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Cytotoxicity and oxidative stress of *Cerastes vipera* venom on human hepatocellular carcinoma (HepG-2)

Aalaa A. Elmeligy[1], Hamdy H. Swelim[1], Ali Abd EL-Aal[1] and Salwa M. El-Hallouty[2]

[1]*Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt,*
[2]*Pharmacognosy Department, National Research Centre, Pharmaceutical and Drug Industries Division, Dokki, Giza, Egypt.*

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ABSTRACT

Snake venoms comprise mixtures of bioactive components, including proteins and peptides, which exhibit diverse biochemical activities. This wide array of pharmacological effects associated with snake venoms has made them attractive sources for research into potentially novel therapeutics, and several venom components-derived drugs are now in use. In the current study, we performed a cytotoxicity test and biochemical analyses to examine the effects of *Cerastes vipera* crude venom toward HepG-2 cells. MTT cell viability assay of cancer cells incubated with crude venom revealed that the venom exhibited significant cytotoxicity potentials on HepG-2 cells. GSH, MDA, and TAC were significantly changed after treatment of HepG-2 cells with the crude venom, as compared to the untreated cells, indicating that *Cerastes vipera* crude venom might contain promising components as antitumor drugs.

Introduction

Elapidae, Crotalidae and Viperidae snake venoms revealed cytotoxicity towards several tumor cell lines such as B16F10 melanoma, chondrosarcoma, human breast carcinoma cell lines MDA-MB-231, human breast adenocarcinoma (MCF-7), human lung squamous carcinoma (SK-MES-1), pancreatic carcinoma Panc-1, and Ehrlich ascites [1,2,3,4,5,6].

The disintegrin protein was purified from *Naja naja* venom [7] and studied for the cytotoxic and apoptotic activity of its protein fraction on the human cancer cell lines; breast cancer (MCF-7), lung cancer (A549), and liver cancer (HepG-2). Another protein fraction called crotoxin isolated from the *Crotalus durissus* venom had antitumor effecton human esophageal carcinoma (Eca-109) cells [8]. Ghazaryan *et al.* [9] investigated the antitumor effect of the *Macrovipera lebetina obtusa* crude venom (MLO) and the obtustatin, a monomeric disintegrin isolated from MLO venom, on the S-180 sarcoma growth *in vitro* and *in vivo*. Nalbantsoy *et al.* [10] evaluated the cytotoxic effect of the crude venoms of *Montivipera raddei* and *Montivipera bulgardaghica* against the cancer cell lines HeLa (human cervical adenocarcinoma), A549 (human lung adenocarcinoma), MCF-7 (human breast adenocarcinoma), CaCo-2 (human colorectal adenocarcinoma), mPANC96 (human pancreatic adenocarcinoma), PC-3 (human prostate adenocarcinoma), and U87MG (human of astrocytoma). Several studies on the antitumor activity of snake venoms were carried out to introduce new avenues for designing and developing novel antineoplastic drugs from unconventional sources [7,11,12,13]. **Chen *et al.*** [13] investigated the apoptosis in human hepatocellular carcinoma cells (HepG-2) induced by a fraction isolated from *Naja naja atra* venom. The inhibition of the proliferation of HepG-2 was in a time- and dose-dependent manner. The cytotoxicity of coral *Micrurus spixii* crude venom on HepG-2 cells was verified by Terra *et al.* [14]. The antitumor activity of *M. spixii* venom against HepG-2 was observed, but with less toxicity towards normal hepatic cells. Bhowmik *et al.* [15] reported that *Naja kaouthia* venom contained a fraction that induced inhibition of the growth of HepG-2. The dose- and time-dependent antiproliferative and cytotoxic properties of the isolated fraction from *Naja kaouthia* venom was found to be via blocking succinate dehydrogenase activity in the mitochondria of the cancer cells.

Materials and Methods

Venom

Cerastes vipera venom was extracted from adult vipers collected from Aswan area. Venom was collected from the vipers every four weeks for a six-month period by

* Corresponding author.
E-mail address: aalaa.elmeligy@yahoo.com

allowing the vipers to bite through a rubber glove covering a glass funnel that emptied into a glass tube in the ice. The venom was lyophilized and stored at 4°C until used.

Cell line

Human hepatocellular carcinoma (HepG-2) cell line was purchased from the Tissue Culture Unit at VACSERA Institute, Agouza, Giza, Egypt.

Chemicals

RPMI 1640 medium (with L-Glutamine), fetal bovine serum and phosphate buffer saline were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA. The antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B), Trypsin/EDTA (1X in PBS), MTT Salt: (3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from the tissue culture unit at VACSERA institute, Agouza, Giza, Egypt. Glutathione reduced (GSH), lipid peroxidation (MDA) and total antioxidant capacity were purchased from Biodiagnostic Co. Egypt. All other chemicals were of the highest commercially available analytical grade and purity.

MTT assay

In vitro cytotoxicity assay was conducted on the HepG-2 cell line cultured and maintained in RPMI 1640 medium, supplemented with 10 % heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic mixture (10000 U/ml potassium penicillin, 10000 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B and 1% L-glutamine) at 37 °C in a humidified 5 % CO_2 incubator (Sheldon, TC2323, Cornelius, OR, USA). For cytotoxicity assay, cells were plated in a 96-well plate at a density of 1×10^4 cells/well and allowed to attach overnight. 100 µg/ml of fresh serum-free medium containing various concentrations of *Cerastes vipera* crude venom (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100.0 µg/ml) was added. Cells incubated with growth medium alone served as a control. All cells were incubated at 37 °C for 24 h. and complete medium without cells was used for blank absorbance readings. After 24 h, cytotoxicity of crude venom was measured using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [16]. A standard curve was generated for the performed assay, and the IC_{50} value was calculated from the regression analysis of growth curves of HepG-2 cells in the presence of the crude venom. All assays were performed in triplicate per treatment and repeated at least three times. The IC_{50} value of the venom was calculated using probit analysis and utilizing the SPSS software program (SPSS Inc., Chicago, USA).

Biochemical Analysis

The cells were seeded with a count of 1 X 10^6 cells /ml in small tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 24 hr. The medium was aspirated and doses equivalent to ½ IC_{50}, 1 IC_{50}, and 2 IC_{50} of the crude venom were added to the flasks. After 1, 3, 6 and 24 hours, cells were harvested by scrapers, washed in PBS and collected by centrifugation at 2000 rpm for 10 min at 4°C. Cell pellets were lysed in 2 ml of cold buffer through freezing and thawing, centrifuged at 4000 rpm for 15 min at 4°C and the supernatant was divided into aliquots and stored at – 80°C. Reduced glutathione (GSH) was measured according to the methods of Beutler *et al*. [17], and lipid peroxidation using the methods of Ohkawa *et al*. [18]. Total antioxidant capacity was determined according to the methods of Koracevic *et al.* [19].

Morphological study

After treatment with different concentrations, three monolayers of HepG-2 per experimental group were photomicrographed at each time interval. The morphological changes of the HepG-2 cells were analyzed using Nikon bright field inverted light microscope (Japan) at 40X magnification and compared to the control group.

Statistical Analyses

All experiments were repeated at least three times, and data were presented as mean + SE. Student's t-test was used to test the significance of differences between controls and experimental values. Any variation with $p < 0.05$ was considered statistically significant.

Results

HepG-2 cells were assayed for cell viability with 24 hours exposure to different doses of *Cerastes vipera* crude venom by the MTT assay. Cells treated for 24 hours with 0.78, 1.56, 3.125, 6.25, 12, 25, 50 and 100 µg venom/ml culture medium showed a significant reduction in cell count when compared with the corresponding controls, indicating the cytotoxic effects of *Cerastes vipera* crude venom (**Fig. 1**). The half maximal inhibitory concentration (IC_{50}) value of the crude venom was 16.3 µg/ml.

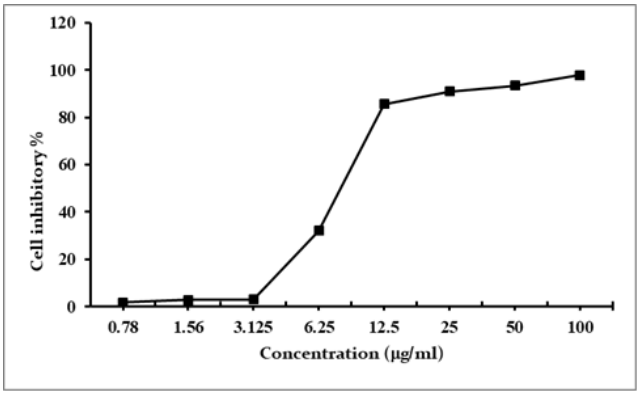

Figure (1): Cytotoxicity of *Cerastes vipera* crude venom to HepG-2 cells (1×10^4 cells/ml) after 24 h incubation at 37 °C, 5 % CO_2. Cell viability was determined by the MTT assay.

HepG-2 cells of the control group that were not treated with the crude venom grew well, arranged tightly and their boundaries were clear. The shape of cells appeared as a polygon or fusiform and their density was high in cultures. These cells developed more processes and increased in size in a time-dependent manner. After incubation for 1,

3, 6 & 24 hours in the media containing ½ IC_{50}, 1 IC_{50} and 2 IC_{50} *Cerastes vipera* crude venom, the morphological changes of HepG-2 cells were examined by an inverted microscope. The morphological changes of cells in the groups treated with the different concentrations of crude venom were in dose- and time-dependent.

The cells treated with ½ IC_{50} showed only a few changes, but after 24 hours, there was a noticeable increase in the number of dead cells (**Fig. 2**). The damage cells became rounded, their boundaries unclear, and were floating in the well. In addition, they lost their fusiform shape and exhibited a clear decrease in density. Cells treated with 1 IC_{50} of the venom showed morphological changes which began to appear after 3 hours, where few dead cells were noticed. After 6 hours and 24 hours, the number of dead cells increased greatly. Moreover, after 24 hours of incubation, the cells shrank, possessed finer processes, and their density decreased obviously (**Fig. 3**). Treatment of cultured cells with the 2 IC_{50} dose induced an increase in the number of dead cells which was time-dependent, when they were few after one hour of incubation and increased considerably after 24 hours. Besides, various features of deformation were noticed after one hour of incubation in the form of fusion of some cells together forming protoplasmic masses along with a decrease in the size and processes of cells (**Fig. 4**).

The level of GSH of HepG-2 cells exposed to ½ IC_{50}, 1 $IC_{50,}$ or 2 IC_{50} dose of *Cerastes vipera* crude venom for 1, 3, 6 and 24 hours showed a significant decrease (**Table 1**). HepG-2 cells exposed to ½ IC_{50}, 1 $IC_{50,}$ or 2 IC_{50} doses of *Cerastes vipera* crude venom for 3, 6 and 24 hours exhibited a significant decrease in the intracellular MDA level, the main product of membrane lipid peroxidation, as compared to the unexposed cells (**Table 2**). The level of MDA of the cells exposed to 1 and 2 IC_{50} of the crude venom showed significant decreases as compared to the unexposed cells. In comparison to the corresponding controls, the total antioxidant capacity (TAC) of HepG-2 cells exposed to ½ IC_{50} (3 hours), 1 IC_{50} (24 hours) and 2 IC_{50} (1 hour) exhibited significant increases (**Table 3**), whereas the level was decreased after 3 hours with the 1 IC_{50}. With the crude venom, there was a significant decrease in the TAC of HepG-2 cells exposed for 3, 6, or 24 hours.

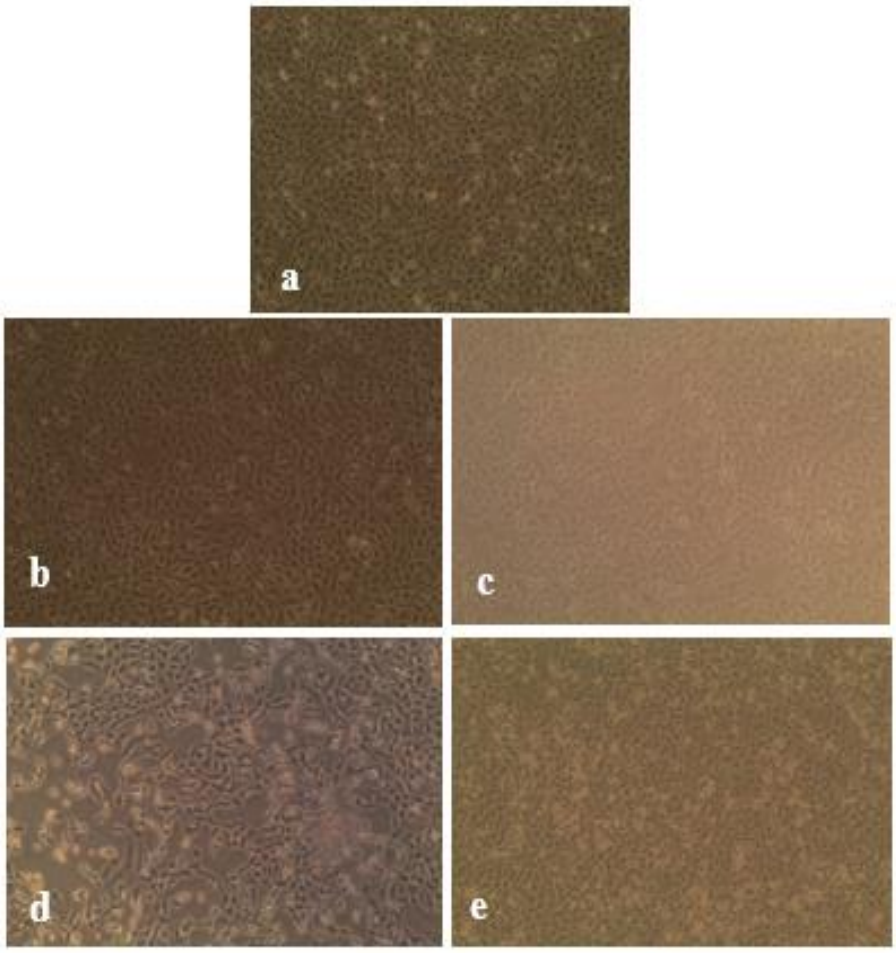

Fig 2: Photomicrographs representing the morphological characterization of HepG-2 cells after exposure to 0.5 IC_{50}. Control cells (a), treated cells for 1 (b), 3 (c), 6 (d) and (e) 24 hours.

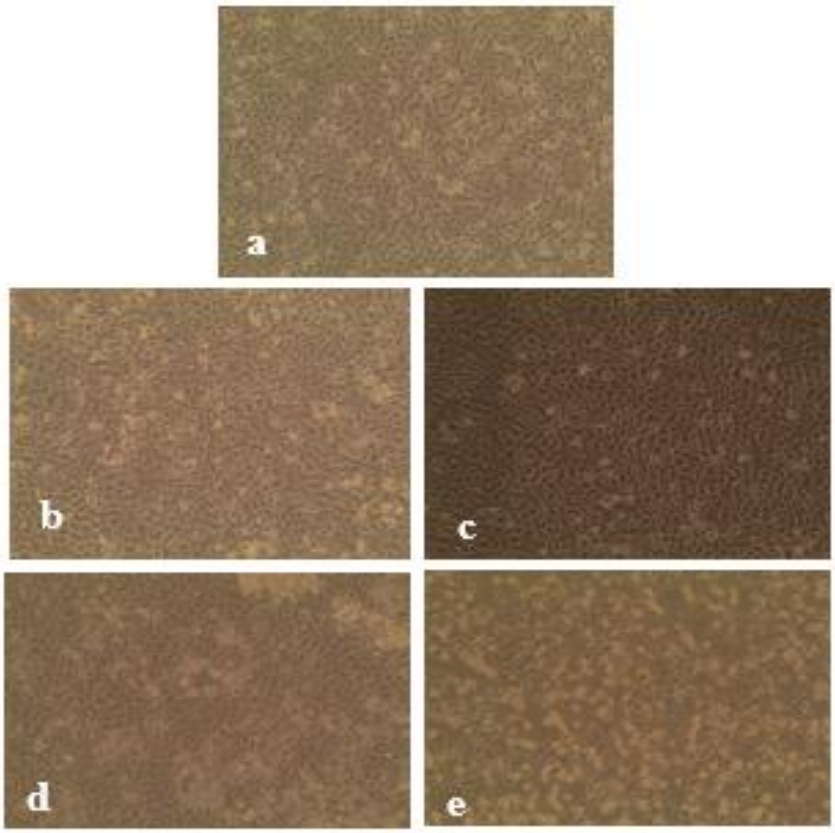

Fig 3: Photomicrographs representing the morphological characterization of HepG-2 cells after exposure to IC_{50}. Control cells (a), treated cells for 1 (b), 3 (c), 6 (d) and (e) 24 hours.

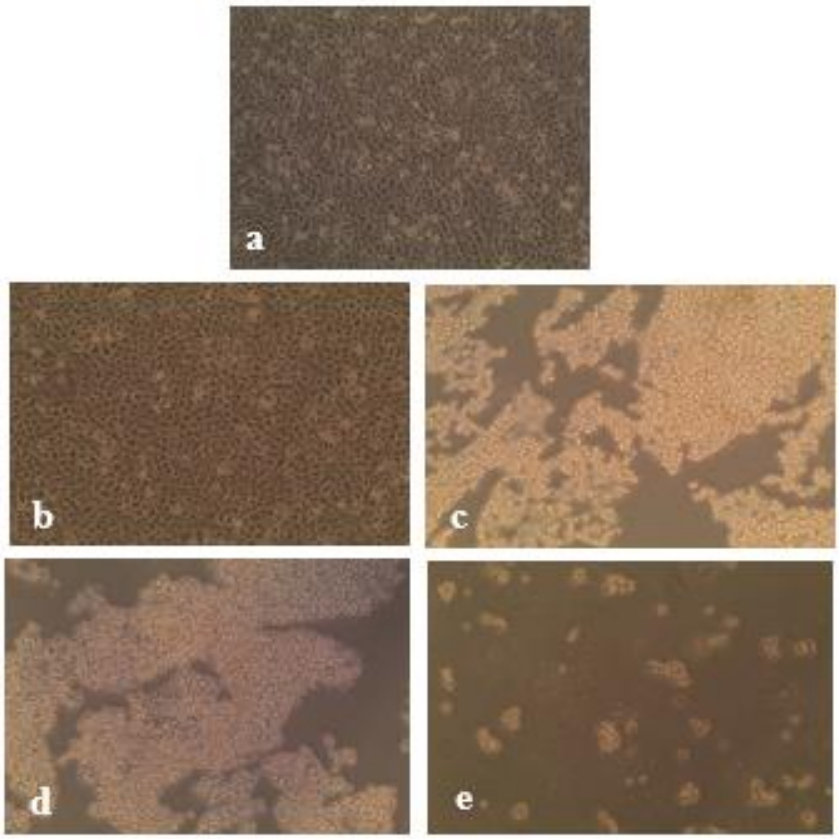

Fig 4: Photomicrographs representing the morphological characterization of HepG-2 cells after exposure to 2 IC_{50}. Control cells (a), treated cells for 1 (b), 3 (c), 6 (d) and (e) 24 hours.

Table 1. Effects of *Cerastes vipera* on GSH of HepG-2 cancer cells

Exposure levels	Experimental Periods (hours)			
	1	3	6	24
Control	487.0±10.63	480.8±9.25	482.2±5.30	488.8±8.16
½ IC_{50}	219.4±3.32[a]	188.5 ±4.84[a]	231.0 ±5.01[a]	225.2±3.33[a]
% Change[@]	- 54.95	- 60.79	- 52.09	- 53.93
IC_{50}	214.7 ±4.10[a]	208.8 ±6.07[a]	172.8±4.85[ab]	220.7[a]±4.17
% Change[@]	- 55.91	- 56.57	- 64.33	- 54.85
2 IC_{50}	220.7±9.73[a]	162.8±3.42[abc]	209.4±11.10[ab]	209.4[a]±4.39
% Change[@]	- 54.68	- 66.14	- 56.57	- 57.16

Data are represented as a mean ± standard error of the mean (SEM).
[@] Percentage of change in relation to the corresponding control.
[a,b,c]Significant in comparison with the control, ½ IC_{50}, and 1 IC_{50} respectively (P<0.05)

Table 2. Effect of *Cerastes vipera* on MDA of HepG-2 cancer cells

Exposure levels	Experimental Periods (hours)			
	1	3	6	24
Control	34.84±1.53	30.7±0.542	30.62±0.961	32.66±1.77
½ IC_{50}	28.21±3.57	20.18 ±1.47[a]	15.86 ±1.95[a]	20.49 ±0.60[a]
% Change[@]	-19.03	-34.27	-48.20	-37.26
IC_{50}	17.1 ±3.31[a]	19.66 ±1.7[a]	18.54 ±1.22[a]	14.28±1.19[a]
% Change[@]	-50.92	-35.97	-39.45	-56.28
2 IC_{50}	16.62±1.78[ab]	15.83±0.844[a]	15.04 ±2.17[a]	23.19±2.51[ac]
% Change[@]	-52.30	-48.44	-50.88	-28.996

Data are represented as a mean ± standard error of the mean (SEM).
[@] Percentage of change in relation to the corresponding control.
[a,b,c]Significant in comparison with the control, ½ IC_{50}, and 1 IC_{50} respectively (P<0.05)

Table 3. Effect of *Cerastes vipera* on TAC of HepG-2 cancer cells

Exposure levels	Experimental Periods (hours)			
	1	3	6	24
Control	0.823±0.11	0.698±0.026	0.788±0.126	0.672±0.046
½ IC_{50}	0.672±0.093	1.289 ±0.047[a]	0.992±0.182	0.366±0.048
% Change[@]	- 18.35	84.67	25.89	- 45.54
IC_{50}	0.514±0.084	0.638 ±0.048 [b]	0.900±0.124	1.462±0.17[ab]
% Change[@]	-37.55	- 8.596	14.21	117.56
2 IC_{50}	1.122±0.099[bc]	0.390±0.057[abc]	0.389[b]±0.028	0.284±0.018[ac]
% Change[@]	36.33	- 44.13	- 50.63	- 57.74

Data are represented as a mean ± standard error of the mean (SEM).
[@] Percentage of change in relation to the corresponding control.
[a,b,c]Significant in comparison with the control, ½ IC_{50}, and 1 IC_{50} respectively (P<0.05)

Discussion

The present results showed that the *Cerastes vipera* crude venom decreased cell proliferation and this effect was dose- and time-dependent. This effect seems to be common with snake venoms as it was reported by Liu *et al.* [20] in their study on cytotoxicity of bee venom on K1735M2 mouse melanoma cells, Omran [21] in his study on cytotoxicity of scorpion (*Leiurus quinquestriatus*) venom on breast and prostate cancer cells *in vitro*, Gao *et al*. [22] in their study on cytotoxicity of spider venom on HeLa cells and El-Refael and Sarkar [23] in their study on cytotoxicity of the *Cerastes cerastes* snake venom on mouse mammary tumor cells, both *in vitro* and *in vivo*. Gao *et al*. [22] reported that spider venom at doses of 40,

20 and 10 mg/L significantly decreased cell proliferation in HeLa cells, in a dose- and time-dependent manner and attributed the cytotoxic effect of the venom to necrosis. The results of the present study are in line with the general consensus of the antiproliferative potentials of animal venoms. Herein, the *Cerastes cerastes* crude venom at doses of 0.5 IC_{50}, IC_{50} and 2 IC_{50} significantly decreased cell proliferation in HepG-2 cells, in a dose- and time-dependent manner.

As an indicator of its high potency against HepG-2 cells, our study revealed that the IC_{50} value of *Cerastes vipera* crude venom (16.3 µg/ml) was significantly lower than those of other snake venoms such as Caspian cobra (*Naja naja oxiana*) venom which was 26.59 µg/ml [24], Corotoxin (extracted from pit viper venom) against human lung adenocarcinoma cell line A549 which was 78 µg/ml [25], and *Naja haje* venom against MCF-7 cells, which was 39.46±3.07 µg/ml [26]. On the contrary, it was reported that IC_{50} value of *Naja haje* venom against HepG-2 cells was 6.52±0.81 µg/ml [26], which indicated that *Naja haje* venom was more potent.

In the present work, photomicrographs revealed prominent morphological alterations in the HepG-2 cells after the exposure to various concentrations of the *Cerastes viper* in comparison to the untreated cells. A Similar study [7] revealed that purified disintegrin protein, isolated from the venom of the Indian cobra snake (*Naja naja*) induce cytotoxic effects of various types of human cancer cell lines such as breast cancer, lung cancer and liver cancer. It was also reported that melittin, as a major peptide component of bee venom has the ability to suppress the growth of human hepatocellular carcinoma HepG-2 cell line [27]. The ability of melittin to kill HepG-2 cells *in vitro* was also observed by Zhao *et al*. [28]. Moreover, the protein toxin (NKCT1) isolated from *Naja kaouthia* snake venom, significantly inhibited the growth of HepG-2 cancer cells as compared with the control cells in a time- and dose-dependent manner [15].

The decreased level of GSH of HepG-2 cells exposed to *Cerastes vipera* crude venom for 1, 3, 6 and 24 hours observed in the present study was probably a major contributor to the cancer cell death and inhibition of growth as a consequence to accumulation of ROS which must have caused a shift in the redox state as was previously documented in transformed cells [29,30]. This situation would eventually lead to increased incidences of DNA fragmentation and apoptosis [31,32,33,34]. GSH depletion is also known to arrest cell proliferation in the G0/G1 phase [35,36]. In support of the present study, a decrease of GSH in the supernatant and homogenate of the isolated hepatocytes treated with *Cerastes cerastes* venom was reported [37].

In the present study, there was a decrease in the MDA level in the HepG-2 cells exposed to different doses of *Cerastes cerastes* venom at all times of exposure when compared with the corresponding control values. These results suggest that the venom, or one of its active components, must have intervened to diminish or hamper the cancer-induced shift in the redox state. Although an increase of oxidative stress has been demonstrated in most of cancer types, the concentration of lipid peroxidation products in cancer cells is still a matter of argumentation. It was found that the level of lipid peroxidation products in the hepatoma cells lower than that the in normal liver cells [38]. According to this study, it was demonstrated that during rat liver carcinogenesis, the activities of the enzymes metabolizing the aldehydes increased, thus may render the cancer cells more protected against the cytotoxic effect of the viper venom [39]. On the contrary, other experimental results demonstrated that malondialdehyde was increased in colorectal cancer tissues [40]. The present study showed significant decreases of TAC in HepG-2 cells exposed to the crude venom. This finding is supported by the studies of Di Giacomo *et al*. [41], Ladas *et al*. [42], Sener *et al*. [43] and Crohns *et al*. [44]. They reported decreases in plasma total antioxidant capacity in different forms of cancer e.g., colon cancer, lung cancer, and breast cancer. In addition, Conus venom was found reduce the total antioxidant capacity of EAC cells, as a consequence to lowering the levels of reduced glutathione [45].

In conclusion, our findings indicate that *Cerastes vipera* crude venom might contain promising components as antitumor drugs.

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