RESEARCH ARTICLE

Viper snake (*Cerastes cerastes*) venom enhances apoptosis in hepatocellular carcinoma in experimental rats: a molecular study

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Hepatocellular carcinoma (HCC) is the second leading cause of death and the fifth most common malignancy worldwide. Conventional treatments of HCC as radiotherapy and chemotherapy have many disadvantages such as various side effects on normal cells (lack of specificity), low success rate, high risk of recurrence, and high degree of mortality. Consequently, the development of new, safe, and effective treatments is extremely needed. One of these alternative approaches is the using of anticancer peptides derived from animal venoms including snakes. The snake's venom has the ability to target cellular metabolism alterations with a major effect on the tumor cells comparing to the normal cells, rendering it a potential anti-oxidizing and anticancer complex. In this study, we assess the potency of *Cerastes cerastes* snake crude venom as anti-HCC in rat using a combination of molecular and immunohistochemical approaches. The results showed the venom induced anticancer effect as evidenced by significant improvement in the final body weight and significant decrease in the relative liver weight. At the molecular level, the venom induced apoptosis which were mediated by up regulation of pro-apoptotic genes (Bcl-2-associated X (Bax), Caspase 3, FS-7-associated surface antigen (FAS), and TNF-related apoptosis-inducing ligand (TRAIL)) and down regulation of anti-apoptotic gene (Bcl-2) in the HCC-venom treated animals without any side effect on the normal animals. By using immunohistochemical techniques, the expression of liver proliferating cell nuclear antigen (PCNA) and KU70 were determined. Interestingly, treatment of 0.1 LD_{50} *Cerastes cerastes* venom decreased the expression of KU70 and PCNA in liver tissues of the HCC-venom treated animals while without any effect on the normal animals that received the venom. Our findings indicated that *Cerastes cerastes* venom might serve as apoptotic stimulator, presenting a novel potential therapeutic pro-drug against HCC.

Keywords: hepatocellular carcinoma; therapeutic strategy; apoptosis; immunohistochemistry; venom; *Cerastes cerastes*; viper snake.

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Introduction

Cancer is a disease that arises from a single abnormal cell with mutated DNA. This mutation gives rise to changes in the expression, activation or localization of the cell's regulatory proteins, affecting the signaling pathways that alter the cell's responses to regulatory stimuli and allow the uncontrolled cell growth of the mutated cell. It is considered to be one of the most common causes of death worldwide. Based on the global cancer statistics of Global burden of cancer

(GLOBOCAN) (https://gco.iarc.fr/), the number of deaths due to cancer was around 8.2 million in 2012 [1] and has risen to 9.6 million in 2018 [2]. Hepatocellular carcinoma (HCC), the most common form of liver cancer, is the second most common cause of cancer-related death worldwide [3] and the fifth most commonly causing cancer globally [4]. More than 80 % of the HCC cases occur in the developing countries. Areas of particularly high incidence are Sub-Saharan Africa and Eastern and South-eastern Asia [5]. The incidence of HCC has increased sharply in the last decade, especially in Egypt, where there has been a doubling of the incidence rate during the last 10 years [6].

Conventional treatments of HCC as radiotherapy and chemotherapy have many disadvantages such as various side effects on normal cells (lack of specificity), low success rate, high risk of recurrence, and high degree of mortality [7]. In addition, tumors often acquire resistance against chemotherapeutics [8]. Consequently, the development of new safe and effective treatments is extremely needed. One of these alternative approaches is the using of anticancer peptides derived from animal venoms including snakes. Snake venom is a complex mixture of bioactive peptides, proteins, enzymes, and toxins including cytotoxins, neurotoxins, and cardiotoxins that function to immobilize or kill the prey as well as to help in the digestion of the prey [9]. Despite its toxic effect, snake venom has the ability to target cellular metabolism alterations with a major effect on the tumor cells comparing to the normal cells, rendering it a potential anti-oxidizing and anticancer complex. It can induce the blockage of some specific ion channels, inhibiting angiogenesis, suppressing metastasis, and activating the intracellular pathways causing apoptosis in tumor cells [10, 11]. Comparing to normal cells, snake venoms are significantly more cytotoxic to malignant cells. As a result, venoms have a variety of therapeutic actions, making them an appealing approach for cancer therapy [9]. Previously, *in vitro* studies on crude snake venoms and purified snake proteins showed cytotoxic effects against various cancerous human cell lines [12-14]. Moreover, *in vivo* investigations were strongly confirmed the *in vitro* results [15-19] without any harmful effect on normal cells.

Cerastes cerastes, commonly known as desert-horned or Egyptian sand viper [20, 21] is one of the most common snakes of the great deserts of North Africa and the Middle East [22-25]. Despite the presence of previous studies on the anticancer effect *C. cerastes* venom, only a limited number of studies specifically assessed its *in vivo* anticancer effects. Previous study by Abu-Sinna, *et al*. reported that treatment of Ehrlich ascites-bearing mice with two intraperitoneal (i.p.) injections of the most lethal fraction (F3) and the non-lethal fraction (F4) of *C. cerastes* snake venom resulted in a significant antitumor activity, which was demonstrated by an increase in the mean survival time of the animals (22.5 and 27.9 days) and in the tumor inhibition ratio of tumor growth (T/C%, 139 and 172), respectively, comparing to the tumor-bearing control mice [26]. Shebl and colleagues reported that *C. cerastes* and *Vipera lebetina* snake venoms induced apoptosis of breast cancer (MCF-7) cells, which was mediated by up regulation of the pro-apoptotic genes p53 and Bcl-2-associated X (Bax) and down regulation of the anti-apoptotic gene B-cell lymphoma 2 (Bcl-2) in the MCF-7 cells [27]. Flow cytometric analysis also showed an increase in the percentage of apoptotic cells post 24 h treatment relative to the venom concentrations. Another study by Akef, *et al*. evaluated the effects of *C. cerastes* snake venom and *Androctonus amoreuxi* scorpion venom on prostate cancer cells (PC3) [28]. They found that the venoms significantly reduced PC3 prostate cancer cells viability in a dose-dependent manner. The venoms significantly decreased the anti-apoptotic Bcl-2 gene expression and significantly increased the Bax/Bcl-2 ratio and the oxidative stress biomarker malondialdehyde (MDA). Effects of *Naja haje* and *C. cerastes* snake venoms on larynx (Hep2) cancer cell line were investigated [29]. The treatment of both venoms decreased the viability percentage of cancer cells, decreased percentage of the DNA content

in G_0-G_1 and S phases, increased percentage of the DNA content in the G_2-M and pre G_1 (total apoptosis) phases, increased percentage of the cells in early and late apoptotic phases in addition to up-regulation of the pro-apoptotic caspase-3 levels in comparing to negative control cells.

Numerous experimental models have been developed in order to define the pathogenesis of HCC. Diethylnitrosamine (DENA) is a strong liver carcinogen, which is well established in animal models and mimics the cancer development as that of humans [30]. Carbon tetrachloride (CCL4) can be used to promote the carcinogenic effect of DENA. This study employed DENA/CCL4-induced HCC animal model to explore the molecular mechanisms of *Cerastes cerastes* viper snake venom against HCC.

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 [29]. 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, i.p. injection, and were monitored for 24 h. The toxicological symptoms as well as the mortality were closely observed and recorded.

Animal experiments
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 [31]. 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 [32, 33] 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 [34]. 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.

Determination of final body weight and relative liver weight

After 3 weeks from the start of venom treatment, which was 11 weeks from the start of the experiment, 6 animals from each group were weighted individually and the average final body weight was determined. The animals were then anesthetized by using diethyl ether and sacrificed by cervical dislocation and exsanguination. The liver samples from different groups were then removed, weighed, and the relative liver weights were calculated.

Immunohistochemical analysis

Portion of the liver tissues of six animals from each group was fixed in 10% neutral buffered formalin, then was processed routinely. One paraffin block was prepared for each sample. The paraffin block was sliced into 4 µm section, and then, was deparaffinized and rehydrated. The antigen epitope retrieval was induced by incubating the slides in the autoclave in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes at the power of 800 Watts. The slides were then cooled down and washed by phosphate buffered saline (PBS) twice at 5 minutes of each. After that, the endogenous peroxidase activity was blocked by incubating the slices with 3% hydrogen peroxidase for 10 minutes. Two to three drops of the working dilution (1:100) of the two primary antibodies, proliferating cell nuclear antigen (PCNA) (Mouse monoclonal: IML-83) and KU70 (IHC World, Woodstock, MD, USA), were applied to the slides and incubated in a humid chamber at room temperature for 60 minutes before washed with PBS 5 minutes for 3 times. The slices were then incubated with the Power-Stain™ 1.0 Poly HRP DAB Kit for Mouse – Rabbit (Genemed Biotechnologies, Torrance, CA, USA) for 30 minutes and washed again with PBS 5 minutes for 3 times. Finally, the bound antibody was detected by using 3, 3'-Diaminobenzidine tetrahydrochloride (DAB) as the chromogen for 5 minutes. Slices were counterstained by hematoxylin and were examined by a light microscope and were photographed.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The portion of the liver tissues that were kept in the RNA later solution was subjected to RNA extraction by using RNeasy mini kit (Qiagen, Düsseldorf, Germany). From the total RNA, complementary DNA (cDNA) was synthesized by using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA).

RT-qPCRs for B-cell lymphoma 2 (Bcl2), Bcl-2-associated X (BAX), Caspase 3, FS-7-associated surface antigen (FAS), and TNF-related apoptosis-inducing ligand (TRAIL) were performed. In brief, the cDNAs were amplified by using QuantiTect Sybr Green PCR Kits (Qiagen, Düsseldorf, Germany). The relative expression levels of genes were analyzed by using the $2^{-\Delta\Delta C_t}$ method by normalizing with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and were presented in fold increase relative to the control group. The sequences of the specific primers used were shown in Table 1.

Statistical analysis

Statistical Package for Social Sciences (SPSS) (version 23) (IBM, Armonk, NY, USA) was employed. The gathered data were expressed as mean ± standard error (SE) of 6 animals for each group. The unpaired Student's t–test and analysis of variance (one-way ANOVA) were used to analyze all the data. *P* values were used to determine statistical significance while $P < 0.05$ as significant difference, $P < 0.01$ as high significant difference, and $P < 0.0001$ as very high significant difference.

Table 1. The sequence of the specific primers used in RT-qPCR.

Bcl-2	Forward primer: 5'- GGG ATG CCT TTG TGG AAC TA -3' Reverse primer: 5'- CTC ACT TGT GGC CCA GGT AT -3'
BAX	Forward primer: 5'- CTG CAG AGG ATG ATT GCT GA -3' Reverse primer: 5'- GAG GAA GTC CAG TGT CCA GC -3'
Caspase-3	Forward primer: 5'- GAG ACA GAC AGT GGA ACT GAC GAT G -3' Reverse primer: 5'- GGC GCA AAG TGA CTG GAT GA -3'
FAS	Forward primer: 5'- TTG CTG TCA ACC GTG TCA GC -3' Reverse primer: 5'- CCA CTT CTA AAC CAT GCC CT -3'
TRAIL	Forward primer: 5'- CAA CTT AGC CTT AAT TCC AAT CTC C -3' Reverse primer: 5'- TCT TTA GCC TCC TTG AAC CGG -3'
GAPDH	Forward primer: 5'- GGT CGG TGT GAA CGG ATT TG -3' Reverse primer: 5'- CGT TGA ACT TGC CGT GGG TA -3'

Table 2. The final body weight and relative liver weight of the normal and the experimental animals.

Groups	Final body weight (g)	Relative liver weight (g)
Normal	255.17 ± 2.61	2.82 ± 0.00
0.1 LD_{50} *C. cerastes*	245.00 ± 2.12$^{\#\#}$	3.00 ± 0.09$^{\#}$
HCC	204.00 ± 1.65$^{\#\#\#}$	5.24 ± 0.00$^{\#\#\#}$
HCC + 0.1 LD_{50} *C. cerastes*	230.00 ± 1.65***	3.91 ± 0.00***
HCC + cisplatin	222.17 ± 1.40***	3.78 ± 0.00***

Notes: data were presented as mean ± SE with 6 rats/group. #: significant difference ($P < 0.05$), ##: highly significant difference ($P < 0.01$), ###: very high significant difference ($P < 0.0001$) comparing to the normal control group. ***: very high significant difference ($P < 0.0001$) comparing to the HCC untreated group.

Results

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

As indicated in the method section, ten groups of weighted albino rats (4 rats in each group) had received increasing doses of the soluble *C. cerastes* venom, i.p., and the morphological changes as well as the mortality rate were recorded. The LD_{50} value of *C. cerastes* venom was estimated as 2.4 mg/kg.

C. cerastes venom improved the final body weight and reduced the relative liver weight

The final body and relative liver weight in all groups were shown in Table 2. There were significant decreases in the final body weight and significant increase in the relative liver weight in 0.1 LD_{50} *C. cerastes* venom treated group and HCC group comparing to that in normal control group. However, administration of 0.1 LD_{50} of *C. cerastes* venom or cisplatin in the DENA/CCL4 induced HCC groups significantly increased the final body weight and significantly decreased the relative liver weight comparing to that in HCC untreated group.

C. cerastes venom downregulated PCNA and KU70 expression

The expressions of PCNA and KU70 in the liver tissues were determined by using the immunohistochemical technique. The immunohistochemical staining of PCNA were illustrated in Figure 1. Photomicrograph for immunohistochemical staining of liver tissues of the normal control group and the 0.1 LD_{50} *C. cerastes* venom treated group using antibody against PCNA showed a weak reaction in the

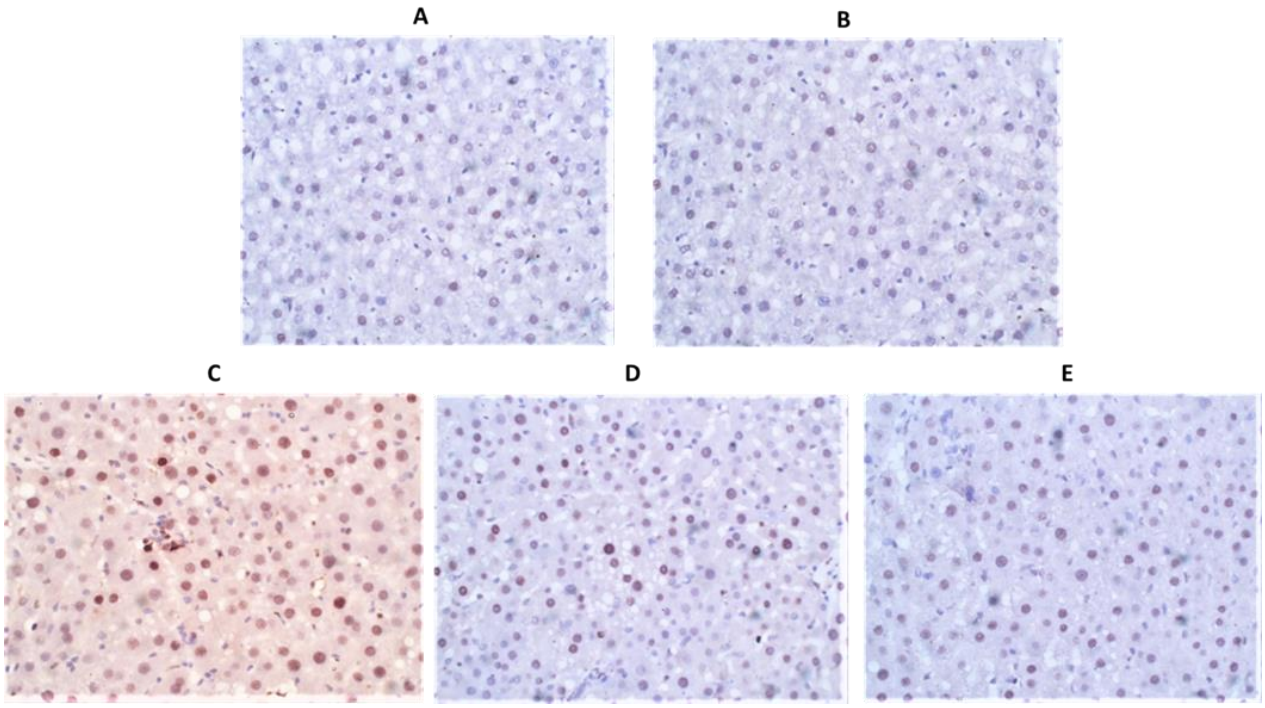

Figure 1. Immunohistochemical analysis of PCNA in the liver tissue of experimental animal groups. **A.** control group. **B.** 0.1 LD_{50} *C. cerastes* venom-treated group. **C.** HCC untreated group. **D.** HCC-0.1 LD_{50} *C. cerastes* venom- treated group. **E.** HCC-cisplatin treated group.

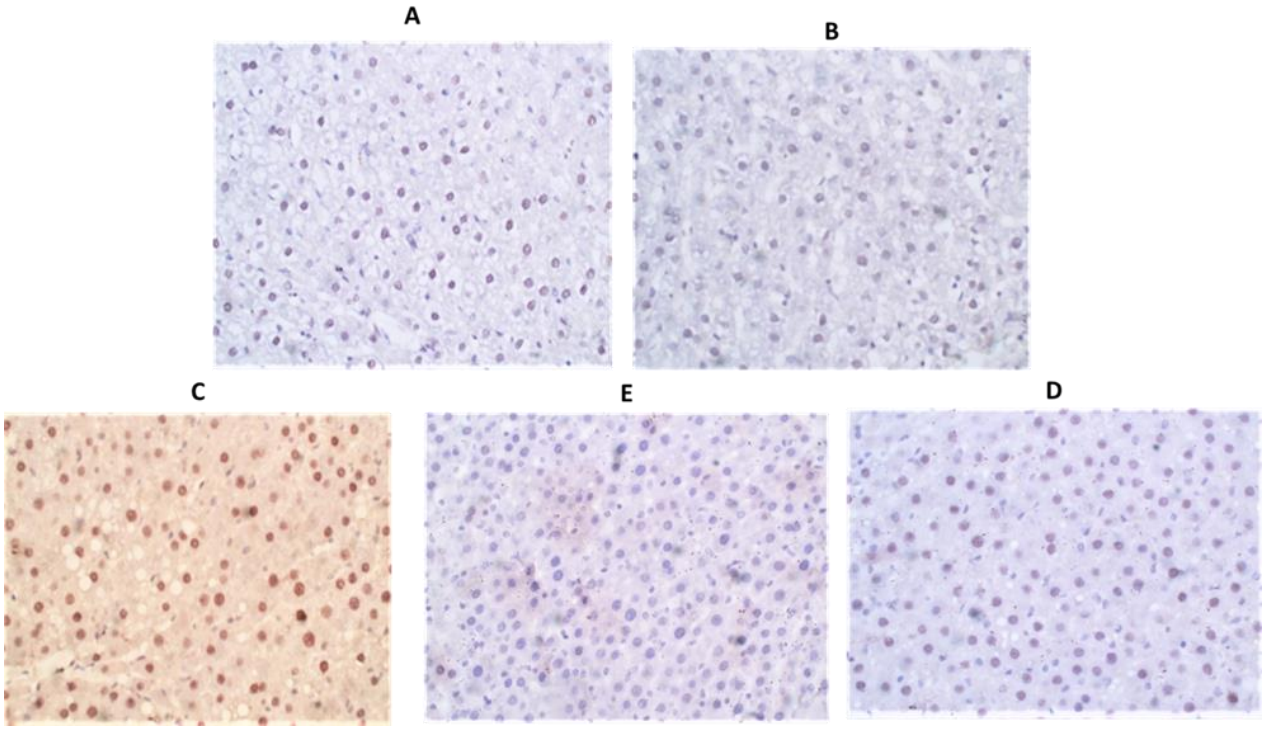

Figure 2. Immunohistochemical analysis of KU70 in the liver tissue of experimental animal groups. **A.** control group. **B.** 0.1 LD_{50} *C. cerastes* venom-treated group. **C.** HCC untreated group. **D.** HCC-0.1 LD_{50} *C. cerastes* venom-treated group. **E.** HCC-cisplatin treated group.

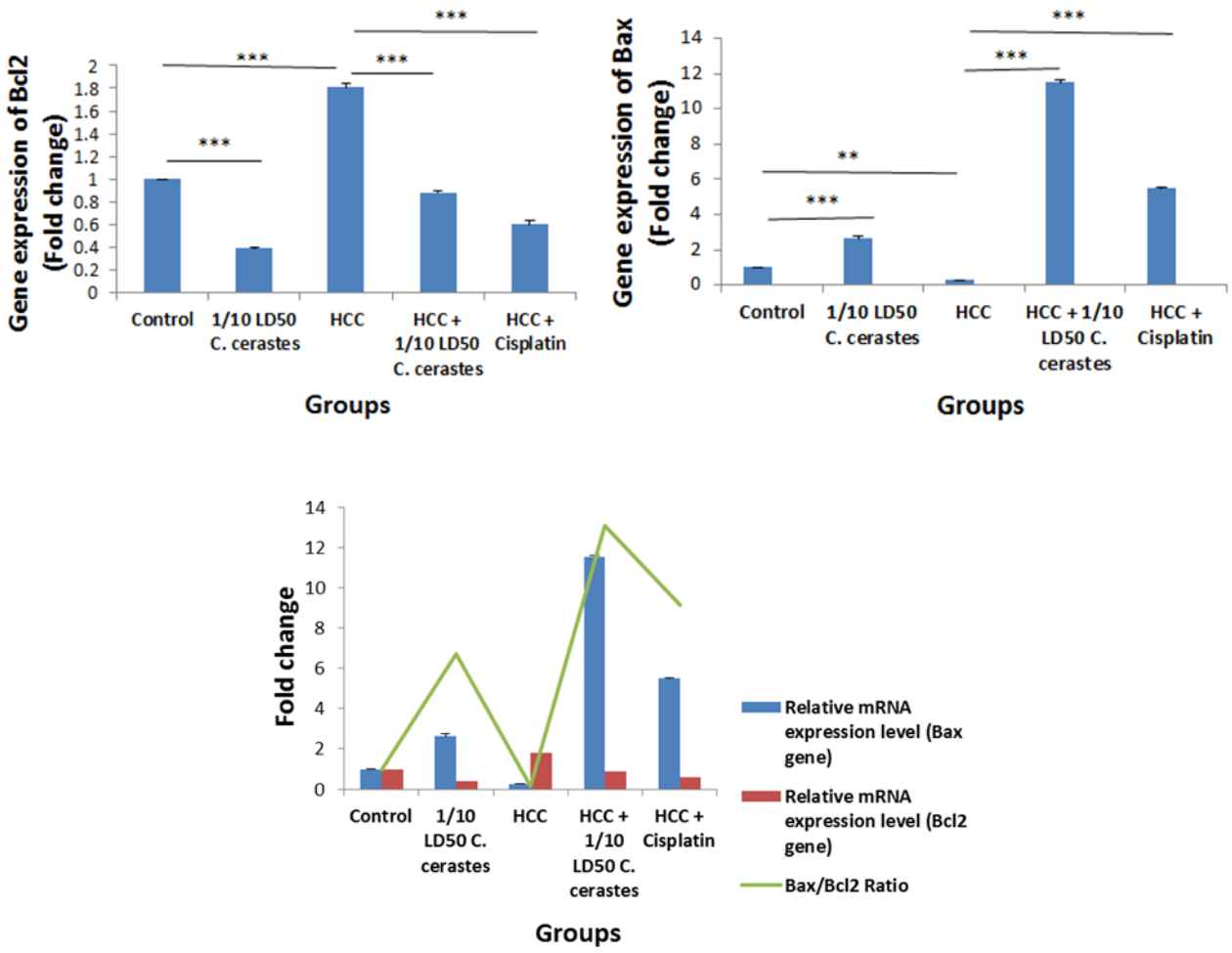

Figure 3. Fold increase in gene expression of Bcl2, Bax, and Bax/Bcl2 ratio. **: high significant difference ($P < 0.01$). ***: very high significant difference ($P < 0.0001$).

nuclei of hepatocytes (Figure 1A, 1B), while the HCC group showed a strong positive reaction for PCNA in the nuclei of hepatocytes (Figure 1C). The HCC-0.1 LD_{50} venom treated group and the HCC-cisplatin treated group showed a moderate positive reaction for PCNA in the nuclei of hepatocytes (Figure 1D, 1E). The KU70 immunohistochemical staining were illustrated in Figure 2. The normal control group and the 0.1 LD_{50} *C. cerastes* venom treated group showed a weak reaction in the nuclei of hepatocytes (Figure 2A, 2B), while the HCC group showed a strong positive reaction for KU70 in the nuclei of hepatocytes (Figure 2C). The HCC-0.1 LD_{50} venom treated group showed weak and focal reaction for KU70 in the nuclei of hepatocytes (Figure 2D), while the HCC-cisplatin treated group showed moderate positive reaction for KU70 in the nuclei of hepatocytes (Figure 2E).

C. cerastes venom induced intrinsic apoptosis *via* inversion of BAX/Bcl2 ratio

The mitochondrial apoptotic proteins Bcl2 and Bax expression were tested. Figure 3 showed the relative mRNA expression levels of the mitochondrial anti-apoptotic gene Bcl2 and the pro-apoptotic gene Bax among the control and the experimental groups. A significant decrease in the relative mRNA expression level of the anti-apoptotic Bcl2 gene was observed in the liver tissues of the 0.1 LD_{50} *C. cerastes* venom treated animals comparing to that of the normal animals, while a significant increase in the relative mRNA expression level of Bcl2 was observed in the HCC

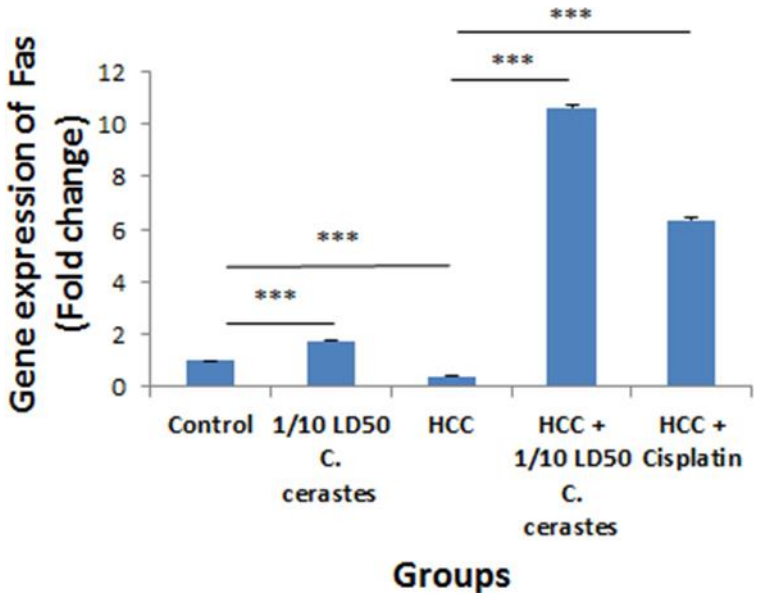

Figure 4. Fold increase in FAS gene expression. ***: very high significant difference ($P < 0.0001$).

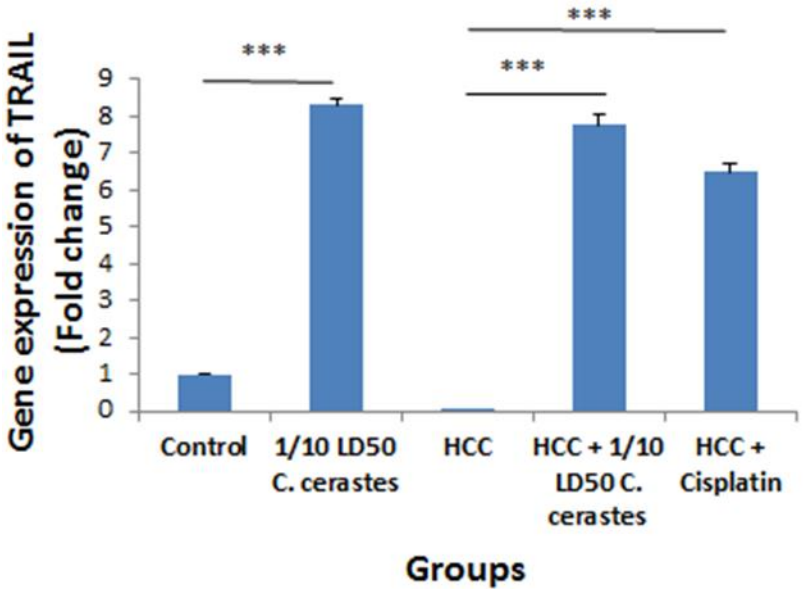

Figure 5. Fold increase in TRAIL gene expression. ***: very high significant difference ($P < 0.0001$).

untreated animals comparing to the normal group animals. Treatment with 0.1 LD_{50} *C. cerastes* venom or cisplatin in the DENA/CCL4 treated animal groups demonstrated the significant decrease of the relative mRNA expression level of Bcl2 comparing to that of the HCC untreated group. On the other hand, significant increased mRNA expression level of Bax was observed in the liver tissues of the 0.1 LD_{50} *C. cerastes* venom treated group comparing to that of the normal group, while a significant decreased mRNA expression level of Bax was quantified in the HCC untreated animals comparing to that of the normal control animals. Administration of 0.1 LD_{50} *C. cerastes* venom or cisplatin in the DENA/CCL4 treated animal groups

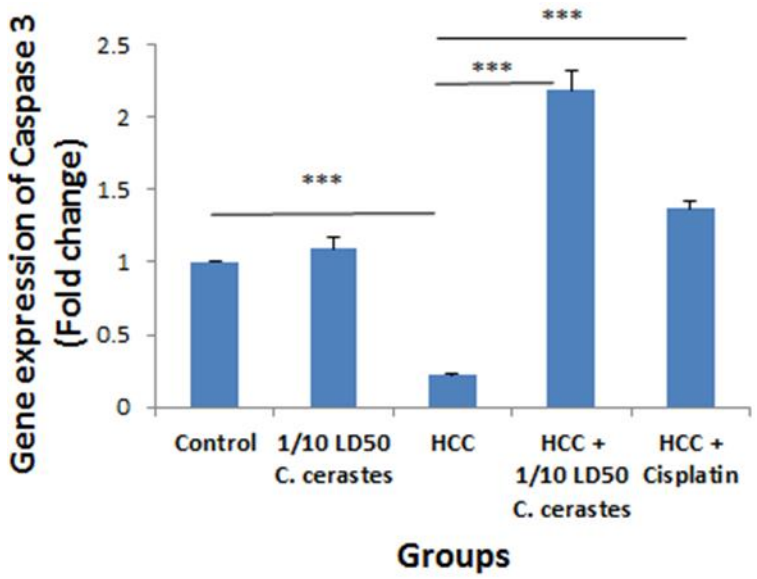

Figure 6. Fold increase in Caspase 3 gene expression. ***: very high significant difference ($P < 0.0001$).

significantly increased the relative mRNA expression level of Bax comparing to that of the HCC untreated group.

***C. cerastes* venom enhanced FAS and TRAIL expression**

Similarly, the extrinsic pathway of apoptosis was examined by measuring FAS and TRAIL expression. Figures 4 and 5 showed the relative mRNA expression levels of FAS and TRAIL among the control and the experimental groups of animals. Significant increase of mRNA expression level of TRAIL was detected in the liver tissues of the 0.1 LD_{50} *C. cerastes* venom treated group comparing to that in the normal group, while a significant decrease of mRNA expression level of FAS (not TRAIL) was observed in the liver tissues of the HCC untreated animals comparing to that of the normal control animals. Administration of 0.1 LD_{50} *C. cerastes* venom or cisplatin in the DENA/CCL4 treated animal groups significantly increased the relative mRNA expression levels of FAS and TRAIL comparing to that in the HCC untreated group.

***C. cerastes* venom upregulated caspase 3 expression**

To evaluate apoptotic effect of *C. cerastes*, the caspase 3 expression levels among the control and the experimental groups were measured (Figure 6). Significant increased mRNA expression level of caspase 3 was observed in the 0.1 LD_{50} *C. cerastes* venom treated group comparing to the normal group, while a significant decreased mRNA expression level of caspase 3 was quantified in the HCC untreated animals comparing to the normal control animals. Administration of 0.1 LD_{50} *C. cerastes* venom or cisplatin in the DENA/CCL4 treated animal groups significantly increased the relative mRNA expression level of caspase 3 comparing to that in the HCC untreated group.

Discussion

Today, cancer is one of the leading causes of death globally. Its development and progression are usually linked to a series of changes in the activity of the cell cycle regulators. Liver cancer represents one of the most common malignancies globally. HCC represents a major form of primary liver cancer in adults. It is the second and the sixth leading cause of the cancer related death in males and females, respectively

[35]. Currently, many therapeutic techniques are used for the treatment of HCC, but unfortunately these therapeutic techniques have not been completely succeeded and cause many toxic side effects [36]. Therefore, the designation of alternative therapy with high efficacy and potency has led to the raised use of anticancer agents developed from natural resources [37]. In the recent years, some studies have provided evidence that the bio-toxins present a high potential as anti-cancer candidates such as snake venom, and therefore, can be used as anticancer agents [38]. Snake venom is a complex mixture of compounds with specific biological and chemical activities and can be used in the treatment of various types of cancer. It was reported that the snake venom was able to induce cell death in different cancer cell line without effect on the normal cells [39]. This study employed DENA/CCL4-induced HCC rat model [40] to investigate the anticancer effect of the Egyptian snake *C. cerastes* venom.

HCC may engender complex metabolic disturbances in both human and experimental animals resulting in rapid loss of body weight. Relative liver weight is an important parameter in judging the pathological conditions of the liver [41]. The results of this study showed that the mean of the relative liver weight was significantly increased in the HCC group comparing to that in the normal control group, which might be due to the increase of the liver weight without increase of the body weight in the DENA/CCL4 treated animals. The increase of liver weight could be due to hyperplasia, hypertrophy, and induction of cirrhosis of the liver by DENA/CCL4 [42]. Similar result has been reported by Zimmerman, *et al*. who demonstrated that in the HCC group of rats [24]. The results showed that mean liver weight was considerably higher than that of the normal group, while the final body weight was significantly lower than that of the normal group, which were also in good agreement with the previous report [41]. Treatment with 0.1 LD_{50} *C. cerastes* venom or cisplatin in the DENA/CCL4 treated animal groups significantly increased the final body weight and significantly decreased the relative liver weight comparing to the HCC untreated group. These data suggested that the venom interfered with the tumor growth, and consistent with the results from Debnath, *et al*. who reported that the venom of Russell's viper inhibited solid tumor growth significantly of sarcoma model in mice comparing to the control untreated group [15]. Another study by Liu, *et al*. also found that melittin, a water-soluble toxic peptide derived from the bee venom of *Apis mellifera*, caused a significant decrease in tumor volume accompanied by an increase in body weight of an orthotopic transplanted nude mouse tumor model of human HCC [43]. On the other hand, Baldi, *et al*. reported results that disagreed with this study, in which there was no any change observed in tumor volume after treating EAC tumor bearing mice with *Crotalus durissus terrificus* snake venom fractions [44].

PCNA functions as a cofactor of DNA-polymerase and is an important marker for evaluating the proliferation of several cancers including HCC [45, 46]. The results showed elevated levels of PCNA in HCC untreated group, which indicated the hyper proliferative activity of tumor cells and was similar to the results from Jagan, *et al*. [47] and Ahmed, *et al*. [48]. Administration of 0.1 LD_{50} *C. cerastes* venom or cisplatin decreased PCNA expression in the liver tissues, which was in agreement with Han, *et al*. who reported that crotoxin (CrTX), the primary toxin in South American rattlesnake (*Crotalus durissus terrificus*) venom, downregulated the expression of PCNA in human lung carcinoma cells *in vitro* [49]. Lee, *et al*. also demonstrated that the snake venom toxin from *Vipera lebetina turanica* inhibited the expression of PCNA in lung cancer cell xenograft bearing nude mice model [50].

The KU protein is a heterodimer of 2 tightly associated sub-units called KU70 and KU80, normally located in the nucleus, where it is involved in nonhomologous end-joining (NHEJ) processes, which is responsible for repairing DNA double strand breaks (DSB) [51]. DNA double-strand breaks can be repaired by two mechanisms, homologous recombination and

NHEJ. The homologous recombination leads to accurate repair whereas NHEJ may be not [52, 53]. NHEJ is an error-prone and non-specific repair mechanism and can be induced before homologous recombination. Its excessive activation is capable of regulating the cell cycle arrest, cell apoptosis, chromosome recombination, and genome instability [54, 55]. The over-expression of KU70 has been found in various human tumors [56]. In the present study, the HCC untreated group showed elevated expression of KU70, which agreed with the findings of Zhang, *et al*. who demonstrated that KU70 expression was significantly increased in the tumor tissues of HCC patients [57]. Differently, Teoh, *et al*. reported that disruption of NHEJ DNA strand break repair by KU70 deficiency predisposed hepatocytes to chromosomal instability and accelerated liver carcinogenesis [58]. In our study, treatment with 0.1 LD_{50} *C. cerastes* venom or cisplatin decreased the expression of KU70 in the liver tissues.

Apoptosis occurs normally during development and aging as a homeostatic mechanism in order to maintain cell populations. Dysregulation of apoptosis can lead to disruption of the equilibrium between cell growth and cell death leading to the development of cancer. Thus, the investigation of new biological apoptotic activators could play a very important role in cancer therapy [27]. Cellular apoptosis signaling could be initiated either by the mitochondrial-mediated intrinsic pathway or by the cell death receptor-mediated extrinsic pathway. Many studies have shown that the activation of the apoptotic pathway in the malignant cells is a main protective mechanism against the development and the progression of cancer [59]. The Bcl2 family proteins, including Bax and Bcl2, contribute to the regulation of intrinsic apoptosis. The anti-apoptotic members of the Bcl2 family, such as Bcl2, act to prevent or delay cell death, whereas the pro apoptotic members, such as Bax, promote apoptosis [60]. It is recognized that the genetic variations in the HCC are leading to an imbalance between the pro-apoptotic and anti-apoptotic members of the Bcl2 family. Therefore, considerable efforts have been made in the recent years to prevent and treat HCC through induction of apoptosis as a promising therapeutic approach for patients with HCC [59, 61]. In the present study, a significant increase in the relative mRNA expression level of Bcl2 and significant decreased relative mRNA expression level of Bax were observed in the liver tissues of the HCC untreated animals comparing to that in the normal control animals. This result agreed with Bishayee, *et al*. who reported that DENA treatment significantly downregulated Bax expression level and significantly upregulated BCl2 expression in the liver tissues of the HCC rats comparing to that in the normal group of rats [62]. Bhatia, *et al*. also reported that liver sections from rats exposed to DENA were shown to have a very limited expression of the pro-apoptotic protein Bax, while the anti-apoptotic protein Bcl2 yielded a very high level of the protein [63]. Administration of 0.1 LD_{50} *C. cerastes* or cisplatin in the DENA/CCL4 treated animals significantly induced intrinsic apoptotic pathway through upregulation of the relative mRNA expression levels of the pro-apoptotic gene Bax and downregulation of the anti-apoptotic gene Bcl2. The results were agreed with Mahmoud, *et al*. who reported that *Echis pyramidum* venom showed upregulation of pro-apoptotic genes (P53, Bax) and downregulation of anti-apoptotic gene (Bcl2) in a significant way in HepG2 and HCT 116 cancer cell lines comparing to that in untreated control cells [64]. Differently, Akef, *et al*. reported that 24 hours treatment of prostate cancer cells (PC3) with *C. cerastes* venom significantly decreased Bax and Bcl2 gene expression levels [28]. This result was consistent with other data that showed the downregulating effect of some venom on the pro-apoptotic and the anti-apoptotic genes [66].

Caspases are proteases of the cysteine family that are commonly apoptosis markers [64]. A significant decreased relative mRNA expression level of caspase 3 was quantified in the liver tissues of the HCC untreated animals comparing to that in the liver tissues of the normal control animals. Administration of 0.1 LD_{50} *C. cerastes* or

cisplatin in the DENA/CCL4 treated animals significantly upregulated the relative mRNA expression level of caspase 3, which was in agreement with El-Fiky, *et al*. who also reported that *Cerastes vipera* venom had apoptotic effect against MDA-MB-231 cell line through elevation of caspase 3, caspase 8, and caspase 9 genes [65].

Selective and specific induction of the extrinsic apoptotic cell death of cancer cells has been increasingly recognized as a promising therapeutic approach for the treatment of many cancers. Tumor necrosis factor-α-related apoptosis inducing ligand (TRAIL), a member of the TNF ligand superfamily, has been shown to selectively induce apoptosis in various tumor cells by engaging its death-inducing receptors (TRAIL-R1 and TRAIL-R2) with no significant side effect on normal cells. Because of its selective induction of apoptosis in tumor cells, TRAIL is a potential target in cancer therapy, and the discovery of agents that induce TRAIL expression and promote apoptosis have received extensive attention [67, 68]. The results of this study demonstrated a significant increase of relative mRNA expression level of TRAIL in the liver tissues of the 0.1 LD_{50} *C. cerastes* venom treated animal group comparing to that in the normal control group. DENA/CCL4 treatment resulted in a decrease in the relative mRNA expression level of TRAIL in the liver tissues of the HCC untreated animals comparing to that in normal control animals. Administration of 0.1 LD_{50} *C. cerastes* or cisplatin in the DENA/CCL4 treated animals significantly increased the relative mRNA expression level of TRAIL comparing to that in the HCC untreated group. Kim and Song reported that *Vipera lebetina turanica* snake venom inhibited the cell growth of human colorectal cancer HCT116 cells through induction of both intrinsic and extrinsic death receptor (DR) mediated apoptosis *via* increased binding of DR4, DR5 with their apoptosis inducing ligand, TRAIL [69], which was agreed with the results of this study. The binding of TRAIL to DR4 and DR5 results in the activation of caspase 8 and 10, which in turn cleaves and activates the executioner caspases that mediate apoptosis [70, 71]. Damasio, *et al*. also demonstrated that TRAIL expression increased approximately by 130% in human colon adenocarcinoma cell line (HT29) incubated with BJcuL (lectin purified from *Bothrops jararacussu* snake venom) comparing to that in the untreated tumor cells [72].

Fas receptor (FasR) and Fas ligand (FasL) are type I and type II members of the tumor necrosis factor (TNF)-receptor superfamily. The autocrine–paracrine interaction between FasR and FasL results in the trimerization and activation of the Fas receptor, which leads to extrinsic apoptotic cell death [73, 74]. The FasR-FasL interaction recruits the Fas-associated death domain adapter protein (FADD) *via* death domain binding, which then interacts with the dimerized procaspase-8 to form the death-inducing signaling complex (DISC) [75]. Caspase-8 catalyzes its autoactivation followed by the proteolytic conversion of downstream effector caspases such as caspase-3 and -7 into their mature forms [73]. Effector caspases direct cell death by apoptosis, which results in nuclear and cytoplasmic condensation followed by cellular fragmentation into membrane-bound apoptotic bodies [76]. In the present study, a significant increased relative mRNA expression level of FAS was observed in the liver tissues of the 0.1 LD_{50} *C. cerastes* venom treated group comparing to that in the normal group. DENA/CCL4 treatment resulted in a significant decrease in the relative mRNA expression level of FAS in the liver tissues of the HCC untreated animals comparing to that in the normal control animals. Administration of 0.1 LD_{50} *C. cerastes* or cisplatin in the DENA/CCL4 treated animals significantly increased the relative mRNA expression level of FAS comparing to that in the HCC untreated group. The results agreed with Zhang and Cui who reported that ACTX-6, a protein isolated from *Agkistrodon acutus* snake venom, induced cell death of the human lung cancer cell line A549 through induction of Fas and FasL protein expression [77]. Chen, *et al*. also reported that phospholipase A (2) from *Naja naja atra* venom treatment increased Fas and FasL protein expression in human leukemia K562 cells [78].

Conclusion

The results of this study indicated that *Cerastes cerastes* venom exhibited anticancer effect against DENA/CCL4 induced HCC experimental rat model as evidenced by improving the final body weight and decreasing the relative liver weight. The venom also induced the intrinsic and extrinsic apoptotic pathways through downregulation of the anti-apoptotic BCl2 gene expression and upregulation of the relative mRNA expression levels of Bax, Caspase 3, FAS, and TRAIL. The immunohistochemical results also run in parallel with the real time qPCR results as the venom downregulated the expression of PCNA and KU70. The results indicated that *Cerastes cerastes* venom might serve as apoptotic stimulator, presenting a novel potential therapeutic pro-drug against HCC.

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