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Brain Biogenic Monoamines in Relation to Brain Histopathology and Plasma Glutathione Shuttle in Rat after Exposure to Sea Anemone *Gyrostoma helianthus* Extract

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Abstract

The extract of sea anemone *Gyrostoma helianthus* (Cnidaria, Anthozoa) has been reported as a source of neuroactive compounds. The current study was designed to explore biogenic monoamine effects of sea anemone crude extract in relation to histopathological changes in brain areas and plasma glutathione shuttling. Mouse bioassays were used to determine the dose response curve and behavioral neurotoxicity. Loss of balance, opaque eyes, tonic convulsions, paralysis, muscle flexing, and exophthalmia were the major behavioral changes observed. The LD_{50} in mice was identified as 29 mg/kg body weight after IP injection, which is calculated to be 20.3 mg/kg body weight in rats using a conversion table.

Serotonin, dopamine, and norepinephrine were significantly increased in the cerebral cortex, cerebellum and pons plus medulla oblongata in rats in our 3-day study after a single IP injection of ½ LD_{50} of the crude extract. The greatest increases in serotonin, dopamine, and norepinephrine were attained in the cerebral cortex. Cells examined in the cerebral cortex and hippocampus showed necrosis, pyknosis, focal gliosis, and congestion of cerebral blood vessels as well as focal cerebral and hippocampus hemorrhage. At the tested dose, the extract caused significant decreases in plasma glutathione and G-reductase, while G-transferase levels were increased.

Keywords: *Gyrostoma helianthus*; Sea anemone; Glutathione; Monoamines; Histopathology

Introduction

In the field of natural products, marine organisms have a rich heritage as therapeutic resources that have been exploited for effective and beneficial use against many human diseases [1], and some have been used to investigate novel drugs [2]. Sea anemones are one of the most ancient predatory marine animals. Pharmaceutical studies have demonstrated that the venom of several species of sea anemones produces different symptoms and modes of toxicity. Studies of inflammation and allergic reactions [3] hemolytic activities [4], immunomodulating activities [5], cardiotoxicity [6], and cytolytic effects [7] have all produced interesting outcomes. Moreover, the incidence of neurotoxicity reactions such as convulsions, paralysis, respiratory failure, cardiovascular reactions, and stroke [6,8] is mostly recognized. Several studies have accumulated data attributing the effect to the diversity of polypeptide toxins [9]. It is increasingly recognized that many marine toxins interact with a large variety of excitable membranes, including myelinated and non-myelinated axons [10], neuronal cells [11] and nerve terminals [12]. Many have also shown activity with diverse receptors and voltage-gated ion channels [9]. Moreover, other investigators have reported that sea anemones' toxin provokes neurotransmitter release from synaptosomes, acts on different receptor structures in the membrane [13], and/or inhibits AChE activity [14].

Although the interest in marine toxins and venoms has increased during the last three to four decades, their mechanism of action at the monoamine level and the histological effect in different brain regions, which may solve unanswered questions related to their neurological effect, are still largely unknown and under debate. Therefore, the present work was designed to study the following: behavioral characteristics of toxic effects; the effect of an acute single dose of *Gyrostoma helianthus* ethanolic aqueous extracts on the cerebellum, cerebral hemisphere and pons and medulla monoamines; and plasma glutathione shuttling in relation to the histopathological changes in the cerebral hemisphere and hippocampal neurons.

Materials and Methods

Sea anemone collection

Specimens of sea anemone Gyrostoma heleanthus were collected from the west coast of the Red Sea at longitudes 38o 45' to 38o 50' E and from 21o 51' to 21o 52' N latitude near the Jeddah province of Saudi Arabia. Specimens were transferred to the laboratory in seawater.

Preparation of the crude extract

Live specimens were rapidly washed and then weighed. Absolute ethanol equal to the specimen weight (1:1 v/w) was added with the anemone and mixed in a blender for 3 minutes. The blended material was centrifuged at 2570 × g for 10 minutes, and the 1st supernatant was reserved for further steps. Pellets were extracted a second time with absolute ethanol (2nd supernatant) and a third extraction with 50% aqueous ethanol (3rd supernatant). The three supernatants were added to each other and were then evaporated under reduced pressure at 40ºC with a rotatory evaporator. Concentrated extracts were dried in a freeze dryer to obtain the final dried crude extract.

Animals

Male albino mice (Mus musculus) with body weight of 20 ± 2 g and rats (Rattus norvegicus) with body weight of 150-170 g were obtained from the animal house at the King Fahd Research at King Abdulaziz University. Laboratory Animal Welfare including environment, housing and management were conducted according to the recommendations for the proper care and use of laboratory animals by the American Association for Accreditation of Laboratory Animal Care (AAALAC) [15] and Al-Hazmi [16]. Rats were kept under good ventilation with 12 hour light and dark cycle, fed a stock diet, and allowed free access to drinking water.

Mouse bioassay and neurobehavioral responses

The mouse bioassay was conducted according to the method of AOAC [17]. Exactly 5 mg of the crude extract was dissolved in 1 ml of ultrapure water, and different dilutions were made to determine the dose response curve using Mouse Units. The median death time of 5 mice was used to calculate the Mouse Units of each dose. Neurobehavioral symptoms were observed during the assay after the IP injection.

LD50 determination in mice

Groups of six mice (18-22 g) each were IP injected with various concentrations of the crude extract (ranging from 5-50 mg/kg body weight). The toxicity was evaluated by determining 50 percent of the lethal dose (LD50) at 48 h according to the method of Litchfield and Wilcoxon [18]. Using the Paget and Barnes [19] conversion table, the LD50 for rats was calculated from the LD50 of mice.

Biogenic monoamine determination

Male rats were treated once by IP administration with ½ LD50 of the lyophilized crude extract dissolved in ultrapure water. The animals were sacrificed at specific time intervals (1, 2 and 3 days). The brain was rapidly and carefully removed and dissected on dry ice according to the method of Glowinski and Iversen [20] into cerebellum, pons plus medulla oblongata, and cerebral hemispheres. The dissected areas were wiped dry with filter paper, weighed and then homogenized in 10 volumes of 0.1 M phosphate buffer (pH 7.8), using an ultrasonic homogenizer. The resulting homogenate was centrifuged at $10,000 \times g$ for 60 min at 4°C. The supernatant was collected and used to determine the levels of serotonin (5-HT), dopamine (DA) and norepinephrine (NE) according to the method of Udenfriend and Wyngaarden [21] as modified by Cliarlone [22]. The fluorescence activity of the tested samples was measured in a Jenway 6200 fluorometer.

Determination of glutathione and related enzyme levels

Following decapitation, blood was collected into clean heparinized centrifuge tubes and plasma was separated by centrifugation. Glutathione (GSH), glutathione S-transferase (GST) and glutathione reductase (GR) were analyzed using the reagent kits purchased from Biodiagnostic.

Histopathology

Another group of animals was IP injected with the tested dose level. At the third day post-injection, the cerebral cortex and hippocampus were rapidly removed and placed in 10% buffered formalin for subsequent processing and histopathological evaluation.

Statistical analysis

One-way ANOVA was used to analyze the results of the biogenic monoamine determination, glutathione, and glutathione-related enzymes using statistical analysis SPSS v20, and LSD was used for mean separation [23].

Results

Dose response curve

The dose response curve (Figure 1) of ethanolic crude extract from the sea anemone *Gyrostoma helianthus* generated a threshold detected following the 2 mg.ml-1 injection with a response of 1.3 Mouse Units (MU). The curve plateaued at the 6 mg.ml-1 dose with a response of 3.8 MU. Increasing the dose to 7 and 8 mg.ml-1 did not increase the response above 3.8 MU. The dose response curve results were used to evaluate the neurological behavior response and to identify the dose that should be used in the LD50 determination.

Neurobehavioral responses

During the mouse bioassay study, the observed behavioral profile in mice after IP administration showed symptoms of convulsions, labored breathing, weakness of limbs, paralysis, walking imbalance, and occasional respiratory and body spasms. The most prominent feature at increasing doses was central nervous system excitation leading to severe convulsions and death.

LD50 determination

The acute toxicity study showed a sigmoidal dose-mortality relationship. Probit values (% mortality) of the crude extract generated a straight line from which the LD50 was extrapolated. The LD50 value of *Gyrostoma helianthus* crude extract in mice was 29 mg.kg-1 body weight after IP injection, which is calculated to be 20.3 mg.kg-1 body weight in rats using the conversion table. To determine the effect of *Gyrostoma helianthus* crude extract on biogenic monoamines, calculated ½ LD_{50} values for rats was used in all experiments. During this experiment, the behavioral changes previously observed in mice were also observed in rats.

Effect on biogenic monoamines

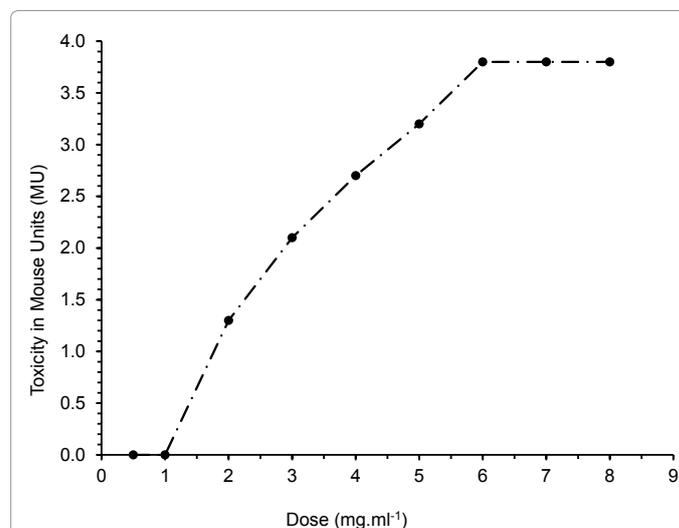**Dopamine (DA) levels:** *Gyrostoma helianthus* crude extract

Figure 1: Dose response curve of the crude aqueous ethanolic extract of the sea anemone *Gyrostoma helianthus* after IP injection in 20 ± 2 g male albino mice.

induced a highly significant increase in DA levels in all brain areas evaluated on the 1st day after injection compared to control rats (Figure 2). DA values increased 123%, 208% and 179% in the pons plus medulla, cerebral hemisphere and cerebellum, respectively. On the 2nd day after injection, DA values were decreased but still significantly higher than control values. DA levels were returned to normal by the 3rd day after injection in all brain areas evaluated, except the cerebral hemisphere, which showed the same elevated value as the 2nd day following injection.

Norepinephrine (NE) levels: NE levels significantly increased in all brain areas compared to control (Figure 3). However, different profiles for the NE increase were observed for the different brain areas evaluated. The pons plus medulla and cerebral hemisphere displayed significant increases (154% and 269%, respectively) on the 1st day after injection with no significant change on the 2nd and 3rd days. A different NE profile was observed in the cerebellum where significant increases were elevated through the 3 days of the study, from 75% on the 1st day to reaching 157% by the 3rd day.

Serotonin (5-HT) levels: The same profile trend observed in NE levels was detected in the 5-HT profile (Figure 4). The 1st day increases for the pons plus medulla (50%) and cerebral hemisphere (64%) were not significantly changed on the 2nd and 3rd days. However, an increase in the cerebellum on the 1st day (130%) was followed by a continuous decrease on the 2nd and 3rd days but remained higher than control by 83% and 62%, respectively.

Glutathione and oxidative enzymes: *Gyrostoma helianthus* crude extract significantly decreased the plasma concentration of glutathione (GSH). The maximum GSH reduction (29%) was observed on the 3rd day after injection (Table 1). Glutathione S-transferase (GST) showed significantly higher values compared to control in the tested period with a maximum increase of 45% on the 3rd day after injection. However, the glutathione reductase (GR) values were significantly decreased compared to control with the maximum decrease (48%) occurring on the 3rd day after injection (Table 1).

Histopathological observations: Histopathological changes in rat brain tissues showed that in contrast to the control group (Plate 1),
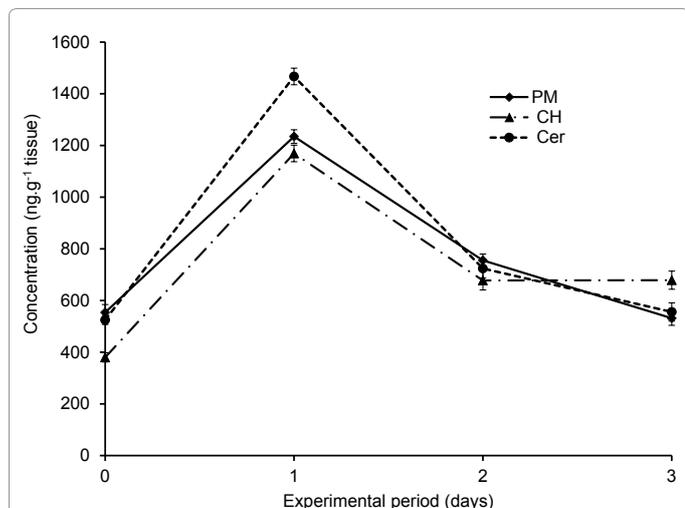

Figure 2: Dopamine concentration in pons plus medulla (PM), cerebral hemisphere (CH), and cerebellum (Cer) of rat brain after IP injection with an acute single dose of *Gyrostoma helianthus* crude extract. Each point represents the mean ± SE (n=6).
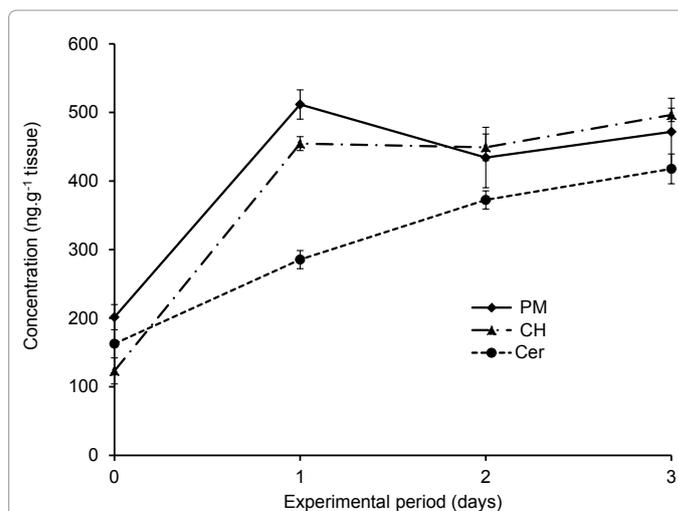

Figure 3: Norepinephrine concentration in pons plus medulla (PM), cerebral hemisphere (CH), and cerebellum (Cer) of rat brain after IP injection with an acute single dose of *Gyrostoma helianthus* crude extract. Each point represents the mean ± SE (n=6).
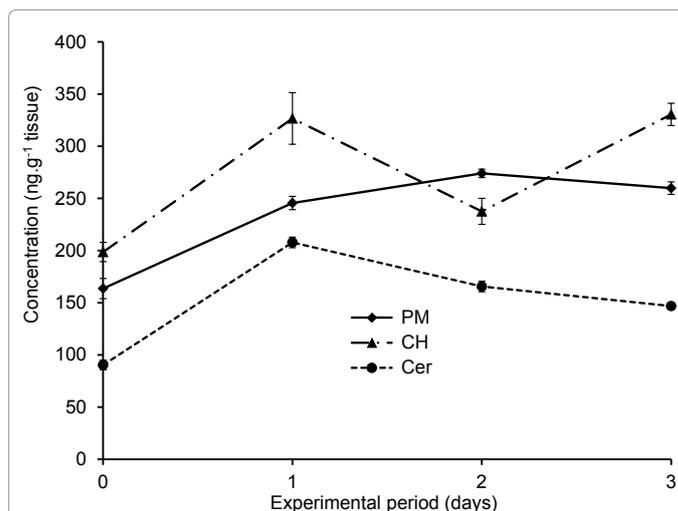

Figure 4: Serotonin concentration in pons plus medulla (PM), cerebral hemisphere (CH), and cerebellum (Cer) of rat brain after IP injection with an acute single dose of *Gyrostoma helianthus* crude extract. Each point represents the mean ± SE (n=6).

Sampling Time after injection (Days)	GSH (μmol/L)	GST (IU/dL)	GR (IU/L)
0	146.00 ± 9.38[a]	394.17 ± 15.82[b]	339.33 ± 11.94[a]
1	112.63 ± 2.95[bc]	545.29 ± 35.40[a]	192.73 ± 11.40[b]
2	126.13 ± 7.85[b]	540.98 ± 21.00[a]	187.40 ± 9.86[b]
3	103.31 ± 2.68[c]	573.36 ± 18.63[a]	176.7 ± 8.42[b]

Data are expressed as the mean ± standard error (n=6).
Means with the same symbol(s) are not significantly different. P<0.001

Table 1: Effects of IP injection of *Gyrostoma helianthus* crude extract at ½LD$_{50}$ on serum glutathione (GSH), glutathione-S-transferase (GST) and glutathione reductase (GR) activity.

the cerebral cortex of treated animals exhibited cellular infiltration, atrophy, pyknosis, necrosis, neurology, congestion of cerebral blood vessels, cellular and perivascular edema as well as focal gliosis and focal

cerebral hemorrhage (Plates 2 and 3). Hippocampal neurons showed similar histopathological observations as the cerebral cortex. The most prominent observations of the examined sections were atrophy, pyknosis, shrunken cells and hemorrhage (Plates 5 and 6) compared to normal hippocampal cells (Plate 4).

Discussion

The investigation of new pharmacological tools and medicines through the study of action mechanisms of strong and highly selective interactions of some marine toxins with specific sites on excitable membranes has revealed a great deal about the physiology of affected tissues and systems [24]. As a result, the study of marine organisms as a source of biologically active compounds has led to the isolation of approximately 10,000 metabolites, many of which are components with pharmacodynamic properties [25]. Sea anemones contain mixtures of a variety of organic substances, including some potent toxins, permitting an improved understanding in the fields of pharmaceutical, neural, and biological sciences and allowing for the development of novel drugs [26].
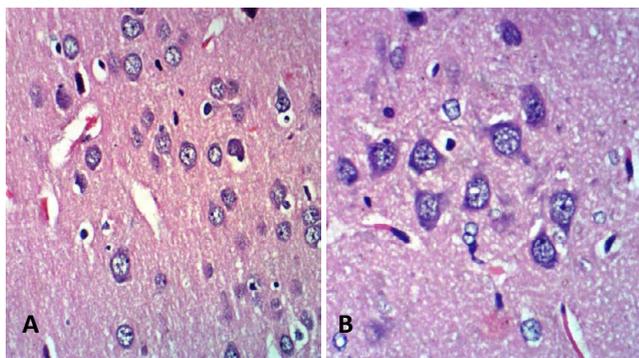

Plate 1: Photomicrographs of cortical cells of normal rat (A-B) [400X].
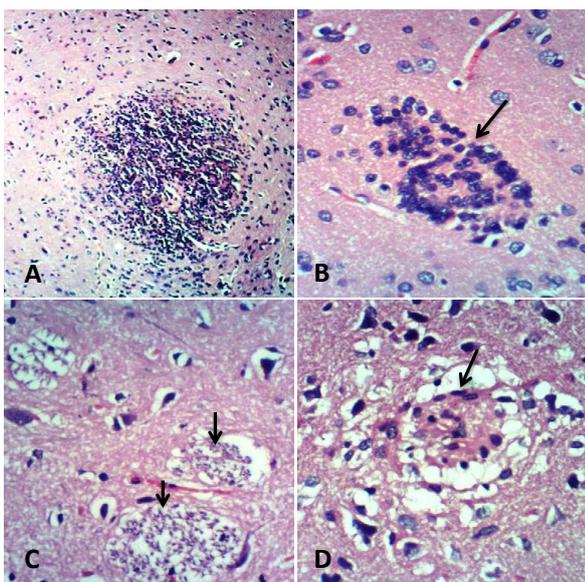

Plate 2: Effect of sea anemone crude extract on cortical neurons of a male albino rat showing focal cerebral necrosis infiltrated with glia cells [A], focal gliosis (arrow) [B], demyelination of nerve fibers (arrows) [C], and focal cerebral necrosis (arrow) [D]. [400X].
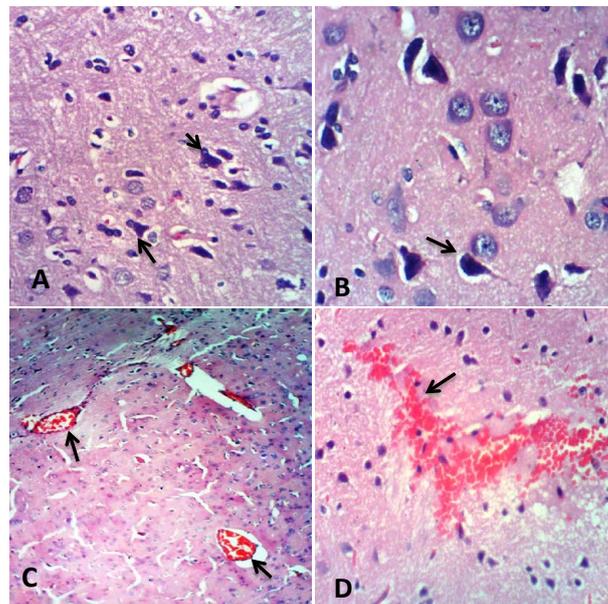

Plate 3: Effect of sea anemone *Gyrostoma hileathus* crude extract on cortical neurons in male albino mice showing necrosis of neurons and neurophagia (arrows) [A], necrosis and pyknosis (arrows) [B], congestion of cerebral blood vessels (arrows) [C] and focal cerebral hemorrhage (arrow) [D]. [Figs A-C, 400X; Fig D, 100X].
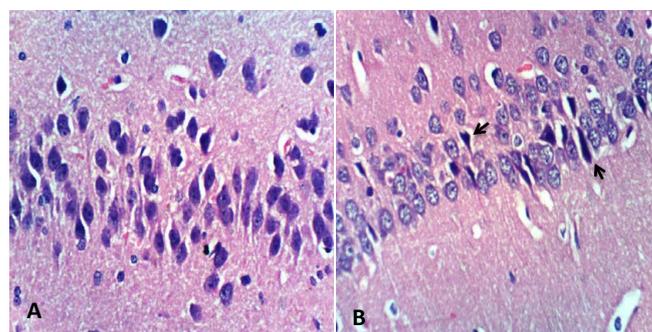

Plate 4: Normal histology of the Pyramidal cell layer of the Hippocampus in male albino mice [A]. Rats treated with sea anemone *Gyrostoma hileathus* crude extract [B] showing pyknosis and atrophy of some pyramidal cells (arrow). [400X].

The ethanolic aqueous extract of the sea anemone *Gyrostoma helianthus* that showed lethality in mice in the current study was inconsistent with the findings of Mebs et al. [27], who stated that *Gyrostoma helianthus* extract had no lethality in mice. However, other reports [28-30] confirmed the lethality of sea anemone extract in mice. The contradiction with Mebs et al. [24] may be due to differences in the extraction protocols. The IP LD50 of sea anemone extract for mice (29 mg.kg^{-1} body weight) reported in this study was within the previously reported LD50 of 700.7, 0.47, 0.71, and 108.24 mg.kg^{-1} body weight (BW) for Bartholomea annulata, Stichodactyla mertensii, Stichodactyla haddoni, and Stichodactyla mertensii, respectively [29-31]. These variations in LD50 may be due to the differences in species and environmental conditions that may lead to differences in chemical compositions.

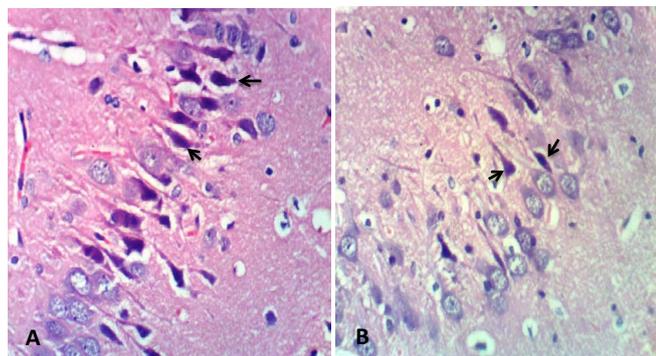The reported neurobehavioral responses observed on mice after

Plate 5: Hippocampal cell layer of a male albino rat following treatment with sea anemone *Gyrostoma hileathus* crude extract showing pyknosis and atrophy with long cytoplasmic processes of some pyramidal cells (arrow) [400X].
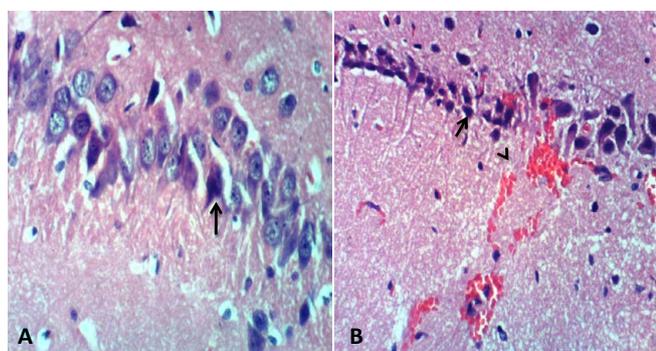

Plate 6: Hippocampal cell layer of a male albino rat following treatment with sea anemone *Gyrostoma hileathus* crude extract showing necrosis of some pyramidal cells (arrow) [A] and multiple focal hemorrhage (arrowhead) and necrosis (arrow) [B]. [200X].

IP injection of the sea anemone extract (convulsions, paralysis, and standing nape hairs) were consistent with those reported by Ramkumar et al. [32]. Different reports explain these neuronal, muscular and cardiovascular hyperexcitabilities as a result of the effect of some sea anemone polypeptides blocking neuronal sodium and potassium channels [3,33,34]; however, the effect of sea anemone extracts on the level of biogenic monoamines and their relation to GSH and related enzymes as well as the potential histopathological changes were not previously studied.

The results of the present study showed that the increase in norepinephrine (NE) dopamine (DA) and serotonin (5-HT) in the cerebellum, cerebral hemisphere, and pones plus medulla following IP injection of sea anemone extract in the rat aligns with the findings of Diochot et al. [35] who showed the enhancement of Ca^{2+} channel gating and catecholamine secretion as a result of sea anemone neurotoxins on voltage-gated sodium and potassium channels via transcription/translation-dependent mechanisms and possibly nicotinic receptors. Additionally, the increase in monoamine levels observed in the current study aligned with the induction of catecholamine secretion by other marine toxin PTX reported by Yoshizumi et al. [36].

Our results suggest that the studied toxin may enhance adrenergic receptor stimulation, which may increase the biogenic monoamine levels. The most likely mechanisms of action are sympatholytic and adrenergic signaling effects, which are among the most pronounced

mechanisms of the hypertension, increased blood pressure, hyperventilation and disrupted animal behavior recognized in the present investigation. This proposed mechanism is consistent with that of Yabe et al. [37] who stated that the isolated toxins from the polyp corals Clavularia sp may act as a neurotrophic factor-like agent in the cholinergic nervous system.

This mechanism is also in line with the histopathological hemorrhage findings in the cerebral hemispheres and hippocampus, which may be related to increased blood pressure. Additionally, Harris and Goonetilleke [38] reported results showing that the essential hypertension accompanying the rise in blood pressure has a significant neurogenic component and is mediated, in part, by over activity of the sympathetic nervous system. In times of high sympathetic nerve activation, adrenal medullary disorders cause very high circulating levels of catecholamine. This led to hypertension and increased blood pressure.

The histopathological changes in the studied brain areas may also be explained by the antioxidant changes observed in the current study. In the nervous system, one out of every three fatty acids (FAs) belongs to the polyunsaturated fatty acid (PUFA) group [39,40]. These PUFAs are especially vulnerable to oxidation, and many brain regions such as the cerebral cortex [41] and hippocampus [42] are highly enriched in non-heme iron. Both of these factors, specifically PUFAs and non-heme iron, increase the susceptibility of brain cells to lipid peroxidation that is catalytically involved in the production of oxygen free radicals and promotes neurodegeneration as well as functional and sensory loss in the brain tissue [43]. In the present study, the responses of cortical and hippocampal neurons were evaluated in relation to the studied toxic stress. Neurons in both areas markedly exhibited severe necrosis, atrophy, pyknosis, perivascular edema, hemorrhage and neuronal demyelination. These changes may be explained by the Mechaly et al. [44] report, which stated that sea anemone toxins affect the permeability of target cells by forming pores in their plasma membranes. Moreover, other investigators have attributed ballooning degeneration and nuclear pyknosis as prominent findings after exposure to sea anemone toxins [45]. Recently, Monroy-Estrada et al. [46] have also suggested that the sea anemone *Bunodeopsis globulifera* induced cytotoxicity through a different cellular mechanism that includes mitochondrial damage and alterations in the cell membrane. The histological changes could also be explained by the induction of oxidative stress. In the present study, administration of *G. healinthus* toxins significantly elevated glutathione transferase and decreased both GSH and glutathione reductase through 3 days post-treatment. One of the proposed mechanisms is glutathione reductase inhibition and glutathione transferase activation. These effects finally lead to increased generation of H_2O_2, which increases oxidative stress in the body, including neurons in the brain. In agreement with these findings, Abdel-Rahman et al. [47] found that the administration of Conus vexillum venom induces several oxidative stress biomarkers and lowers glutathione. According to Parihar et al., [48], regions such as the cortex and hippocampus are highly susceptible to oxidative damage. The present findings are in accordance with Uttara et al. [49], who reported that antioxidant enzymes and glutathione are an essential part of the cellular defense against cellular stress, including toxin exposure. Decreased activity of glutathione reductase and increased levels of glutathione transferase parallel the decrease in GSH, indicating an increase in oxidative stress in toxin-exposed animals. As reported recently by Mariottini and Pane [7], several cnidarian venoms have been found to have cytotoxic properties. In this regard, Soletti et al. [50] showed that the cytolysins EqTx-II and Bc2 from the sea anemone

Sagartia rosea potentiated cytotoxicity in human glioblastoma cells through a necrosis-like cell death. Moreover, Monroy-Estrada et al. [46] proposed that the crude extract from the sea anemone *Bunodeopsis globulifera* induces reactive oxygen species, which damage the cellular elements of lung cancer cells. Moreover, there is recent evidence suggesting that sea anemone toxins interact with the arylhydrocarbon receptor (AhR) [51] and activation of cytochrome P450 [52].

Conclusion

The sea anemone *Gyrostoma helianthus* induced the release of monoamines in brain regions, severe histopathological effects, and affected glutathione levels as well as related oxidative enzyme activity. Additional studies are needed to fully detail the neurologic effects of individual sea anemone compounds.

Acknowledgements

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