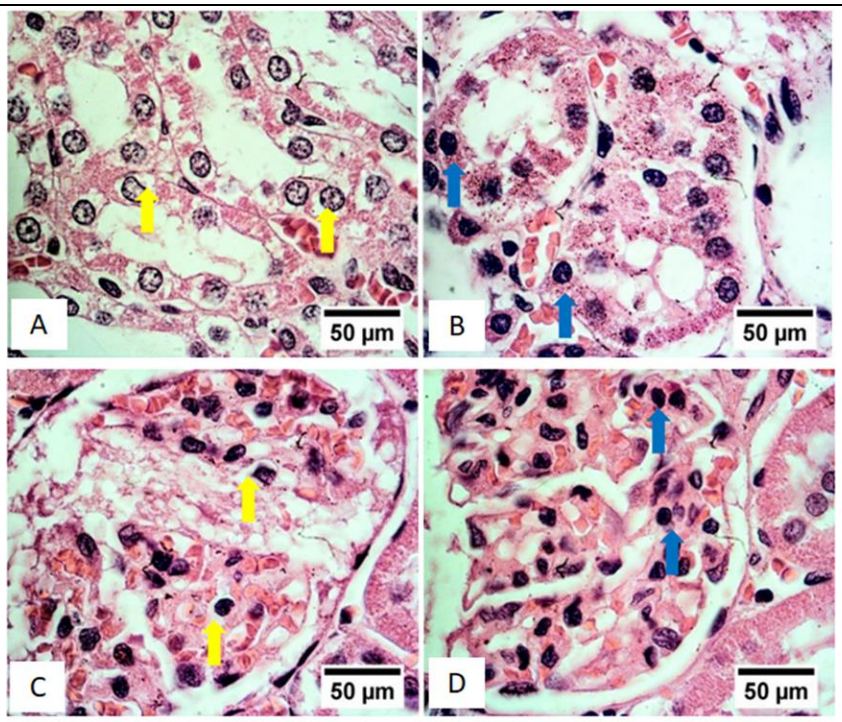

Figure 2.
Microphotography of degenerating cells (A, yellow arrows) and necrosis (B, blue arrows) in the tubules and degenerating cells (C) and necrosis (D) in the glomeruli

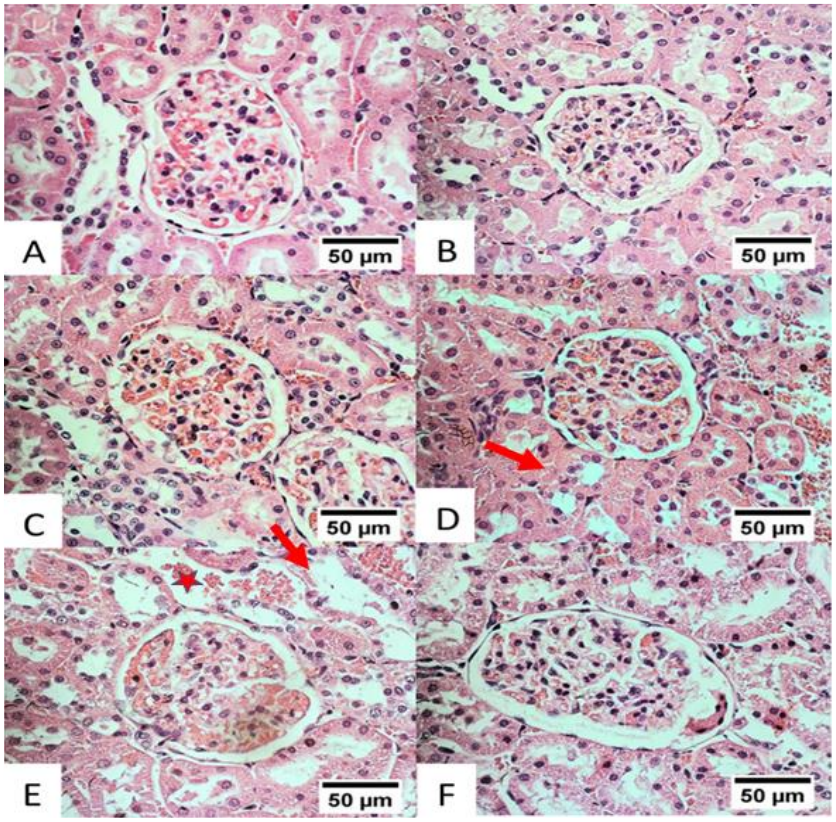

Figure 3.
The kidney histopathology of each group after being treated with *H. atra* extract is shown with HE staining at 40x magnification (bar 50 µm). Group A: distilled water (control); Group B: dose 25 mg/kg BW; Group C: dose 50 mg/kg BW; Group D: dose 100 mg/kg BW; Group E: dose 200 mg/kg BW; Group F: dose 400 mg/kg BW
Red arrows (Figure D) showed degenerated tubular cells, red arrows (Figure E) indicated tubular dilatation and the asterisk (Figure E) showed protein deposits

Decreased functional cells in the glomerulus can cause reduced kidney function; this was indicated by the presence of protein in the lumen of the proximal tubules. Protein is generally not removed, but recirculated to meet the body's needs. Leakage in the glomerulus also indicated the occurrence of cell damage. The leak causes the protein to not recirculated. According to Kumar *et al*. [21], protein deposits in the lumen of the tubules are caused by the presence of plasma proteins that escape from the glomerular capillaries. The protein that escapes the tubule and stays in the lumen exceeds the absorptive capacity of the cell. Protein deposits in the tubular lumen are then brought into the cell to be phagocytized by lysosomes.

As in the liver, the number of normal cells decreased drastically in the renal tubules and glomeruli after being given a dose of extracts E and F (200 and 400 kg/kg BW). At these two doses, degenerating and necrotizing cells were also increased (Table II). At high doses (200 mg and 400 mg), high blood flow to the kidneys in drug and chemical excretion can cause accumulation and damage [22]. Since *H. atra* contains high amounts of saponins [15], the extract can cause cell damage in the tubules and glomerulus.

Although saponins have promising pharmacological properties, they may cause toxicity at high doses. Saponin causes an increase in the permeability of the lipid bilayer of cells to macromolecules that will cause reversible or irreversible damage [23]. Saponins form complexes with cell-membrane cholesterol, which cause pore formation and cell permeabilization, leading to cell membrane damage [24, 25]. Saponin compounds from *H. atra*, such as echinoside and holothurin, are cytotoxic against several cancer cells [12, 26].

The increase in cell degeneration and necrosis at high doses (groups E and F) could also be influenced by the high protein content of sea cucumbers. Dried sea cucumbers contain 40.7 - 63.3% of protein [27], while the crude extracts contain 13.19% protein [28]. Aparicio *et al*. [29] reported that the kidneys play an essential role in protein metabolism. The amount and composition of protein intake directly impact kidney function. This finding agrees with Jia *et al*. [30], which stated that kidney damage could occur in high-protein diets in rats.

This study showed that administration of *H. atra* extract caused significant changes to the liver and kidneys of white rats in groups E and F. These changes increased degeneration and inflammatory cells. On the other hand, the administration of *H. atra* extract was safe to use at doses of up to 100 mg/kg BW because the changes are minimal in these organs.

An interesting result from this study was that at low doses (doses of 50 and 100 mg/kg BW), normal cells in the liver were the highest in number compared to those of the control group ($P < 0.05$). The number of apoptotic and necrotic cells was significantly reduced at this dose, including fatty degeneration, hydropic degeneration, and inflammatory cells ($P < 0.05$). This result suggested that *H. atra* extract at specific doses has positive effects on the liver. The positive effects of *H. atra* extract may be related to the high content of nutrients, such as vitamins, minerals and bioactive compounds that have unique biological activities [4]. Further research is needed to determine the positive effect of *H. atra* extract, particularly related to its function as a hepato-protector.

Conclusions

The administration of *H. atra* sea cucumber extract for 90 days at 50 and 100 mg/kg BW showed positive effects on the liver cells of white rats. At doses of 200 mg/kg BW and 400 mg/kg BW, *H. atra* extract showed opposite results compared to those of 50 mg and 100 mg/kg BW. During 90 days of oral administration, extracts at up to 100 mg/kg BW were safe for the kidney. In contrast, extracts at doses of 200 and 400 mg/kg BW resulted in degeneration and necrosis of kidney cells.

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Conflict of interest

The authors declare no conflict of interest.

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