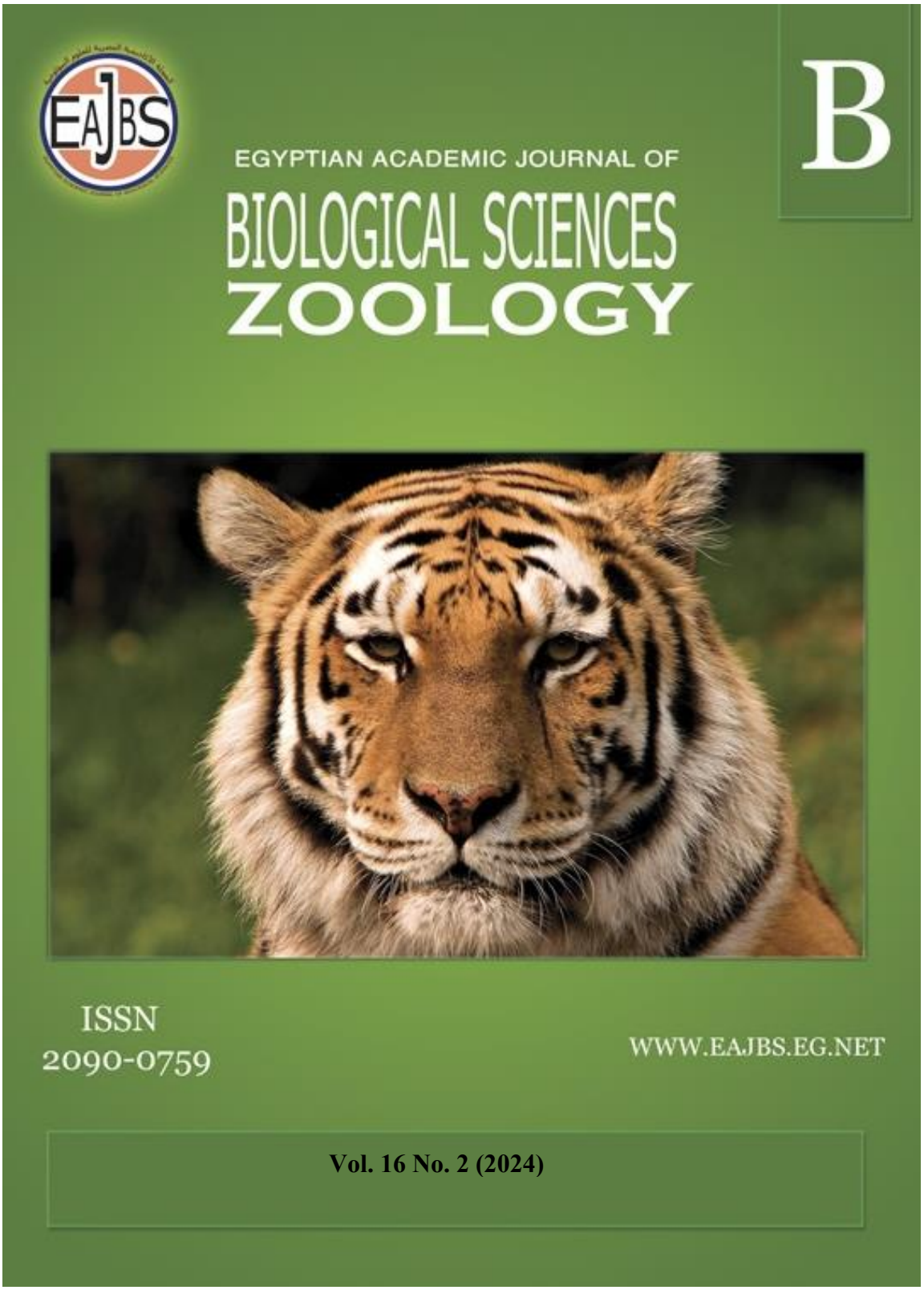

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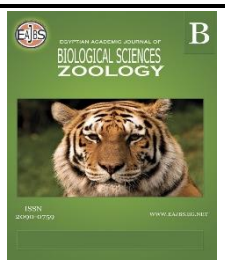

Isolation and Characterization of a Neurotoxic Fraction from *Cerastes cerastes cerastes* Snake Venom

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Cerastes; phrenic nerve hemi-diaphragm; postsynaptic neurotoxin; behavior.

Abstract

Cerastes cerastes cerastes is an Egyptian viper which belongs to the family of Viperidae and the genus *Cerastes*. There is no available information on the neurotoxic action of *C.c.cerastes*. The present work aimed to test the neurotoxicity of *Cerastes cerastes cerastes* crude venom and to isolate and purify the neurotoxic fraction(s). The neurotoxicity was verified by neuromuscular techniques (rat phrenic nerve hemi diaphragm, RPNH), and neurobehavioral experiments. The present results showed that *Cerastes* venom (20 µg/ml) and a purified caseinolytic fraction (10 µg/ml) inhibited the contractile responses of indirect RPNH. The contractile responses to acetylcholine (10^{-3} M), carbachol ($2x10^{-6}$ M) and KCl (60 mM) were inhibited by the venom and the purified fraction. However, i.p. injection of the fraction (73 µg/100gm b.w) elicited a group of behavioral changes which included some stereotypes, abnormal movements and postures, some respiratory disturbances, rats became quiet and sluggish in movements and there were periods of immobility. The observed hypo-locomotion was quantitatively verified by using two different types of activity cages. The fraction caused a significant decrease in the locomotor activity in both cages, the decrease was more remarkable in the activity wheel cage. The results suggested that *C.c.cerastes* venom has neurotoxic activity which is mainly concentrated in fraction 1. Both the crude venom and the purified fraction act primarily postsynaptically to depress muscle contractility.

INTRODUCTION

Snake venoms are rich sources of pharmacologically active polypeptides and proteins. Some of these proteins exhibit enzymatic activities. These enzymes include phospholipase A_2, proteinase, nucleotidase, phosphodiesterase, and L-amino acid oxidase (Kini, 1997; Bittenbinder *et al.*, 2024). Several other snake venom proteins and polypeptides do not exhibit enzymatic activities and thus are described as non-enzymatic proteins. These proteins include neurotoxins, cardiotoxins, myotoxins, ion channel inhibitors, and anticoagulant proteins (Harvey, 1991; Tu, 1991). The present study aimed to test if the crude venom of *Cerastes cerastes cerastes* and isolated fractions have a neurotoxic effect.

MATERIALS AND METHODS

1. Venom Collection:

Cerastes cerastes cerastes venom was extracted from adult vipers collected from the Toushka Region at Aswan, Egypt. They were kept at the serpentarium of the Zoology Department, Faculty of Science, Ain Shams University. The venom was obtained from the vipers by allowing them to bite naturally through a layer of rubber covering a glass beaker. The venom was then lyophilized (Labconco lyophilizer, USA) and stored desiccated at room temperature in a dark vial until used.

2. Fractionation of *Cerastes cerastes cerastes* Venom:

The method was described by Batzri-Israel and Bdolah (1982). Samples of lyophilized *Cerastes cerastes cerastes* venom (100 mg) were reconstituted in 5 ml 0.03 M ammonium acetate buffer, pH 4.6 and applied to a 2.5x75 cm column (Pharmacia Biotech, Piscataway, NJ, USA) packed with Sephadex G-100 equilibrated with the same buffer. The elution was at a flow rate of 18 ml per hour using an EP-1 Econo Pump (Bio-Rad Laboratory, Hercules, CA, USA). Five millilitre fractions were collected by an automatic fraction collector (Pharmacia, Sweden). The total protein content of each tube was determined by absorbance measurements at 280nm (A_{280}). The resulting optical densities were drawn against the elution volume on millimeter paper. Tubes representing each fraction were pooled together and then lyophilized.

3. *In Vivo* Toxicity Test:

Toxicity of the individual fractions of *C. c. cerastes* venom was studied. For that, six groups of white rats (70 – 100 g) were used, each one composed of 5 animals. Each group received an i.p. injection of the venom fractions at a dose of 7 x LD_{50} of the crude venom (7 × 146 µg/100g). According to Abu-Sinna *et al*. (1993) the LD_{50} for *C. c. cerastes* is 146 µg/100g for the rat. The rate of mortality was monitored and recorded throughout 24 hr after the injection. According to Meier and Theakston (1986), the fraction which causes no mortality in excess of 10 µg / g body weight was considered non-lethal.

4. Neurotoxicity Symptoms:

The venom and toxic fractions can act through their hemotoxic, myotoxic, neurotoxic etc. effects or both together. To verify the mode of action of these fractions, the morphological changes and the symptoms which appear on animals after the injection were observed and recorded till the death of animals. There are some important signs that should be recorded to verify the neurotoxic effect of the venom fractions (Luiz *et al*., 1988; Silveira *et al*., 1988; Breton *et al*., 1997), they are listed below.

- Complex stereotyped behaviour such as: biting, grooming, compulsive muzzle, gnawing, head nodding, wet dog shakes and rearing (CNS effect).
- Salivary and lacrimal secretions (autonomic effect).
- Tremors (mild, moderate or severe).
- Ataxia and uncoordinated gait.
- Paralysis (incomplete or complete).
- Convulsions.
- Coma and death.

5. *In vitro* Neuromuscular Preparations (Rat phrenic nerve-hemi-diaphragm preparation):

This preparation, first described by Bülbring, (1946) was used for studying the effects of drugs on the neuromuscular transmission of the striated muscles in response to electrical stimulation of the phrenic nerve.

6. Neurobehavioral Experiments:

Activity experiment:

The effect of the neurotoxic fraction on locomotion and activity was assessed using two types of cages. The first one is an activity cage whereas the other one is an activity wheel cage.

a) Activity Cage:

As shown in figure 1a, the cage (UGO BASILE 7431 activity cage, Italy) was designed to record the spontaneous co-ordinate activity of rats and mice (individual or groups). The cage dimensions are 36 × 24 × 25 cm. After 15 minutes habituation period in the activity cage, 10 rats were chosen. The animals were randomly divided into two equal groups control and treated groups, n=5. The rats of the treated group were injected i.p. with the neurotoxic, F1 (73 µg/ 100 µl/ 100gm b.w), while animals of the second group were injected with the same volume of physiological saline. For each animal, count was started 15 min after injection and counts of locomotor activity were collected in 10-min intervals for 60 min. The cage was firmly cleaned with wet cotton after every individual reading to exclude the effect of odour.

b) Activity Wheel Cage:

As shown in Figure 1b, the activity wheel cage (UGO BASILE 1800 activity wheel cage, Italy) was designed to provide an easy convenient method for measuring laboratory physical activity of rodents. The rats were individually allowed to habituate in the cage. The chosen animals were randomly divided into two equal groups control and treated groups, n=5. The rats of the treated group were injected i.p. with the neurotoxic fraction F1 (73 µg/ 100 µl/ 100gm b.w), while animals of the second group were injected with 0.9% NaCl (100 µl). For each animal, counting started 15 min after injection and the count was collected in 4-hour intervals for 24 hours. The cage was firmly cleaned with wet cotton after every individual reading to exclude the effect of odor.

(a) (b)

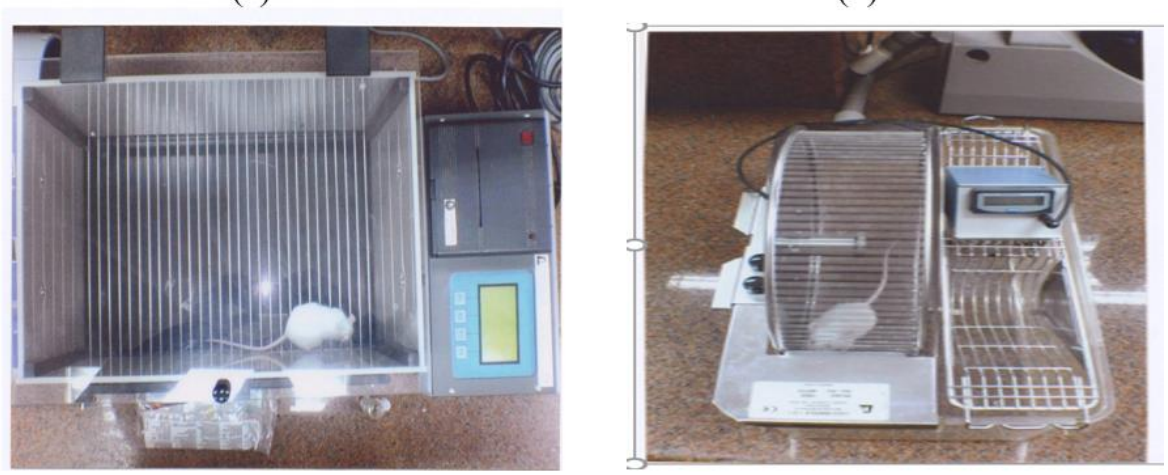

Fig. 1. Photograph showing UGO BASILE mouse activity cage (a), Photograph showing UGO BASILE mouse activity wheel cage (b).

7. **Determination of Venom Phospholipase A_2, Acetylcholinesterase (AChE), and Protease Activities:**

Crude venom and each of the lyophilized fractions were assayed for phospholipase A_2 activity according to the method of Augustyn and Elliot (1969). AChE activity was estimated using the colourimetric method of Ellman *et al*. (1961) as modified by Gorun *et al*. (1978). Protease activity was estimated by the ultraviolet method of Labib *et al*. (1980).

8. Statistical Analysis/Graph Plotting.

Statistical comparisons were made using student's t-test and one-way ANOVA with repeated measures using the Minitab Statistical Software Package. The differences were considered significant at $P<0.05$. Data were graphed using GraphPad Prism version 4 and Origin version 6.1.

RESULTS

1. Fractionation of *Cerastes cerastes cerastes* Venom:

As shown in the Figure 2 the venom was resolved into 6 fractions. The collected fractions were lyophilized using freeze drying.

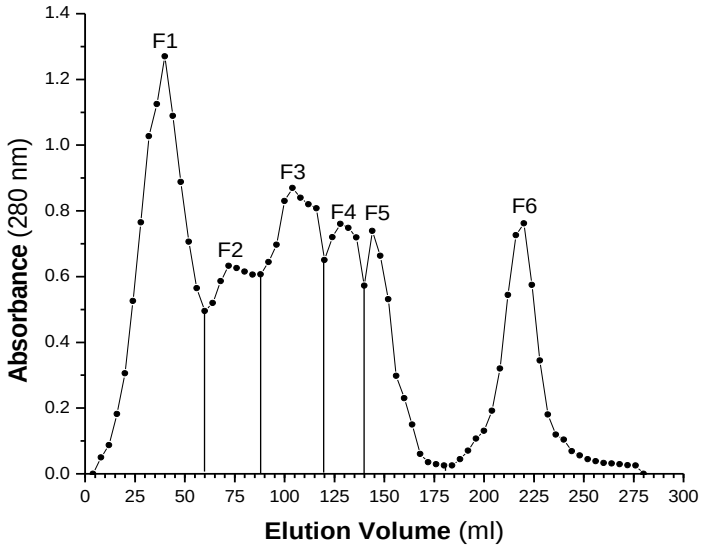

Fig. 2. Fractionation of *C. c. cerastes* crude venom using Sephadex G-100.

2. Toxicity Test:

As shown in Table 1, only fractions 1, 2 and 6 had toxic effects on the white rats, while fractions 3, 4, and 5 were nontoxic and didn't record any signs of toxicity. Each of F1 and F2 recorded an 80% mortality ratio (four animals died from five), while F6 recorded only a 60% mortality ratio (three animals died from five). All the animals died 24 hours after the injection.

Table 1. Mortality percentage after i.p injection of F1, F2, F3, F4, F5 and F6 isolated from the venom of *C.c.cerastes* at a dose of 7 x 0.146 mg/100 g b.w**.**

Group no.	No. of dead animals	% of mortality
I (F1 injected)	4	80%
II (F2 injected)	4	80%
III (F3 injected)	--	0%
IV (F4 injected)	--	0%
V (F5 injected)	--	0%
VI (F6 injected)	3	60%

3. Toxicity Symptoms:

After i.p. injection of rats with a dose of 1022 µg/100gm body weight of F1, the toxicity symptoms of animals were observed by two different observers. As shown in Table 2, irritability and nervousness were the first symptoms observed after injection of F1, followed by hyperventilation which included deep breathing and an increase in the breathing rate (tachypnea). Some stereotyped behaviour was observed such as head nodding, chewing movements, muzzle grooming and wet dog shakes. This group of symptoms was mainly observed during the first 60 min. New symptoms appeared in the next 60 min such as paralysis in the right hind limb (appeared in one animal only), ataxia and uncoordinated gait (imbalance in the movement), general weakness and delayed response to any stimulus such as touch stimulus. Another group of signs was recorded in the third hour such as immobility periods which were accompanied by periods of intermittent tremors, the rate of breathing became very slow (bradypnea), and the animal completely lost its ability for movement, intermittent convulsions were recorded before death. On the other hand, chewing movements, and abnormal postures such as rearing position were observed after 60 min of F2 injection. No new symptoms were observed during the next 120 min. In the fourth hour, the animals had undergone general weakness and complete immobility. Most of the animals died in about 5 hours after injection. Similarly, irritability, nervousness, chewing movements, deep breathing and high breathing rate were observed in the first hour following F6 injection. After five hours, general weakness was observed followed by complete immobility and finally death occurred in less than 6 hours. From all the symptoms observed following F1, F2 and F6 intoxication, it is probable that F1 caused death in rats due to its neurotoxic effect.

Table 2. Main behavioral signs elicited by intraperitoneal injection of F1, F2, and F6 (1022 µg /100 g b.w) of the *C.c.cerastes* crude venom on white rats.

	F1		F2	F6
Stage	**Time (min)**	**Predominant signs**	**Predominant signs**	**Predominant signs**
I	<60	- Irritability and nervousness - Deep breathing and tachypnea. - Stereotyped behavior (head nodding, chewing movements and wet dog shakes.	- Irritability and nervousness. - Chewing movements. - Abnormal postures such as rearing position.	- Irritability and nervousness. - Chewing movements. - Deep breathing and tachypnea.
II	<120	-Paralysis in the right hind limb. Uncoordinated gait and ataxia -Slow and delayed response to stimulus.	No new symptoms	No new symptoms
III	<180	-Periods of immobility with intermittent tremors. -Complete immobility. -Intermittent convulsions. -Comma and death.	General weakness	General weakness
IV	<360		- Death.	-Complete immobility. -Death.

4. *In vitro* Neuromuscular Preparations (Rat phrenic nerve hemi-diaphragm):

Crude venom and F1 reduced the responses of rat phrenic nerve hemidiaphragm to indirect stimulation (nerve stimulation) in a time and concentration-dependent manner. Figure 3, shows the effects of two doses of venom on muscle contraction. 5 µg/ml had an insignificant effect on muscle twitch after 60 min. On the other hand, 20 µg/ml was a potent dose and produced a significant ($p<0.05$) decrease in the twitch height. Figure 4, shows the effects of two doses of F1 on muscle contraction. 5 µg/ml had an insignificant effect on

muscle twitch after 60 min. On the other hand, 10 µg/ml was a potent dose and produced a significant ($p<0.05$) decrease in the twitch height. That decrease reached about 86.6 ± 3.5%, 59.8 ± 5.7% and 22 ± 5.81% of the non-venom treated hemidiaphragm contraction in 20, 40 and 60 min respectively.

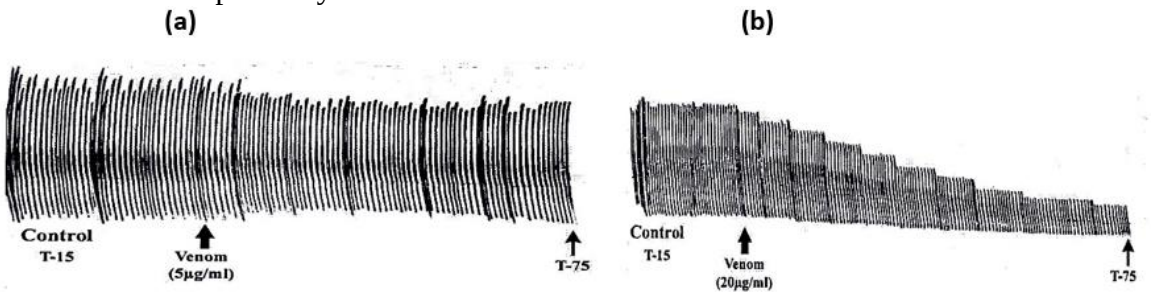

Fig. 3. Typical tracings showing the effects of the venom *C.c.cerastes* on twitch responses of the rat phrenic nerve hemidiaphragm preparation evoked via nerve stimulation (0.1 HZ x 0.2 msec). (a) 5 µg/ml venom; (b) 20 µg/ml venom. T, time (min).

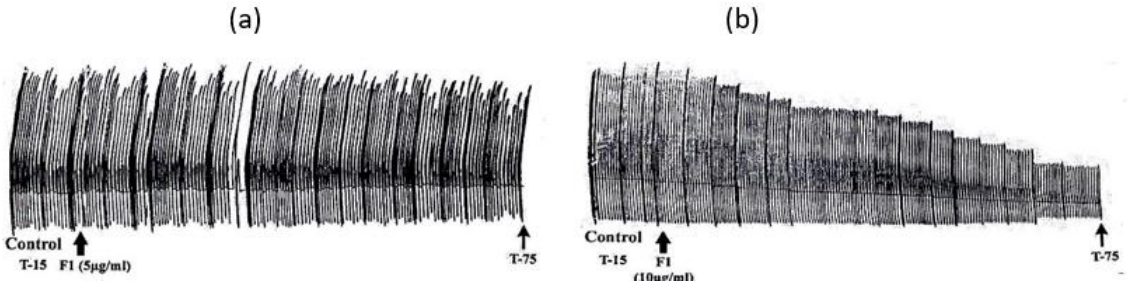

Fig. 4: Typical tracings showing the effects of F1 of the venom *C. c. cerastes* on twitch responses of the rat phrenic nerve hemidiaphragm preparations evoked via nerve stimulation (0.1 HZ x 0.2 msec). (a) 5 µg/ml venom; (b) 10 µg/ml venom, T, time (min).

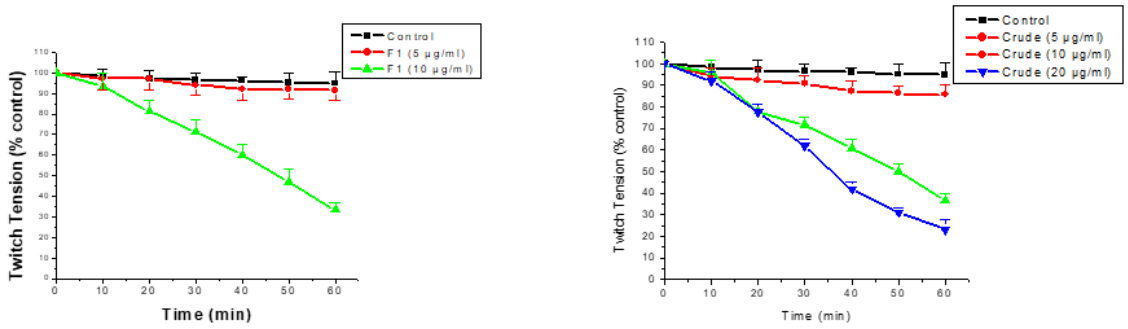

Fig. 5: Effects of *C.c.cerastes* crude venom (5, and 20 µg/ml) and F1 on responses of phrenic nerve hemidiaphragm preparation to nerve stimulation. Each point represents the mean of values obtained from four experiments and SEM is indicated by bars unless these bars are smaller than symbols.

5. Neurobehavioural Experiments:

(1) Activity Cage:

To quantitatively verify the results produced from the *in vivo* observation experiment, the effects of F1 on the locomotor activity of rats were examined. As shown in Table 3 and Figure 6, F1 at a dose of 73 µg/100gm b.w induced hypolocomotion after intraperitoneal injection. The treated animals recorded an average activity of 76.5 ± 2.6 (movements/10min) compared to 92.6 ± 1.3 (movements/10min) produced by the control animals. The decrease in locomotor activity was statistically significant (-17.4; $p<0.05$). There was no statistically significant time effect of treated animals for the whole

experimental duration of 1 hour as indicated by one-way ANOVA. This suggests that the hypo-locomotion effect was not yet recovered.

Table 3. Differences in activity between control and treated animals with F1 (73 µg/100gm) of *C.c.cerastes* crude venom using activity cage. Values are expressed as movement / 10min**.**

Time interval (min)	Control	Treated
1st 10	93 ± 2.3	83 ± 4
2nd 20	92 ± 3.5	83 ± 4.1
3rd 30	92 ± 2.4	75 ± 2.5
4th 40	88 ± 3.1	77 ± 2.9
5th 50	98 ± 2.4	75 ± 3.2
6th 60	93 ± 3.5	66 ± 3.4
Mean **% of change**	92.6 ± 1.3	76.5 ± 2.6 -17.4

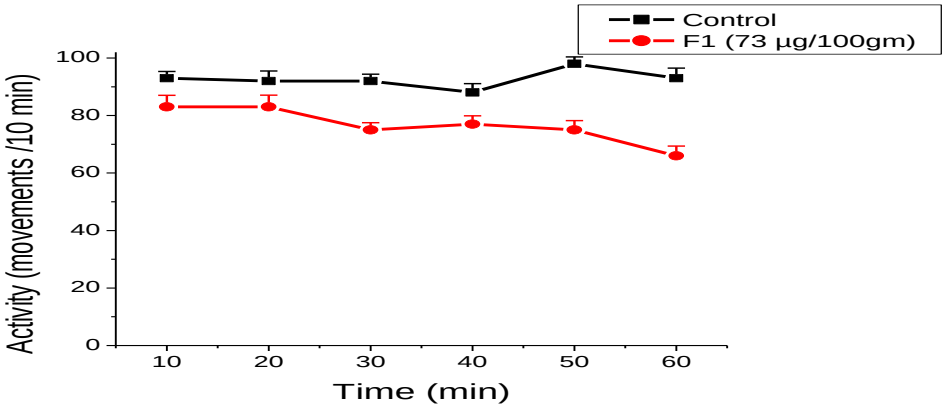

Fig. 6: Time response curve of the locomotor activity of rats following i.p. injection of F1 (73 µg/100gm) of F1 of *C. c. cerastes* venom using UGO BASILE 7431 activity cage.

(2) Activity Wheel Cage:

Pronounced significant decrease in locomotor activity of animals (rats are not interested in rolling the wheel) after F1 injection compared with the control group. The data presented in Table 4 and Figure 7 revealed a significant decrease (-90%; $p<0.05$) in the activity of animals after F1 injection. The treated animals recorded a mean activity of 22.8 ± 3.5 (rounds / 4hrs) compared to 217.3 ± 8.5 (rounds / 4hrs) for the control animals. There was no statistically significant time effect within the treated animals for the whole experimental duration of 24 hr as indicated by one-way ANOVA. This suggests that the inhibition effect is irreversible and cannot be recovered.

Table 4. Differences in activity between control and treated animals with F1 (73 µg/100gm) of F1 of *C. c. cerastes* venom using activity wheel cage. Values are expressed as rounds/ 4hr.

Time interval (hr)	Control (rounds/4 hr)	Treated (rounds/4 hr)
4	240 ± 8.5	28 ± 10.5
8	200 ± 10.2	32 ± 12.3
12	208 ± 12.3	12 ± 6.5
16	216 ± 14.3	28 ± 10.6
20	196 ± 20	25 ± 14.4
24	244 ± 22.2	12 ± 6.3
Mean % of change	217.3 ± 8.5	22.8 ± 3.5 -90

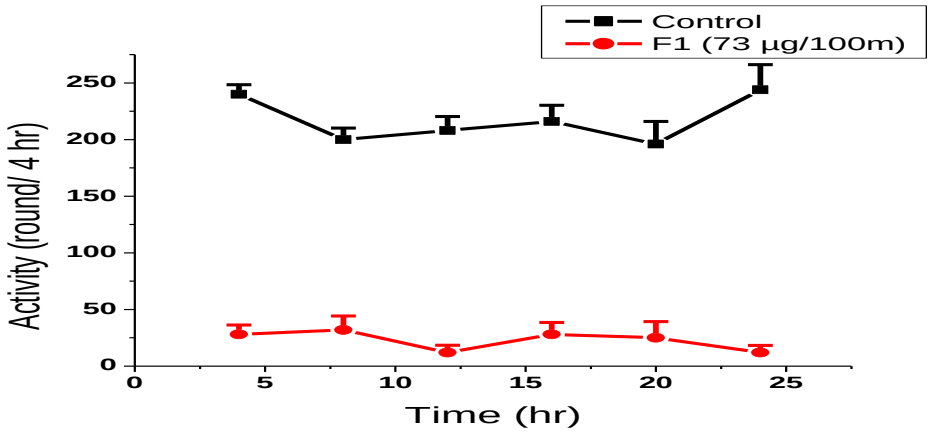

Fig. 7: Time response curve of the locomotor activity of rats following i.p. injection of sF1 (73 µg/100gm) of *C.c.cerastes* venom using UGO BASILE 1800 activity wheel cage**.**

6. **Determination of Venom Phospholipase A_2, Acetylcholinesterase (AChE), and Protease Activities:**

The data presented in Table 5, revealed that the neurotoxic fraction (F1) possesses only caseinolytic activity, while it lacks PLA_2 and AChE activities.

Table 5. Determination of phospholipase A_2 (PLA_2) activity (µg hydrolyzed lecithin /hr/mg protein), proteinase, and acetylcholinesterase (AChE) in *C.c.cerastes* crude venom and its six gel filtration fractions.

Sample	PLA_2 Activity µg lecithin hydrolyzed/hr/mg protein	Proteinase activity (ΔA_{280} /hr/ mg)	AChE activity
Crude venom	**400 ± 25**	**2.03 ± 0.3**	**-ve**
F1	**-ve**	**2.9 ± 0.26**	**-ve**
F2	**760 ± 44**	**-ve**	**-ve**
F6	**-ve**	**7.2 ± 1.3**	**-ve**

DISCUSSION

The viper, *Cerastes cerastes cerastes* was gathered from the Toushka region around the High Dam area in the south of Aswan governorate. No detailed studies were carried out on the venom of this viper especially those concerned with neurotoxicity effects. Further, other biological effects were of more interest (Abu-Sinna *et al*., 1992, 1993 & 2003). the best separation was achieved when Sephadex G-100, 0.02 M ammonium acetate buffer pH 4.6 and column dimensions 2.6 x 74 cm were used, as 6 fractions (F1, F2, F3, F4, F5 and F6) were obtained. The neurotoxicity of the six fractions was tested using different techniques (behavioural and neuromuscular techniques). F1 recorded apparent neurotoxicity.

More than 100 α-neurotoxins have been isolated and purified from various snake venoms and their amino acid sequences have been determined. They have the ability to block nerve transmission by binding specifically to the nicotinic acetylcholine receptors on the postsynaptic membranes of skeletal muscles and/or neurons. This made it possible to elucidate the mode of action and the toxicity of their individual components (Servent *et al*., 1997; Gong *et al*., 1999; Phui-Yee *et al*., 2004; Wickramaratna *et al*., 2004; Kuruppu *et al*., 2005; Marcon *et al*., 2013; Osipov and Utkin, 2023). These findings are in harmony with the present results.

In the present study, the toxicity of the six gel filtration fractions was tested by injecting rats i.p. with the fractions at a dose of 7 x LD_{50} of the crude venom. According to Meier and Theakston (1986), the fraction which causes no mortality in excess of 10 µg / gm body weight is considered non-lethal. From the results, it was clear that fractions 1, 2 and 6 were toxic while the toxicity was lacking from the other fractions. The main symptoms preceding the death of animals were monitored and recorded. Many signs of neurotoxicity were obvious after the F1 injection. Some were related to paralysis and convulsions like wet dog shakes, myoclonus and complete immobility. Other signs included uncoordinated gait, general weakness and attacks of intermittent tremors were also recorded. The appearance of some signs of complex behavior which included stereotypes like head nodding, chewing movements, wet dog shakes and abnormal movements and postures was distinct. The high level of neural integration required to perform such a complex behavior suggests an involvement of the CNS in F1-elicited behavior (Barbeito *et al*., 1982; Silveira *et al*., 1985; Gandolfo *et al*., 1996). Finally, the rats underwent complete paralysis interrupted with intermittent convulsions and death.

This neuromuscular action apparently involved interference with the neurotransmission as shown by the reduced response to ACh and CCh after incubation with the venom (Harvey *et al*., 1994; Wickramaratna *et al*., 2004; Kuruppu *et al*., 2006). Such inhibition of neurotransmission could involve damage to the nicotinic receptors by proteases present in this venom as well as damage to muscle fibers. The action on muscle is based on the observation that the venom depressed the responses to K^+ (Prianti *et al*., 2003; Al-Shammari *et al*., 2023). These explanations are supported by several reports. Souza *et al*. (2002) worked on *Bothrops jararacuss* venom, Kuch *et al.* (2003) worked on *Bungarus candidus* venom, Lumsden *et al.* (2005) worked on *Boiga dendrophila* venom and Kuruppu *et al.* (2006) working on *Demansia papuensis* venom, Žužek *et al*. (2024) working on ammodytoxin and Stazi *et al*. (2024) working on *Vipera aspis* venoms.

The twitch response of the rat phrenic nerve hemidiaphragm was significantly reduced in the presence of F1 of crude venom. At a concentration of 10 µg/ml, the fraction reduced the twitches by more than 75% after 75 min. A significant reduction in the contractile response to exogenous ACh (10^{-3} M), CCh ($2x10^{-6}$ M) and KCL (60mM) was remarkable after F1 treatment. As shown from the results, F1 was effective at a small dose

(10 µg/ml) compared with 20 µg/ml in crude venom. So it is concluded that the effectiveness and potency of neurotoxicity increases with fractionation and purification.

On the other hand, the other fractions F2, F3, F4, F5 and F6 lack any effect on neuromuscular transmission. They did not show any significant change in the twitch response of the RFNH to nerve stimulation. Even with the large dose (100 µg/ml), the fractions had no significant effects on the twitch tension throughout 120 min and did not cause any change compared with the control. Furthermore, no one of these fractions caused a remarkable change in the contractile response to nicotinic agonists (ACh and CCh) or to KCL.

From the above results, it is clear that most of the neurotoxicity of the crude *C.c.* cerastes venom is concentrated in F1, as it was the only fraction that has the ability to block neuromuscular transmission and nerve conductance. F1 was

Conclusion

1) C. c. cerastes venom showed neurotoxic action which was evidenced by different techniques. i) It produced a blocking effect on neuromuscular transmission in RPNH preparations. ii) Some behavioral signs were related to the effect of venom on the nervous system.

2) A neurotoxic (caseinolytic protein) was purified from the crude venom. The protein is potent neurotoxic as indicated by its blocking effect on RPNH. The fraction has a suppressing effect on activity as indicated by the remarkable hypolocomotion of rats in the neurobehavioral experiments.

3) Some of the behavioral signs following i.p injection of the toxin suggest the involvement of neurotoxicity in CNS, but this finding needs further work to prove it.

Declarations:

Ethical Approval: Not applicable.

Competing interests: The authors declare no conflict of interest.

Availability of Data and Materials: All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

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