RESEARCH ARTICLE

Viper snake (*Cerastes cerastes*) venom overcomes the hepatocellular carcinoma in experimental rats: histological evidence

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Hepatocellular carcinoma (HCC) is one of the world's most serious health issues. It is ranked as the third leading cause of cancer death worldwide. Conventional HCC treatments such as radiotherapy and chemotherapy have numerous drawbacks including a wide range of side effects on normal cells (lack of specificity), low success rate, high risk of recurrence, and high mortality rate. As a result, the development of new safe and effective treatments is extremely needed. One of these alternatives is the use of anticancer peptides derived from animal venom such as snake venom. The venom of the snake has the ability to target cellular metabolism alterations with a greater effect on tumor cells then it on normal cells, making it a potential anti-oxidizing and anticancer complex. In this study, we assess the potency of *Cerastes cerastes* snake venom as anti-HCC crude in rat model using a combination of biochemical and histological approaches. The results demonstrated that the liver functions were improved as indicated by significant decrease in the serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), creatinine, urea, malondialdehyde (MDA), and nitric oxide (NO) levels with significant improvement in the serum total protein, albumin, and the antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), levels comparing to that in the HCC untreated animals. Moreover, the histopathological examinations of liver tissues also showed remarkably improvement in the venom-treated group comparing to that in HCC untreated group. The results indicated that *Cerastes cerastes* venom might serve as a novel potential therapeutic pro-drug against HCC.

Keywords: cancer; hepatocellular carcinoma; anticancer therapy; lipid peroxidation; histopathology.

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Introduction

Cancer is one of the leading causes of morbidity and mortality throughout the world. According to the statistics from Global burden of cancer (GLOBOCAN) (https://gco.iarc.fr), the number of cancer deaths was around 8.2 million in 2012 [1] and increased to 9.6 million in 2018 [2]. Hepatocellular carcinoma (HCC) is the world's fifth most common cancer with 0.25-1 million new cases diagnosed each year [3, 4]. Hepatitis viral infection, aflatoxin infection, cirrhosis of any cause, excessive alcohol consumption, and cigarette smoking are all major risk factors for HCC [5]. The incidence of HCC has risen dramatically in the last decade particularly in Egypt where incidence rates have more than doubled in the last decade [6].

Although chemotherapy and radiotherapy are widely used to treat various types of tumors, they both have numerous drawbacks including harmful side effects on normal cells, low success rate, and high risk of recurrence. Furthermore, tumors frequently develop resistance to chemotherapy [7]. Therefore, the development of new safe and effective treatments is critical [8]. Recently, compounds isolated and purified from snake venom have demonstrated tremendous promise as agents targeting specific molecular pathways in cancer cells [9, 10]. Snake venoms are complex mixtures of proteins, peptides, and other bioactive molecules secreted by snake's venom gland and injected by unique snake fangs to weaken and digest their prey. Despite its toxicity, the venom of the snake has the ability to target changes in cell metabolism with a greater effect on tumor cells than that in normal ones. As a result, it has the potential to act as an antioxidant and anti-cancer complex [10, 11]. *In vitro* studies on crude snake venoms and purified snake proteins revealed that they were cytotoxic to a variety of cancerous human cell lines [12-14]. The results of the *in vivo* experiments were completely consistent with the results obtained *in vitro* [15-19]. *Cerastes cerastes*, also known as the desert-horned or Egyptian sand viper [20, 21], is one of the most well-known snakes of North Africa and the Middle East's great deserts [22, 23]. It is the most poisonous and widely distributed snake in Africa and lives in Egypt's sandy deserts [24, 25]. Despite the presence of previous research on the anticancer effect of *C. cerastes* venom, only a few studies specifically assessed its anticancer effects *in vivo*.

Plethora of experimental models have been developed to better understand HCC pathogenesis. Diethylnitrosamine (DENA) is one of the most potent hepatotoxins used to induce hepatocarcinogenesis in experimental animals through repeated administration and has been used to mimic the development of cancer in humans [26]. The carbon tetrachloride (CCL4) has been used to promote DENA's carcinogenic effect. This study applied DENA/CCL4-induced HCC experimental rat model to investigate how *Cerastes cerastes* viper snake venom works to against HCC on both biochemical and histopathological levels.

Materials and methods

Collection of snakes and venom preparation

Eight *C. cerastes* viper snakes were collected from Aswan governorate, Upper Egypt, and were transferred to Zoology Department, Faculty of Science, Suez Canal University (Ismailia, Egypt). The snakes were kept carefully in large wooden boxes and were fed on mice and provided with water every 15 days. The venom was mechanically collected in a sterile 50 mL glass beaker. The collected venom was then lyophilized by using Labconco 77500 Freeze Dryer (Labconco, Kansas City, MO, USA) at Suez Canal University, Center for Environmental Studies and Consultations, then stored at -20°C.

Determination of the median lethal dose (LD_{50}) value of *C. cerastes* venom

The LD_{50} of *C. cerastes* venom was calculated according to the method described by Meier and Theakston [25]. Accordingly, ten groups of albino rats (4 rats per group) received graded doses of *C. cerastes* venom ranging from 0.1 to 3.6 mg/kg intraperitoneal injection (i.p.) and were monitored for 24 h. The toxicological symptoms as well as the mortality were closely observed and recorded.

Animal experimental groups

A total of 60 adult male albino rats (120-150 g/each) were used in this study. The animals were obtained from the National Research Center for Experimental Animals (Cairo, Egypt). All animals were housed in plastic cages (6 rats/cage) at room temperature and were kept under a 12 h light–dark cycle with free access to food and water. The animals were left for acclimatization for one week before the start of the experiments. At the end of the experiments, animals were anaesthetized before scarification. The experiment was carried out according to the

standard procedures laid down by the Organization for Economic Cooperation and Development (OECD) guidelines 414 (OECD, 2001) for testing chemical and prenatal development.

Animals were randomly divided into five experimental groups. Group 1 was the normal control group (n=12). All animals in this group received a single i.p. injection of sterile 0.9% saline (vehicle). After two weeks, they received i.p. injections of olive oil at 3 mL/kg/week for 6 weeks followed by i.p. injections of saline, twice a week for 3 weeks. Group 2 was *Cerastes cerastes* venom-treated group (n=12). All animals in this group received a single i.p. injection of sterile 0.9% saline. After two weeks, they received i.p. injections of olive oil at 3 mL/kg/ week for 6 weeks followed by i.p. injections of 0.1 LD_{50} of the soluble *C. cerastes* venom in 0.9% saline, twice a week for 3 weeks. Group 3 was HCC group (n=12). All animals in this group received a single i.p. injection of DENA (Sigma-Aldrich, Saint Louis, MO, USA) at a dose of 200 mg/kg [27]. Two weeks later, they received i.p. injections of CCL4 dissolved in olive oil at 3 mL/kg/week for 6 weeks to promote the carcinogenic effect of DENA [28, 29] followed by i.p. injections of saline twice a week for 3 weeks. Group 4 was HCC-*Cerastes cerastes* venom-treated group (n=12). All animals in this group received the carcinogenic combination DENA + CCL4 first, followed by i.p. injections of 0.1 LD_{50} of the soluble *C. cerastes* venom dissolved in saline twice a week for 3 weeks. Group 5 was HCC-cisplatin treated group. The animals received the carcinogenic combination DENA + CCL4 first, followed by i.p. injections of cisplatin (Sanofi, Paris, France) at a dose of 1.5 mg/kg, twice a week for 3 weeks [30]. Cisplatin is a well-known chemotherapeutic drug and has been used for treatment of numerous human cancers. Its mode of action has been linked to its ability to crosslink with the purine bases on the DNA, interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells.

Biochemical assessment of liver and kidney function markers

Blood samples were collected from the retro-orbital venous plexus of six anesthetized rats from each group using a fine capillary tube introduced into the medial epicanthus of the rat's eye at the end of the experiment. Two milliliters (mL) of the blood were collected from each rat in a clean and dry tube without anticoagulant. The blood samples were left for clotting at 25°C for 15 minutes. The supernatant serum was then collected in another clean and dry tube and was centrifuged at 4,000 rpm, 4°C, for 15 minutes to separate the serum from the remaining blood cells. The clean non-hemolyzed serum was then pipetted into clean Eppendorf tube. The serum biochemical parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, creatinine, and urea were measured by using commercial kits (Biodiagnostic Company, Cairo, Egypt) according to the previous reported methods, respectively [31-38].

Measurement of oxidative stress biomarkers

The level of serum malondialdehyde (MDA), an end product of lipid peroxidation, nitric oxide (NO), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were measured by using Biodiagnostic Company kit (Cairo, Egypt) according to the reported methods, respectively [39-42].

Tissue collection and histopathological examination

Livers and kidneys were immediately excised after decapitation of animals and were rinsed in saline. Portions from the liver and kidney tissues were preserved in 10% neutral buffered formalin for histopathological examinations according to the reported method [43]. Hematoxylin and eosin (H&E) stained sections were examined by using a light microscope and were photographed.

Statistical analysis

Statistical Package for Social Sciences (SPSS) for Microsoft Windows (version 23) (IBM, Armonk, NY, USA) was used for data analysis. The data

Table 1. The levels of serum AST, ALT, TP, albumin, urea, and creatinine of the normal control and the experimental groups of animals.

Groups	AST (U/L)	ALT (U/L)	Total protein (g/dL)	Albumin (g/dL)	Urea (mg/dL)	Creatinine (mg/dL)
Normal	50.24 ± 1.36	84.81 ± 0.91	7.20 ± 0.07	3.68 ± 0.02	25.30 ± 0.09	0.58 ± 0.013
0.1 LD_{50} *C. cerastes*	60.83 ± 0.95 ###	90.50 ± 0.86	6.88± 0.094	3.80 ± 0.01	26.2 ± 0.10 ###	0.60 ± 0.011
HCC untreated	135.14 ± 1.25 ###	189.98 ± 1.37 ###	4.90 ± 0.12 ###	2.08 ± 0.01 ###	35.71 ± 0.10 ###	1.21 ± 0.014 ###
HCC + 0.1 LD_{50} *C. cerastes*	79.83 ± 1.08 ***	114.00 ± 0.97 ***	5.90 ± 0.09 ***	4.02 ± 0.09 ***	28.72 ± 0.17 ***	0.78 ± 0.011 ***
HCC + cisplatin	70.00 ± 1.18 ***	102.11 ± 1.56 ***	6.20 ± 0.1 ***	3.12 ± 0.01 ***	27.92 ± 0.15 ***	0.72 ± 0.013 ***

Notes: data were presented as mean ± S.E. ###: very high significant difference comparing to the normal control group. ***: very high significant difference comparing to the HCC untreated group.

Table 2. The levels of serum MDA, NO, SOD, and GPx of the normal and the experimental groups of animals.

Groups	MDA (nmol/mL)	NO (µmol/L)	SOD (U/mL)	GPx (IU/L)
Normal	13.60 ± 0.11	18.00 ± 0.58	4.52 ± 0.19	108.45 ± 1.4
0.1 LD_{50} *C. cerastes*	13.32 ± 0.27	24.17± 0.94$^{\#}$	4.25± 0.15	101.53 ± 1.04$^{\#\#}$
HCC	38.43 ± 1.08$^{\#\#\#}$	135.00 ± 1.88$^{\#\#\#}$	0.85 ± 0.01$^{\#\#\#}$	68.12 ± 0.65$^{\#\#\#}$
HCC + 0.1 LD_{50} *C. Cerastes*	19.55 ± 0.54***	62.17 ± 1.42***	2.21 ± 0.14***	95.75 ± 1.45***
HCC + cisplatin	16.81 ± 0.28***	55.17 ± 1.81***	2.53 ± 0.18***	98.35 ± 1.39***

Notes: data were presented as mean ± S.E. #: significant difference; ##: high significant difference; ###: very high significant difference comparing to the normal control group. ***: very high significant difference comparing to the HCC untreated group.

were expressed as mean ± standard error (S.E.). The unpaired Student's t–test and one-way ANOVA were used to analyze all the data. *P* value was used to determine statistically significant difference between two groups of data with $P < 0.05$ as significant difference, $P < 0.01$ as high significant difference, $P < 0.0001$ as very high significant difference.

Results

The LD_{50} value of *C. cerastes* venom was determined as 2.4 mg/kg.

Effect of *C. cerastes* venom on serum biochemical parameters

Table 1 showed the levels of liver and kidney function test markers among the control and the experimental groups of animals. Amplified levels of liver markers enzymes (AST, ALT) and kidney function markers (creatinine and urea) were detected in the serum of the HCC untreated group comparing to that in the normal group. Administration of 0.1 LD_{50} *C. cerastes* venom and cisplatin in the DENA/CCL4 treated animals significantly decreased the levels of serum AST, ALT, urea, and creatinine comparing to that in the HCC untreated animals. In addition, the serum total protein and albumin levels were significantly decreased in the HCC untreated group while they were significantly increased in the DENA/CCL4 treated groups with the administration of 0.1 LD_{50} *C. cerastes* venom and cisplatin (Table 1).

C. cerastes venom reduces lipid peroxidation and improves the antioxidant status

Data in Table 2 demonstrated the levels of serum MDA, NO, SOD, and GPx in the different groups of animals. Amplified levels of serum MDA and NO were observed in the HCC untreated animals,

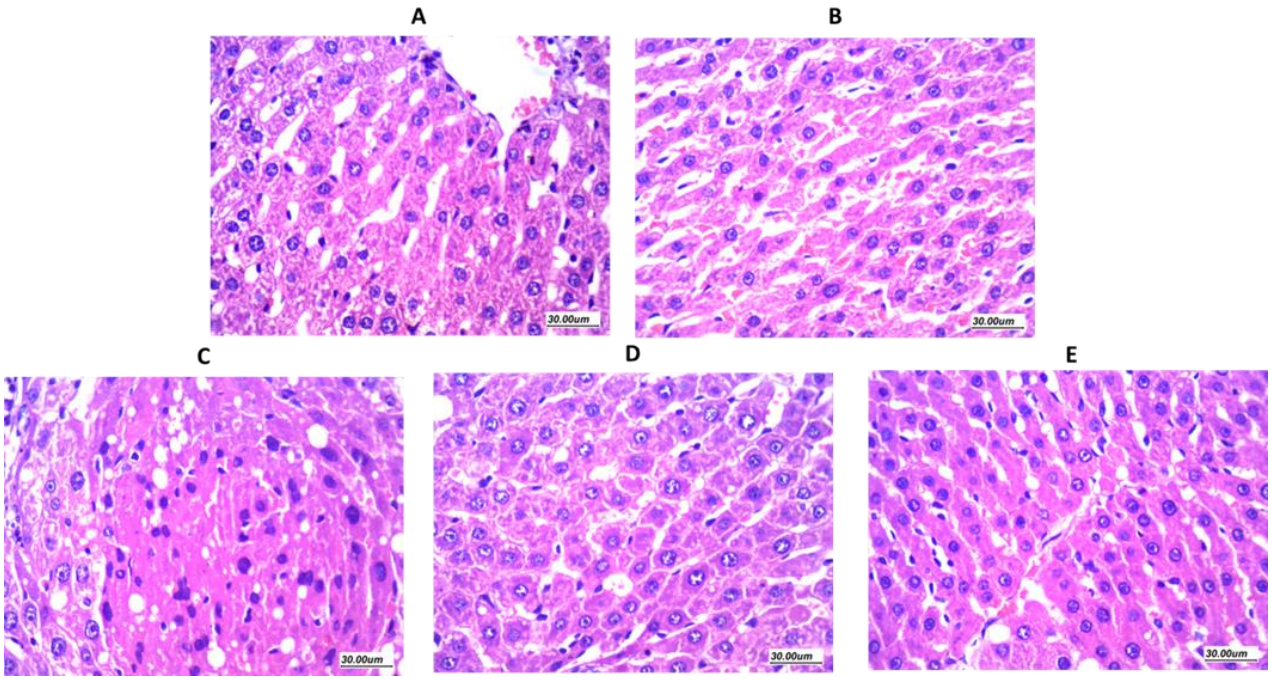

Figure 1. Representative photomicrographs of H&E staining liver sections of experimental animal groups. **A and B**: the normal group (A) and the 0.1 LD_{50} *C. cerastes* venom treated group (B). Both showed preserved hepatic lobular architecture. **C**: the HCC untreated group showing loss of normal hepatic architecture. **D and E**: the HCC-0.1 LD_{50} venom treated group (D) and the HCC-cisplatin treated group (E) showed remarkably improvement of changes in comparison to the HCC untreated group.

while the significant decrease in the levels of serum SOD and GPx were also observed in the same group. Administration of 0.1 LD_{50} of *C. cerastes* venom and cisplatin in the DENA/CCL4 treated animal groups significantly decreased the serum levels of MDA and NO while significantly increased the serum levels of SOD and GPx comparing to the HCC untreated animals.

C. cerastes venom improves histopathological features of HCC animals

Histological examination of liver and kidney tissues in various experimental groups of rats were observed by H&E staining (Figure 1 and 2). Figure 1 showed that the liver tissues from the normal and the 0.1 LD_{50} venom treated animals demonstrated the preserved hepatic lobular architecture with thin-walled central veins and the hepatocytes arranged in thin plates formed of one to two cells thickness surrounded by thin sinusoids. Each cell showed abundant eosinophilic cytoplasm with central vesicular nucleus showing pale chromatin with occasional nucleolus and preserved nucleocytoplasmic ratio. Whereas the liver tissues from the HCC group animals showed disturbed hepatic architecture with hepatocytes arranged in thick plates of more than 3 cells thickness and the cells showed a significant increase in nucleocytoplasmic ratio with large nuclei showing remarkable hyperchromasia and eosinophilic cytoplasm, irregular nuclear membranes, and smudged chromatic details. Some cells were bi- and multi-nucleated. There was prominent steatosis of hepatocytes surrounding tumor areas with occasional apoptotic cells. The presence of thick plates, high nucleocytoplasmic ratio, and nodular, nested, or acinar arrangement is consistent with neoplastic proliferation of hepatocytes. However, the liver tissues from the HCC-*C. cerastes* venom treated group and the HCC-cisplatin treated group showed remarkably improvement of changes in comparison to the HCC untreated group with hepatocytes arranged mostly in thin plates with two or one cell thickness, preserved

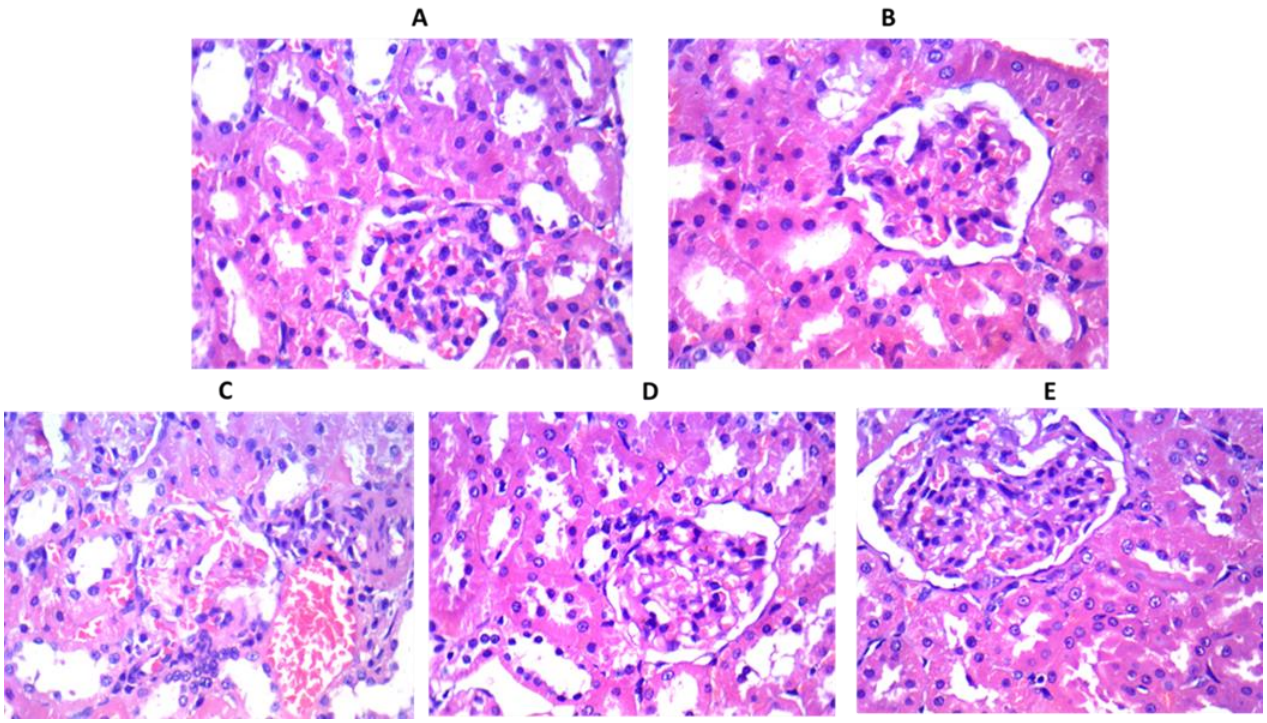

Figure 2. Representative photomicrographs of H&E staining sections of the renal tissues of experimental animal groups. **A and B**: the normal group (A) and the 0.1 LD_{50} *C. cerastes* venom treated group (B). **C**: the HCC untreated group. **D**: the HCC-0.1 LD_{50} venom treated group. **E**: the HCC-cisplatin treated group.

nucleocytoplasmic ratio, vesicular nuclei, and occasional nucleoli. Few scattered hepatocytes showed vacuolar degeneration.

On the other hand, the histopathological examination of the kidney tissues was also performed by H&E staining (Figure 2). Sections of the renal tissues from the control and 0.1 LD_{50} *C. cerastes* venom treated group showed tubules and glomeruli with tubules lined by cubical epithelial cells containing round nuclei and eosinophilic cytoplasm and the glomeruli formed of capillary tufts surrounded by mesangial cells with patent bowman's space. The interstitial tissues were formed by very thin fibrovascular stroma with thin-walled vessels (Figure 2A and 2B). The representative H&E staining sections from the HCC untreated group showed remarkably interstitial inflammatory infiltrates formed by small lymphocytes and plasma cells surrounding vessels and tubules. The tubular epithelial cells showed moderate to significant hydropic degeneration (vacuolar degeneration of the cytoplasm) with significant congested vessels with foci of thickening of walls and hemorrhage. (Figure 2C). Section in kidney tissue of the HCC-0.1 LD_{50} venom-treated group showed remarkably improvement of changes with residual mild vacuolar degeneration of tubules with no to minimal glomerular increase in cellularity. Mild congestion with no inflammatory infiltrate was observed (Figure 2D). Representative H&E staining sections from the HCC-cisplatin treated group showed moderate improvement of changes with residual mild vacuolar degeneration of tubules with no to minimal glomerular increase in cellularity. Moderate residual inflammatory infiltrate was seen with mild congestion (Figure 2E).

Discussion

HCC is the most common type of primary liver cancer in adults. It is the second and sixth leading cause of cancer-related death in both men and women, respectively [44]. Many therapeutic techniques are currently used to treat HCC, but they have not been completely succeeded and have resulted in numerous toxic side effects [45]. As a result, the designation of choice therapy with high efficacy and potency has increased the use of anticancer agents derived from natural resources [46]. This study focused on the anticancer effect of the Egyptian snake *Cerastes cerastes* venom against DENA/CCL4-induced HCC rat model [47].

ALT and AST enzymes serve as the indexes of liver cell injury and can be used to identify or confirms liver diseases. Elevation of the level of these enzymes is considered as the most sensitive markers in the diagnosis of hepatocellular damage and loss of functional integrity of the cell membrane. The carcinogenesis of the liver affects liver cells with the breakdown of cell membrane architecture, which leads to the release of these enzymes into the serum where their levels go high [48]. On the other hand, serum total protein and albumin are the most important factors for evaluating the secretory capacity of the liver.

The results showed that there was no significant difference observed in serum ALT, total protein, and albumin levels between the normal group and the 0.1 LD_{50} *Cerastes cerastes* venom treated group. However, a significant increase in the level of serum AST was observed in the 0.1 LD_{50} *C. cerastes* venom treated group comparing to that in the normal control group. Previous study reported that treatment of mice with two i.p. injections of 0.2 mg/kg of *Echis coloratus* crude venom caused no significant change in serum levels of total proteins and ALT, but significantly increased the levels of AST and LDH comparing to that in the normal control mice [49], which supported the results of this study. DENA and CCL4 treatment resulted in a significant amplified levels of serum AST and ALT comparing to that in the normal control group, while the levels of serum total protein and albumin were significantly decreased. This result agreed with previous studies which reported that the serum levels of AST and ALT in the HCC animals were significantly higher than that in the normal control animals, while the serum levels of total protein were reduced significantly [44, 50, 52, 53]. Administration of 0.1 LD_{50} *C. cerastes* venom and cisplatin significantly decreased the serum levels of ALT and AST, while significantly increased the levels of the serum total protein and albumin comparing to that in the HCC untreated animals. The increased levels of the serum total protein and albumin indicated the restoration of liver function. The results agreed with Gawade, *et al*. who reported that two viper snake venom Photoproducts (SV1 and SV2) significantly decreased the levels of AST, but differently decreased the level of total protein and increased the level of ALT in EAC bearing mice [51].

Our data also showed that DENA and CCL4 treatment resulted in a significant increase in the serum creatinine and urea levels comparing to the normal control group. This might be attributed to the catabolic effect of the tumor and elevation in the urea production. In the meantime, increased creatinine level in the HCC control group might be due to muscle necrosis [54]. Administration of 0.1 LD_{50} of *C. cerastes* and cisplatin significantly decreased the serum creatinine and urea levels comparing to that in the HCC control group. These results agreed with Nafie, *et al*. who reported that treatment with the *Androctonus australis* scorpion venom significantly decreased the serum urea level, while it caused a non-significant decrease in the serum creatinine level of EAC bearing mice [55]. Salem, *et al*. also reported that *Androctonus amoreuxi* venom treatment decreased the level of serum creatinine comparing to that in the control EAC bearing mice [8].

Oxidative stress occurs when the balance between the formation of reactive oxygen species (ROS) and their removal by oxidoreductase enzymes is disrupted. One of the

consequences of the uncontrolled oxidative stress is the injury of cells, tissues, and organs. Lipid peroxidation can be described as a process under which oxidants such as free radicals attack the lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids [56, 57]. NO is a free radical generated during the conversion of amino acid L-arginine to L-citrulline by the NO synthase [58]. It readily reacts with superoxide to form peroxynitrite which is a potent oxidant capable of attacking and modifying proteins and also depleting antioxidant defenses. In this study, NO level and the biomarker of lipid peroxidation (MDA) were measured in the control and the experimental groups. DENA/CCL4 treatment resulted in a significant increase in the serum levels of NO and MDA while a significant decrease in the levels of serum SOD and GPx enzymes comparing to that in the normal control animals. This result agreed with the previous report that administration of DENA led to increase in the levels of lipid peroxidation and decrease in catalase, SOD, glutathione peroxidase, and glutathione S-transferase in the liver homogenate of the HCC animals [48]. Other studies also reported that the significant diminished contents of antioxidant parameters were showed in the HCC animals comparing to that in the normal animals [53, 59]. Administration of 0.1 LD_{50} *C. cerastes* venom and cisplatin significantly decreased the serum levels of NO and MDA while significantly increased the serum levels of the antioxidant enzymes SOD and GPx. This result agreed with Gawade, *et al*. who reported that two viper venom photoproducts SV1 and SV2 significantly reduced the MDA level and significantly increased the GSH level towards normal value in EAC bearing mice in comparison to the saline control group [49]. However, a different result was reported by Akef, *et al*. [60], which reported that a 24 hour's treatment of PC3 (human prostate cancer cells) with the IC_{50} value of the *Cerastes cerastes* snake venom caused a significant increase in the MDA level in the cell lysate comparing to that in the control cells. On the other hand, they reported that the venom increased the activity of the antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase, which was in agreement with our results.

The histological examination results of the liver and kidney tissues in various experimental groups showed that the liver and renal tissues of the control normal group and the *C. cerastes* venom treated group demonstrated normal hepatic lobular and renal architecture. In contrast, the animals treated with DENA/CCL4 showed disturbed kidney and hepatic architecture with hepatocytes arranged in thick plates of more than three cells thickness. The tissues treated with 0.1 LD_{50} *C. cerastes* venom showed remarkably improvement of changes in comparison to the HCC untreated group. The results agreed with the previous studies which reported remarkably improvement of liver and kidney sections of EAC tumor bearing animals after venom treatment [8, 55].

This study showed the antitumor effect of the venom, which significantly improved the liver and kidney functions as well as the antioxidant enzymes levels in serum. By using histological examination, the venom also showed remarkably improvement of liver tissues. The results indicated that *Cerastes cerastes* venom might serve as novel potential therapeutic pro-drug against HCC.

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