# Short Communication
# Notes on the role of *Echis coloratus* and *Naja nigricollis* Snake venoms on neuronal cell death

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## ABSTRACT

*In the present study murine hippocampal HT22 cells were employed to investigate the role of Echis coloratus and Naja nigricollis snake venom on cell death. Monitoring of the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the culture medium after treatment of the cells with different concentrations (50ug/ml, 100 µg/ml) from four fractions (F1, F2, F3 and F4) that obtained from each venom after purification. The time variation (6, 12, 18 and 24 hours) of the LDH concentration in the medium was used to indicate the total amount of lysed and, hence, the specific rates of cell death. In the first 6h from treatment of cells with F2( 50 µg/ml ) from Naja nigricollis venom( which is the most effective fraction in both venoms), LDH released into cell culture media more than treatment of cells with F3 and crude venom. Treatment of cells with a concentration of 50ug/ml and 100 µg/ml F3 Naja nigricollis (after 12h and 24h) snake venom, LDH elevated more than F2 and crude venom. Otherwise, lactate dehydrogenase (LDH) was released into cell culture media by treatment of the cell by 50 µg/ml F3 more than treatment of cells by F4 and crude venom from Echis coloratus.*

***Key words:*** *Echis coloratus - Naja nigricollis - HT22 cells- LDH*

## INTRODUCTION

Snake venoms are complex mixtures of protein and non-protein components[1,2]. Snake envenomation constitutes a medical hazard in most regions of the world[3]. It is considered as a subcutaneous/ intradermal injection of venom into the prey or human victims. The pathophysiology of envenomation may include only local effects (hemorrhage, edema, myonecrosis, and extracellular matrix (ECM) degradation) or may include systemic effects (neurotoxicity, myotoxicity, cardiotoxicity, and alterations in hematological systems)[4,5]. The latter depends on the concentration, efficiency, and rate of diffusion of target-specific toxins from the site of the bite into the general circulation.

It has been reported that venoms from the snake families Elapidae, Viperidae, and Crotalidae caused lysis of different cells[6]. *Echis Coloratus* is a snake species that belongs to the Viperidae family that have common

clinical features on the other hand, *Naja nigricollis* belong to Elapiae family. *Naja nigricollis* snake is able to propel the venom through the air to hit the eyes of the prey. As example, the neurological disturbances observed in *E. coloratus* envenomated animals in the absence of bleeding in the brain[7]. Neurotoxic activity of *Echis Coloratus* venom in dogs and guinea pigs was observed with histochemical methods showing a diffuse breakdown of the blood-brain barrier[8].

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid[9]. *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. As a result, the release of lactate dehydrogenase has proved to be a popular and reliable test for cytotoxicity in both immunological studies, where it has superseded the radioactive chromium release test as an assay for cellular cytotoxicity[10,11], and in biocompatibility studies, where it has now become an important *in vitro* screening test[12,13]. In order to assess the effects of a biomaterial on mammalian cell cultures, cells are exposed to varying concentrations of test material over a period of days. The release of LDH into culture supernatant correlates with the amount of cell death and membrane damage, providing an accurate measure of the cellular toxicity induced by the test substance.

LDH assays have been used to quantify cell-mediated cytotoxicity induced by cytotoxic T cells, natural killer cells, lymphokine-activated killer cells, and monocytes[10,11], as well as to identify mediators that induce cytolysis[11].

The present study aimed to investigate the most effective fraction in both snake venoms on cell death.

## MATERIALS & METHODS

**Chemicals**:

L-Glutamate, Triton X-100, BAPTA-AM and all cell culture media and reagents were obtained from InVitrogen (Paisley, UK). Lactate dehydrogenase (LDH) detection kit was obtained from Roche (Lewes, UK).

**Cell culture** HT22 cells were cultured[14] in DMEM containing 10% FBS, 2 mM L-glutamine and 50 U/ml penicillin and 50 μg/ml streptomycin under a humidified atmosphere (5% $CO_2$, 37°C). Unless indicated otherwise, $8\times10^3$ cells (counted by hemocytometer) were seeded into 24-well plates and incubated overnight, prior to media renewal and treatment of the cells with the compounds of interest in Dulbecco's modified Eagle's medium (DMEM) containing 2% FBS. For the measurement of cell viability, 100 μl from cell culture were used and aliquots of the media were removed at the indicated time points and assayed for lactate dehydrogenase (LDH) release, according to the manufacturer's instructions (Roche, Lewes, UK). Each sample of LDH was compared to the total, Triton X-100 releasable

LDH from the same well at the end of each experiment.

**The crude venoms**. It has been milked from *Naja nigricollis*, and *Echis coloratus* snakes. Freeze-dried snake venoms were obtained from Faculty of Science- Department of Zoology, King Saud university-KSA and Faculty of Medicine - Ain-shams university- Egypt.

**Purfficatlon of venoms fractions.**

*Naja nigricollis* crud venom an d *Echis coloratus* crude venom were fractiated and partially purified by gel filtration on sephadex G-70 and sephadex G-75 respectively, using 0.03M sodium phosphate buffer pH 7.4, at flow rate of 20mm/hr, 3 ml/tube for 80 tubes. The protein concentration was estimated at OD of 280 nm. It was noticed that 4 fractions have been obtained which were named F1, F2, F3 and F4 from each venom.

Protein concentrations were measured by Bio-RAD Dc protein assay and by Lowry method in 96 well plates. All fractions (both venoms) were dissolved in sodium phosphate buffer (pH 7.4).

## RESULTS & DISCUSSION

Experimental models have been attempted in order to clarify the role of *Echis coloratus* and *Naja nigricollis* snake venom on cell death by monitoring of the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) in the culture medium under different concentrations of two different venoms by incubation of HT22 cells to different levels snake venoms.

Separation and purification of both snake venoms were carried out as described under Materials and methods section.

The intracellular LDH content of living attached cells is checked to be constant during the culture. However, cells detached from the microcarriers, and counted dead, have only released part of their intracellular LDH. Lactate dehydrogenase has proved to be an extremely useful in vitro marker for cellular toxicity. LDH assays are of great value both in immunology for the quantification of cytotoxicity, and in the evaluation of biomaterial biocompatibility.

Previous studies done on that species (*E. coloratus*) were focused on serum and tissue (*in vitro*) profile of experimental animals. Snake venom components, especially those of vipers, either activate, inhibit or liberate enzymes by cellular organelles destruction[15,16]. The different toxic effects exhibited by venoms of vipers were due to their contents of proteolytic and lipolytic enzymes[17].

The protein concentration in crouds and all fractions were measured (Table 1 and Table 2). The highest protein contents were in both crude venoms. The lowest protein content was F2 in *Echis coloratus* venom. Taking advantage of a commonly used procedure, we detected the cell death by measuring the release of lactate dehydrogenase (LDH) into cell culture media. The time variation of the LDH concentration in the medium was used to calculate the total amount of lysed and actually produced cells in the reactors, hence, the actual specific

rates of cell growth and death. LDH release measured at different time points following of venom incubation. As shown in all figures, venom incubation induced a time-dependent increase in LDH release compared control, indicating injury of HT22 cells.

As shown in Tables 1.1, 1.2 and 1.3, the release of LDH increases as a function *Naja nigricollis* venom fraction concentration and time of incubation. Moreover, LDH released in the presence of 50 µg\ml from F2 in the first 6 hours from incubation of *Naja nigricollis* venom was more than that released by crude venom and other fractions. These results agree with those of Isac de Castroa[18] who funnd that the most intense effects were obtained with venom concentration of 30 µg/m by using Bothrops venom.

On the other hand, incubation of cells in presence of F3 (100 µg\ml and 50 µg\ml) release nearly the same LDH in incubation 6-18 hours, but 100 µM\ml slightly increase. The maximum release of LDH from all cells (100%) after 24 hours incubation these indicate that the all cells were dead.

Moreover, from tables and figures 1.1, 1.2 and 1.3, it was noticed that, the effect of *Naja nigricollis* crude venom in elevation of LDH from the cells is less effective than that of F2 and F3. These means that the separated fractions are more toxic than the crude venom. Moreover, F2 is more toxic than F3 and crude venom, as reported by Gamal Morkos[19] who reported that F2 was the most lethal fraction in these venom. But by increasing time of incubation in case of F3 (100 µg\ml) after 24h all cells died and all LDH elevated compare with the effect of F2 and crude venom. Previous study was carried out by exposed epithelial MDCK cells and mesangial cells to viper's venom concentrations of 100–1000 µg/ml for 5 min up to 48 h, resulting in cells injuries[20].

A concentration of 50 µg\ml of F1, F3 and F4 from *Echis coloratus* venom with 6, 12, 18 and 24 h of incubation period caused cell death and increase in LDH release (Figures and Tables 2.1, 2.2, 2.3 and 2.4) compared with concentration of 100 µg\ml i.e. low concentration of separated fractions from *Echis coloratus* venom is increase of rate of cell death and increase than the high concentration. Otherwise, a concentration of 100 µg\ml of crude venom (Figure 2.1) elevated LDH and increase cell death more than 50µg\ml from crude venom.

In addition to that the increase in LDH observed in the present study is in agreement with the finding of others[16,21] in viper snakes *Echis carinatus* and *Cerastes cerastes.* Furthermore, increase of LDH activity over the control value was observed in the supernatant from undiluted PRP treated with 100µg/ml of the *Eristocophis macmahoni* venom[22]. On the other hand, the incubation of 10 µg of the crude venom (*Echis coloratus*,) and its fractions with fibroblast sonicates or with freshly prepared serum showed no effect on LDH.

That finding suggests that the venom does not act directly on the enzyme protein itself but may act as mediator[23].

Bothrops asper Snake Venom affects collecting lymphatic vessels through the action of myotoxic Phospholipase A2 on the smooth muscle of these vessels, inducing cell contraction and irreversible cell damage[24,25]. The different toxic effects exhibited by venoms of vipers were due to their contents of proteolytic and lipolytic enzymes[17].

From the previous result, it is noticed that F3 from *Echis coloratus*, that showed phospholipase A2 activity[23] is the most cytotoxic fraction than crude venom and other fractions as reported by Hessa[26], who found that F3 was the most lethal fraction and the toxicity normally depending on the LDH releases[27,28].

**Table 1- *protein concentration in Naja nigricolus* venom**

| Fraction no. | Protein con. *mg/ml* |
|---|---|
| Crude venom | 58.87 |
| F1 | 5.36 |
| F2 | 12.898 |
| F3 | 4.384 |
| F4 | 0.595 |

**Table 2- *protein concentration in Echis coloratus* venom**

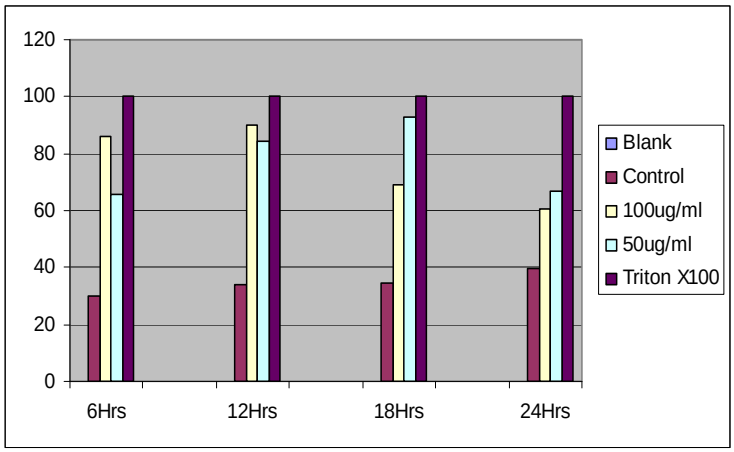
| Fraction no. | otein con. *mg/ml* |
|---|---|
| Crude venom | 53.663 |
| F1 | 0.259 |
| F2 | 0.005 |
| F3 | 0.371 |
| F4 | 0.923 |

**Time course of *Naja nigricolus* and *Echis coloratus* venoms for LDH assay**

***Naja nigricolus* venom.**

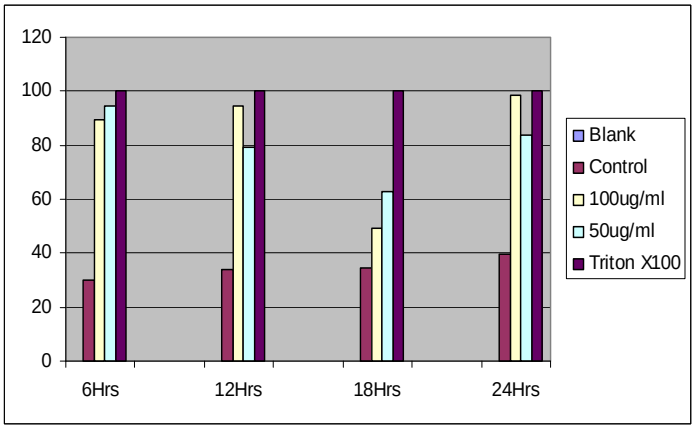
***1.1 Naja nigricolus* crude venom**

| | 6Hrs | 12Hrs | 18Hrs | 24Hrs |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 29.9659 | 33.93006 | 34.79782 | 39.46952 |
| 100ug/ml | 85.80563 | 90.09159 | 68.8958 | 60.53048 |
| 50ug/ml | 65.60102 | 84.59617 | 92.80715 | 66.86461 |
| Triton X100 | 100 | 100 | 100 | 100 |



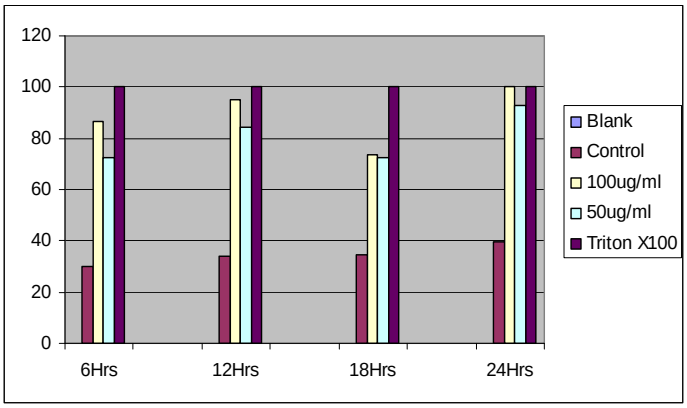

**1.2- F2 from *Naja nigricolus* venom**

| | 6Hrs | 12Hrs | 18Hrs | 24Hrs |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 29.9659 | 33.93006 | 34.79782 | 39.46952 |
| 100ug/ml | 89.55669 | 94.71274 | 49.10575 | 98.77276 |
| 50ug/ml | 94.5439 | 79.43381 | 63.02488 | 83.96675 |
| Triton X100 | 100 | 100 | 100 | 100 |



**1.3- F3 from *Naja nigricolus* venom**

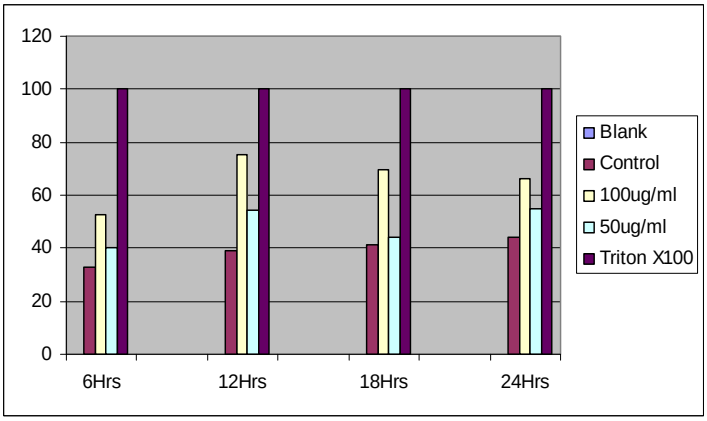
| | 6Hrs | 12Hrs | 18Hrs | 24Hrs |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 29.9659 | 33.93006 | 34.79782 | 39.46952 |
| 100ug/ml | 86.87127 | 95.08743 | 73.56143 | 100.0174 |
| 50ug/ml | 72.20801 | 84.51291 | 72.47278 | 92.67617 |
| Triton X100 | 100 | 100 | 100 | 100 |




***2. Echis coloratus* venom**

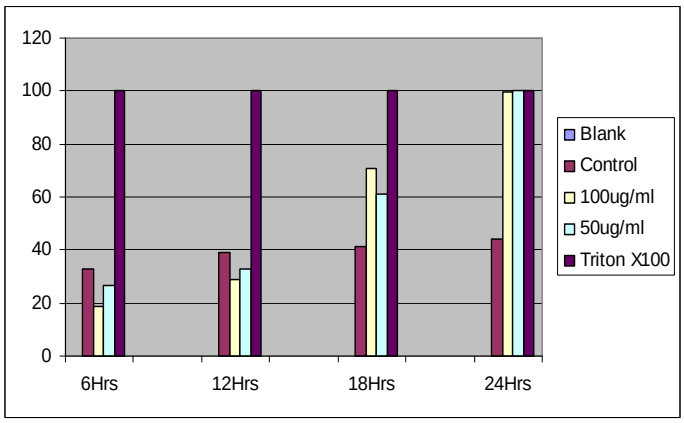
**2.1- *Echis coloratus* crude**

| | **6Hrs** | **12Hrs** | **18Hrs** | **24Hrs** |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 32.9497 | 38.96753 | 41.29082 | 44.18052 |
| 100ug/ml | 52.77067 | 75.10408 | 69.36236 | 66.34996 |
| 50ug/ml | 40.2387 | 54.37136 | 44.12908 | 54.86936 |
| Triton X100 | 100 | 100 | 100 | 100 |



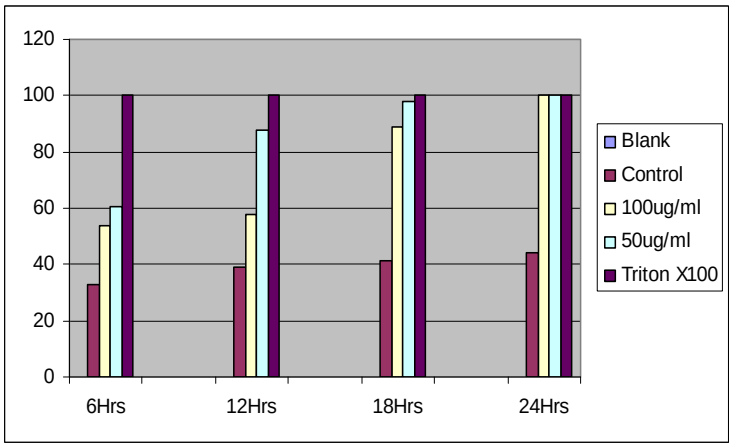
**2.2-*Echis coloratus F1***

| | **6Hrs** | **12Hrs** | **18Hrs** | **24Hrs** |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 32.9497 | 38.96753 | 41.29082 | 44.18052 |
| 100ug/ml | 18.84058 | 28.85096 | 71.03421 | 99.61822 |
| 50ug/ml | 26.38534 | 32.6811 | 61.15863 | 100 |
| Triton X100 | 100 | 100 | 100 | 100 |



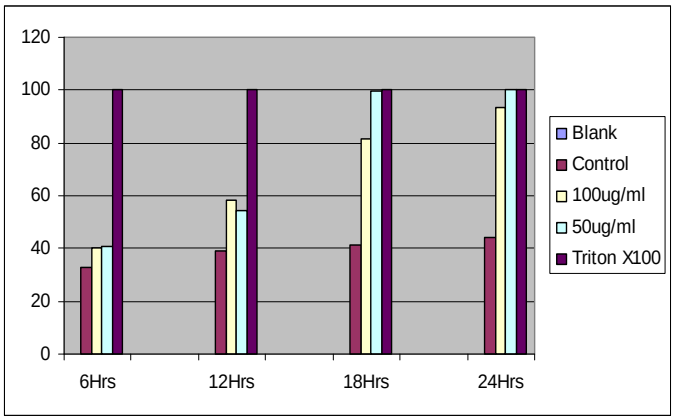

**2.3-*Echis coloratus F3***

| | 6Hrs | 12Hrs | 18Hrs | 24Hrs |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 32.9497 | 38.96753 | 41.29082 | 44.18052 |
| 100ug/ml | 53.53794 | 57.91007 | 88.95801 | 100 |
| 50ug/ml | 60.40068 | 87.46878 | 97.82271 | 100 |
| Triton X100 | 100 | 100 | 100 | 100 |



**2.4-*Echis coloratus F4***

| | 6Hrs | 12Hrs | 18Hrs | 24Hrs |
|---|---|---|---|---|
| Blank | 0 | 0 | 0 | 0 |
| Control | 32.9497 | 38.96753 | 41.29082 | 44.18052 |
| 100ug/ml | 40.15345 | 58.03497 | 81.64852 | 93.34917 |
| 50ug/ml | 40.57971 | 54.20483 | 99.6112 | 100 |
| Triton X100 | 100 | 100 | 100 | 100 |

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## REFERENCES

1- **Bougis PE, Marchol P, Rochat H. (1986)**: Charecterization of Elapidae snake venom components using optimization reverse-phase high performance liquid chromatographic conditions and screening assays for neurotoxin and phospholipase A2 activities. Biochemistry 25: 7235-7243.

2- **Ownby CL, Colberg TR. (1987)**: Characterization of the biological and immunological properties of fractions of Prairie rattle snake (Crotalus viridis viridis) venom. ***Toxicon*** 25: 1329-1342.

3- **Chippaux, J. P. (1998)**: Snake-bites: appraisal of the global situation Bull. World Health Organ. 76 (5):515-24. Review

4- **Kini, R. M. (1997)***: Venom Phospholipase A2 Enzymes: Structure*, *Function and Mechanism*, J. Wiley, New York.

**5- Aird, S. D. (2002)**: Ophidian envenomation strategies and the role of purines. *Toxicon* **40 (4)**: 335-393

**6- TU A., GILTNER JB. (1974)**: Cytotoxic effects of snake venoms on KB and Yoshida cells. *Res. Commun. Chem. Pathol. Pharmacol.*, 9,783-6.

**7- Gitter, S., Levi, G., KocHwa, S., DE Vries, A., RECHNIC, J. and Casper, J. (1960)**: Studies on the venom of *Echis colorata*. Am. J. trop. Med. Hyg., 9: 391.

**8- Sandbank U and Djaldetti M (1966)**: Effect of *Echis colorata* venom inoculation on the nervous system of the dog and guinea pig. Acta Neuropathol., 6(1): 61

**9- Rae, T. (1977)**: Tolerance of mouse macrophages in vitro to barium sulfate used in orthopedic bone cement. *J. Biomed. Mater. Res.,* 11: 839.

**10- Decker, T. and Lohmann-Matthes, ML. (1988)**: A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity *J. Immunol. Methods* 115 (1): 61.

**11- Korzeniewski, C. and Callewaert, D.M. (1983)**: An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods* 64 (3): 313.

**12- Rae, T. (1975)**: A study on the effects of particulate metals of orthopaedic interest on murine macrophages in vitro. *J. Bone Jt. Surg Br.* 57(4): 444.

**13- Rae, T. (1981)**: The toxicity of metals used in orthopaedic prostheses. An experimental study using cultured human synovial fibroblasts. *J. Bone Jt Br. Surg*. 63-B (3): 435.

**14- George E.N. Kass, Lucy M. Elphick, Mohammad Hawat , Nick J. Toms, Annika Meinander, Andrey Mikhailov, John E. Eriksson. (2008):** Opposing roles for caspase and calpain death proteases in L-glutamate-induced oxidative neurotoxicity. Toxicology and Applied Pharmacology (232) 258–267

**15- Moustafa, F.A., Y.Y. Ahmed and M.F. El-Asmer, (1974)**: Effect of scorpion *Buthus minax* L. Koch venom on succinic dehydrogenase and cholinesterase activity of mouse tissue. Toxicon 12: 237-240.

**16- Abdel-Nabi, I., R. Awadalla and I. El-Shamy (1997)**: Biochemical effects of intraperitoneal injection of rats with the venom of the snake *Echis carinatus*. Egypt. J. Zool., 29: 195-205.

**17- Tan, N. H. and G. Ponnudurai, (1990)**: A comparative study of the biological properties of venoms from snakes of the genus Vipera (true adders). Comp. Biochem. Physiol., 96: 683-688.

**18- Isac de Castroa, Emmanuel de A. Burdmannb, Antonio Carlos Seguroa, Luis Yu. (2004)**: Bothrops venom induces direct renal tubular injury: role for lipid peroxidation and prevention by antivenom. Toxicon 43 : 833–839

**19- Gamal T. G. Morkos (1992):** Toxicological studies on a snake

venom in Egypt. Thesis. Benha, Faculty of medicine**.**

20- **Willinger, C.C., Thamaree, S., Schramek, H., Gstraunthaler, G., Pfaller, W**., **(1995):** In vitro nephrotoxicity of Russell's viper venom. Kidney Int., 47(2): 518–528.

21- **Hodhod, S., Swelam, N., Tash, F. and El-Asmar. M. F (1989):** Effect of Cerates cerates (Egyptian Sand Viper) Venom on Rat Pancreas. Egypt. J. Biochem., 7: 1-8.

22- **Syed Abid Ali, Faiza Hamid, Atiya Abbasi, Zafar H. Zaidi, Darakhsh and Shehnaza (1999):** Pharmacological effects of the leaf-nosed viper snake (Eristocophis macmahoni) venom and its HPLC fractions. Toxicon 37: 1095-1107

23- **Al-Saleh S.S., H. K. Ghneim, H.Y Haddad and S.U. Khan (2002):** Separation and purification of Echis coloratus venom and some biological and biochemical effects of the proteins. Cell Biochemistry and function 20: 153-162.

24- **24-Mora J, Mora R, Lomonte B, Gutièrrez JM (2008)**: Effects of Bothrops asper Snake Venom on Lymphatic Vessels: Insights into a Hidden Aspect of Envenomation. PLoS Negl Trop Dis., 2(10): e318

25- **Junaid Mahmood Alam and Rashida Qasim (1993)**: Changes in serum components induced by venoms of marine animals. *Pakistan Journal of Pharmaceutical* Sciences 6(1): 81-87

26- **26 - Hessa El-mozafer**. **(2000)**: Fractionation and Purification of *Echis coloratus* Venom from Saudia Arabia and Study of its Biological Activities. Thesis. Girls College of Science .Dammam . Saudi Arabia

27- **Rodwell, V.M. (1990)**: Enzymes: General properties. In: *Harper's Biochemistry,* pp. 58-67 (Murray, R.K, Granner, D.K, Mayes, P.A. and Rodwell, V.M. eds.) Connecticut: Appleton and Lange.

28- **Williamson, J.A., Burnett, J.W., Fenner, P.J. Hach-Wunderls, V., Hoe, L.Y. and Adige, K.M. (1988)**: Acute regional vascular insufficiency after jellyfish envenomation. *Med. J. Aust*., **149**: 698-701.

# ملاحظات على تاثير سم افعى السجاد الشرقي و سم الثعبان البخاخ المصرى على موت الخلايا العصبية

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تم استخدام الخلايا العصبية (murine hippocampal HT22) فى هذا البحث لدراسة تاثير سم افعى السجاد الشرقي و سم الثعبان البخاخ المصرى على موت هذه الخلايا . و بقياس تركيز LDH) فى الوسط البيئى بعد اضافة تركيزات مختلفة من السموم(50ug/ml, 100 µg/ml ) بعد فصلها ( F1, F2, F3 and F4) من كلا السمان، يمكن دراسة تاثير هذه السموم على الخلايا. كذلك يشير تركز LDH فى الوسط باختلاف زمن تحضين السم مع الخلايا لكمية الخلايا التى تتكسر و تموت و كذلك معدل موت الخلايا. و يلاحظ انه فى الست ساعات الاولى من اضافة F2 , 50 µg/ml من سم الثعبان البخاخ المصرى فان معدل خروج LDH للوسط يكون اكثر من كمية LDH المقاسة عند معاملة الخلايا بكل من F3 وكذلك السم الخام. و من جه اخرى فعند معاملة الخلايا بتركيز 50ug/ml و 100 µg/ml من F3 المنقى من سم الثعبان البخاخ المصرى بعد فترة تحضين (١٢ و ٢٤ ساعة) فان كمية LDH التى تم قياسها كانت اكبر من مثيلاتها عند معاملة الخلايا بكل من F2 و السم الخام تحت نفس الظروف.و فى النهاية فان كمية LDH التى خرجت من الخلايا للوسط عند معاملة الخلايا بتركيز 50 µg/ml من F3 فان كمية LDH المقاسة تكون اكثر من التركيز الذى ينتج عند معاملة الخلايا بكل من F4 و السم الخام الذى تم الحصول علية من سم افعى السجاد الشرقي.