Original Research

Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/42/1/MRN-1431

Toxicity studies of sea anemone (*Gyrostoma helianthus*) crude extract and fractionated proteins on liver and kidney in rats

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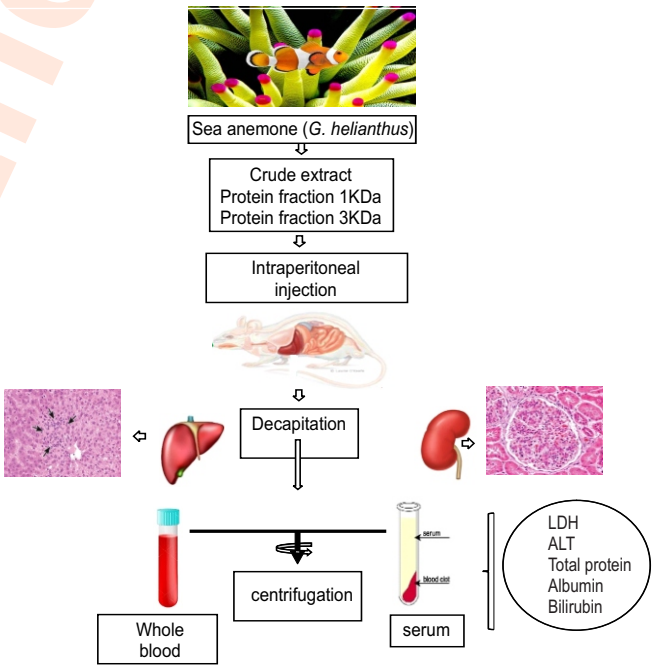
Received: 20.02.2020 *Revised*: 25.04.2020 *Accepted*: 30.09.2020

Abstract

Aim: To study the poisonous effects of crude extract and fractionated protein of sea anemone *Gyrostoma helianthus* on histological and biochemical parameters in male rats.

Methodology: Live specimens of *Gyrostoma helianthus* were collected from Jeddah coast, fixed in ethanol, dried and powdered to obtain the crude residue extract. Some of the crude extract were ultra-filtered to produce protein fractions of 1 and 3KDa. The LD_{50} of crude extract powder was estimated for male rats and momentary repeated subacute dosing (1/4 LD_{50}) for 7 days was carried out to obtain toxicity data. Histological examination of liver and kidney sections were carried out. Serum whole protein, total albumin, ALT and LDH were estimated by standard protocol.

Results: The LD_{50} of crude extract was 20.32 mg kg^{-1} for male rats. In acute and subsequent sub-acute toxicity, neurological symptoms such as convulsions, paralysis, tremors, and ataxia were observed overdose exposure. At the end of exposure to subacute dose histopathological changes like hemolyzed blood and atrophy of glomerular tuft in kidney and fatty changes, vacuolation, necrosis, and infiltration in liver was noted. Furthermore, vital significant increase in total protein, ALT and LDH and reduced bilirubin in serum of treated groups was observed as compared to the control.



Interpretation: The present study emphasizes the toxicological, behavioral, biochemical, and histological bioactivity of crude extract and protein fractions of 1 and 3 KDa of *Gyrostoma helianthus* sea anemone, which is commonly found in the Red Sea. The tested extracts were found to be active at a concentration 5.08 mg kg^{-1}. The yielded effects may interpret treatment strategies of toxicological and pharmacologic intervention.

Key words: Biochemistry, *Gyrostoma helianthus*, Histopathology, Toxicity

How to cite : Al-Shaikh, Turki M.: Toxicity studies of sea anemone (*Gyrostoma helianthus*) crude extract and fractionated proteins on liver and kidney in rats. *J. Environ. Biol.*, **42**, 135-143 (2021).

Introduction

Sea anemone, *Gyrostoma helianthus* is a solitary-living organism from Phylum Cnidaria, Class Anthozoa. They produce distinctive poisonous proteins. According to Frazão *et al.* (2012) nematocysts of sea anemones possess at least four classes of excessive poisonous supplies whose composition and outcomes have now not been confirmed yet. As reported by Rojko *et al.* (2016) and Madio *et al.* (2019) sea anemone venom with more than type of one toxin and most have effective cytolytic effects on invertebrate and vertebrates. In mammals, sea anemone toxins motive distinctive kinds of syndromes, which include hepatic failure (Garcia *et al.,* 1994), nephrotoxicity (Mizuno *et al.,* 2012), cardiotoxicity (Bruhn *et al.,* 2001) and neurotoxicity (Madio *et al.,* 2019). These hazard outcomes vary notably due to difference in susceptibility amongst individuals, toxin awareness and how toxin injures the body. The toxicity of sea anemone toxins has been related with the binding to cell receptor and interrupting cell channel (Suput, 2009), that provoke cell membrane pore-formation and cell lysis (Ramírez-Carreto *et al.,* 2019).

Among the exceptional toxicological results of sea anemone venoms, research on the histological effects is still meager mainly on the groundwork of venom protein fractions. Ravindran *et al.* (2012), reported that the crude extracts and isolated proteins of sea anemone induces sever histopathological changes in heart, kidney, liver and brain tissue of mice. Further, Al-Hazmi *et al.* (2015) demonstrated that *G. helianthus* toxins as crude and fractionated protein extracts actively damage neural cells of cerebral cortex. Besides, the histopathological effect, the cytotoxicity and cytolysis impact of exclusive sea anemone toxins attracted tremendous activity (García-Linares *et al.,* 2016; Ramírez-Carreto *et al.,* 2019).

The fundamental characteristic of cell cytolysins via sea anemone toxins in most of the stated information is their capability to shape pores in cell membranes, which lead to cell lysis and death (Anderluh and Macek, 2002; Madio *et al.,* 2019). Frazão *et al.* (2012), postulated that the destructive cytolytic consequences depending on the toxin type, concentration and molecular characterization. Until recently, the toxicological influence of *G. helianthus* has not been yet fully characterized in liver and kidney, which are important organs of metabolism, detoxification, storage, and excretion of toxic sources and are specifically prone to damage. In view of the above, this study was carried out assess, the histopathological and biochemical effects of crude and two protein fractions from *G. helianthus* amassed from the Red sea coast, Saudi Arabia, Jeddah effects.

Materials and Methods

Sample collection and preparation of crude extract: Live specimens of sea anemone (*G. helianthus*) were collected, rapidly washed, and weighed and finely ground for 3 min in absolute ethanol in a ratio of equal weight per volume. The blended material was once centrifuged at 15,000 rpm for 10 min at 4°C, and the first supernatant used to be preserved for following steps. Pellets have been extracted for 2nd time in an absolute ethanol (2- supernatant) and the third step of extraction was once executed in 50% aqueous ethanol (3- supernatant). The three supernatants have been delivered to each other and then the supernatants had been evaporated below pressure at 40°C in an evaporator. Then the got extracts had been dried in a freezer to get the final crude extract.

Isolation and partial purification of crude extract: Fractionation of ethanolic extract of *G. helianthus* and its purification was carried out by Molecular Weight Exclusion Ultrafiltration: Crude extract was filtered once via membrane filters with cut off 3 kDa and 1 kDa (76 mm in diameter).

Animal maintenance and treatments: Adult male albino rats (*Rattus norvegicus*) weighing (150-170 g) had been chosen and housed underneath hygienic conditions and a photoperiod of 12 hr (day/night).The animals had been divided into 5 groups, the first was used for estimating the lethal dose (LD_{50}) of the crude extract by intraperitoneal injection with successive increasing doses (3-30 mg kg^{-1}). After administration of the treatment,dead animals were counted 24 hr. latter and the LD_{50} value was calculated as described by Litchfield and Wilcoxan method(1949). Throughout toxicity test, events statement of neuropathology and motor activity was recorded.

As Jeddah University is a newly established university in Saudi Arabia, animal ethical committee is not fully functional. However, animal remedies and handling during the experiment was carried out following the codal formalities of Jeddah University Ethical Committee.

After estimating LD_{50}, 4 groups of animals (10 animals each) were divided as follows: Control group was once intraperitoneally injected with 0.5 ml of saline solution for seven days. Group two were intraperitoneally injected with the crude extract (5.08 mg kg^{-1}) (1/4 LD_{50}) for seven days. The animals of 3rd and 4th groups were intraperitoneally injected with the protein fraction of either 1KDa or 3KDa at all identical dose of crude extract and for the same experimental period.

Histopathological examination: As described by Humason (1978) and after completion of treatment, the experimental animals were sacrificed via cervical dislocation. Slices of right liver lobe and right kidney from 7 control and treated groups were dissected cautiously and fixed in 10% formalin for 24 hr. Washing was done with tap water and then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene, embedded in paraffin at 56°C in warm air oven for 24 hr. Paraffin wax tissue blocks were organized for sectioning at 5 µm thickness by way of sliding microtome, routinely stained with hematoxylin and eosin (H&E) and histological commentary were assayed and photographed under a light microscope (Labomed, CXII).

Serum biochemical analysis: At the end of the experimental period, blood was collected in tubes, centrifuged at 5000 rpm for 15 min and stored at 4°C for biochemical assay analyses. The serum range of total bilirubin, total protein and total albumin, as well as ALT and LDH activities (U/L) were determined by a diagnostic kit (Dia Sys Diagnostic Systems, Germany). The optical density was measured with Perkin Elmer UV/VIS spectrophotometer Lambda EZ201.

Statistical analyses: Statistical analyses was carried out with SPSS model 17.0. for windows. The specific variables statistical significances was assessed by one-way ANOVA. P values < 0.05 were considered to be significant.

Results and Discussion

To date more than 32 species of sea anemones have been reported to produce lethal peptides and proteins (Ferreiro *et al.,* 2015; Visciano *et al.,* 2016). For various special characteristics, sea anemone toxins are still desired for understanding the novel bioactive compounds focusing on their biological activity, characterization, and their mode of action (Ramezanpour *et al.,* 2014; Utkin, 2015). In the present study, lethality appeared in rats once injected intraperitoneally with crude extract as well as fractionated proteins of 1kDa and 3kDa, injecting higher showed dose of venom. The rats distinct neurotoxic signs and symptoms as restlessness, flexing of muscles, palpitation and hairs stand nape. As time advanced, paralysis of forelimbs, dragging of hind limbs, tonic convulsions, violent convulsions were observed, eventually leading to death. This finding suggests the notably neurotoxicological activity similar neurological observations have additionally been located in different cnidarian (Moghadasi *et al.,* 2018; Madio *et al.,* 2019). Till now the current study of marine toxins represent a structurally and toxicological diverse action. As recorded, most, but not all, exert their toxic effects by disrupting the nervous system synaptic transmission by the way of sodium channels blocking, Honma and Shiomi, (2006), protease inhibition(Alvarez *et al.*, 2009) and AChE inhibition and brain biogenic amine levels disturbances (Honma and Shiomi, 2006) as well as cellular and structural brain damage in exposed animals (Prentis *et al*., 2018). In the cutting-edge study, histological observation from liver tissue of treated animal confirmed sinusoids enlargement, hepatocytes vacuole formations, white blood cells infiltrations and blood vessels congestion with hemorrhage (Fig. 2-5).

The results of the present study is in agreement with the findings of Yadappa *et al.* (2017) and Rjeibi *et al.* (2018), who reported that, crude extract and partially purified protein fractions intoxication results in deleterious disorganization and necrosis of hepatic cells and the diagnosed impact is in general established on the type of toxins, purification as well as dose level. Here, the hepatic histopathological impact was diagnosed in all the studied toxins and with extra powerful impact after intoxication with protein fraction of 1kDa (Fig. 4-5) and the crude extract has the much fewer effects (Fig. 2). The variable effects, however, can be defined on the basis of cytolytic impact on the examined toxins protein. Rojko *et al.* (2016) and Ramírez-Carreto *et al.* (2019), elucidated that distinct kinds of protein toxins that have been recognized from sea anemones and most can produce cytotoxicity due to the formation of pores. The pores produced alter the integrity of membrane and produce ionic imbalance that may lead to death and cell necrosis. However, the necrotic action exhibited by the toxins can also no longer be the only mechanism that causes cell death in view that there is elevating evidence by Yap and Hwang (2018) that pore-formation by toxins may set off apoptosis in the cells in a dose-dependent manner. In the current study, hepatic steatosis is one of the most identified commentary in the liver of animals that treated with fractionated protein.

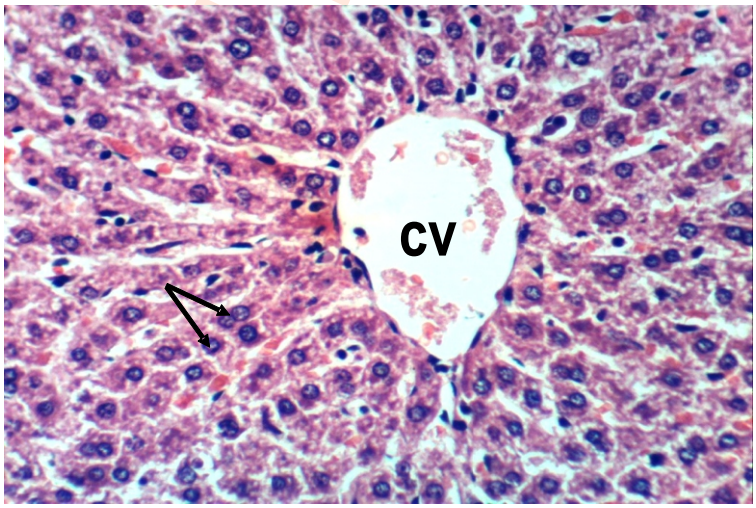


Fig. 1: Photomicrograph of liver section of male rats (control) showing normal hepatic structure, central vein (CV) and hepatocyte cell plates. The cells showed active vesicular nuclei and normal hepatic sinusoids (solid arrow) (H&E X400).

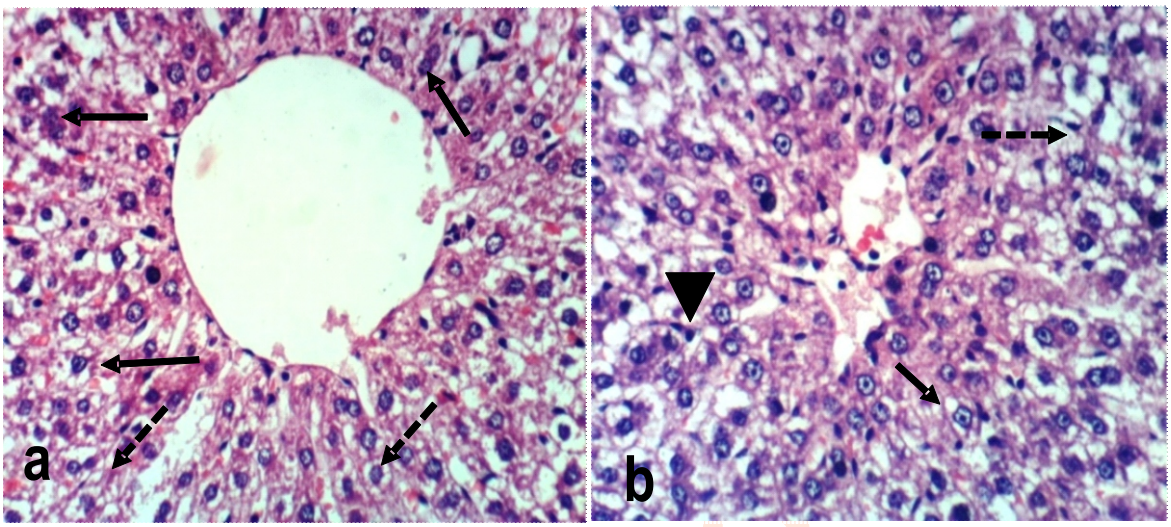


Fig. 2: Photomicrographs of liver sections of male rats post 7th dose treatments with *G. helianthus* crude extract (5.08 mg/kg body), arrows indicating (a) polymorphic nuclear material in hepatocytes; (b) cytoplasmic vacuolation, necrosis (dash arrows) and fatty change (solid arrow) (H&E X400).

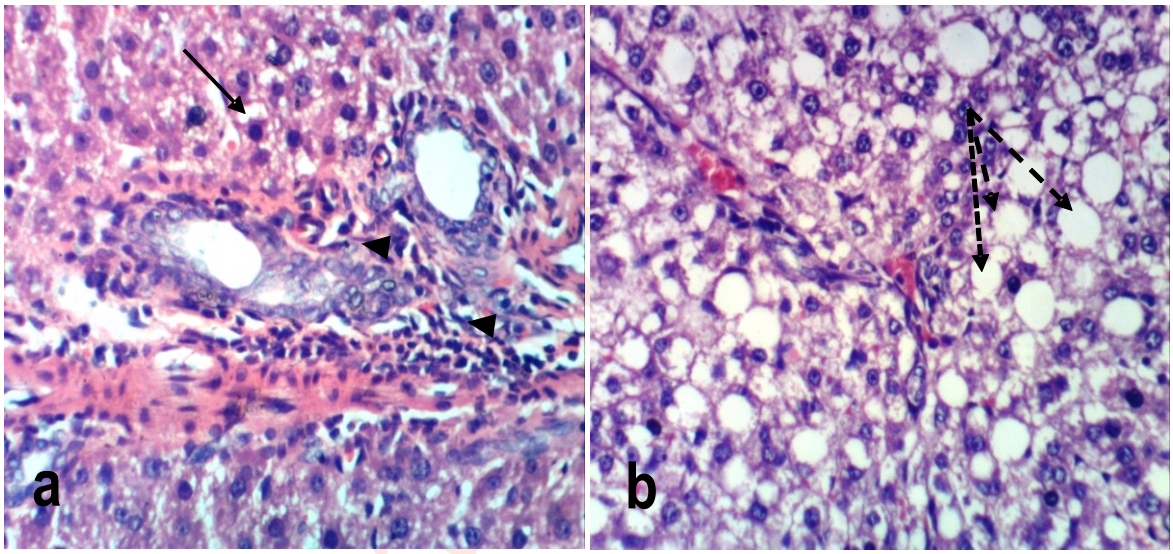


Fig. 3: Photomicrograph of liver sections of male rats of 7th dose treatments with *G. helianthus* fractionated protein (3KDa), arrows indicating (a)focal hepatic necrosis (arrows) and inflammatory cells infiltration (head arrows), (b) apoptosis associated with hyperplasia of epithelial lining bile duct (dash arrows) (H&E X400).

These findings are in agreement with Wahlang *et al.,* (2013), who postulated that exposure to some chemical compounds may additionally lead to hepatic steatosis that can lead to inflammatory responses and lead to fibrosis and cirrhosis. Further, the exposure to toxins additionally produces harmful impact on the renal tissue. The most identified observations observed were atrophy and congestion of glomerular tuft, vacuolation of epithelial lining renal tubules, proliferation, cellular cast in the lumen of renal tubules, interstitial inflammatory cells infiltration, vacuolation and focal renal hemorrhage (Fig.7-9). These findings corroborated with the findings of previous study on the toxins isolated from different types of sea anemones (Mizuno *et al.,* 2012). According to these investigators, the actual cell processes underlying the sea anemone toxins triggered nephrotoxicity are not but fully understood. Different indirect mechanisms of renal failure with sea anemone toxins have been

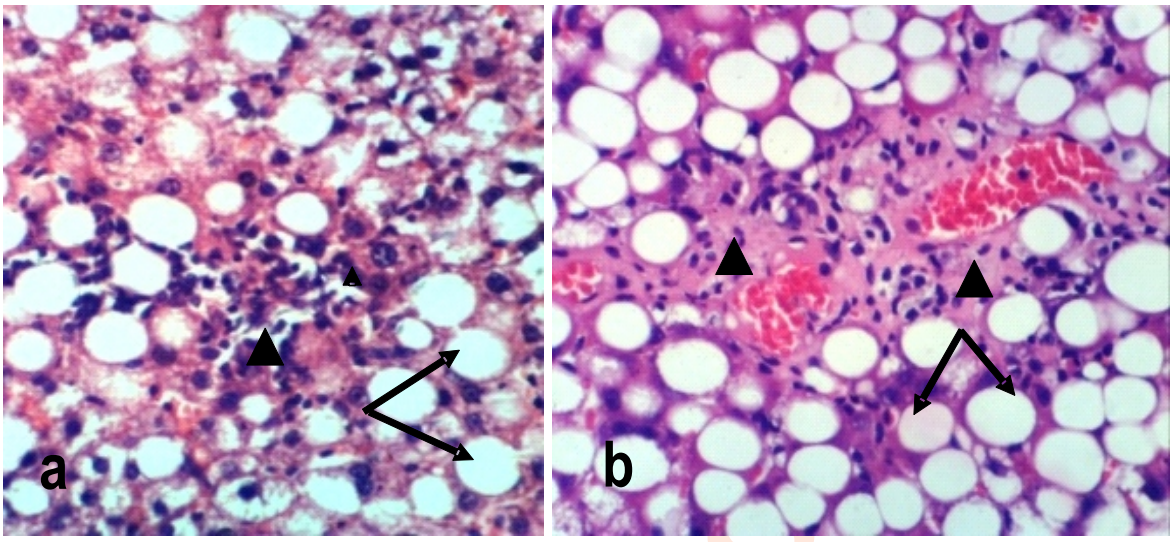


Fig. 4: Photomicrographs of liver of male rats of 7th dose treatments with *G. helianthus* fractionated protein (1KDa) arrows indicating macrovesicular steatosis, focal inflammatory cells infiltration(head arrow),and focal hepatic necrosis (dash lines) (H&E X400).

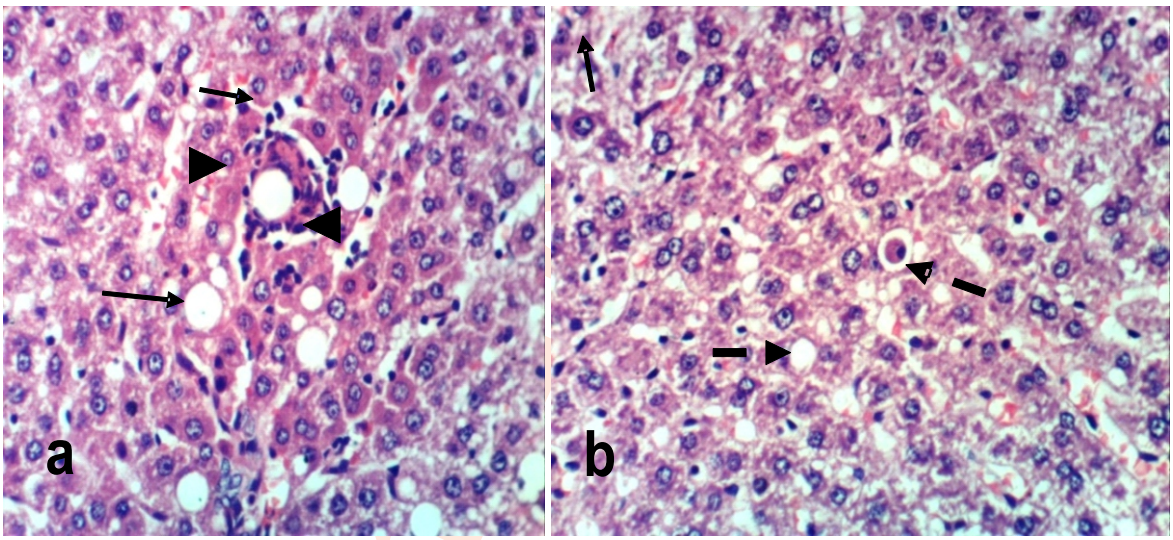


Fig. 5: Photomicrograph of liver sections of male rats post 7th doses treatments with *G. helianthus* fractionated protein (1KDa) arrows indicating (a) fatty change of hepatocytes, cytoplasmic vacuolization, and oval cells proliferation (head arrow); (b) cytoplasmic vacuolization and apoptosis of hepatocytes (dash arrows) (H&E X400).

discussed. Oshiro *et al.* (2004), reported that renal damage is related with complement activating factors in the venoms that indirectly contribute to renal tissue damage. Ramkumar *et al.* (2012), demonstrated that nephrotoxicity can also be due to glomerular filtration which leads to excessive localization of venom in the kidney. Additionally, it may be associated to heart hemolysis that may motive renal block and subsequently inflicting kidney cells injury (Berger *et al.,* 2015).

In the existing study, with the exception of total bilirubin which is highly and significantly lowered throughout the tested toxins, ALT and LDH and total protein are significantly elevated. Moreover, the degree of change varies as the type of the tested toxin. In this regard elevated ALT and total protein are seen post treatments 3KDa and LDH after intoxication with protein fraction of 1KDa (Table 1).These changes may be due the action of anemone toxins on the organ systems and varying detoxification

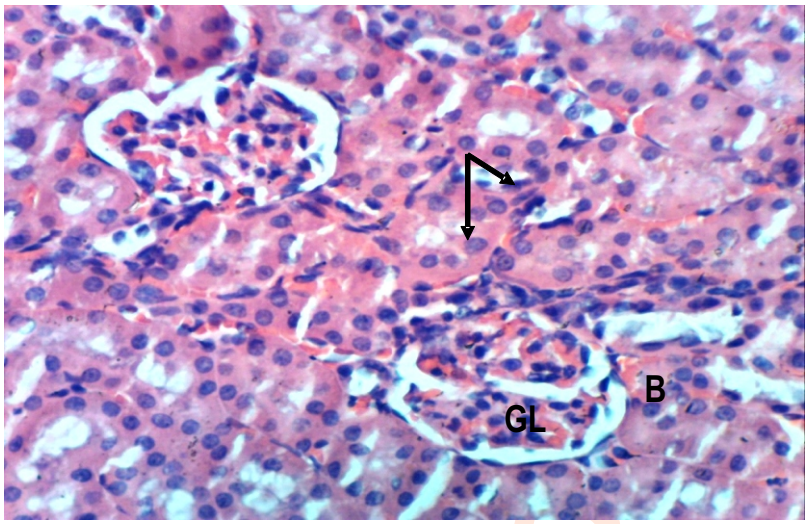


Fig. 6: Photomicrograph of kidney section of male rats of control group showing normal histological structure of renal parenchyma, normal Bowman's capsule (BC), glomeruli (GL) and normal renal lobules (arrows) (H&EX400).

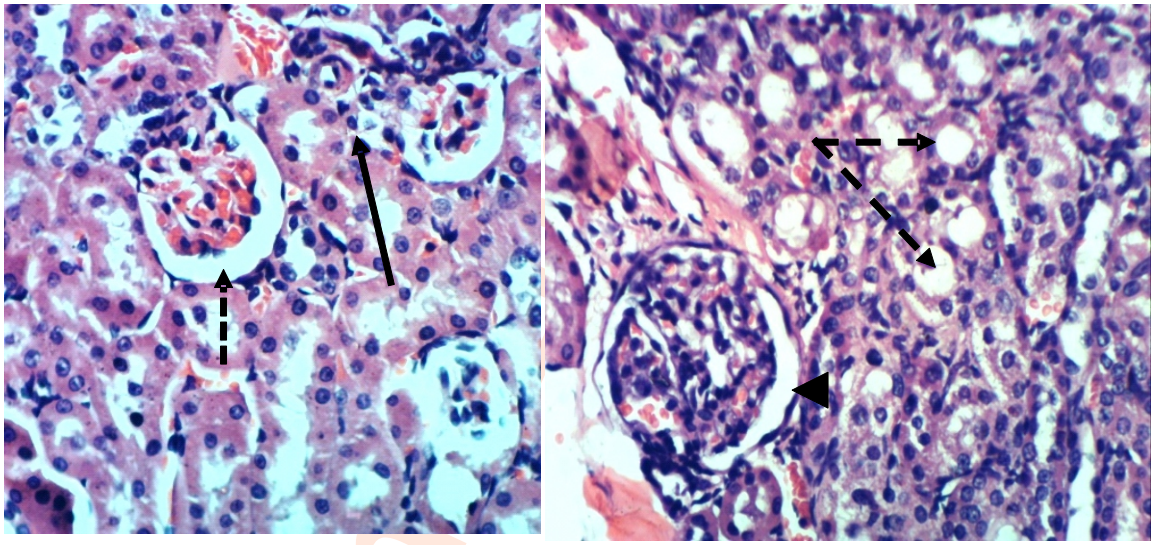


Fig. 7: Photomicrograph of kidney sections of male rats post treatments with crude extract of *G. hileanthus* arrows indicating (a) atrophy of glomerular tuft, vacuolation of epithelial lining renal tubules (dash arrow) and (b) thickening of parietal layer of Bowman's capsule(head arrow) and fatty change (dash arrows) (H&E X400).

processes. As described by several investigators the change in the studied serum bio-chemicals reflect liver and kidney tissue damage (Luo *et al*., 2014; Qin *et al*., 2016). As stated by Hu *et al.* (2011), actinoporin protein remoted from the sea anemone *Stichodactyla Gigantea* motives significant increase in ALT activity. Also, as mentioned recently by Ramírez-Carreto *et al.* (2019), the venom isolated from the sea anemone *Anthopleura dowii Verrill* causes release of LDH in proportion to the quantity of poisonous dose level and protein fraction type. Moreover, much of research have indicated the possible relationship between low bilirubin range and toxicity injured impact on of sea anemone toxins on cardiac (Ravindran *et al.,* 2010) and neuronal cells (Kvetkina *et al.,* 2020).

In addition, the studied toxins raised serum whole protein levels which in accordance with Lukačínová *et al.,* 2011; Ekam *et al.,* 2012 and Ye *et al*., 2020 working on different hepatoxic and nephrotoxic agents. Elevations in ALT and LDH and low bilirubin denotes injured hepatic and renal cells and reflect a consequence

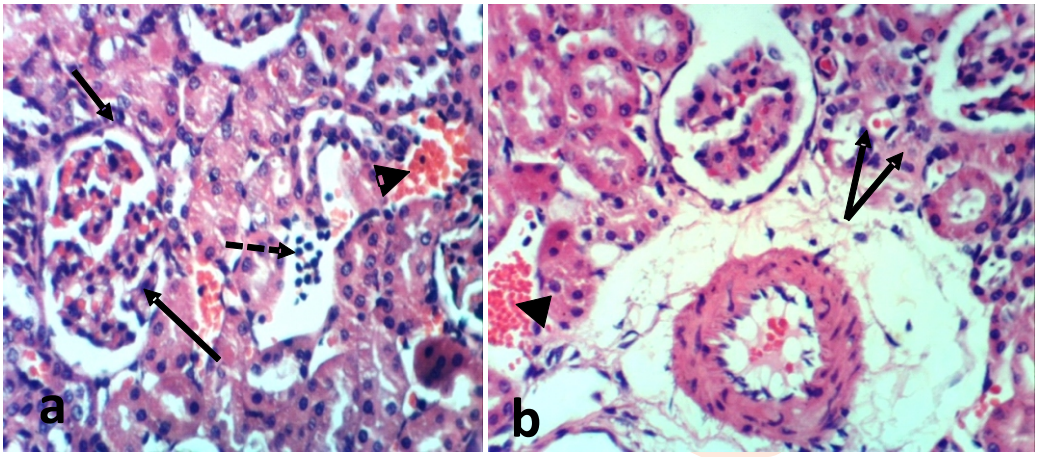


Fig. 8: Photomicrograph of kidney sections of male rats post treatments with 3Kda extract of *G. hileanthus* extract arrows indicating (a) slight atrophy and congestion of glomerular tuft and renal blood vessel, presence of cellular cast in the lumen of renal tubules (dash arrow)and occlusions with hemolyzed blood (head arrow), (b) interstitial fibroblasts proliferation , vacuolation of epithelial lining renal tubules and perivascular oedema (head arrow) (H&E X400).

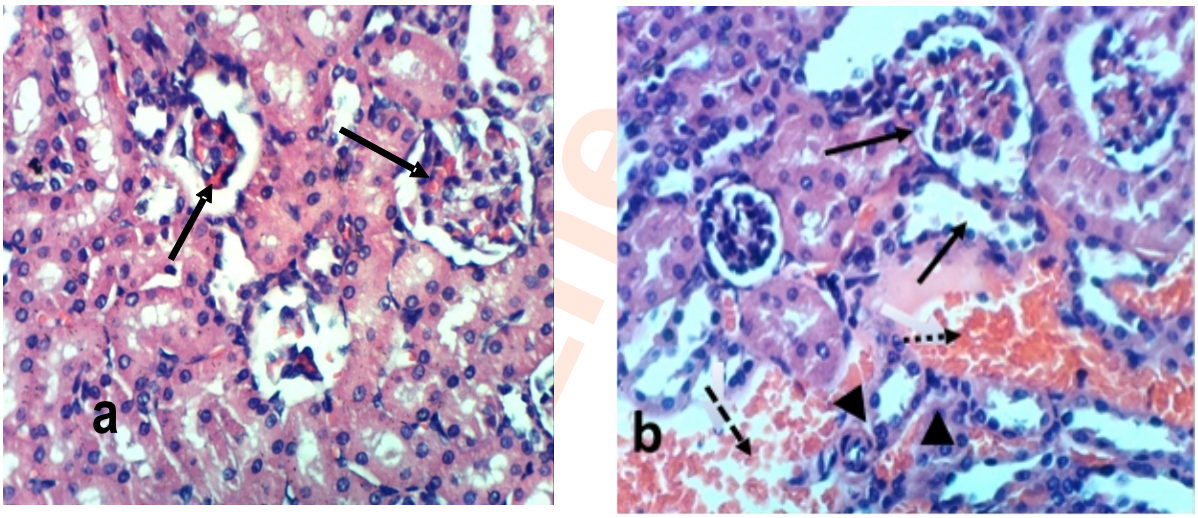


Fig. 9: Photomicrograph of kidney sections of male rats post treatments with 5.08 mg kg^{-1} of sea anemone *G. helianthus* fractionated protein (1KDa) arrows indicating (a) hypertrophy of glomerular tuft and interstitial nephritis, (b) interstitial cells infiltration (head arrows) and focal renal hemorrhage with hemolyzed blood (dash lines) (E and H X400).

Table 1 : Effect of crude and partially purified protein of *G. helianthus* on the levels of some biochemical parameter

Parameter	control	Crude extract	Protein fraction(1kDa)	Protein fraction (3kDa)
ALT(U dL^{-1})	16.973±1.44	21.65±1.44*	20.30±1.46*	24.441±1.43*
LDH (U L^{-1})	1579.01±87.30	2429.450±52.14**	2434.44±55.35**	1279.38±84.65**
Bilirubin (mg dL^{-1})	1.93±o.44	0.248±0.047**	0.46875±0.04**	0.49375±0.08**
Protein (g dL^{-1})	5.042±0.26	6.376±0.73*	7.287=0.41**	8.585±0.35**
Albumin (g dL^{-1})	3.299±0.18	3.402±0.16	3.474±0.15	3.221±0.16

Values are as means of six replicate ± S.E.; ANOVA was used for determination of significance (*p≤0.05). *significant, ** highly significant

of inflammation, injury and cell death as diagnosed in the present histological examinations. Apoptotic cell death, inflammation and necrosis can be idea of as cell implosion; that leads to loss of cell membrane and leakage of proteins into the extracellular space and plasma. Overall, even though cell dying, and plasma membrane damage may additionally recommend the dominant reasons of serum biochemical modifications post treatments, it is additionally feasible that different mechanisms can also play a role in the recorded biochemical results.

Acknowledgments

I would like thank to Prof. Sayed Rawi University of Jeddah and Dr Osama Mohamed Ahmed, Beni-Suef University, for their useful planning and careful reading. In addition, my honest perception goes to the participants of micro-technique units, National Cancer Institute Egypt for amenities provided and technical histological preparations.

Add-on Information

Authors' contribution: Turki M. Al-Shaikh: Author designed and performed the research, collected, analyzed and interpreted data and drafted manuscript. The author read and approved the final version.

Research content: The research contents is original and has not been published elsewhere

Ethical approval: Not Applicable.

Conflict of interest: The author declares that there is no conflict of interest.

Data from other sources: Not Applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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