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Cytotoxic effect of three fractions isolated from *Cerastes cerastes* venom on MCF-7 breast cancer cells *in vitro*

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ABSTRACT

In the present study fractionation of the *Cerastes cerastes* venom by gel filtration on Sephadex G-75 gave four protein fractions. All the four fractions exhibited phospholipase PLA_2 and proteolytic activities. SDS-polyacrylamide gel electrophoresis revealed that the crude venom and its four fractions F_1, F_2, F_3, and F_4 were not homogenous. The fraction F_1 was further separated into twelve bands. F_2 and F_3 were separated into nine bands, while F_4 yielded seven bands. The morphological alterations of the MCF-7 cells after the exposure to various concentrations of the crude venom and fractions were examined. The results revealed that the crude venom and its fractions F_1, F_2 and F_3 possessed an appreciable cytotoxic effect on the MCF-7 cells, whereas F_3 had a weak effect. Fraction F_4 was found to be without a detectable antitumor effect. The 50 % inhibitory concentration (IC_{50}) of the crude venom and fractions were estimated. The IC_{50} of the crude venom was 0.963 µg/ml. The IC_{50} of the three fractions F_1, F_2 and F_3 were 1.09, 2.09 and 9.78 µg/ml, respectively.

Introduction

Snake venoms contain many pharmacological proteins and peptides, with anti-inflammatory, anti-tumor, analgesic and other pharmacological effects [1,2,3]. Previous studies reported that many snake venoms exhibited significant anti-tumor effects both *in vivo* and *in vitro*. For example, Indian cobra (*Naja kaouthia*) and Russell's viper (*Vipera russelli*) venoms extended the survival period of Ehrlich Ascites Carcinoma (EAC)-bearing mice [4]. It was found that many components in snake venoms, such as disintegrins [5], cytotoxins [6], L-amino acid oxidase [7], phospholipases A_2 [8] and metalloproteinase are accountable for these antitumor potentials.

Viperidae snake venoms including the viper *Cerastes* sp. are complex mixtures of toxic non-enzymatic proteins and enzymatic proteins that induced a wide range of toxic effects [9-12]. The major active components of these venoms were identified as neurotoxins, hemolysins, cardiotoxins, metalloproteinases, in addition to different phosphatases including phospholipases A_2. The use of snake venoms in the treatment of cancer has been known for decades. In this regard, the whole venoms of the Indian monocle cobra (*Naja kaouthia*) and Russell's viper (*Vipera russelli*) were reported to exhibit significant levels of anticancer activity against Ehrlich's Ascites Carcinoma (EAC) cells [13,14].

Thangam *et al.* [15] investigated the cytotoxic activity of a fraction from *Naja naja* venom against human breast cancer MCF-7 cells, liver cancer HepG2 cells and lung carcinoma A549 cell. This fraction exerted an inhibitory effect on tumor cell growth after 24 hours. Al-sadoon *et al.* [16] showed that *Walterinnesia aegyptia* venom in combination with nanoparticles induced apoptosis in human breast carcinoma cell lines MDA-MB-231 and MCF-7 without effect on normal MCF-10 cells.

Mukherjee & Mackessy [17] and Mukherjee *et al.* [18] isolated a cytotoxin from the venom of *Daboia russelii russelli* and studied its cytotoxic properties against several tumor cell lines such as mammalian cells Colo-205 (human colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma) and 3T3 (mouse embryo fibroblast). The cytotoxin showed an inhibitory effect on the growth of MCF-7 cells via stimulating the apoptotic process. Moreover, Thakur *et al.* [19] studied the antiproliferative activity of a small peptide isolated from *Daboia russelii russelii* venom on various cell lines including MCF-7 cells. After incubation of MCF-7 cells with the peptide for 24 hours, induction of apoptosis in MCF-7 cells was observed and the inhibition of MCF-7 cell proliferation was dose-dependent. The venom. fraction

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induced DNA condensation confirming the apoptotic induction.
Local tissue damage is one of the main physiological effects of *Cerastes cerastes* venom. Electrophoretic analysis of the venom revealed the presence of multiple protein bands with proteolytic activities [14]. The study of Boumaiza *et al.* [20] showed that *Cerastes cerastes* venom had a high phospholipase activity that induced necrosis and extracellular matrix degradation. The hyaluronidases and proteases of the venom played a dominant role in tissue damage due to their spreading properties.
Treatment of tumor-bearing mice with lethal fractions and a non-lethal fraction of *Cerastes cerastes* venom at the maximum tumor inhibitory concentrations gave rise to a smaller increase in the mean total body weight at the end of the experiment as compared to tumor-bearing controls. The cytotoxic activity of a non-lethal fraction was demonstrated by a significant reduction in viable EAC cell count associated with a significant elevation in dead EAC cell count as compared to the tumor-bearing controls [21]. Shebl *et al.* [22] evaluated the cytotoxic effects of *Cerastes cerastes* and *Vipera lebetina* snake venoms on MCF-7 cells and reported that the two venoms increased cellular irregularities of MCF-7 cells with severe shrinkage and condensation of cellular content.
The aim of this study was to fractionate *Cerastes cerastes* venom and to evaluate the potency of the antitumor activity of the fractions.

Materials and methods

Venom

The horned vipers *Cerastes cerastes* were collected from Toushka region in Aswan and maintained at the Venom Research Laboratory of the Zoology Department, Faculty of Science, Ain Shams University. Venom was obtained by allowing adult vipers to inject their venoms through a rubber-covered glass beaker, the venom was then lyophilized and stored desiccated at 4 ºC in the dark until used. A fresh solution of the dry crystalline powdered venom was reconstituted in RPMI-1640 media to the desired concentration just before use.

Cell line

MCF-7 cell line (Human breast adenocarcinoma) was purchased from the Tissue culture unit at VACSERA institute, Agouza, Giza, Egypt.

Chemicals

Sephadex G-75, N, N-Methylene bis acrylamide (bis), N, N, N, N- tetramethylethylenediamine (TEMED), sodium dodecyl sulphate, ammonium persulphate, Folin reagent, ammonium persulphate (APS) and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich Chemical Company, St. Louis, USA. RPMI-1640 media, fetal bovine serum, and phosphate buffer saline were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA. All other chemicals were of an analytical grade and highest purity available.

Fractionation of the crude venom

The method used for gel filtration was previously described [23]. Where, 0.2 g of lyophilized crude venom from *Cerastes cerastes* viper was dissolved in 1 ml of 0.2 M ammonium acetate buffer, pH 4.6, then centrifuged at 1000 rpm for 15 minutes at 4℃ to remove the insoluble materials. The supernatant was loaded onto a gel column (1.5 x 20 cm). Seven grams of Sephadex G-75 were dissolved in 200 ml of 0.2 M ammonium acetate buffer, pH 4.6 and allowed to swell for 48 hours at room temperature. Fractions (2 ml/fraction) were collected at a flow rate 0.4 ml/min. The absorbance of the fractions was read at 280 nm, a curve was constructed, and the peaks of the highest protein content were monitored, those fractions were then kept at -20℃. After determination of the proteolytic and phospholipase activities, the fractions having the highest protein content, proteolytic and phospholipase activities were pooled together.

Gel electrophoresis

The crude venom and fractions were applied to the SDS-PAGE electrophoresis according to previous methods [24]. A vertical slab gel electrophoresis apparatus (Bio/Phoresis^{TM}– Bio Gene Limited, England) of 10 x 8.3 cm glass plates and 10 wells comb was used with a buffer reservoir of 1 L. Electrophoresis was carried out at room temperature at a constant current of 20 mA/gel (90-120 minutes). The gel was stained overnight in Coomassie Brilliant Blue R-250. After staining, the gel was de-stained by several changes of the de-staining solution until the gel background became clear.

Determination of Phospholipase A_2 activity

Phospholipase A_2 (PLA_2) activity was determined by indirect hemolysis on washed rabbit erythrocytes in presence of an egg yolk suspension. Hemolysis was detected in the supernatant and the amount of hemoglobin liberated in the supernatant was read in the spectrophotometer at 540 nm and taken as the measure of the phospholipase A_2 activity [25].

Determination of the Proteolytic activity:

Proteolytic activity was carried out using casein as the substrate [8]. One unit of caseinolytic activity was defined as the amount of venom/protease that produces an increase in absorbance of 0.001 U/min.

Determination of the 50 % inhibitory concentration (IC_{50})

For the antitumor activity assays on the Human breast carcinoma cell line (MCF-7), the IC_{50} value was determined for each of the *Cerastes cerastes* crude venom and its fractions. MCF-7 cells were grown on RPMI-1640 medium supplemented with 10 % inactivated fetal calf serum and 50 µg/ml gentamycin. The cells were maintained at 37 ºC in a humidified atmosphere with 5 % CO_2 and were subcultured two to three times a week. The tumor cell lines were suspended in the medium at a concentration of $5x10^4$ cells/well in Corning® 96-well tissue culture plates and incubated for 24 hours. Media was removed, and cells were exposed to ascending concentrations of the crude venom (50–500 µg/ml) or its fractions. After incubation for 24 hours, the numbers of viable cells were determined by the MTT test [15]. The percentage of viability was calculated as:

$$\frac{\text{optical density of treated cells}}{\text{optical density of control cells}} \times 100$$

The relation between surviving cells and the concentration of the crude venom or its fraction concentration was plotted to get the survival curve of the tumor cell line after treatment with the specific compound.The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve for each concentration using Graph Pad Prism software (San Diego, CA. USA).

Morphological study

After treatment at each time interval with different concentrations, the MCF-7 cells were stained with crystal violet stain. Three monolayers per experimental group were photo micro-graphed. The morphological changes of the MCF-7 cells were analyzed using Nikon (Japan) bright field inverted light microscopy compared to the control group.

Results and Discussion

The crude *Cerastes cerastes* venom was separated into four fractions **(Fig. 1)**, F_1 (Tube 6-Tube 12), F_2 (Tube 13-Tube 17), F_3 (Tube 18-Tube 21) and F_4 (Tube 22-Tube 34) with the first two fractions (F_1 and F_2) having the highest protein content. After the fractionation process, the phospholipase activity of each of the obtained individual fraction was assessed. It was found that the fractions having phospholipase A_2 activity formed five peaks **(Fig. 2)**. The proteolytic activity of each individual fraction was also evaluated, and the results showed that the fractions containing the activity formed ten peaks as shown in **(Fig. 3)**.

SDS-polyacrylamide gel electrophoresis showed that the four fractions F_1, F_2, F_3, and F_4 are not homogenous as shown in **(Fig. 4)**, the fraction $F_1$was further separated into twelve bands with molecular weights of 1.875, 5.125, 9.5938, 10.548, 12.808, 15, 19.265, 22, 27.25, 31.364, 36.591 and 50.556 kDa. The fraction F_2 was separated into nine bands with molecular weights of 1.0625, 3.9063, 9.5938, 10.548, 13.014, 13.699, 32.5, 38.409 and 52.778 kDa. The fraction F_3 was separated into nine bands with molecular weights of 0.65625, 2.2813, 9.1875, 10.479, 13.288, 14.041, 32.5, 38.864 and 52.5 kDa. The fraction F_4 was separated into seven bands with molecular weights of 3.9063, 10.479, 11.575, 13.836, 32.5, 39.091 and 51.389 kDa.

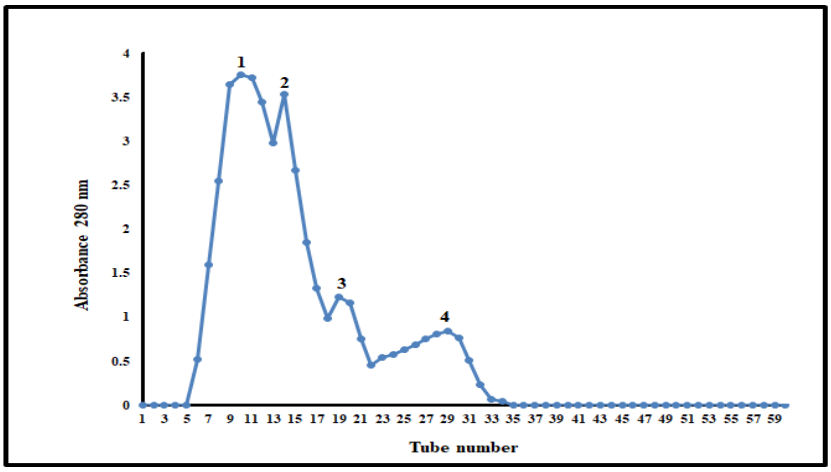

Fig 1: Fractionation of the crude venom of *Cerastes cerastes* on Sephadex G-75: 1) First fraction, 2) Second fraction, 3) Third fraction, 4) Fourth fraction.

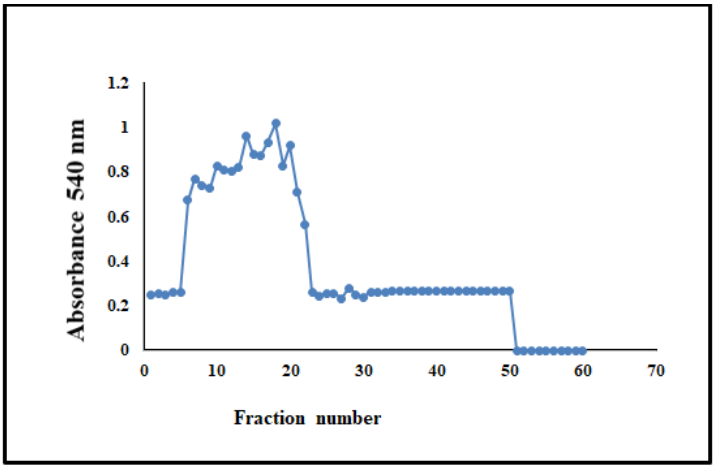

Fig 2: The phospholipase A_2 activity of the fractions obtained from fractionation of the crude venom of *Cerastes cerastes* on Sephadex G-75.

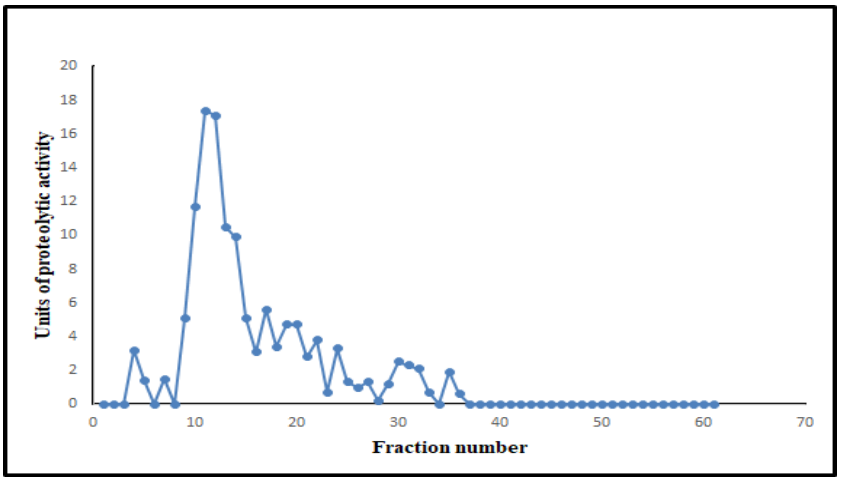

Fig 3: The proteolytic activity of the fractions obtained from fractionation of the crude venom of *Cerastes cerastes* on Sephadex G-75.

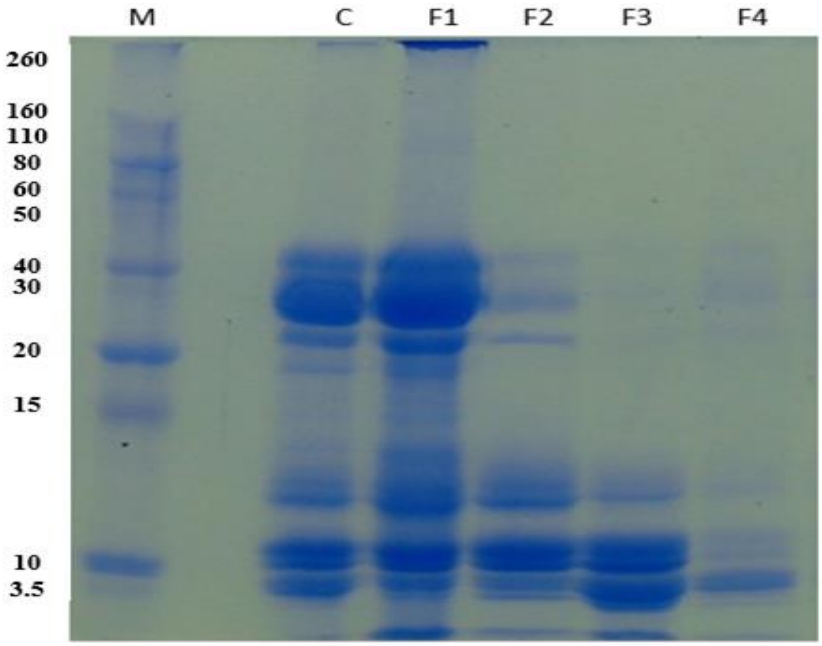

Fig 4: SDS-PAGE of the four fractions obtained from the fractionation of the crude venom of *Cerastes cerastes*. M) molecular weight marker, C) crude venom, F_1) fraction 1, F_2) fraction 2, F_3) fraction 3, F_4) fraction 4.

In the present study gel filtration of *Cerastes cerastes* venom on Sephadex G-75 yielded 4 protein fractions **(Fig. 1)**. Phospholipase A_2 (PLA_2) activity was determined in the crude venom and its fractions because of its fundamental role in the snake venom lethality [26,27]. The PLA_2 activity was detected in F_1, F_2, F_3, and F_4 **(Fig. 2)** and has been found to be relatively low in the *Cerastes cerastes* venom in comparison to other snake venoms such as *Naja haje* and *Najanaja atra* [28,29,21]. This suggests that PLA_2 is not necessarily the main enzyme that contributes to the *Cerastes cerastes* venom toxicity and that other components such as proteases could be implied. Our findings are in harmony with those of Mebes and Ownby [30], who reported that viperid venoms contain PLA_2 enzymes devoid of lethal or hemorrhagic activity though they produce local as well as generalized myonecrosis in mice. The non-toxic PLA_2 activity was also reported in the *Vipera ammodytes* venom [31].

In the present work, the morphological alterations in the MCF-7 cells after the exposure to various concentrations of the *Cerastes cerastes* crude venom and its fractions were examined using the inverted light microscope after staining with the crystal violet stain. The photomicrographs presented in (**Figs. 5 and 6)** revealed that the MCF-7 cells treated with different concentrations of *Cerastes cerastes* venom exhibited morphological alterations in comparison to the untreated cells in the form of detachment of the cells from the dish, cell rounding and shape irregularities. MCF-7 cells treated with different concentrations of the venom fractions showed a decrease

in the cell counts and the most effectiveness was obtained with the crude venom and the fractions at 24 hours.

The IC_{50} of the crude venom was 0.963 µg/ml and for the three fractions F_1, F_2 and F_3 were 1.09, 2.09 and 9.78 µg/ml respectively. F_1 and F_2 exhibited a strong antitumor effect, whereas fraction F_3 revealed a weak effect and F_4 was devoid of a detectable antitumor effect.

The MCF-7 breast cancer cells appear to be more susceptible to lectin exposure, with an increase in the number of cell fragments and cells displaying apoptotic characteristics. In support of the present results, Ponraj *et al*. [32] reported morphological changes in lectin treated breast cancer cells in the form of cytoplasmic condensation, cell shrinkage, and production of numerous cell surface protuberances at the plasma membrane blabbing. The cell membrane disintegrate were observed using phase-contrast light microscopy to measure the level of cell shrinkage at the IC_{50} concentration of protein disintegrin isolated from snake venom after both 24 h and 48 h of incubation.

In the present study, treatment with *Cerastes cerastes* venom or its fractions resulted in damaging, rounding up and detachment of the MCF-7 cells accompanied with multicellular aggregate formation. In addition, disorganization of the cells and large areas lacking cells were observed in the wells. Interestingly, our results showed that the venom fractions possess proteolytic activity, but there is limited information regarding the *in vitro* or *in vivo* anticancer effects of the venom and its fractions on MCF-7 cells. Generally, serine and metalloproteases are involved in the degradation of extracellular matrix proteins and facilitate tumor growth, invasion, metastasis and angiogenesis [33].

Abu Sinna *et al*. [21] reported the cytotoxic activity of three lethal fractions of *Cerastes cerastes cerastes* venom demonstrated by an increase in the mean survival time of the Ehrlich ascites-bearing mice, the significant tumor inhibitory effect on tumor growth, reduction in the viable tumor cell count and elevation in the dead tumor cell count. These observations might be due to the presence of certain protein cytotoxin(s) that inhibited the DNA synthesis. This assumption is supported by the significant reduction in the tumor cell count. However, further studies are warranted for a better understanding of the underlying reason of this effect. The exact mechanisms which cause tumor regression in experimental animals after treatment with the crude snake venoms and/or their fractions are still unclear. In this regard, several possibilities may emerge to explain how snake venoms act on tumor cells. In general, it is acceptable that venoms' effects are mediated by either direct or indirect mechanism. The direct effects involve lytic action on the tumor cells whereas the indirect ones focus on destroying the favorable microenvironment that is produced by the tumor cells to ensure their growth [34].

In conclusion, the present findings have shown that three fractions isolated from *Cerastes cerastes* venom were cytotoxic to MCF-7 breast cancer cells in vitro. Further studies are required to elucidate the precise molecular mechanism and targets for cell growth inhibition, which will allow for the rationale design of more effective molecules analogous to the protein fractions for the eventual use as a cancer chemopreventive and/or therapeutic agents.

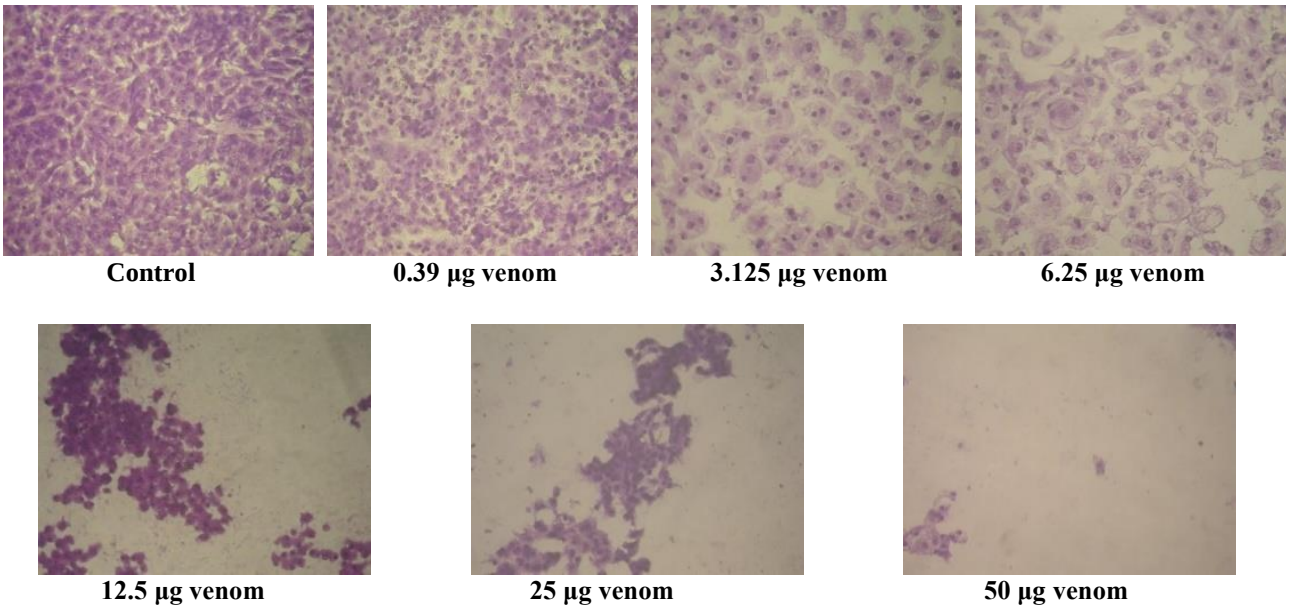

Fig 5: Photomicrograph representing the morphological characterization of MCF-7 cells after the exposure to different concentrations of the crude venom of *Cerastes cerastes*.

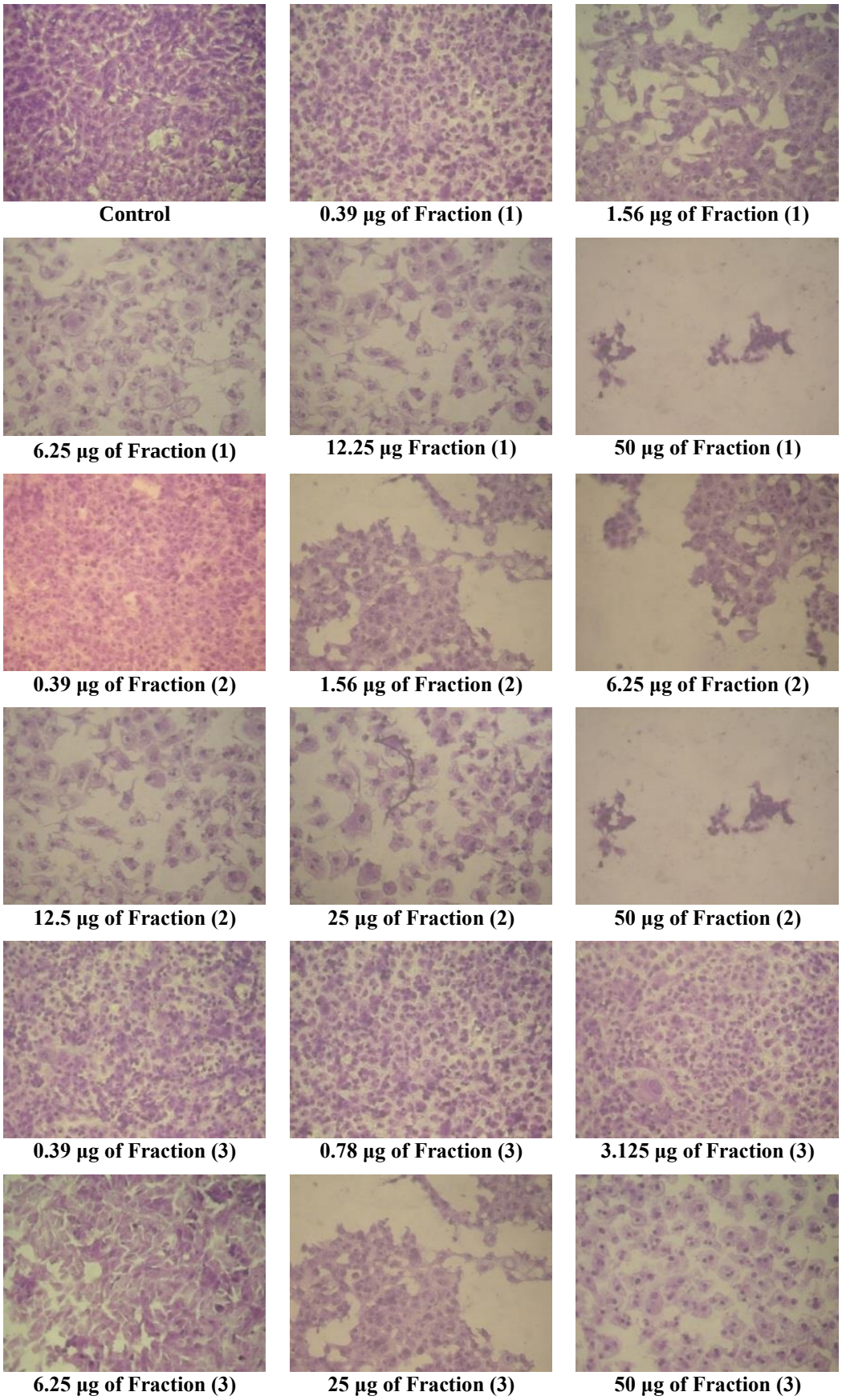

Fig 6: Photomicrograph representing the morphological characterization of MCF-7 cells after the exposure to different concentrations of three fractions of *Cerastes cerastes* venom.

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