

Acute Oral Safety Profile of GA: SPI-Encapsulated *Holothuria atra* as a Candidate Antimalarial: An Integrated Clinical, Biochemical, and Histopathological Evaluation in Wistar Rats

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Abstract

Introduction: Malaria remains a significant challenge to public health, reinforcing the importance of identifying new antimalarial candidates with an adequately defined preclinical safety profile. Previous studies have shown antimalarial activity and hepatorenal protective effects of encapsulated *Holothuria atra*, but its safety after a single oral exposure has not yet been comprehensively established. Accordingly, this study evaluated the acute oral toxicity of a 4:1 gum Arabic–soy protein isolate/GA-SPI microencapsulated formulation of *H. atra* in male Wistar rats.

Materials & Methods: Thirty-five animals were assigned to one vehicle-control group and four treatment groups that received a one-time oral administration of 5, 50, 300, or 2,000 mg/kg body weight and were monitored for 14 days. Evaluations included clinical and behavioral observations, food consumption, body weight, hematological indices, serum biochemical markers, absolute organ weights, and histopathology of the heart, brain, liver, and kidneys.

Results: Food consumption did not show progressive suppression with increasing dose, and changes in body weight were not significantly different among groups. Hematological findings were generally stable, whereas biochemical responses varied across parameters without a uniform dose-related trend. Clinical and behavioral changes were more apparent at higher exposures, particularly at 2,000 mg/kg. Histopathological scores differed among groups for all examined organs and were consistently greatest at 2,000 mg/kg.

Conclusion: Taken together, acute exposure to encapsulated *H. atra* was associated with generally maintained systemic tolerability, although the highest dose produced more evident clinical and microscopic tissue changes. Repeated-dose and toxicokinetic studies are needed to delineate the therapeutic safety window more precisely.

Keywords: Acute oral toxicity; Antimalarial candidate; Histopathology; *Holothuria atra*; Microencapsulation

1. Introduction

Malaria remains an important public-health problem in Indonesia, particularly in regions with sustained transmission and geographic and health-system challenges such as Papua and West Papua [1]. Artemisinin-based combination therapy (ACT) remains a standard treatment, but the emergence and spread of *Plasmodium* resistance highlight the need for alternative antimalarial candidates [2]. Marine organisms represent a valuable source of bioactive compounds, including the black sea cucumber *Holothuria atra*, which contains triterpene glycosides and other compounds with potentially relevant biological activities [3].

Previous studies provide evidences supporting the potential of *H. atra* as an antimalarial candidate. Triterpene glycosides, which are among the major bioactive constituents of *Holothuria* species, have been investigated for their potential activity against *Plasmodium*. In particular, an *in silico* study of triterpene glycosides from *H. atra* suggested their potential interaction with orotidine 5'-monophosphate decarboxylase (PfOMPDC), an enzyme involved in pyrimidine biosynthesis in *Plasmodium falciparum*, providing a possible molecular basis for antiplasmodial activity [4]. Importantly, experimental studies have further demonstrated that microencapsulated *H. atra* retains biological activity after formulation. A 2024 study reported that *H. atra* microcapsules exhibited antiplasmodial activity in addition to antioxidant and antibacterial activities, indicating that microencapsulation can preserve multiple bioactive properties of the extract [5]. Furthermore, a subsequent preclinical study using a 4:1 gum Arabic (GA)-based encapsulated formulation demonstrated that administration at 100 mg/kg in *Plasmodium berghei* ANKA-infected mice receiving chloroquine was associated with reduced hepatic and renal histopathological injury [6]. These findings suggest that GA:SPI encapsulation may offer a practical delivery strategy by improving the physicochemical stability, dispersibility, and gastrointestinal handling of *H. atra* bioactive constituents, while potentially preserving their antiplasmodial and tissue-protective effects [5–8]. These findings support further development of the formulation and emphasize the need to establish its safety profile.

Microencapsulation may improve the physicochemical stability and delivery of bioactive constituents. Gum Arabic provides favorable encapsulating and controlled-release properties, whereas soy protein isolate (SPI) can improve encapsulation efficiency and dispersibility [7,8]. Combining GA and SPI may therefore protect *H. atra* constituents during processing and gastrointestinal exposure. However, encapsulation can also modify biological availability and tissue exposure, making safety evaluation of the final formulation essential.

Acute oral toxicity assessment should not rely solely on mortality but should integrate clinical observations, body weight, food intake, hematological and biochemical parameters, organ weights, and histopathology [9–11]. Such an integrated approach can identify both overt systemic effects and organ-specific alterations that may not be reflected by conventional functional biomarkers. Guided by this multidimensional approach, the present study evaluated the acute oral safety of the GA:SPI-encapsulated *H. atra* formulation across a broad dose range, with the aim of defining its overall tolerability and identifying dose levels associated with clinically or microscopically detectable alterations.

2. Materials and Methods

2.1. Experimental design, ethical considerations, and animal subjects

A posttest-only control-group experimental study was conducted to evaluate the acute oral toxicity of GA 4:1-encapsulated *H. atra* in male Wistar rats (*Rattus norvegicus*). Thirty-five rats aged 8–10 weeks and weighing approximately 150–200 g were allocated into five groups (n=7/group): vehicle-control group (G0) and four treatment groups receiving 5 mg/kg (G1), 50 mg/kg (G2), 300 mg/kg (G3), or 2,000 mg/kg body weight (G4) of the encapsulated formulation.

2.2. Preparation of encapsulated *Holothuria atra*

Dried *H. atra* material was milled and sieved through a 40-mesh screen. Approximately 2 kg of the resulting powder was macerated in 70% ethanol using a 1:5 solid-to-solvent ratio. The solvent extract was subsequently concentrated in a rotary evaporator operated at 500 rpm and 70 °C. The concentrated extract was combined with 1% gum Arabic and 1% soy protein isolate to achieve a GA:SPI ratio of 4:1. After homogenization by stirring for 120 min at 25 °C, the preparation was freeze-dried to produce encapsulated *H. atra* powder. Selection of the 4:1 GA:SPI formulation was based on its earlier preclinical development and reported antimalarial potential [12].

2.3. Acute oral toxicity procedure and clinical monitoring

Acute oral toxicity was examined using a fixed-dose procedure guided by OECD Test Guideline 420 [13]. After dosing, all animals were monitored for 14 days for mortality and treatment-associated clinical changes. Observations covered general activity, responsiveness to stimuli, posture, tremor, seizures, ocular appearance, respiration, salivation, and diarrhea. Following acute oral exposure to encapsulated *Holothuria atra*, clinical and behavioral signs

were evaluated using a 0–3 scoring system. Parameters included spontaneous activity, response to stimuli, posture, tremor, seizure, eye condition, respiration, salivation, and diarrhea. A score of 0 indicated a normal or absent finding, while scores of 1, 2, and 3 indicated progressively increasing severity. Higher total scores represented more severe clinical and behavioral abnormalities.

2.4. Hematological, biochemical, and histopathological assessments

The hematology panel comprised white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT) count, and leukocyte differential counts. Serum biochemical parameters included glucose, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, blood urea nitrogen (BUN), alkaline phosphatase (ALP), total cholesterol, and triglycerides. Histopathological lesions in the heart, liver, kidney, and brain were evaluated using established scoring criteria [14-17].

2.5. Statistical analysis

Data were analysed statistically. A two-sided $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Clinical and behavioral observations

Kruskal-Wallis testing identified significant between-group differences for all post-treatment clinical and behavioral domains ($p < 0.05$). Spontaneous-activity scores increased from 0.97 ± 0.21 in G0 to 1.89 ± 0.36 in G3 and 1.91 ± 0.28 in G4. Dunn-Holm comparisons showed higher scores in G3 and G4 than in G0 and G1, while G2 remained intermediate and was not significantly different from either lower- or higher-scoring groups (Table 1).

Table 1. Post-treatment behavioral scores following administration of encapsulated *H. atra*

Parameter	G0	G1	G2	G3	G4	<i>p</i> value
Spontaneous activity	0.97 ± 0.21^a	1.14 ± 0.15^a	1.51 ± 0.16^{ab}	1.89 ± 0.36^b	1.91 ± 0.28^b	<0.001

Response to stimuli	0.66±0.36 ^a	0.86±0.15 ^a	0.83±0.35 ^a	1.23±0.35 ^{ab}	1.97±0.24 ^b	<0.001
Body posture	0.40±0.16 ^a	0.66±0.46 ^a	0.63±0.24 ^a	0.89±0.34 ^{ab}	1.49±0.28 ^b	<0.001
Tremor	0.46±0.22 ^a	0.66±0.28 ^a	0.69±0.20 ^a	0.74±0.30 ^{ab}	1.40±0.23 ^b	<0.001
Seizure	0.46±0.34 ^a	0.66±0.19 ^a	0.74±0.15 ^{ab}	1.31±0.20 ^{bc}	1.57±0.18 ^c	<0.001
Eye condition	0.51±0.11 ^a	0.71±0.28 ^{ab}	0.94±0.44 ^{ab}	1.09±0.65 ^{ab}	1.23±0.51 ^b	0.037
Respiration	0.51±0.23 ^a	0.71±0.40 ^{ab}	1.03±0.29 ^{ab}	1.09±0.36 ^{ab}	1.37±0.39 ^b	0.005
Salivation	0.37±0.34 ^a	0.54±0.22 ^{ab}	0.74±0.25 ^{ab}	0.74±0.40 ^{ab}	1.00±0.43 ^b	0.054
Diarrhea	0.29±0.11 ^a	0.54±0.30 ^{ab}	0.60±0.23 ^{ab}	0.66±0.19 ^{ab}	0.91±0.16 ^b	0.001

NB: G0, untreated vehicle control; G1, 5 mg/kg BW; G2, 50 mg/kg BW; G3, 300 mg/kg BW; G4, 2,000 mg/kg BW. Within a row, groups that share at least one superscript letter are statistically comparable, whereas groups without a common superscript differ according to Dunn post-hoc comparisons with Holm correction ($p < 0.05$).

3.2. Food intake

Table 2 showed that mean food intake was 17.00±3.54 g/day in the vehicle-control group and ranged from 19.87±3.96 to 22.47±3.40 g/day among rats receiving the GA:SPI 4:1 formulation. The overall group effect was significant (one-way ANOVA, $F(4,30)=2.719$, $p=0.048$, $\eta^2=0.266$). Tukey analysis identified higher intake in G1 than in G0 ($p=0.040$), while G2, G3, and G4 were comparable with the control group ($p > 0.05$). Intake did not decline with increasing dose, and animals exposed to 2,000 mg/kg continued to consume amounts similar to the other treated groups. Thus, no progressive dose-associated suppression of feeding was evident during follow-up.

Table 2. Mean daily food intake during the 14-day observation period

Group	Dose (mg/kg BW)	Food intake (g/day) mean ± SD	Tukey
G0	control group	17.00 ± 3.54	a
G1	5	22.47 ± 3.40	b
G2	50	21.47 ± 2.80	ab
G3	300	19.87 ± 3.96	ab
G4	2,000	21.26 ± 3.21	ab

Superscript letters denote Tukey post-hoc groupings. Groups sharing at least one superscript letter are not significantly different, whereas groups with no superscript letter in common differ significantly at $p < 0.05$.

3.3. Body weight observation

Table 3 showed that the body-weight change was not significantly different among groups ($p=0.209$). Although G0 showed a numerical decrease over the observation period,

animals in G1-G4 generally maintained or gained weight. No progressive loss of body weight was evident across the dose range, including at 2,000 mg/kg.

Table 3. Change in body weight of rats after treatment with encapsulated *H. atra*

Group	Initial Observation (g)	Final Observation (g)	Change (g)
G0	134.29 ± 14.13	122.29 ± 20.80	-12.00 ± 24.59
G1	153.00 ± 20.61	164.29 ± 23.82	11.29 ± 16.17
G2	156.29 ± 23.77	156.43 ± 32.84	0.14 ± 25.06
G3	152.43 ± 18.75	156.29 ± 16.16	3.86 ± 13.43
G4	138.86 ± 17.65	147.29 ± 22.61	8.43 ± 12.93

Data are shown as mean±SD (n=7/group). G0, control; G1, 5 mg/kg BW; G2, 50 mg/kg BW; G3, 300 mg/kg BW; G4, 2,000 mg/kg BW. Between-group differences in body-weight change were analyzed using one-way ANOVA and were not statistically significant (F(4,30)=1.57, p=0.209). Because the omnibus test was not significant, post-hoc pairwise comparisons were not performed.

3.4. Absolute organ weight

Table 4 showed no significant group differences were detected for absolute kidney, liver, pancreas, or lung weight (p>0.05). Brain weight differed overall (p=0.035), but the only significant pairwise contrast was between G1 and G3, and neither differed from G0. Heart weight also showed an overall difference (p=0.018), although no pairwise contrast remained significant after Holm correction. A group effect was found for spleen weight (p=0.002), with higher values in G2 and G4 than in G0 and intermediate values in G1 and G3. Collectively, organ-weight findings did not display a uniform dose-related pattern.

Table 4. Absolute organ weights following encapsulated *H. atra* administration

Organ	G0	G1	G2	G3	G4	p-value
Brain	1.16±0.18 ^{ab}	0.92±0.12 ^a	1.18±0.30 ^{ab}	1.19±0.12 ^b	1.08±0.31 ^{ab}	0.035
Heart	0.46±0.13 ^a	0.48±0.10 ^a	0.63±0.18 ^a	0.65±0.11 ^a	0.76±0.35 ^a	0.018
Kidney	1.01±0.24 ^a	1.06±0.19 ^a	1.18±0.25 ^a	1.17±0.21 ^a	0.96±0.09 ^a	0.217
Liver	4.46±0.36 ^a	5.05±0.76 ^a	4.24±1.52 ^a	4.95±1.02 ^a	4.77±0.85 ^a	0.505

Data are presented as mean ± SD in grams (n=7/group). G0, control; G1, 5 mg/kg BW; G2, 50 mg/kg BW; G3, 300 mg/kg BW; G4, 2,000 mg/kg BW. Brain and heart weights were analyzed using the Kruskal–Wallis test followed by Dunn–Holm post-hoc comparisons, whereas the remaining organs were analyzed using one-way ANOVA followed by Tukey's post-hoc test when appropriate. Superscript letters indicate the results of post-hoc pairwise comparisons: groups sharing at least one superscript letter are not significantly different, whereas groups with no superscript letter in common differ significantly at p<0.05.

3.5. Hematological and serum biochemical findings

Albumin, ALT, AST, ALP, and creatinine differed significantly among groups, whereas BUN did not. The direction and magnitude of these biochemical changes did not form a consistent dose-response pattern. G4 showed the highest ALP value, while BUN, as a conventional renal-function indicator, remained statistically unchanged, as presented in Table 5.

Table 5. Serum biochemical parameters

Parameter	G0	G1	G2	G3	G4	p
Albumin	3.08 (2.28–3.47) a	2.61 (2.49–2.90) a	2.47 (2.05–2.86) a	1.01 (0.97–1.20) ^b	2.32 (1.91–2.33) ab	<0.001
ALT	24.81 ± 6.90 ^{ab}	29.89 ± 5.66 ^a	25.08 ± 6.80 ^{ab}	43.02 ± 10.06 ^c	15.10 ± 8.69 ^b	<0.001
AST	132.36 ± 52.25 ^a	153.23 ± 32.71 ^a	146.75 ± 37.48 ^a	64.13 ± 11.63 ^b	117.47 ± 25.01 ^{ab}	<0.001
ALP	68.17 (51.57–78.84) ^{abc}	57.46 (56.11–64.08) ^{ab}	53.78 (45.63–60.21) ^a	78.28 (74.42–93.63) ^{bc}	173.71 (138.33–267.82) ^c	<0.001
Creatinine	1.56 (1.36–1.76) a	1.31 (1.25–1.43) a	1.33 (1.13–1.46) a	0.51 (0.48–0.60) ^b	1.14 (0.94–1.17) ab	<0.001
BUN	17.12 (12.58–27.10)	19.18 (12.43–19.86)	19.86 (18.05–20.57)	13.10 (13.09–13.87)	18.14 (16.62–18.43)	0.077

Results are expressed as mean±SD for normally distributed variables and as median (Q1–Q3) for non-normal variables. G0, control; G1, 5 mg/kg; G2, 50 mg/kg; G3, 300 mg/kg; G4, 2,000 mg/kg BW. Depending on distributional assumptions, group comparisons used one-way ANOVA with Tukey testing or Kruskal-Wallis analysis with Dunn-Holm adjustment. Superscript letters indicate the results of post-hoc pairwise comparisons. Groups sharing at least one superscript letter are not significantly different, whereas groups with no superscript letter in common differ significantly at p<0.05.

Table 6 showed that hemoglobin, hematocrit, RBC count, and platelet count were statistically similar across groups. Taken together, the hematological data did not reveal a consistent pattern of increasing alteration with dose.

Table 6. Hematological parameters

Parameter	G0	G1	G2	G3	G4	p
Hemoglobin	9.58±5.78 ^a	14.76±1.67 ^a	15.89±4.39 ^a	14.14±4.83 ^a	12.79±6.82 ^a	0.189
Hematocrit	30.70±16.75 ^a	42.32±2.42 ^a	43.17±7.76 ^a	38.91±9.80 ^a	36.01±17.00 ^a	0.318
RBC	5.52	6.25	6.30	6.14	6.39	0.589

	(3.55–6.28) ^a	(6.22–6.79) ^a	(6.20–6.33) ^a	(6.08–6.57) ^a	(5.04–6.89) ^a	
WBC	12.37±7.73 ^a	19.25±2.05 ^a	15.10±2.72 ^a	18.48±5.67 ^a	13.39±7.24 ^a	0.034
Platelets	489.89±383.70 ^a	879.52±75.43 ^a	1020.83±424.16 ^a	1008.93±378.22 ^a	887.74±504.88 ^a	0.090

Values are summarized as mean±SD for normally distributed variables or median (Q1-Q3) for non-normal variables. G0, control; G1, 5 mg/kg BW; G2, 50 mg/kg BW; G3, 300 mg/kg BW; G4, 2,000 mg/kg BW of GA:SPI 4:1-encapsulated *Holothuria atra*. Depending on the data characteristics, comparisons used one-way ANOVA with Tukey post-hoc testing, Welch ANOVA with Games-Howell comparisons, or Kruskal-Wallis testing followed by Dunn-Holm adjustment. Superscript letters indicate post-hoc groupings. Groups sharing the same superscript letter are not significantly different from each other, whereas groups with no superscript letter in common differ significantly at $p < 0.05$. All groups sharing the superscript ^a indicate that there were no significant pairwise differences among the groups for the respective hematological parameter.

3.6. Histopathological findings

Table 7 showed that the composite histopathological scores differed among groups for every organ examined. Overall, microscopic tissue alterations were most consistently expressed at the highest administered dose. Histopathological examination of the kidney, liver, heart, and were presented in Figures 1–4.

Table 7. Composite histopathological scores following administration of encapsulated *H. atra*

Organ	G0	G1	G2	G3	G4	p-value
Heart	1.49 ± 0.14 ^a	1.67 ± 0.13 ^{ab}	1.76 ± 0.13 ^{abc}	1.89 ± 0.18 ^{bc}	2.21 ± 0.15 ^c	<0.001
Brain	1.39 ± 0.11 ^a	1.67 ± 0.16 ^{ab}	1.77 ± 0.15 ^{abc}	1.90 ± 0.16 ^{bc}	2.17 ± 0.23 ^c	<0.001
Liver	1.46 ± 0.07 ^a	1.96 ± 0.22 ^b	1.86 ± 0.22 ^{ab}	1.90 ± 0.27 ^b	2.17 ± 0.22 ^b	0.001
Kidney	1.55 ± 0.20 ^a	1.57 ± 0.25 ^a	1.66 ± 0.10 ^{ab}	1.77 ± 0.23 ^{ab}	1.99 ± 0.27 ^b	0.017

Data are presented as mean±SD (n=7/group). Composite scores were calculated by averaging lesion-component scores obtained from five microscopic fields per animal. G0, control; G1-G4, encapsulated *H. atra* at 5, 50, 300, and 2,000 mg/kg BW, respectively. Group effects were assessed with the Kruskal-Wallis test and significant omnibus findings were followed by Dunn comparisons with Holm adjustment. Within a row, a shared superscript letter indicates statistical similarity; groups without a common letter differ at $p < 0.05$.

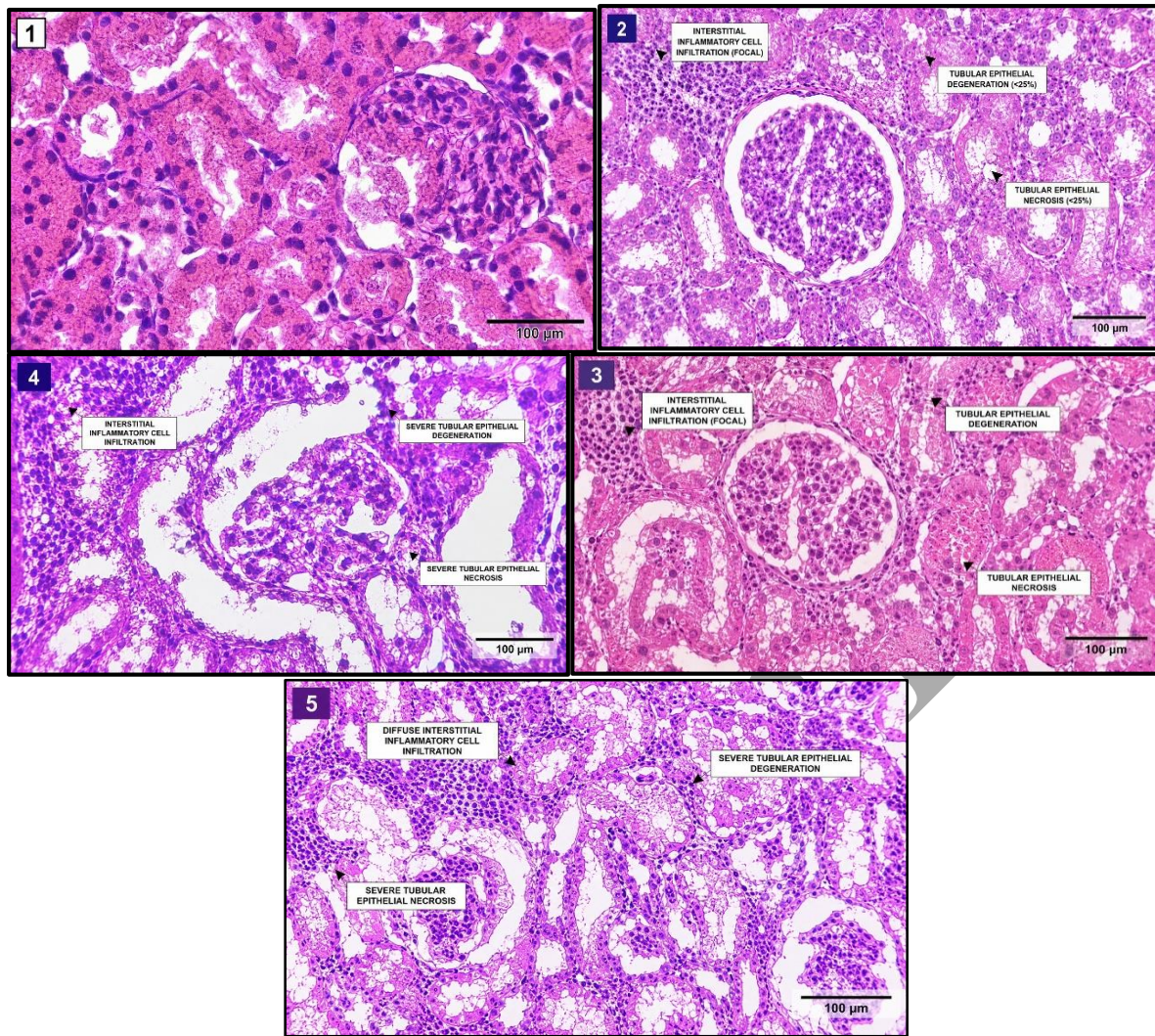


Figure 1. Characterisation of the kidney by histopathological changes at different stages of injury.

The panels illustrate a continuum of renal histopathological change from near-normal morphology to severe injury. Panel 1 shows largely preserved renal architecture. Panel 2 demonstrates mild tubular epithelial degeneration and necrosis with limited focal interstitial inflammatory-cell infiltration. Panel 3 shows more evident degeneration, necrosis, and interstitial inflammation. Panel 4 displays broader degenerative and necrotic involvement accompanied by a more extensive inflammatory infiltrate. Panel 5 represents the most severe pattern, with marked disruption of tissue architecture, diffuse tubular epithelial degeneration and necrosis, and the most prominent interstitial inflammatory-cell infiltration. H&E staining; scale bar = 100 µm.

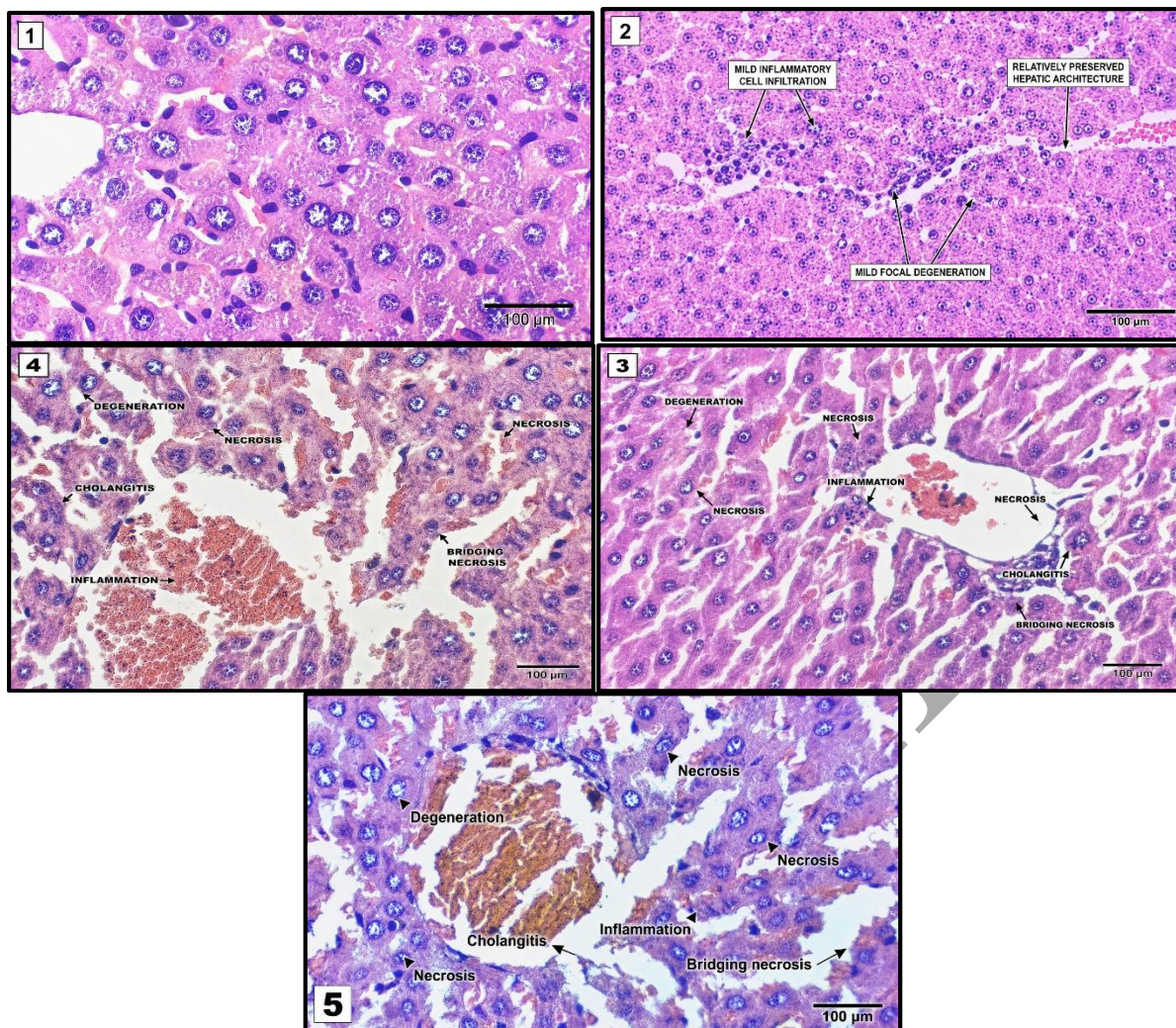


Figure 2. Histopathological findings of the liver at various levels of injury.

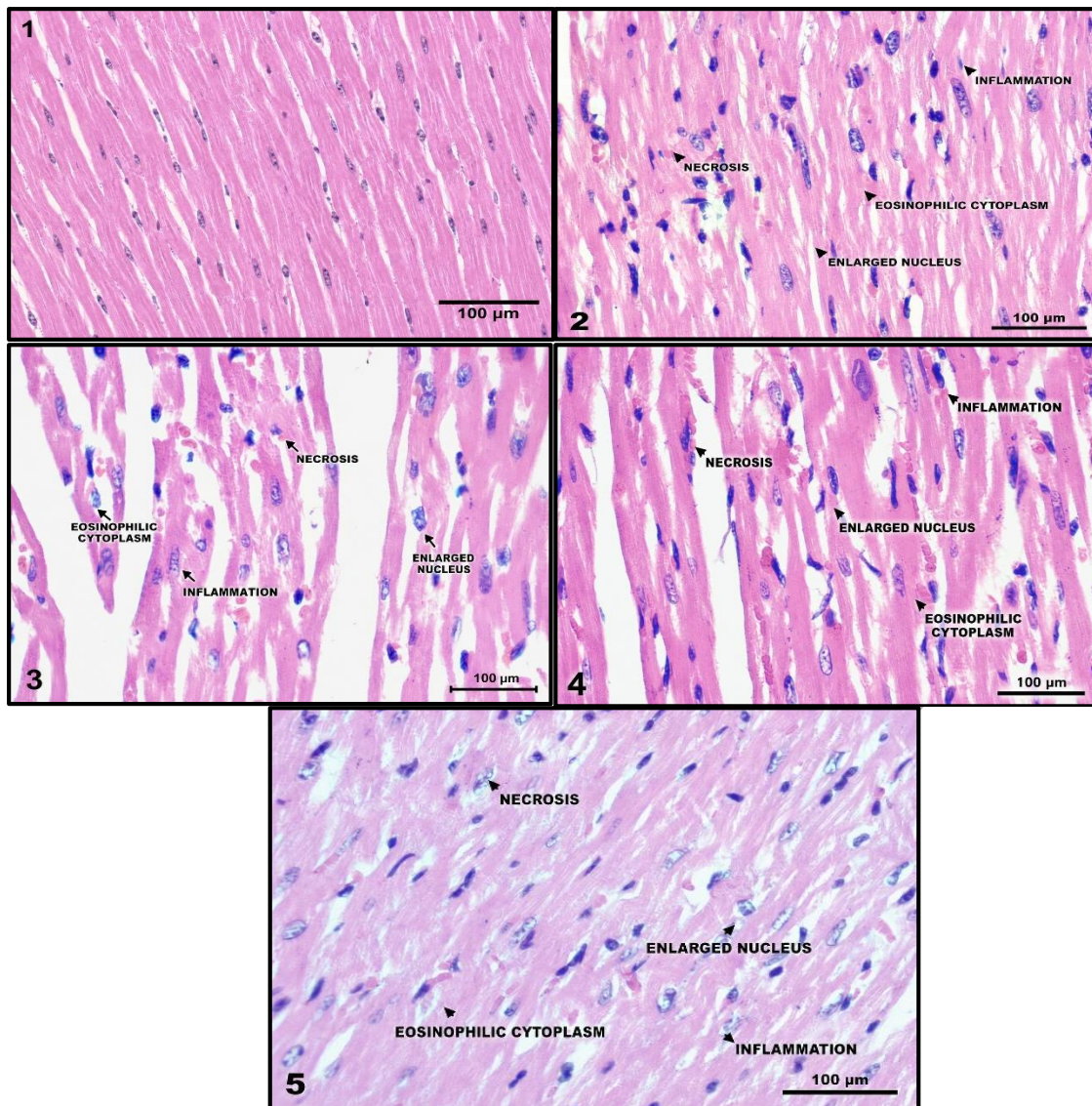


Figure 3. Historical aspects of heart after varied degrees of injury

Panel 1 demonstrates largely preserved myocardial architecture without major histopathological abnormalities. Panel 2 shows mild myocardial injury characterized by eosinophilic cytoplasmic change, nuclear enlargement, focal necrosis, and sparse inflammatory-cell infiltration. Panel 3 demonstrates moderate injury with more evident cellular and structural abnormalities. Panel 4 shows more extensive myocardial damage with broader inflammatory changes, eosinophilic cytoplasmic alteration, nuclear enlargement, and necrosis. Panel 5 represents the most severe pattern among the graded images. H&E staining; scale bar = 100 µm.

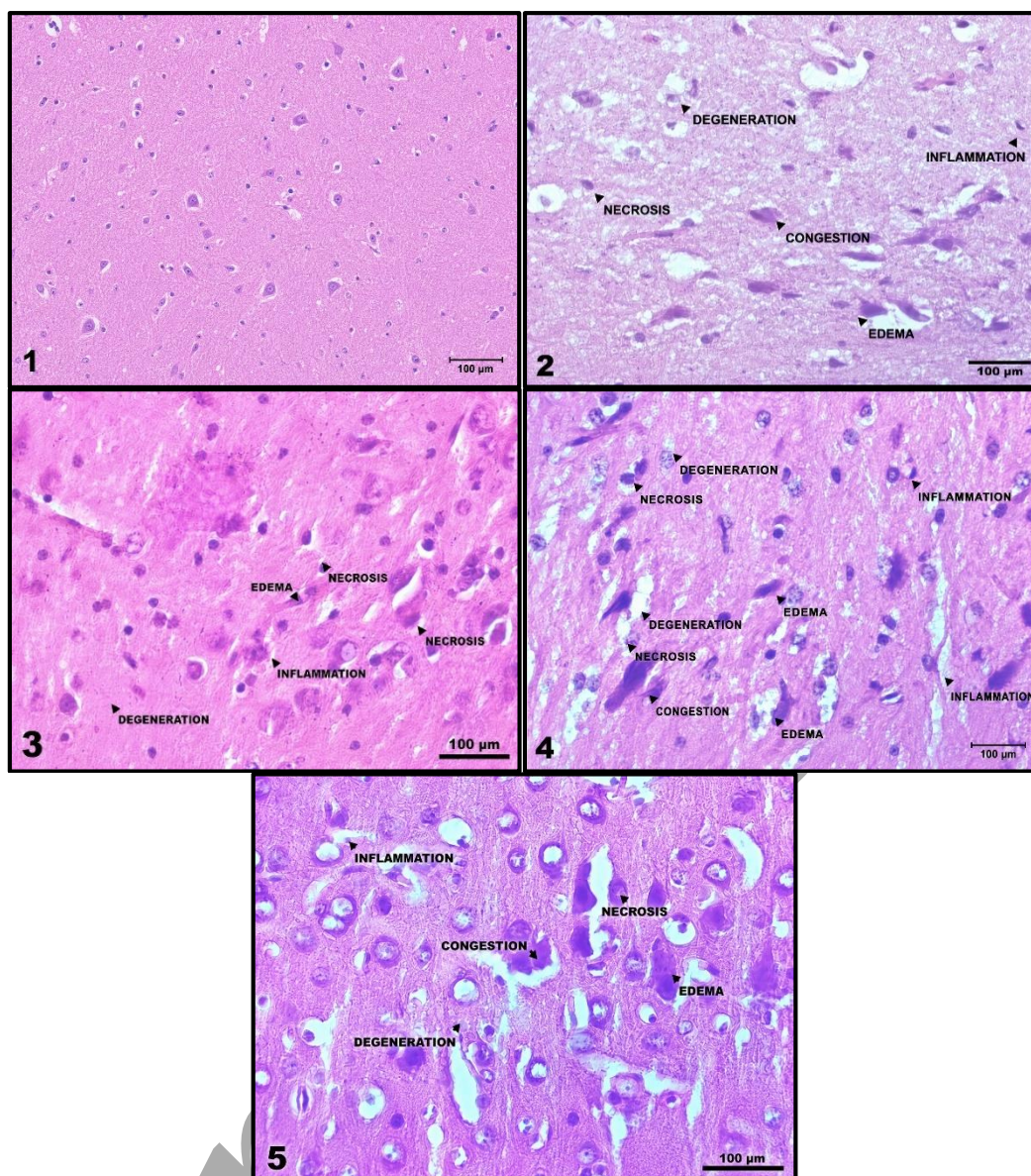


Figure 4. Representative histopathological features of the brain at increasing degrees of tissue injury.

Panel 1 demonstrates relatively preserved brain tissue with minimal microscopic abnormality. Panel 2 shows mild injury with degeneration, focal necrosis, edema, congestion, and inflammatory-cell infiltration. Panel 3 demonstrates moderate tissue injury with more apparent degeneration, necrosis, edema, and inflammation. Panel 4 shows a broader distribution of degenerative, necrotic, edematous, congestive, and inflammatory changes. Panel 5 represents the most severe pattern, with the greatest overall extent of these lesions among the graded images. H&E staining; scale bar = 100 µm.

Figure 5 illustrates the composite histopathological scores of the heart, brain, liver, and kidney across the experimental groups. Overall, the mean histopathological scores tended to increase with increasing doses of GA:SPI 4:1-encapsulated *Holothuria atra*, with the highest values consistently observed in G4 (2,000 mg/kg BW). For the heart, the score increased from

1.49 in G0 to 2.21 in G4 ($p < 0.001$), while the brain score increased from 1.39 to 2.17 ($p < 0.001$). The liver showed scores ranging from 1.46 in G0 to 2.17 in G4 ($p = 0.001$), although the increase was not strictly dose-dependent, as G1 showed a higher score than G2 and G3. Similarly, kidney scores increased from 1.55 in G0 to 1.99 in G4 ($p = 0.017$). These findings indicate that histopathological alterations were most pronounced at the highest dose, particularly in the heart, brain, and liver.

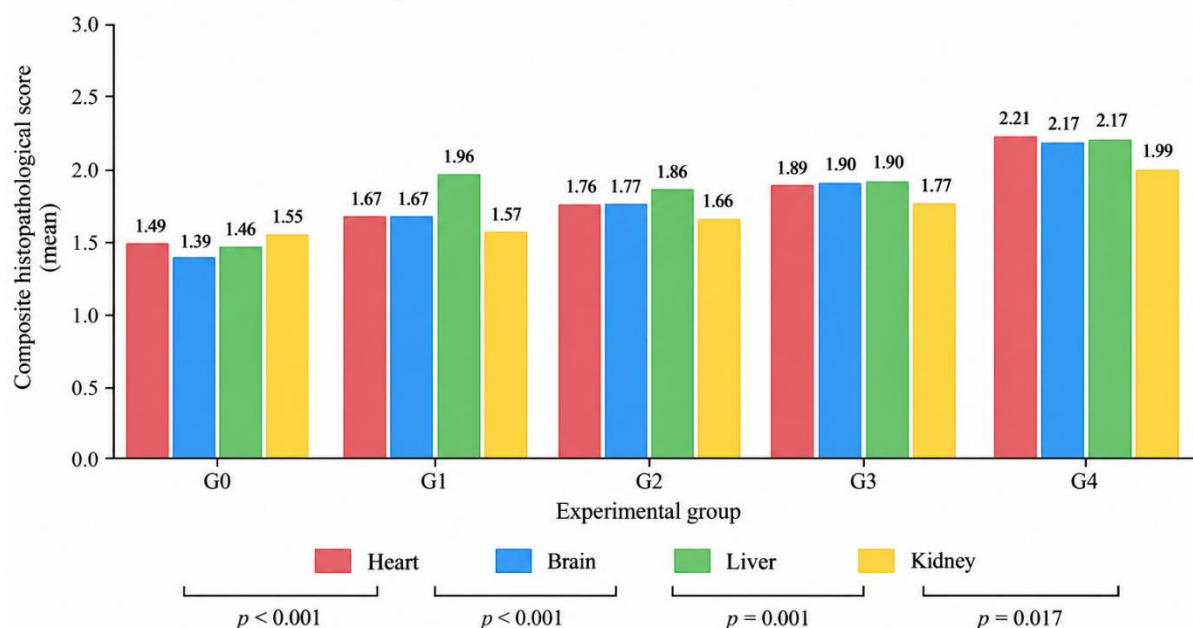


Figure 5. Histopathological safety profile across experimental groups. Bars represent mean composite histopathological scores for the heart, brain, liver, and kidney following administration of GA:SPI 4:1-encapsulated *Holothuria atra*.

Values are mean composite histopathological scores. G0 = vehicle-control; G1 = 5 mg/kg; G2 = 50 mg/kg; G3 = 300 mg/kg; G4 = 2,000 mg/kg BW of GA:SPI 4:1-encapsulated *Holothuria atra*. Higher scores indicate greater histopathological alteration. Overall group differences were significant for the heart ($p < 0.001$), brain ($p < 0.001$), liver ($p = 0.001$), and kidney ($p = 0.017$).

4. Discussion

This study provides an integrated assessment of the acute oral safety of GA 4:1-encapsulated *H. atra*. Overall tolerability was maintained with respect to food intake and body weight, indicate that the formulation did not produce sustained anorexia or generalized deterioration during the 14-day observation period. Nevertheless, clinical and behavioral changes became more apparent with increasing exposure, particularly at 300 and 2,000 mg/kg. Such findings emphasize that preserved body weight does not necessarily indicate the absence

of biological effects during acute toxicity assessment. Similar observations have been reported for other natural-product nanoparticle formulations [18,19].

Hematological results likewise suggested a selective response rather than generalized systemic toxicity. Hemoglobin, hematocrit, erythrocyte count, and platelet count remained comparable across groups, while the overall WBC difference did not translate into significant pairwise contrasts. Serum biochemical responses were variable rather than uniformly dose related. Albumin, ALT, AST, ALP, and creatinine showed significant between-group differences, whereas BUN did not accumulate with exposure. ALT peaked in G3, while ALP reached its highest value in G4, illustrating the lack of a single monotonic pattern. Individual enzyme changes should therefore be interpreted cautiously and in the context of structural evidence from the target organ. Conventional serum markers remain useful for screening but provide limited tissue specificity and mechanistic resolution; integration with histopathology and complementary biomarkers is consequently more informative [20,21].

ALT reached its maximum in G3, whereas G4 showed the highest ALP concentration and the greatest composite liver histopathology score; absolute liver weight, however, was unchanged. This suggests that serum enzymes and tissue morphology may reflect distinct aspects or time points of hepatic response rather than a single uniform sequence of injury. Because conventional serum markers have limited tissue specificity, they are most informative when interpreted together with histopathological evidence of structural change [21].

Histopathology provided the most consistent indication of a tissue response at high exposure. Although changes were not strictly monotonic for every organ or dose, composite scores were greatest in G4, and cardiac and cerebral alterations were already detectable at 300 mg/kg. Microscopic examination therefore appeared more sensitive than several systemic indicators for identifying tissue-level effects. The overall pattern is best described as increasingly evident structural alteration at high exposure rather than as a uniformly linear toxic response. The biological basis of these findings may relate to the chemical complexity of *H. atra*. Sea cucumbers contain multiple classes of bioactive constituents, including triterpene glycosides, sulfated polysaccharides, peptides, and other metabolites with distinct biological properties. Reviews indicate that activity varies substantially according to compound class, species, and tissue source [22,23].

Encapsulation adds further complexity because the carrier system can modify gastrointestinal stability, release, and systemic availability. Studies of natural compounds have repeatedly shown that polymer-based delivery systems can alter oral bioavailability, sometimes increasing exposure several-fold relative to the unencapsulated compound [24,25].

5. Conclusion

This study advances the preclinical evaluation of GA:SPI 4:1-encapsulated *Holothuria atra* by characterizing acute oral safety across exposures from 5 to 2,000 mg/kg body weight. Overall systemic tolerance was largely maintained: food intake and body-weight trajectories showed no consistent deterioration with dose, and most hematological variables remained comparable among groups. In contrast, clinical and behavioral changes became more evident as exposure increased, while serum biochemical responses were heterogeneous and did not follow a uniform dose-response pattern. Histopathological assessment provided the most consistent evidence of high-exposure effects, with the greatest alterations in the heart, brain, liver, and kidneys at 2,000 mg/kg.

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Compliance with ethical guidelines

This study was approved by the Health Research Ethics Committee, Faculty of Medicine, Hang Tuah University, Surabaya, Indonesia (Ethical Approval No. I/029/UHT.KEPK.03/VII/2026).

Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' contributions

Conceptualization, study design, and writing—original draft, PDU; administrative, technical, and material support, and writing—review and editing, ETP and INAP; data analysis and interpretation, and writing—review and editing, PDU and HS; data acquisition and writing—review and editing, DRTS.

Declaration of AI-assisted tools

The authors used artificial intelligence (AI)-assisted tools solely for English grammar correction and language polishing. All scientific content, data analysis, interpretations, and conclusions were independently prepared and verified by the authors, who take full responsibility for the accuracy and integrity of the manuscript.

Conflict of interest

The authors declared no conflict of interest.

List of abbreviations

ACT, artemisinin-based combination therapy; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BUN, blood urea nitrogen; GA, gum Arabic; GA:SPI, gum Arabic–soy protein isolate; *H. atra*, *Holothuria atra*; H&E, hematoxylin and eosin; PfOMPDC, *Plasmodium falciparum* orotidine 5'-monophosphate decarboxylase; PLT, platelet; RBC, red blood cell; SPI, soy protein isolate; and WBC, white blood cell.

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Graphical abstract:

