Intersexual Variation in Tail Length, Venom Composition, Toxicity, and Anticancer Activity of *Cerastes cerastes* (Viperidae)

Moustafa Sarhan[1], Ahmed Mostafa[1], Serag Eldin Elbehiry[3], Abdelbaset M. A. Abd el Reheem[1] and Samy A. Saber[2]

[1] Zoology Department, Faculty of Science, Al Azhar University, Assiut,[2]Zoology Department, Faculty of Science, Al Azhar University, Cairo, [3]The Egyptian Holding Company for Biological Products and Vaccin (VACSERA), Cairo, Egypt

ABSTRACT

Background: The medically important desert-horned or Egyptian sand viper (*Cerastes cerastes*) is one of the most familiar snakes of the great deserts of North Africa and the Middle East. It is a poisonous and widely distributed snake in Africa and inhabits the sandy deserts of Egypt. Male and female specimens of *C. cerastes* have been compared from different aspects. **Results:** Morphologically, tail length relative to snout-vent length was compared. Males significantly showed longer tails than do females. From the venom aspect, males showed a significant ($p<0.005$) higher concentration of protein in the venom (133mg/ml) compared to females (106 mg/ml). Female venom was significantly more toxic than male, with a median lethal dose (LD_{50}) in mice of 0.6µg venom protein/gm body weight whereas LD50 for males was 0.7 µg/gm. Protein analyses by means of electrophoretic technique revealed differences in venom composition between males and females. We have detected individual variability and highlighted sex-specific protein similarities and differences among snake venoms. SDS-polyacrylamide gel electrophoresis showed protein bands of 42 and 39 kDa specific to male venoms while bands of 46 and 44 kDa are specific to female venoms. Moreover, we evaluated the antitumor efficacy of male and female snake venoms in liver (HEPG-2), breast (MCF-7), colon (HTC-116) and normal cell lines and IC_{50} was calculated. Interestingly, both male and female venoms had anti proliferative effects on the tumor cell lines with different potency. Female venom had a higher cytotoxicity against colon cells (IC_{50}=0.006 µg/ml) than male venom (IC_{50}= 0.019µg /ml). In contrast, male venom had a higher cytotoxicity against breast cells (IC_{50}=0.005 µg/ml) than female venom (IC_{50}=0.024µg /ml). These results indicate that males and females of *C. cerastes* produce venoms with different composition and activity, which may have epidemiological implications.

Keywords: *Cerastes cerastes*, venom composition, toxicity, anticancer activity, intersexual variation.

INTRODUCTION

Snake venoms are complex mixtures of hundreds of proteins and peptides that function to immobilize or to kill the prey as well as to assist in the digestion of the prey [1, 2]. The toxic effects of snake venoms are complex because their different components have various actions. These components may concert with other venom toxins in a synergistic way to boost their activities [2, 3]. Venom variability has long been investigated and it is a documented phenomenon [4, 5]. It occurs at several levels including interfamily, intergenus, interspecies, intersubspecies and intraspecies variation [6]. Moreover, venom composition may be influenced by the geographical and habitat of the snake origin [6-9]. Intraspecific venom variation occurs between individual specimens, and also in individual specimens, due to seasonal variation [10], diet [11], age [12], and sexual dimorphism[13]. Venom variation is important to basic venom research to consider such variation for the choice of snake donors that are used for venom production. Venom variation is also important to the management of snake envenomation in the selection of snake donors for antivenom production and subsequently the selection of anti-sera for therapeutic use [6, 14]. Moreover, venom variation at different levels is also an important issue of studies on the evolutionary aspects of venomous snakes. At the level of intraspecific variation, the individual contribution to the venom composition is important but the effects contributed by environmental conditions, age and feeding habits also influence the venom protein picture exhibited by each specimen [15]. Studies of captive-bred snakes indicated that the intraspecific variation in venom is genetically

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inherited rather than environmentally induced [11]. However, according to Sasa [16], micro-evolutionary forces other than selection for local prey should also be considered as a source of the high variation of venom components among populations.

C. cerastes is commonly known as desert-horned or Egyptian sand viper [6, 17]. *C. cerastes* is one of the most familiar snakes of the great deserts of North Africa and the Middle East [18, 19]. It is a poisonous and widely distributed snake in Africa [20] and inhabits the sandy deserts of Egypt [21]. The Horned Viper *C. cerastes* is of major clinical importance as a cause of snakes' bite in Egypt. Although *C. cerastes* venom has been the focus of extensive studies on the effects of viper venoms on man and animals [17, 22-28], the degree to which the venom varies between individuals or between sexes is poorly understood. The objective of this investigation was to analyze venom samples from male and female viper (*C. cerastes*) at the laboratory in order to evaluate possible sex-based differences in venom composition and effects "*in vivo* and *in vitro"*. Electrophoretic technique was used to analyze proteins of the individual venoms; we have highlighted sex-specific protein similarities and differences among different *C. cerastes* sexes. Moreover, protocols for measuring the toxicity and anticancer activities supported the sex-based difference in the venom of *C. cerastes.*

MATERIALS AND METHODS

Snakes were collected from Aswan (Khor Megahed and Jarf Hussin- Abu Steit Mountain). Twenty -two snakes were identified, and then we measured snout to ventral length (SVL) and tail length.

Venom collection:

The venom was collected separately from twenty-two adult *C. cerastes* of both sexes (11 males and 11 females). To collect venom, the head of the snake was held and made to bite on the rubberized synthetic sheet, stretched and tied at the mouth of a sterilized glass beaker, to allow voluntary injection of the venom into a receptacle through a rubber membrane [29]. Collected venom was immediately centrifuged at 15000 rpm, 4°C, divided into aliquots and finally kept at -20°C until use.

Total protein estimation:

Protein in venom was determined by measuring the absorbance at 540 nanometer using spectrophotometer and commercial kit (vitroscient) according to manufacturer's instructions.

Determination of LD_{50}:

Male albino mice weighting 18-25g. were used for the present study. All animals were housed in polyethylene (65cm×25cm×15 cm) home cages, with sawdust-covered floors. Animals were maintained at 25±2 °C, with free access to standard laboratory mice food and water. All procedures on care and maintenance of the experimental animals were in accordance with International Guiding Principles for Animal Research. "Ethical standards and guidelines for toxicological research" were adopted to all the present experiments. The lethal toxicity (LD_{50}) for *C. cerastes* male and female venoms was determined after injecting intraperitoneally different known concentrations of each male or female venoms according to the method described by Meier and Theakston [30].

SDS-PAGE and determination of molecular weight:

Molecular weights of venom proteins were determined using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a "Vertical Electrophoresis Unit", according to Sarhan *et al* [31]. The gel was photographed and analyzed using Gel-Pro analyzer (3.1).

Cytotoxicity assays:

Cytotoxic effects of snake venoms were tested against Human hepatocellular carcinoma (HEPG-2) and Human colon carcinoma cells (HTC-116), Human breast adenocarcinoma (MCF-7) and Human amnion epithelium (WISH, normal cells) in vitro cell lines by the MTT assay as previously described by Alley *et al* [32]. Negative cell control was included. IC_{50} was calculated by using Masterplex 2010 hitachia (GIRSS). **The study was done after approval of ethical board of Al-Azhar university.**

Statistical analysis:

Data were statistically analyzed using SPSS Software and presented as means and standard error (Mean ±S.E.). The statistical significance was examined using Student's t test. P value of <0.05 was considered significant.

RESULTS

C. cerastes vipers display noticeable sexual dimorphism in tail length relative to body length. At the same SVL, males have longer tails than do conspecific females (Fig. 1 A). Accordingly, the tail/body length ratio is significantly higher in males than females ($p<0.001$) (Fig.1 B).

Protein concentration was measured in female and male *C. cerastes* venom. A significantly lesser amount of concentration in terms of protein was produced by females compared to males. In female venom, protein concentration was 106 mg/ml whereas males contained 133 mg/ml (Fig. 2).

To check the possible difference between male and female *C. cerastes* venoms at the composition level, SDS-PAGE was carried out. Results showed that, there are two specific bands at 42 and 39 kDa in male venom samples, while there are another two specific bands at 46 and 44 kDa detected in the female venom samples (Table 1 & Fig. 3).

To go further, we checked the lethal effect of venoms of both sexes of snakes. The LD_{50} was calculated after the interprotenial injection of the venom and count the percentage of mortality after 24 hours. As shown in Figure 4, results indicated that the female venom was potent than that of males. The LD_{50} for female was 0.6 µg/gm and 0.7 µg/gm for male venom.

To explore the possible different effects of venoms on cancer cell lines, the inhibition of cancer cell proliferation post snake venom treatment was performed by using MTT assay. The result showed a significant reduction of cell viability against all checked cancer cell lines, when compared to the normal cell line. Both venoms of male and female of *C. cerastes* have anti-proliferative effects on MCF-7 cell lines in a concentration-dependent manner (Fig. 5C). Male venom showed a significant high cytotoxic activity against cancer cells exceeds that of female with IC_{50} 0.005±0.001 and 0.024±0.01 µg/ml respectively (Table 2).

We also evaluated the effects of the snake venom toxin from *C. cerastes* on the growth of liver cancer cells; we analyzed the cell viability against liver cancer cell line HEPG-2. The results showed that, the inhibition of HEPG-2 viability was in a concentration-dependent manner (Fig. 5B). Moreover, the IC_{50} values of snake venom toxin of both male and female were 0.02±0.01 and 0.018±0.01 µg/ml, respectively and showed a significant reduction of cell viability when compared to normal cell line (Table 2).

With a view to an extensive analysis of these anti-cancer activities, we further check the effect of *C. cerastes* venom against colon cancer cells HTC-116 to obtain the broadest data set of this study. The venom showed a reduction of cell viability against HTC-116 in a concentration-dependent manner (Fig. 5D), with IC_{50}, 0.019±0.002 and 0.006±0.004 µg/ml for male and female respectively and also showed a significant reduction of cell viability when compared to normal cell line (Table 2). As expected, the results showed growth inhibition against WISH cell line (normal cells) in a concentration-dependent manner (Fig.5A). The IC_{50} value of the normal cells was 0.215±0.045 and 0.199±0.018 µg/ml for male and female respectively as shown in table 2. Taken together, the harvested data clearly represented that female and male venoms of *C. cerastes* had variable effects not only on both normal and cancerous cell lines but also among cancerous cell lines.

DISCUSSION

Sexual dimorphism in size, shape, color, and behavior is a widespread phenomenon among animals, and in most snake taxa the females exceed males in body size[33, 34]. The primary causes for sexual dimorphism in body size may be due to selection for fecundity or ecological requirements [35, 36]. Our study provides direct evidence on the consequences of tail length differences in male and female *C. cerastes*. Sex differences in relative tail length are very widespread in snakes [34, 37]. Accordingly, male *Thammophis sirtalis* snakes have longer tails than females. Males with relatively longer tails had longer hemipenes and accidental loss of part of the tail was associated with three fold decrease in male mating success [38]. Relative tail length in male snakes is a biologically relevant trait, which affects male mating success. In this regard, King [37] postulated two hypotheses: (1) The morphological constraint hypothesis; the longer tails enhance male fitness by providing space for larger hemipenes and (2) The male mating ability hypothesis; enhance male's ability to obtain mating. SDS-PAGE analysis of the venom of male and female *C. cerastes* showed protein variation in individual venom samples related to sex. The number of protein bands found in the

venom of males and females was compared in this study. Using SDS-polyacrylamide gel electrophoresis under non-reducing conditions, Menezes *et al*. [15] showed protein bands of 100 kDa specific of male venom of *Bothrops jararaca* snake. Accordingly, acetic acid-urea gel electrophoresis, used to study toxin composition in Tityus scorpion venoms *Tityus discrepans*. Borges *et al*. [39] demonstrated the presence of female-specific components. Same results were observed in the venom of another Tityus scorpion venoms *Tityusnor orientalis* [40]. Other arachnids, such as rainforest tarantula, *Coremiocnemis tropix* and *Phoneutria nigriventer*, demonstrate intersexual toxicological differences, but the potency of their venoms is not only related to protein content but also to the species affected and the victim´s physiological condition. Herzig and Hodgson [41] have shown that venom from *C. tropix* males contains components more potent on insects when compared with females. Analogously, vertebrate active toxins were more common in *C. tropix* female venom. Similar results were obtained when testing venom from female Brazilian wandering spider, *Phoneutria nigriventer* on insects [42]. Abdel-Rahman *et al*. [43] analyzed male and female venom of the Egyptian scorpion *Scorpio maurus palmatus* and found that female venom appears to be more complicated than the male venom and female venom samples showed 9 protein bands which were absent in the male venom. Our toxicity results showed that female venom is more potent than male when injected in mice. An explanation for female *C. cerastes* venom potency could be related to the fact that females with young react aggressively to any disturbance and that maternal care could involve venom with higher toxicity to defend offspring and to provide nutrients during embryonic development. It will be interesting, as one of future directions, to analyze the venom samples using highly sophisticated proteomic techniques such as LC-MS. Investigated venoms showed variable cytotoxic effect to breast cancer cell line MCF-7 cells, liver cancer cell line HEPG-2 and colon cancer cell line HTC-116. The differences among venoms of male and female of the same snake litter showed here by various protocols may be attributed to variation of environmental conditions, age or diet of the specimens analyzed since these were born and raised under uncontrolled conditions. Another possibility, the diversity observed in the venom could be genetically inherited and imposed by evolutionary forces. One important aspect of the intraspecific variability of snake venoms is the variability of specific venom components. Metalloproteinases, serine proteinases and phospholipases A2 are among the most abundant enzymes found in snake venoms [44-48]. Diverse isoforms of these snake venom enzyme families have been identified which vary in their biophysical and biochemical properties. These enzymes have diversified amino acid sequences and display a variety of physiological activities.

Several studies reported the presence of peptides and proteins with cytotoxic activity against tumor cells in venoms for a wide range of snake species, particularly those belonging to the Viperidae family [49, 50]. So, it is possibly that each sex of *C. cerastes* snakes possess different kinds of antitumor peptides and/or concentrations. If so, it would be interesting to test the crude snake venom of both sexes separately with chemotherapeutic drugs. Lipps [51] reported the synergetic activity of snake venom proteins atroporin and kao tree against cancer cells. On the other hand the most important benefits of using the combination of a chemotherapeutic drug with venom or venom derived component is to reduce the required therapeutic dose of either one, minimize their side effects and also decrease the cancer cell resistance; this information was recently confirmed [52]. It would be of interest to determine whether these differences in toxicity and anticancer activity can also be found in other Egyptian venomous animals. Antivenom preparation could benefit from these findings as to compensate for such intersexual variations when milking snake venom for immunization.

In conclusion, intersexual variations were found in venom toxicity and anticancer activity in the snake species, *C. cerastes*, suggesting the existence of sex-related differences in venom composition and activity of other venomous animals, which is important issue of studies on the evolutionary aspects of venomous snakes as well as to the management of snake envenomation.

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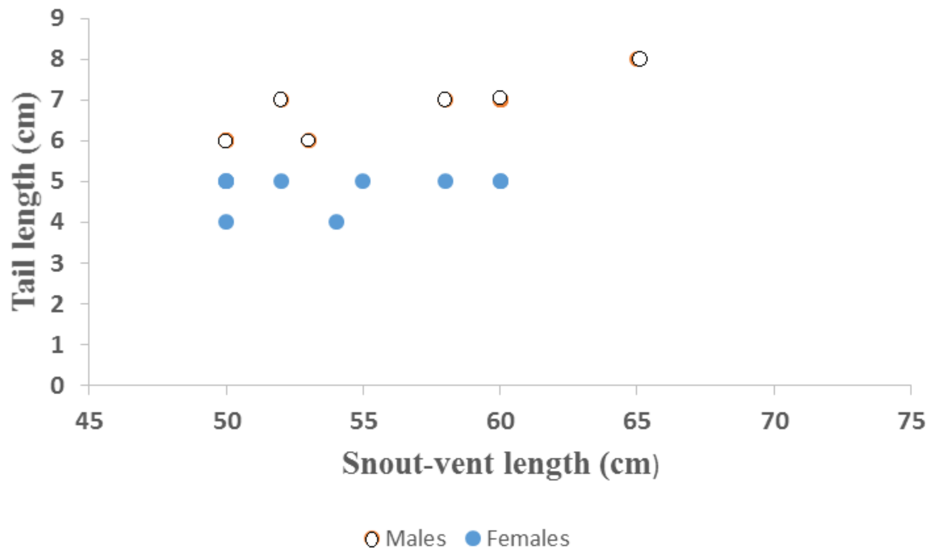

Figure 1.A. Sex divergence in tail length relative to snout-vent length in horned viper snake (*C. cerastes*) from southern Egypt (Aswan).

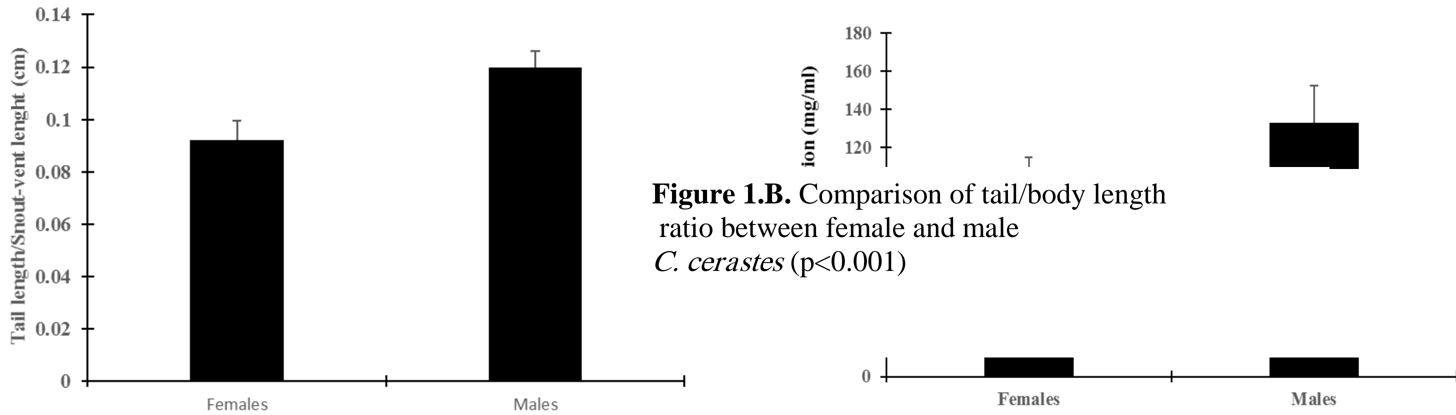

Figure 1.B. Comparison of tail/body length ratio between female and male *C. cerastes* ($p<0.001$)

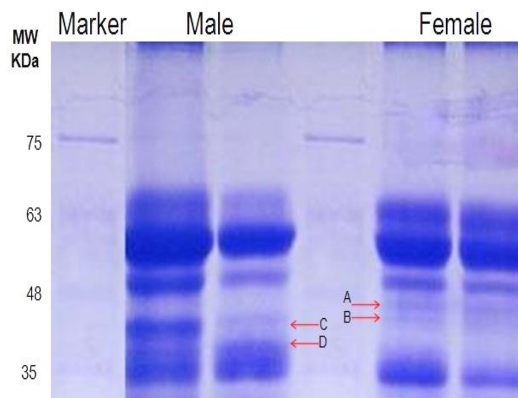

Figure 3. SDS-PAGE analysis of male and female *C. cerastes* venom. Arrows denote specific bands at 46 kDa (A) and 44 kDa (B) detected in the female venom, while there were two specific bands at 42 kDa (C) and 39 kDa (D) found in male venom.

Table 1. Comparison of protein bands in male and female *C. cerastes* venom using 12% SDS-PAGE.

Sex / MW(kDa)	Female	Male
66	+	+
49	+	+
46	+	-
44	+	-
42	-	+
39	-	+
36	+	+
32	+	+
29	+	+
25	+	+

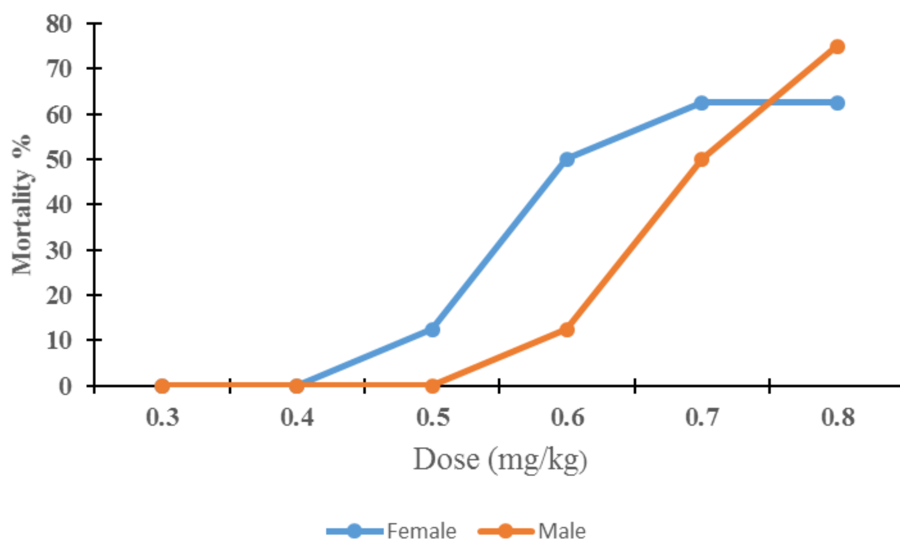

Figure 4. Determination of LD_{50} of female and male *C. cerastes* venom

Table 2. IC_{50} values of venoms in Normal, MCF-7, HEPG-2 and HTC-116 treated cell lines.

Cell line	IC_{50} (μg/ml)		P value	Histological origin
	Male	Female		
WISH (Norma cell line)	0.215±0.045	0.199±0.018	0.74	Human amnion epithelium cells
HEPG-2	0.02±0.01	0.018±0.01	0.73	Human hepatocellular carcinoma cells
HTC-116	0.019±0.002	0.006±0.004	0.043*	Human colon carcinoma cells
MCF-7	0.005±0.001	0.024±0.01	0.0045*	Human breast adenocarcinoma cells

*Statistically significant P value <0.05

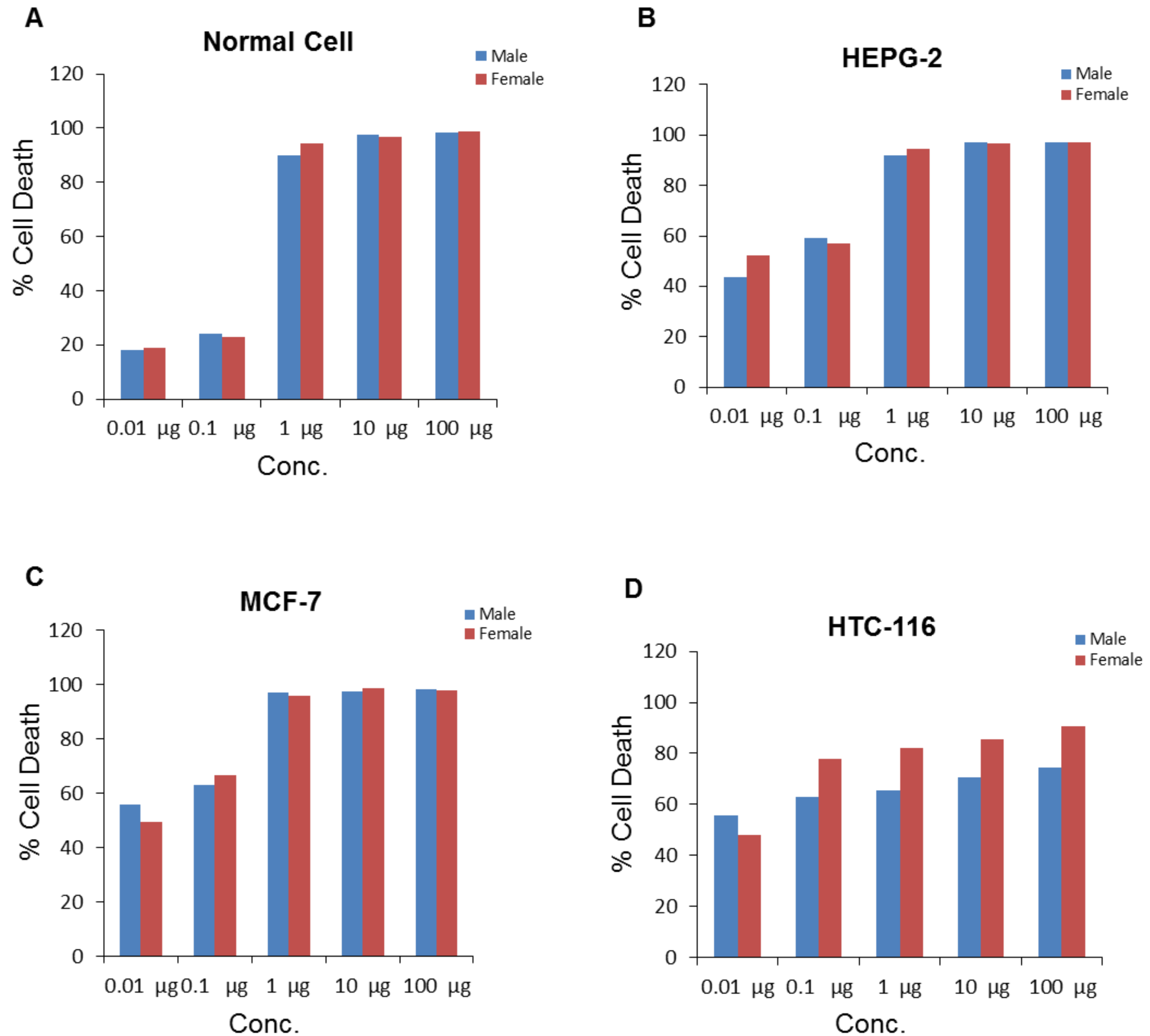

Figure 5. Percentage of cell death in Normal cell line (A), liver cell line "HEPG-2"(B), breast cancer cell line "MCF-7 "(C) and colon cancer cell line "HTC-116 " (D) induced by male and female *C. cerastes* venom at different concentrations (0.01, 0.1, 1, 10 and 100 µg)