

***Crotalus durissus cascavella* VENOM TOXICITY TO MAMMALIAN CELLS**

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ABSTRACT

Snakes from the genus *Crotalus* gained significant space in the scientific field since the development of researches with their venom involving cytotoxic activity. *Crotalus durissus cascavella* is the only subspecies recorded of the Caatinga biome of Northeastern Brazil and it's noticed a lack of studies on its venom properties. The present study evaluated the crude venom of *C. d. cascavella* for its cytotoxic activity *in vitro* against both tumor cell lines and normal cells. The mechanism of cell death induced by this venom was also investigated. *C. d. cascavella* venom presented high cytotoxicity (IC₅₀ values ranging from 2.7 to 6.9 µg/mL) against five tumor cells lines: OVCAR-8 and SKOV3 (ovarian carcinomas), PC-3M (metastatic prostate carcinoma), MCF-7 (breast carcinoma), and SF-268 (glioblastoma) and PBMC as well. The cells treated with *C. d. cascavella* venom triggered the apoptotic pathway as confirmed by several methods. These results underline the biomedical potential of toxins from the venom from this species.

Keywords: apoptosis, *Crotalus durissus cascavella*, cytotoxicity, tumor cells, snake venom.

TOXICIDADE DO VENENO DE *Crotalus durissus cascavella* EM CÉLULAS DE MAMÍFEROS**RESUMO**

Serpentes do Gênero *Crotalus* ganhou espaço significativo no campo científico, desde o desenvolvimento de pesquisas com seu veneno envolvendo atividade citotóxica. A *Crotalus durissus cascavella* (*C.d.cascavella*) é uma subespécie característica da Caatinga do Nordeste do Brasil e faltam estudos sobre as propriedades de seu veneno. O presente estudo avaliou o veneno bruto de *C. d. cascavella* para a sua atividade citotóxica *in vitro* contra as linhagens de células tumorais e células normais. O mecanismo de morte celular induzida por este veneno também foi investigado. Observou-se alta citotoxicidade (valores de IC₅₀ entre 2,7-6,9 µg/mL) contra cinco linhagens de células tumorais, dentre elas: OVCAR - 8 e SKOV3 (carcinoma ovariano), PC-3M (carcinoma metastático de próstata), MCF-7 (carcinoma mamário), SF - 268 (glioblastoma) e PBMC. As células tratadas dispararam a cascata apoptótica, como confirmado por vários métodos demonstrados neste trabalho. Esses

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resultados reforçam, assim, o potencial biomédico e farmacológico das toxinas do veneno dessa espécie.

Palavras-chave: apoptose, *Crotalus durissus cascavella*, citotoxicidade, células tumorais, veneno seperente.

TOXICIDAD DEL VENENO DE *Crotalus durissus cascavella* EN CÉLULAS DE MAMÍFEROS

RESUMEN

Las serpientes del género *Crotalus* ganaron espacio significativo en el campo científico desde el desarrollo de investigaciones con su veneno que involucran la actividad citotóxica. *Crotalus durissus cascavella* es la única subespecie grabadas del bioma de la Caatinga del nordeste de Brasil y los estudios carecen en sus propiedades del veneno. El presente estudio evaluó el veneno crudo de *C. d. cascavella* por su actividad citotóxica *in vitro* contra líneas celulares tumorales y las células normales. El mecanismo de la muerte celular inducida por este veneno también se investigó. *C. d. cascavella* mostró una alta citotoxicidad (valores de IC50 que oscila desde 2,7 hasta 6,9 g/mL) contra cinco líneas de células tumorales, entre las cuales: OVCAR - 8 y SKOV3 (carcinoma de ovario), PC-3M (carcinoma metastásico de próstata), MCF-7 (carcinoma de mama), SF - 268 (glioblastoma) y PBMC también. Las células tratadas con *C. d. cascavella* disparó la cascada apoptótica, según lo confirmado por diversos métodos que se muestran en este trabajo. Estos resultados refuerzan así el potencial biomédico y farmacológico de las toxinas del veneno de esta especie.

Palabras clave: apoptosis, *Crotalus durissus cascavella*, citotoxicidad, células tumorales, veneno de serpiente.

INTRODUCTION

Venoms from snakes of the genus *Crotalus* gained significant interest due to their unique effects. This genus occurs in almost all countries of South America (except Chile and Ecuador). In Brazil, so far, there is only one specie of rattlesnake (cascavel) recognized, *Crotalus durissus* [Linnaeus, 1758] (1). However, according to the list of reptiles of Brazilian Society of Herpetology (SBH), six subspecies are considered: *C. d. cascavella*, *C. d. collilineatus*, *C. d. dryinas*, *C. d. marajoensis*, *C. d. ruruima*, and *C. d. terrificus* (2). *C. d. cascavella* is the only subspecies recorded for the Caatinga biome of Northeastern Brazil (1,3).

Most pharmacological properties of snake venoms are determined by the presence of specific and biologically active substances, and approximately 95% of the dry weight of snake venoms is composed by proteins, which are responsible for almost all of the biological effects attributed to snake venoms (4,5). The venom of *Crotalus* presents itself as a complex-toxic enzyme, in which are found phosphodiesterase enzymes, amino oxidase I, 5-nucleotidase and toxins, such as, crotoxin, convulxin, crotamine and gyroxin (6). The crotoxin is considered the major component of the venom from this genus, and depending on the snake subspecies, differences in the protein isoform may be found, as well as differences in their activities (6).

Only few studies have been conducted by testing the crude venom and some isolated fractions from *C. durissus* species in mammalian cell lines, to access their toxicity and related antitumor potential (7,8). For *C. d. cascavella*, subspecies typical from the northeast of Brazil, until the present date there are no studies about the cytotoxic properties from its venom.

Therefore, the aim of the present study was to analyze the venom from *C. d. cascavella* for its cytotoxic activity against tumor cell lines and normal cells, as well as, its related mechanism of cell death induction.

MATERIAL AND METHODS

Venom of Crotalus durissus cascavella Wagler, 1824

The venom from the snake *Crotalus durissus cascavella* Wagler, 1824 was extracted in the Regional Center of Ophiology of Federal University of Ceará (NUROF-UFC), a scientific breeding which maintains species of venomous and non-venomous snakes (Registry CTF-IBAMA No. 480572). For this study, the specimens used were from the State of Ceará, with taxonomic identification of subspecies adequately confirmed.

Cell lines

The cytotoxic activity of the *C. d. cascavella* venom was tested against both tumor cell lines and primary culture of peripheral blood mononuclear cells (PBMC). The tested tumor cell lines were OVCAR-8 and SKOV3 (ovarian carcinoma), PC-3M (metastatic prostate carcinoma), MCF-7 (breast carcinoma), and SF-268 (glioblastoma). All these cell lines were kindly donated by the National Cancer Institute U.S. (Bethesda, MD). Cells were maintained in RPMI 1640 medium (Gibco® Invitrogen, Carlsbad, CA/USA) supplemented with 2% glutamine containing 10% fetal bovine serum (Gibco® Invitrogen, Carlsbad, CA/USA), 100 µg/mL streptomycin and 100 U/mL penicillin (Gibco® Invitrogen, Carlsbad, CA/USA) and incubated at 37 °C under a 5% CO₂ atmosphere. Cells were regularly split to keep them in a logarithmic phase of growth.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood from healthy, non-smoking donors who had not taken any drugs for at least 15 days prior to sampling through a standard method of density-gradient centrifugation over Ficoll-Hypaque (Sigma Aldrich Co. St. Louis, MO/USA). PBMC were washed and re-suspended at a concentration of 3×10^5 cells/ml in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. At the beginning of the experiment, 4% phytohemagglutinin (Gibco® Invitrogen, Carlsbad, CA/USA) was added to stimulate cell growth.

Cytotoxicity assay

Cytotoxicity was determined using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma Aldrich Co., St. Louis, MO) assay (9). For that, cells were seeded (4×10^4 cells/mL for tumor cells and 3×10^5 cells/ml for PBMC) in 96-well plates 24 h prior to treatment with the venom (0.19 - 25 µg/mL) or vehicle control (saline) for 72 h. Three hours before the end of the incubation, medium was replaced by 150 µL of 0.5 mg/mL MTT (Sigma Aldrich Co. St. Louis, MO/USA) of each well. Then the formazan product was dissolved by replacing the later solution for 150 µL of DMSO and absorbance was measured at 595 nm using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, CA).

Hemolytic activity

A total of 3 Swiss female mice (*Mus musculus*) were obtained from the central animal house of Universidade Federal do Ceará, Brazil. The animals were kept under a 12:12 h light-dark cycle with food and water *ad libitum*. Animals were treated according to the ethical principles for animal experimentation of CONCEA (Conselho Nacional de Controle de

Experimentação Animal) and CEUA (Comitê de Ética no Uso de Animais). The test was performed in 96-well plates using a 2% mouse erythrocytes suspension in 0.85% NaCl containing 10 mM CaCl₂, following the method described by Jimenez et al. (10). The crude venom was tested at concentrations ranging from 3.9 µg/mL to 250.0 µg/mL. After incubation for 1h, 2h or 4h at room temperature the cells were spun down and the supernatant was transferred to another plate to measure the hemoglobin released at 540 nm using a multiplate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc. Fullerton, CA/USA).

Analysis of mechanisms involved in the cytotoxic activity of C. d. cascavella venom

OVCAR-8 cell line was chosen to perform experiments to investigate the mechanism of action of *C. d. cascavella* venom. Briefly, the cells were seeded at a density of 4x10⁴ cells/mL and treated with the venom at concentrations of 1, 2 and 4 µg/mL during 24h. The vehicle (0.85% NaCl) was used as negative control and paclitaxel at 0.05 µM (Sigma Aldrich Co. St. Louis, MO/USA) or doxorubicin at 0.5 µM (Sigma Aldrich Co. St. Louis, MO/USA) were used as positive controls.

Morphological changes analysis

To evaluate cell morphology, after the incubation period, cells were harvested, transferred to cytospin slides, fixed with methanol for 1 min and stained with hematoxylin and eosin. The morphological characteristics from untreated and treated cells were observed using a light microscope (Motic BA310, Moticam 2000, Causeway Bay, Hong Kong). In order to investigate nuclear alterations, the cells were incubated with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) Life Technologies). Briefly, after the incubation period, cells were fixed with 3.7 % formaldehyde and incubated with DAPI (5 µg/mL). After extensive washing with PBS, images were captured by confocal microscopy (LSM 710 Zeiss) excitation wave length 350 nm.

Flow cytometry analysis

Five thousand events were analyzed for each replicate in three independent experiments and cellular debris was omitted from the analysis. OVCAR-8 cells fluorescence was then determined by flow cytometry in a Guava EasyCyte Mini System cytometer using the CytoSoft 4.1 software (Guava Technologies, Hayward, CA/USA). Five thousand events were acquired on a gated region to exclude debris from the analysis.

Cell membrane integrity

The cell membrane integrity was evaluated using propidium iodide dye (Sigma Aldrich Co., St. Louis, MO/USA). Briefly, 100 µL suspension of treated and untreated OVCAR-8 cells were incubated with 5 µg/mL propidium iodide for 10 min. Fluorescence was measured and analyzed by cell number and membrane integrity.

Internucleosomal DNA fragmentation

DNA fragmentation and cell cycle distribution were analyzed by flow cytometry after DNA staining with propidium iodide. Briefly, 100 µL of treated and untreated OVCAR-8 cells were incubated for 30 min, in the dark, with a solution containing 5 µg/mL propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Fluorescence was measured and DNA fragmentation and cell cycle distribution were analyzed.

Caspase activation

Treated and untreated cells were incubated with Fluorescent Labeled Inhibitor of Caspases (FLICA) 3 and 7 (Guava® Caspase 3/7 FAM kit, EMD Millipore Corporation

Billerica, MA/USA) for 1h at 37° C under 5% CO₂ atmosphere. After washing with PBS, the cells were resuspended in the working solution (7-AAD 1:200 in 1× washing buffer) and analyzed immediately using flow cytometry.

Statistical analysis

For the MTT assay the inhibition concentration mean (IC₅₀) values of three independent experiments were determined by nonlinear regression using the software GraphPad® Prism 5.0 (Intuitive Software for Science, San Diego, CA). The data from the flow cytometry analysis are presented as mean values ± S.E.M. of three independent experiments performed in triplicate. *p<0.05 compared to the negative control by ANOVA followed by Dunnett's test.

Ethical aspects

This project was submitted to the Ethics Committee on Animal Research at the State University of Ceará (number of the submission process: 11516651-3). All care were taken to preserve the health of the researchers and other professionals involved in this work and the environment in the areas where it conducts the research project.

RESULTS

Cytotoxicity assay

The cytotoxicity of the venom was assessed against five tumor cell lines of different histotypes and PBMC using the MTT assay [9]. As shown in Table 1, the venom did not presented any selectivity, and its IC₅₀ values ranged from 1.5 µg/mL in PBMC to 6.9 µg/mL in SKOV3 cells, respectively. The lack of selectivity could imply in membrane damaging effects.

Table 1. Cytotoxic activity of *C. d. cascavella* venom on mammalian cells.

	Cell lines				
	OVCAR-8	PC-3M	MCF-7	SF-268	SKOV3
Venom	2.7 (2.6 – 2.9)	2.4 (2.2 – 2.6)	4.9 (4.2 – 5.8)	4.1 (3.5 – 4.7)	6.9 (5.9 – 8.0)
Doxorubicin	0.3 (0.1 – 0.4)	0.8 (0,7 – 0.9)	0.4 (0.3 – 0.6)	0.6 (0.4 – 0.9)	n.d.

Data are presented as IC₅₀ values in µg/mL along with its respective confidence interval of 95% obtained by nonlinear regression from two independent experiments, performed in duplicate, after 72 h of incubation. n.d.= not determined.

Hemolytic activity

The analysis of hemolytic effects demonstrated that the venom did not cause any significant membrane damage to the erythrocytes (data not shown), suggesting that cytotoxicity should be related to more specific cellular pathways.

Morphological changes analysis

In a second set of experiments, *C.d. cascavella* venom apoptosis-inducing properties were assessed in OVCAR-8 ovarian carcinoma cells. This cell lineage was chosen due to the

preliminary results, which showed in this particular lineage, the venom presented better activity.

Treated cells displayed apoptotic morphological features such as reduction in cell volume, nuclear fragmentation and formation of apoptotic bodies (Fig 1A-1D). At the highest tested concentration (4 $\mu\text{g/mL}$), the effects of the venom were more intense and characteristics of late apoptosis or necrosis could also be seen (Fig 1D). Negative control cells stained with DAPI showed a round nucleus, typical of interphase, while in doxorubicin treated cells the nucleus appears to be already fragmented. At the lowest concentration tested (1 $\mu\text{g/mL}$), the nucleus of the cells looks similar to the negative control cells (data not shown), being considered viable, and as the concentration increased, the number of cells with DNA fragmentation increased (Fig 1G and 1H).

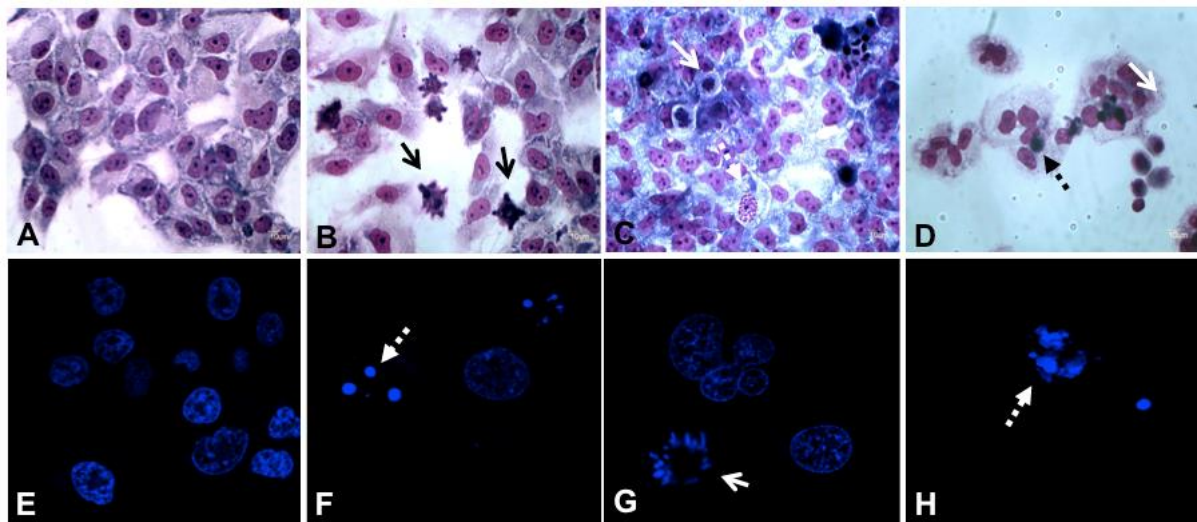


Figure 1. OVCAR-8 cells treated with *C. d. cascavella* venom show apoptosis characteristics. Control cells were incubated during 24 h without treatment (A and E); or treated with doxorubicin 0.5 μM (B and F); or treated with *C. d. cascavella* venom at 2 $\mu\text{g/mL}$ (C and G) or at 4 $\mu\text{g/mL}$ (D and H). Arrows: black, cell membrane blebbing; black with dashed line, pyknosis; white, atypical mitosis; white with dashed line, nuclear fragmentation. 400X

Cell membrane integrity

After observing the morphological features, we then decided to analyze some cellular and biochemical events to assess the mechanisms involved in cell death induced by *C. d. cascavella* venom. The antiproliferative activity of the venom was confirmed by flow cytometry (Fig 2A), with a significant reduction of cell number at concentration of 4 $\mu\text{g/mL}$. The inhibition of cell proliferation can be associated with loss of cell membrane integrity and at 4 $\mu\text{g/mL}$, 36 % of treated cells presented membrane damage (Fig 2B), corroborating data from morphological analysis.

Internucleosomal DNA fragmentation

The analysis of DNA content through flow cytometry showed an increasing number of subdiploid population, indicating DNA fragmentation (Fig. 2C), while the distribution of cell cycle profile was not affected by *C. d. cascavella* venom (data not shown).

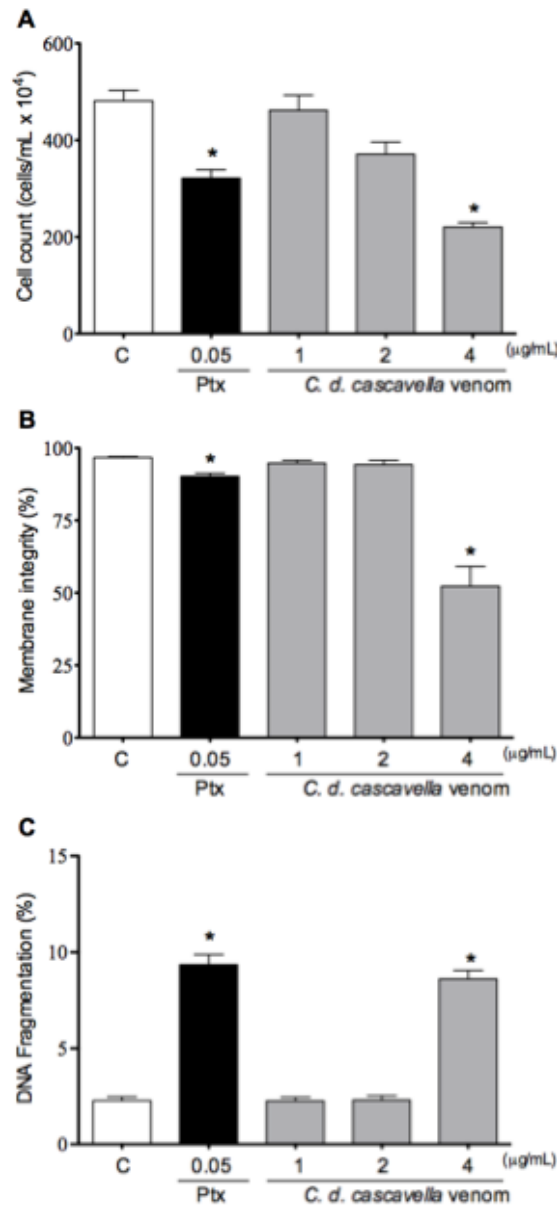


Figure 2. *C. d. cascavella* venom reduces cell proliferation accomplished by membrane damage and DNA fragmentation. Effect of *C. d. cascavella* venom on OVCAR-8 cell counting (A), membrane integrity (B) and DNA fragmentation (C) determined by flow cytometry, using propidium iodide after 24 h incubation. The negative control was treated with the vehicle saline (C). Paclitaxel (Ptx) at 0.05 μ M was used as the positive control. Data are presented as mean values \pm S.E.M. of three independent experiments performed in triplicate. * $p < 0.05$ compared to the negative control by ANOVA followed by Dunnett's test.

Caspase activation

The apoptosis induction by *C. d. cascavella* venom was later confirmed through the activation of caspases 3 and 7 (Fig. 3). At the concentration of 4 μ g/mL, 32 % of the cells showed caspases activation. The caspases are one of the key executors of apoptotic process, generally being activated by several anti-tumoral drugs. This activation involves mainly two signaling pathways, one is the mitochondrial and the other is the cell death receptor pathway, which activate caspases 9 and 8 respectively.

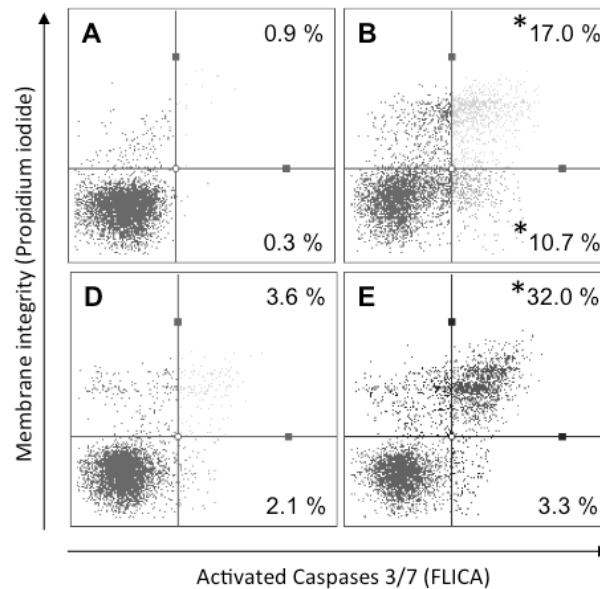


Figure 3. *C. d. cascavella* venom activates caspases 3 and 7. Caspases activation was determined by flow cytometry after 24 h incubation. **A**, Control; **B**, Doxorubicin 0.5 μ M; **C**, *C. d. cascavella* venom 2 μ g/mL; **D**, *C. d. cascavella* venom 4.0 μ g/mL. Percentage of cells in early and late apoptosis are indicated. Data are presented as mean values \pm S.E.M. of three independent experiments performed in triplicate. *p<0.05 compared to the negative control by ANOVA followed by Dunnett's test.

DISCUSSION

Snake venoms are complex, being composed of a mixture of toxins, enzymes, growth factors and other proteins with several biological properties (11,12). The knowledge of snake venoms composition and activities have emerged as key factor to characterize envenomation and drawn the best therapeutic approach. Additionally, snake toxins have been considered a starting point in the development of highly specific biotechnological tools to treat diseases (13-15). In such context, cellular systems could be useful to rapidly evaluate direct effects on membrane integrity or cellular pathways to allow the understanding of venom local effects and possible biotechnological applications.

In fact, the major differences in *Bothrops* and *Crotalus* envenomation are related to the severity of local effects in the former, and major systemic myotoxicity and neurotoxicity in the later (16). The presence of hemolytic toxins in *Bothrops* venom has previously been reported (17), while *Crotalus* venom, generally, does not present a significant hemolytic activity (18).

The effects of snake venoms in tumor cells have been described in the literature (19-21). For *Crotalus* venom, Tamietti et al. (8) showed *C. d. terrificus* venom cytotoxic effects in CHO-K1 hamster ovarian carcinoma cells and suggested apoptosis induction as the main mechanism. Further, Soares et al. (7) confirmed apoptosis-inducing effects for *C. d. terrificus* venom in RT-2 glioblastoma cells (RT2) and GH3 benign pituitary adenoma cells. Crotoxin, the main component of *Crotalus* venom, have been described as the active principle against tumor cells (22), although the venom seemed to be much more active than crotoxin itself, suggesting the presence of other active cytotoxins.

Cell membrane integrity serves as a parameter of the viability of cells and at the initial stages of apoptosis. The cell shrinks while its membrane remains intact, however both cells undergoing late apoptosis or necrosis loose membrane integrity (23).

In order to differentiate wich pathway was responsible for the cells death, if the apoptotic or the necrotic pathway, the caspase assay would provide better evidences. Both

caspases may end up activating pathway 3 and 7 (24,25). Yan and collaborators (26) had already shown that crotoxin induces caspase 3 activation, that is accordance with our results.

In summary, it was showed that *C.d. cascavella* venom is cytotoxic against mammalian cells, causing cell death by apoptosis induction. Moreover, this study represents the first report on the biological activity of the venom from *C.d. cascavella* and highlights the biomedical importance of this species. Further studies are necessary to elucidate the exact mechanism involved in the cytotoxicity of *C. d. cascavella* venom as well as to identify the active components.

CONFLICT OF INTEREST

The authors of this article declare that there is no potential conflicts of interest including employment, consultancies, stock ownership, honoraria, paid expert testimony and patent applications/registrations related to the current manuscript.

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