RESEARCH ARTICLE

In vitro anticancer effect of venom from Cuban scorpion *Rhopalurus junceus* against a panel of human cancer cell lines

Alexis Díaz-García[α]*, Luis Morier-Díaz[β], Yahima Frión-Herrera[¥] Hermis Rodríguez-Sánchez[β], Yamira Caballero-Lorenzo[β], Dianeya Mendoza-Llanes[β], Yanelis Riquenes-Garlobo[α] and José A Fraga-Castro[α]

[α]Laboratories of Biopharmaceuticals and Chemistry Productions (LABIOFAM), Havana, Cuba, [β]Microbiology Department, Cell culture Laboratory, Tropical Medicine Institute "Pedro Kouri", Havana, Cuba, [¥]Food and Pharmacy Institute, Havana University, Cuba.

*Correspondence to: Alexis Díaz-García, Email: alexisdg@infomed.sld.cu, Tel: +537 6830326 or +537 6849662, Fax: +537 6830326

ABSTRACT

In Cuba the endemic species of scorpion *Rhopalurus junceus* has been used in traditional medicine for cancer treatment. However, there is little scientific evidence about its potential in cancer therapy. The effect of a range of scorpion venom concentrations (0.1, 0.25, 0.5, 0.75 and 1mg/ml) against a panel of human tumor cell lines from epithelial (Hela, SiHa, Hep-2, NCI-H292, A549, MDA-MB-231, MDA-MB-468, HT-29), hematopoietic origins (U937, K562, Raji) and normal cells (MRC-5, MDCK, Vero) was determined by the MTT assay. Additionally, the effect of venom on tumor cell death was assayed by Fluorescence microscopy, RT-PCR and western blot. Only the epithelial cancer cells showed significant cell viability reduction, with medium cytotoxic concentration (IC_{50}) ranging from 0.6-1mg/ml, in a concentration-dependent manner. There was no effect on either normal or hematopoietic tumor cells. Scorpion venom demonstrated to induce apoptosis in less sensitive tumor cells (Hela) as evidenced by chromatin condensation, over expression of *p53* and *bax* mRNA, down expression of *bcl-2* mRNA and increase of activated caspases 3, 8, 9. In most sensitive tumor cells (A549), scorpion venom induced necrosis evidenced by acridine orange/ethidium bromide fluorescent dyes and down-expression of apoptosis-related genes. We concluded the scorpion venom from *R. junceus* possessed a selective and differential toxicity against epithelial cancer cells. This is the first report related to biological effect of *R. junceus* venom against a panel of tumor cells lines. All these results make *R. junceus* venom as a promise natural product for cancer treatment.

KEYWORDS: *Rhopalurus junceus*, scorpion venom, cancer cells, apoptosis, necrosis

INTRODUCTION

Cancer is one of the leading causes of death around the world. Cancer is a complex multifactorial disease, which is dependent on cellular accumulation of various genetic and epigenetic events (Blagosklonny, 2005). Specifically, solid tumors are the most common worldwide with higher mortality rate. Radiotherapy, chemotherapy and surgical are the key tools for cancer treatment (Fritz et al, 2013).

However, these treatments also cause severe systemic side effects. For this reason, several therapeutic approaches have been developed to overcome the complexities of different cancers (Meiyanto et al, 2012). Among them, searching and discovering new drugs against cancer, especially derived from the natural products, is increasing throughout the world. One of them, the scorpion venom, is now considered an interesting natural source for cancer therapy (Gomes et al, 2010).

The use of scorpion venom from species *Leiurus quinquestriatus* and *Buthus martensii* Karsh (BMK) as a potential natural product for cancer treatment has been shown previously (Xiao, 1990; Debin et al, 1993). BMK scorpion and its venom have been used as a traditional and folk therapy for cancer treatment and others pathophysiological conditions (Goudet et al, 2002). Additionally, Das Gupta and colleagues established the cytotoxic activity of Indian black scorpion (*Heterometrus bengalensis*) venom on human leukemic U937 and K562 cells (Das Gupta et al, 2007).

The scorpion *Rhopalurus junceus (R. junceus)* is an endemic species from Cuba belonging to *Buthidae* family. This scorpion is widespread and there is no report of scorpionism from this or other species in the country. For this reason, they are not considered dangerous to humans. For a long time, venom from *R. junceus* has been used in Cuban traditional medicine for treatment of some illnesses, including cancer, and has shown beneficial effects for some people. However, there is scarce scientific evidence about the biological activity and spectrum of action of this scorpion venom against cancer cells. Thus, we evaluated the anticancer effect of *R. junceus* scorpion venom on a panel of cancer cell lines from different histological origins including normal cells.

MATERIALS AND METHODS

Reagents
RPMI-1640 and Dulbecco's modified Eagle's medium were purchased from GIBCO/BRL (Caithershurg, MD). Fetal bovine serum (FBS) was purchased from Hyclone. TRIzol reagent was obtained from Invitrogen (Invitrogen, USA). dNTPs, GoTaq DNA polymerase and M-MLV reverse transcriptase system were purchased from Promega (Promega Inc, USA). The 3-[4,5-dimethylth-iazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) reagent was purchased from Sigma. All of other chemicals and reagents were obtained from Sigma (St Louis, MO).

Venom source
Adults *Rhopalurus junceus* scorpions were maintained in individual plastic cages in laboratories belonging to The Entrepreneurial Group of Biopharmaceuticals and Chemistries Production (LABIOFAM). Venom from scorpions kept alive in the laboratory was extracted by electrical stimulation. Venom was dissolved in distilled water and centrifuged at 15000x*g* for 15min. The supernatant was filtered by using a 0.2μm syringe filter and stored at -20ºC until used. The protein concentration was calculated by the Lowry modified method (Herrera et al, 1999).

Cell lines and culture
The human cancer cell lines used in the experiments were obtained from ATCC culture collection. Cell lines used included epithelial cell lines Hela (**cervix** adenocarcinoma ATCC CCL-2™), SiHa (cervix squamous cell carcinoma grade II ATCC HTB-35™), NCI-H292 (mucoepidermoid pulmonary carcinoma ATCC CRL-1848™), A549 (lung carcinoma ATCC CCL-185™), Hep-2 (larynx carcinoma ATCC CCL-23™), MDA-MB-468 (mammary gland adenocarcinoma ATCC HTB-132™), MDA-MB-231(mammary gland adenocarcinoma ATCC HTB-26) and HT-29 (colorectal adenocarcinoma ATCC HTB-38™); hematopoietic cancer U937 (histiocytic lymphoma ATCC CRL-1593.2™), K562 (chronic myelogenous leukemia ATCC CCL-243™) and Raji (Burkitt's lymphoma ATCC CCL-86™) cell lines. Besides were used the MRC-5 (normal human lung fibroblast ATCC CCL-171™); MDCK (normal canine kidney ATCC CCL-34™) and Vero (normal african green monkey kidney ATCC CRL-1586™) cell lines. The cells Hela, SiHa and Hep-2, were maintained in Eagle's Minimum Essential Medium in Earle's BSS with non-essential amino acids, 90% (w/v) and heat inactivated fetal bovine serum (FBS), 10% (v/v), penicillin (100U/ml), and streptomycin (100μg/ml). The cells NCI-H292, A549, MDA-MB-231, MDA-MB-468, HT-29, Vero and MDCK were maintained in Dulbecco's modified Eagle's medium, 90% (w/v) with heat inactivated fetal bovine serum (FBS), 10% (v/v), penicillin (100U/ml), and streptomycin (100μg/ml). The MRC-5 cell line was maintained in RPMI-1640 supplemented with 10% (v/v) FBS, penicillin (100U/ml), and streptomycin (100μg/ml).

In vitro cell viability assay (MTT assay)
The effect of scorpion venom on cell viability was determined by the MTT assay (Mosmann, 1983). SiHa Cells ($5x10^3$/well) and the remaining cell lines ($1x10^4$/well) were plated in 50μl of medium/well in 96-well culture plates (Costar Corning, Rochester, NY) and incubated overnight to recovery and cell adhesion in a humidified atmosphere of 5% (v/v) CO_2 at 37ºC. After incubation, 50μl of different scorpion venom amounts dissolved in medium were added at final concentration of venom at 0.1, 0.25, 0.5, 0.75 and 1mg/ml in each well. Cells with culture medium and without scorpion venom were used as untreated growth control. Five wells were included in each concentration. After treatment for 72hr, 10μl of 5mg/ml of sterile MTT (pH 4.7) was added per well and cultivated for another 3hr. The supernatant was carefully removed, 150μl DMSO was added per well and shaken for 15min at 37ºC. The absorbance was measured with a microplate reader (ELISA MRX Revelation Dynex Technologies 560nm with 630nm as reference). Absorbance from untreated cells was considered as 100% of growth and used for viability calculation. The effect of scorpion venom on the viability for human cell lines panel was expressed as the % viability, using the formula: %viability= A_{560} of treated cells/A_{560} of control cells x 100%. The IC_{50} values (venom concentration that causes 50% reduction of cell) from cancer cells were determined. The experiments were performed five times.

ISOLATION OF TOTAL RNA AND RT-PCR ANALYSIS OF *P53*, *BAX* AND *BCL-2* GENES EXPRESSION IN HELA AND A549 CELLS

Hela and A549 cells (2×10^5/well) seeded on 24-well plate were cultured for 24hr. The concentration of scorpion venom used with fresh medium was 0.75mg/ml and triplicate cell cultures wells were exposed included vehicle (control cells). Treated and control cell cultures were incubated for a further 8hr, 24hr and 48hr. At the end of the incubation period cells were harvested and used for total RNA extraction and reverse transcription PCR (RT- PCR).

Total RNA was isolated from cells, using TRIzol reagent according to the manufacturer's specifications (Invitrogen, USA). Total RNA concentration in the final elutes

was determined by using a Biophotometer plus (Eppendorf, Germany). Each sample of isolated RNA (1μg) was reverse transcribed by M-MLV reverse transcriptase system (Promega Inc. USA) in a 50μl volume reaction. Each PCR was carried out in a master mix containing 1X Green Go Taq Flexi Buffer, 2mM $MgCl_2$, 10mM dNTPs and 1.25U GoTaq DNA polymerase (Promega Inc. US) with 0.2mM of respective forward and reverse primers and 5μl of DNA_C in 25μl reaction mix. The PCR amplification was carried out in a Thermal cycler (AUXILAB, Spain). GAPDH (glyceraldehydes 3-phosphate dehydrogenase) amplification was performed as a housekeeping gene. The primer sequence for RT-PCR were 5´-ACACCCACTCCTCCAC-CTTT-3´ and 5´-TAGCCAAATTCGTTGTCATACC-3´ for GAPDH; 5´-GGGTTAGTTTACAATCAGCCACATT-3´ and 5´-GGCCTTGAAGTTAGAGAAAATTCA-3´for p53; 5´-GGACGAACTGGACAGTAACATGG-3´ and 5´-GCAAAGTAGAAAAGGGCGACAAC-3´ for bax; and 5´-CAGGTCCTCTCAGAGGCAGATAC-3´ and 5´-CCTCTCCAGGGACCTTAACG-3´ for bcl-2. PCR conditions for GAPDH were 30 cycles at 94ºC for 30sec, at 54ºC for 30sec at 72ºC for 1min. PCR conditions for Bcl-2 were 30 cycles at 94ºC for 1.5min, at 56ºC for 30sec at 72ºC for 1min. PCR conditions for Bax were 35 cycles at 94ºC for 1.5min, at 55ºC for 30sec, at 72ºC for 1min. PCR conditions for p53 were 30 cycles at 94ºC for 1.5min, at 56ºC for 30sec at 72ºC for 1min. Amplified PCR products were subjected to electrophoresis at 70V in 1.5% (w/v) agarose gel for 1.5hr. A 50 bp DNA ladder marker was used as a molecular marker. Gels were visualized with ethidium bromide in 1xTBE (Tris-borate-EDTA) buffer. The gels were examined and the intensity of each band was measured by using Gel Doc imaging system UVIsave D-55/20M version 15.08 (UVItec, England). Size of the amplified PCR products were 120bp for GAPDH, 220bp for Bcl-2, 150bp for Bax and 130bp for p53.

Morphological assessment and measurement of apoptotic and necrotic cells

To investigate the cell death event, Hela and A549 cells (2×10^5/well) were grown in 24 well-culture plates overnight and treated for 48hr with 0.75mg/ml of scorpion venom. At this periods, cells were incubated with a mixture of acridine orange and ethidium bromide in PBS (100μg/ml acridine orange/100μg/ml ethidium bromide). The nuclei of living cells were stained by the membrane-permanent dye acridine orange, while necrotic cells were stained by the highly fluorescent ethidium bromide. Following the addition of fluorochromes, 200 cells were analyzed and counted in each of three independent experiments using fluorescence microscopy IX-71 (Olympus, Japan) at 480nm and 520nm filters. Additionally, scorpion venom-treated and non-treated cells were stained for 5min with 4´, 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (1μg/ml) to identify apoptotic bodies.

Determination of Caspase 3, 8 and 9 activity by western blotting

Hela cells (2×10^5/well) were plated in 24-well culture plates and treated with 0.75mg/ml scorpion venom. Untreated control cells and scorpion venom treated-cells were then incubated for 24hr and 48hr. After these time, cells were incubated in lysis buffer containing 50mM Tris-HCl, 150mM NaCl, 1mM ethylene glycol tetra-acetic acid (EGTA), 1mM EDTA, 20mM NaF, 100mM Na_3VO_4, 1% (v/v) NP-40, 1% (v/v) Triton X-100, 1mM phenylmethylsulfonyl fluoride (PMSF), 10μg/ml Aprotinin and 10μg/ml Leupeptin on ice for 30min to lyses the cells. After centrifugation, total protein was determined using the method of Lowry modified by Herrera et al. (Herrera et al, 1999). Protein was resolved (50μg) by 12% (w/v) SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membranes. The membranes were blocked in 5% (w/v) skim milk dissolved in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween (PBS-Tween), pH7.5 for 1hr at room temperature. After washing steps, membranes were probed with an appropriate primary antibody for caspase 3, 8 and 9 in blocking buffer for additional 2hr at room temperature. Finally, membranes were incubated with the respective secondary antibodies for 1hr and was detected using the enhanced chemiluminescence blotting detection system.

Statistical analysis

The IC_{50} values were determined by interpolation of tendency line from linear regression curve and they were compared using Mann-whitney U test. In case this parameter could not be obtained it means no effect and ¨NE¨ (No Effect) was stated. Analysis of main cell death event was made using Mann-whitney U test. Band intensity of each gene from scorpion venom-treated and non-treated cells were compared using Mann-whitney U test. For all analysis we used the GraphPad Prism version 5.01 for Windows, (GraphPad Software, San Diego California, USA).Significant differences were considered for $p<0.05$.

RESULTS

MTT assay and IC_{50} values

In order to explore the effect of the scorpion venom on cell viability, increased concentration were applied to a panel of human cell lines. The result evidenced a significant reduction of cell viability against epithelial cancer cell lines (carcinomas and adenocarcinomas), when compared to respective control cells ($p<0.05$) after 72hr of treatment (Figure 1A and 1B).

Significant reduction of cancer cell viability ($p<0.05$) was obtained between successive venom concentration used in the study showing the growth inhibition was in concentration-dependent manner. On contrary, *R. junceus* scorpion venom do not affect the viability in normal neither hematopoietic cell lines (Figure 1C and 1D) at the concentration used.

The IC_{50} parameter was used to measure the effect of scorpion venom on cell viability in human cell lines. Table I shows the IC_{50} value which permits the comparison from each individual cell lines. The IC_{50} showed that the venom exerts a selective viability reduction against epithelial cancer cell compare to normal and hematopoietic cells where no effect was observed (Table 1). A549 and NCI-H292 lung cancer correspond to cells with lowest IC_{50} of 0.63±0,04mg/ml and 0.68±0,01mg/ml, respectively (Table 1). While Hela and Siha represent the cell with the highest IC_{50} values from the panel with 1.05±0,02mg/ml and 0.91±0,01mg/ml, respectively (Table 1).
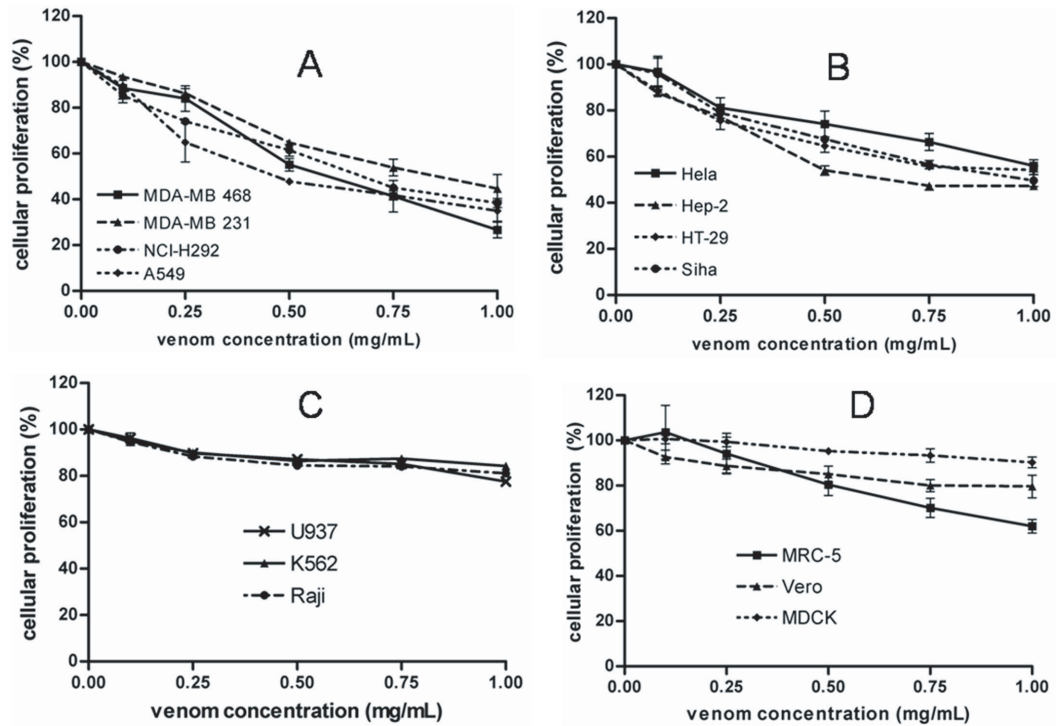
Figure 1. Graphics of cytotoxicity of scorpion venom against different cell lines. The cells were incubated for 72hr with scorpion venom at concentrations of 0.1-1mg/ml. The percentage of cell proliferation was measured by MTT cytotoxicity assay. A. Most sensitive human epithelial tumor cell lines. B. Less sensitive human epithelial tumor cell lines. C. Human hematopoietic tumor cell lines. D. Normal cell lines.

Table 1. IC_{50} values obtained after R. *junceus* scorpion venom treatment for 72hr in all panel cell line. ¨NE¨ means No Effect as described in Materials and Methods. Values represent the mean ±SD obtained from five independent experiments.

Cell line	IC_{50} (mg/ml)	Histological origin
A549	0.63 ± 0.04	Lung carcinoma
NCI-H292	0.68 ± 0.01	mucoepidermoid lung carcinoma
Hep-2	0.79 ± 0.03	larynx carcinoma
Hela	1.05 ± 0.02	cervix adenocarcinoma
Siha	0.91 ± 0.01	cervix squamous cell carcinoma
MDA-MB 231	0.7 ± 0.02	mammary gland adenocarcinoma
MDA-MB 468	0.64 ± 0.01	mammary gland adenocarcinoma
HT-29	0.89 ± 0.02	colorectal adenocarcinoma
Raji	NE	Burkitt's lymphoma
K562	NE	chronic myelogenous leukemia
U937	NE	histiocytic lymphoma
MRC-5	NE	normal human lung fibroblast
Vero	NE	normal african green monkey kidney
MDCK	NE	normal canine kidney

Effect of scorpion venom on apoptosis-related genes in Hela and A549 cells

For analysis of apoptosis cells were selected with the lowest and highest IC_{50} values from the panel which correspond to A549 and Hela. In this study, we examined the effect of scorpion venom concentration (0.75mg/ml), in Hela and A549 cells, on expression rates of apoptosis-related genes. The concentration of scorpion venom used represents a value lower than IC_{50} from Hela cells and higher than IC_{50} from A549 (Table 1). The expression levels of *p53, bax, bcl-2* mRNA and GAPDH as internal control, at 8hr, 24hr and 48hr of venom application are presented in Figure 2.

In Hela cells, we found that *p53* gene was significantly over-expressed from 8hr and increased (p<0.05) in a time-dependent manner until 48hr (p<0.001) of venom
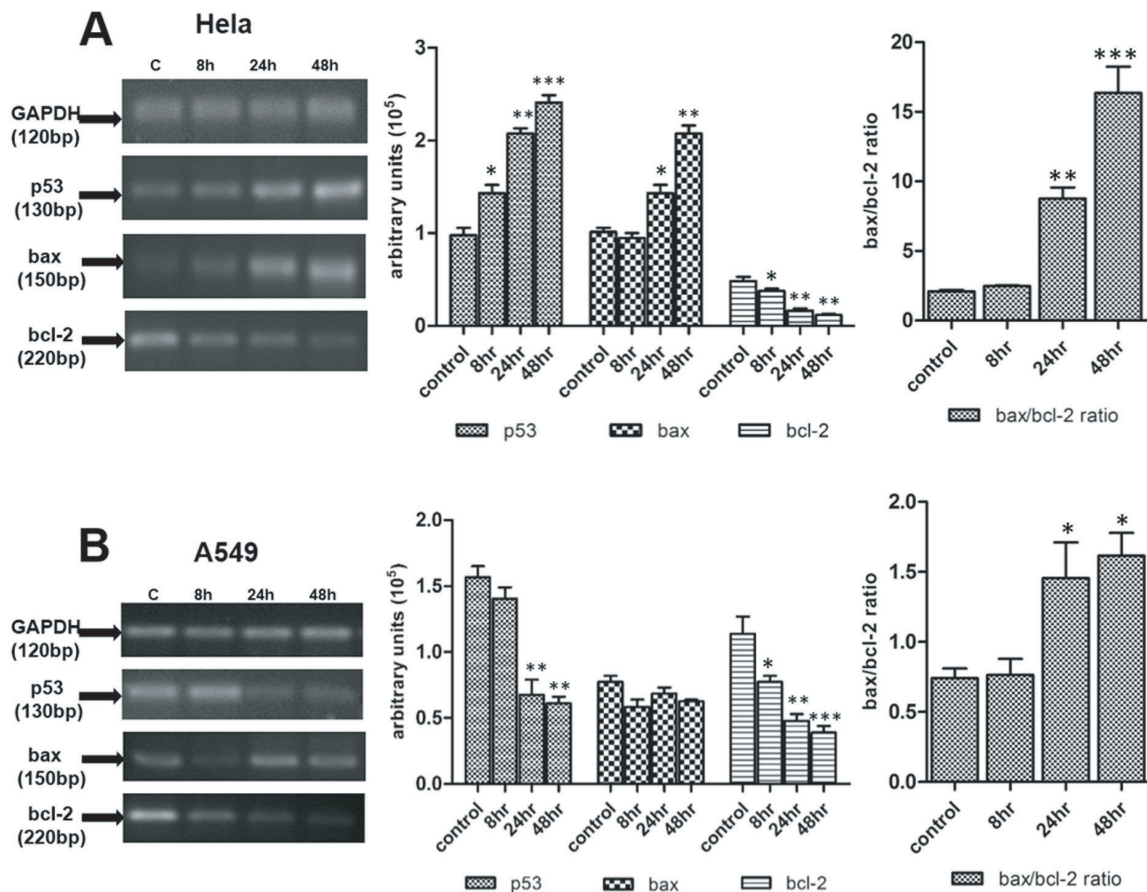
Figure 2. Effect of scorpion venom treatment on p53, bax and bcl-2 genes expression in Hela and A549 cells. A. p53, bax and bcl-2 mRNAs expression were detected by RT-PCR after 8hr, 24hr and 48hr. B. Relative signal intensities of p53, Bax, Bcl-2 and Bax/Bcl-2 ratio mRNAs expression levels compared with GAPDH. Values represent the mean+SD obtained from at least three independent experiments. The p values were obtained comparing the control group versus every group of treatment by Mann-Whitney U test. Significant differences *$p<0.05$, ** $p<0.01$, ***$p<0.001$.
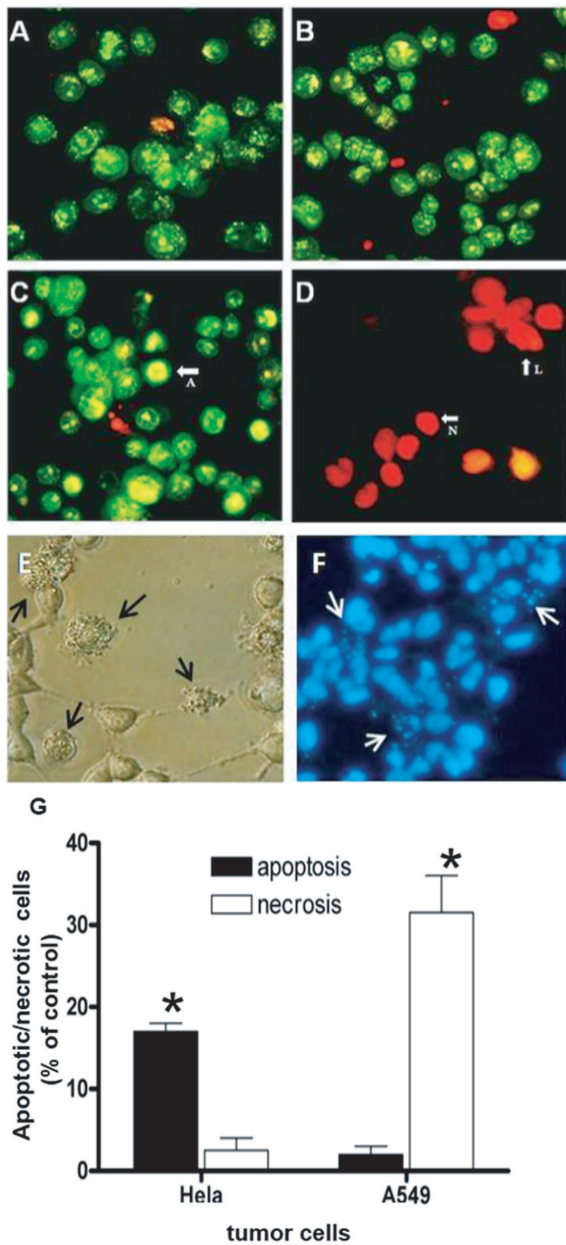
treatment compare to control without treatment (Figure 2A). The expression of *bax* gene increased significantly at 24hr ($p<0.05$) and 48hr ($p<0.01$) of scorpion venom treatment. On the contrary, *bcl-2* gene expression was significantly down-regulated from 8hr ($p<0.05$) to 24hr ($p<0.01$), as compared to untreated control (Figure 2A). Additional time points did not show a significant decrease of expression of *bcl-2* genes (Figure 2A). The Bax/Bcl-2 ratio was significantly increased between untreated control and scorpion venom-treated Hela cells for 24hr ($p<0.01$) and 48hr ($p<0.001$) (Figure 2A). The increase of Bax/Bcl-2 ratio was due to increase of *bax* mRNA and decrease of *bcl-2* mRNA expression.

In A549 cells, the expression of *p53* mRNA was significantly down-regulated after 24hr and 48hr of venom treatment compared to untreated controls ($p<0.01$) (Figure 2B). Additionally, *bcl-2* mRNA expression was also significantly down-regulated from 8hr ($p<0.05$) to 48hr ($p<0.001$) compared with untreated control (Figure 2B). Scorpion venom did not alter the *bax* mRNA expression level (Figure 2B). In A549 cells a significant increase of Bax/Bcl-2 ratio was found at 24hr and 48hr in the scorpion venom treated groups compared with controls ($p<0.05$). The increase of Bax/Bcl-2 ratio was entirely due to *bcl-2* mRNA decrease (Figure 2B).

Apoptosis/Necrosis analysis by fluorescence staining dyes in Hela and A549 cells

To determine whether the growth inhibitory activity of scorpion venom was related to the induction of apoptosis or necrosis, morphological assay of cell death was investigated using the AO/EB and DAPI staining. After Hela and A549 cells were exposed to scorpion venom for 48hr, different morphological features were observed. Nuclear morphology from control cells were seen uniformly green with normal morphology (Figure 3A and 3B).
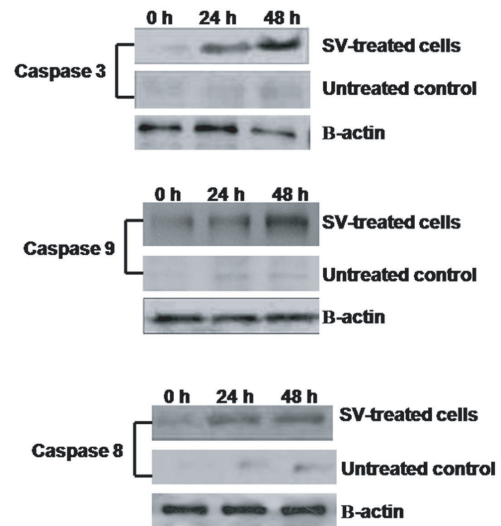
In scorpion venom-treated Hela cells morphological alterations like bright green early apoptotic cells with nuclear margination and chromatin condensation (Figure 3C) was observed. Evidences of induction of apoptosis in Hela included membrane blebbing (Figure 3E) and apoptotic bodies (Figure 3F). In this cells scorpion venom caused significant apoptosis over necrosis ($p<0.05$; apoptotic index 17%) (Figure 3G). In contrast, the most sensitive tumor cell line A549, displayed a high number of necrotic cells upon incubation with scorpion venom (Figure 3D). Orange/red stained cells were very prominent over bright green nucleus, indicating high necrotic cell death ($p<0.05$; necrotic index 32 %) (Figure 3G). The results suggest that scorpion venom is able to induce apoptosis or necrosis depending on the cancer cell type.

Figure 4. Representative western blot showing the expression of Caspase 3, 8 and 9 in untreated control and scorpion venom-treated Hela cells (sv-treated cell). Hela cells (2×10^5/ml) were treated with 0.75mg/ml scorpion venom for 24hr and 48hr then harvested for apoptosis-associated protein expressions estimated using western blotting as described in Materials and Methods.

Figure 3. Morphological evidence of apoptosis/necrosis in Hela and A549 cancer cells after 48hr of scorpion venom treatment. A. AO/EB stain in Hela untreated control cells. B. AO/EB stain in A549 untreated control cells. C. AO/EB stain in scorpion venom treated-Hela cells, arrows next to 'A' indicate apoptotic cells. D. AO/EB stain in scorpion venom treated-A549 cells, arrows next to 'N' indicate necrotic cells; arrows next to 'L' indicate late apoptosis; E. Arrows indicate membrane blebbing in scorpion venom treated-Hela cells. F. DAPI stain of nuclei in scorpion venom treated-Hela cells, arrows indicate apoptotic bodies. G. The histogram shows that there was a significant increase of apoptosis and necrosis compare to control cells. The apoptotic and necrotic cell number were compared with that of the untreated control, *significant difference (p<0.05).

Determination of caspases activity in Hela cells

We studied the expression of main caspases related with the extrinsic and intrinsic apoptotic pathway in Hela cells. Active forms of Capase-3, 8, 9 were detected at 24hr and 48hr after treatment, while in untreated control cells there

was not observed increment of active form of caspases 3, 8 and 9 (Figure 4).

DISCUSSION

Scorpion venom represents an interesting natural source for cancer therapy (Gao et al, 2008; Gomes et al, 2010). The present work was conducted to evaluate the effect of *R. junceus* scorpion venom on a panel of human cancer cells. The exposure of scorpion venom evidenced a significant cytotoxicity and selectivity against epithelial cancer cells while no effect was observed on epithelial normal counterpart. Among all epithelial cancer cells, lung (A549, NCI-H292) and breast (MDA-MB-213, MDA-MB-468) cell lines were slightly more sensitive. We observed that treatment with scorpion venom between 0.1-0.75mg/ml reduced cell viability below 50% in this group. While in the remainder epithelial cancer cells more than 50% of viability reduction was observed only at venom concentration higher than 0.75mg/ml. It is possible that this differential susceptibility could be related to differential expression of cellular targets usually recognized by scorpion venoms (Jehle et al, 2011). Additionally, whilst the epithelial cancer cells were all susceptible to the scorpion venom treatment, hematopoietic cancer cells were not sensitive. This feature suggests that susceptibility of cancer cells to scorpion venom treatment is histologically origin-dependent. This is the same feature that might account for the absence of toxicity in normal cells (Cherubini et al, 2000; Brevet et al, 2008). Additional experiments identifying potential pharmacological targets in these cells would help to characterize the spectrum of action considering the histological source.

Some reports have demonstrated the anticancer effect of different scorpion venom. BMK venom has shown significant cytotoxicity towards tumor brain cell line U251-MG while

it was not observed on normal cells (Wang and Ji, 2005). Venom and peptides from *Tytius discrepans* exhibited anti-cancer effect against the breast cancer cell line SKBR3 and no effect was observed on normal monkey kidney cell line MA104 (D'suze et al, 2010), both histological types included in our study. Additionally, venom from *Odontobuthus doriae* induces a cytotoxic effect against human neuroblastoma cells SH-SY5Y in a concentration dependent manner (Zargan et al, 2011). This evidence, similar to those obtained in this work, supports the potentiality of scorpion venom against epithelial cancers.

In our study, *R. junceus* scorpion venom induced a significant effect on cells viability against Hela and A549 cells but the mode of cell death differed. The scorpion venom-induced apoptotic cell death in Hela cells involved up-regulation of *bax* and down-regulation of *bcl-2* genes probably as a result of *p53* up-regulation and included caspases activation, chromatin condensation and formation of apoptotic nuclei.

Multiple signal pathways are involved in the regulation of apoptosis. The mechanism of p53-mediated cell death is associated with its function as transcriptional modulator and this may influence the activation of pro-apoptotic genes like *bax* and suppression of anti-apoptotic like *bcl-2* genes (Mirzayans et al, 2012; Ouyang et al, 2012). *bcl-2* gene family play a central role in the activation of caspases and dominate the intrinsic pathways of apoptosis (Wu et al, 2001).

(Mirzayans et al, 2012). Our results showed that Bax/Bcl-2 ratio increased indicating that mitochondria-mediated apoptosis is involved in scorpion venom-induced cell death in Hela. The Bax/Bcl-2 ratio can be recognized as a key factor for apoptotic process by regulating cytochrome c release from mitochondria to cytosol (Adams and Cory, 2007). In mitochondrion-dependent apoptosis, increase of *bax* and *bcl-2* down-expression induce Bax relocation in the outer mitochondrial membrane. The formation of homodimers Bax-Bax creates pores in mitochondrial membrane allowing the release of apoptogenic factors such as cytochrome c (Renault et al, 2013). This step activates the initiator caspase-9 and the effector caspase-3, which plays key role in both intrinsic and extrinsic pathways (Mirzayans et al, 2012; Ouyang et al, 2012). Caspases, a family of cysteine proteases, are known to form integral parts of the apoptotic pathway. Effectors caspases, such as caspase 3 are usually activated proteolytically by the upstream caspases 8 and 9 (Cohen, 1997). Activated caspase-3 cleaves DNA and initiates DNA fragmentation which represents the hallmark of apoptosis (Cohen, 1997). The increase of activated caspase 8 and 9 suggest that *R. junceus* venom can act trough both extrinsic and intrinsic apoptotic pathways in Hela cells.

Our results indicate that *R. junceus* scorpion venom induces necrotic cell death preferentially in A549 cells. Necrosis could be detected in AO/EB fluorescence upon scorpion venom treatment. The *p53* and *bcl-2* mRNA were both down-regulated upon scorpion venom treatment. Contrary to Hela cells the effect of scorpion venom on *bcl-2* expression was not due primarily to *p53*. Additionally, *bax* expression level do not varied in all time evaluated, then Bax/Bcl-2

ratio in this cell line was increased mainly due to *bcl-2* down expression suggesting at this level necrotic cell death. Sasi et al indicated that Bcl-2 over-expression is responsible for many drug-resistant or apoptotic-resistant cancers such as B-cell lymphoma and small cell lung cancer (Sasi et al, 2009). Attenuation of Bcl-2 may prove favorable in certain clinical settings to enhance alternative modes of cell death and drug efficacy.

Different reports have shown Bcl-2, to be the mediator of not only apoptosis, but also programmed necrosis (Poliseno et al, 2004; Sasi et al, 2009). Shikonin, a naturally-occurring compound found in the roots of *Lithospermum erythrorhizon* has been shown to directly induce necrosis. This compound has also been found to down-regulate Bcl-2 and Bcl-xL levels. Authors hypothesize that these findings could have true clinical impact, because shikonin may be able to inhibit Bcl-2/xL levels, while inducing necrosis during Bcl-2/xL over-expression, and stands out as a strong candidate for the suppression of apoptosis-resistant cancer cells (Han et al, 2007).

In A549, the venom concentration used (higher than IC_{50} value) induced necrotic cell death while in Hela the same venom concentration (lower than IC_{50} value) induced apoptotic cell death. It is quite probable that venoms can induce necrotic or apoptotic cell death depending on venom concentration. Additional studies must be done at different venom concentrations to confirm the hypothesis.

The ability to induce apoptosis and/or necrosis has been observed in different scorpion species. BMK venom has shown to inhibit glioma cell growth by apoptosis induction. This was evidenced by DAPI fluorescence staining and FACS analysis (Wang and Ji, 2005). Venom from *Heterometrus bengalensis* scorpion inhibits U937 and K562 cell growth through apoptosis. The event was characterized by membrane blebbing, chromatin condensation and DNA fragmentation evidenced by confocal, fluorescence, scanning electron microscopy and Flow-cytometry assay (Gupta et al, 2007). Furthermore, Omran observed that *Leiurus quinquestriatus* scorpion venom applied to the cell line C2C12 at higher concentrations was able to induce necrosis while in 293T cells at lower concentration can induce apoptosis (Omran, 2003). *Odontobuthus doriae* scorpion venom induces change in mitochondrial membrane potential in neuroblastoma cells; this provokes the activation of intrinsic via of apoptosis and cancer cell death at lower concentration while at higher concentration necrotic cell death has been suggested (Zargan et al, 2011).

At present, there are an increased number of cancers that become refractory or intrinsically resistant to chemotherapy failing to die by apoptosis (Plati et al, 2008; Liu, 2009). The dual mechanisms of cell death of *R. junceus* scorpion venom could represent an interesting alternative to overcome drug resistance.

CONCLUSION

Venom from *R. junceus* induces selective and differential anticancer effect against epithelial cancer cells. This study demonstrated that the apoptotic and necrotic effect of

venom was probably due to the sensitivity of cancer cells; however, further studies involving more cell lines at different doses of natural products are needed to firmly establish these observations. To our knowledge this is the first scientific evidence demonstrating the effect on cell viability and cell death events of this scorpion venom in a panel of human cancer cell lines. This makes *R. junceus* venom a favourable candidate for future studies in *in vivo* antitumor experiments

ACKNOWLEDGMENTS

REFERENCES

Adams JM and Cory S. 2007. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol, 19, 488–896.

Blagosklonny MV. 2005. Molecular theory of cancer. Cancer Biol Ther, 4, 621–127.

Brevet M, Ahidouch A, Sevestre H et al. 2008. Expression of K+ channels in normal and cancerous human breast. Histol Histopathol, 23, 965–572.

Cohen GM 1997. Caspases: the executioners of apoptosis. Biochem J, 326, 1–16.

Cherubini A, Taddei GL, Crociani O et al. 2000. HERG potassium channels are more frequently expressed in human endometrial cancer as compared to non-cancerous endometrium. Br J Cancer, 83, 1722–2729.

D'Suze G, Rosales A, Salazar V et al. 2010. Apoptogenic peptides from *Tytius discrepans* scorpion venom acting against SKBR3 breast cancer cell line. Toxicon, 56, 1495–5505.

Das Gupta S, Debnath A, Saha A et al. 2007. Indian black scorpion (*Heterometrus bengalensis* Koch) venom induced antiproliferative and apoptogenic activity against human leukemic cell lines U937 and K562. Leukemia Res, 31, 817.

Debin JA, Maggio JEStrichartz GR. 1993. Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. Am J Physiol, 264, 361–169.

Fritz H, Seely D, Kennedy DA et al. 2013. Green Tea and Lung Cancer: A Systematic Review. Integr Cancer Ther, 12, 7–74

Gao R, Zhag YGopalakrishnakone P. 2008. Purification and N-terminal sequence of a serine proteinase-like protein (BMK-CBP) from the venom of the Chinese scorpion (*Buthus martensii* Karsch). Toxicon, 52, 348–853.

Gomes A, Bhattacharjee P, Mishra R et al. 2010. Anticancer potencial of animals venom and toxins. Indian J Exp Bio, 48, 93–303.

Goudet C, Chi CWTytgat J. 2002. An overview of toxins and genes from the venom of the Asian scorpion *Buthus martensi* Karsch. Toxicon, 40, 1239–9258.

Gupta SD, Debnath A, Saha A et al. 2007. Indian black scorpion (*Heterometrus bengalensis* Koch) venom induced antiproliferative and apoptogenic activity against human leukemic cell lines U937 and K562. Leukemia Res, 31, 817–725.

Han W, Li L, Qiu S et al. 2007. Shikonin circumvents cancer drug resistance by induction of a necroptotic death. Mol Cancer Ther, 6, 1641–1649.

Herrera Y, Heras N and Cardoso D. 1999. Adaptación a microplacas y validación de la técnica de Lowry. VacciMonitor, 3, 7–71.

Jehle J, Schweizer PA, Katus HA et al. 2011. Novel roles for hERG Kþ channels in cell proliferation and apoptosis. Cell Death Dis, 2, 2–2.

Liu FS. 2009. Mechanisms of chemotherapeutic drug resistance in cancer therapy--a quick review. Taiwan J Obstet Gynecol, 48, 239–944.

Meiyanto E, Hermawan AAnindyajati. 2012. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pac J Cancer Prev, 13, 427–736.

Mirzayans R, Andrais B, Scott A et al. 2012. New Insights into p53 Signaling and Cancer Cell Response to DNA Damage: Implications for Cancer Therapy. J Biomed Biotechnol, 2012, 1–16.

Mosmann T. 1983. Rapid colorimetric assay for cellular grow and survival: application to proliferation and citotoxicity assays. J Immunol Meth, 65, 55–53.

Omran MA. 2003. Cytotoxic and apoptotic effects of scorpion Leiurus quinquestriatus venom on 293T and C2C12 eukaryotic cell lines. J Venom Anim Toxins incl Trop Dis, 9

Ouyang L, Shi Z, Zhao S et al. 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif, 45, 487–798.

Plati J, Bucu RO and Khosravi-Far R. 2008. Dysregulation of Apoptotic Signaling in Cancer: Molecular Mechanisms and Therapeutic Opportunities. J Cell Biochem, 104, 1124–4149.

Poliseno L, Bianchi L, Citti L et al. 2004. Bcl2-low-expressing MCF7 cells undergo necrosis rather than apoptosis upon staurosporine treatment. Biochem J, 379, 823–332.

Renault TT, Teijido O, Antonsson B et al. 2013. Regulation of Bax mitochondrial localization by Bcl-2 and Bcl-x(L): keep your friends close but your enemies closer. Int J Biochem Cell Biol, 45, 64–47.

Sasi N, Hwang M, Jaboin J et al. 2009. Regulated cell death pathways: New twists in modulation of BCL2 family function. Mol Cancer Ther, 8, 1421–1429.

Wang WX and JI YH. 2005. Scorpion venom induces glioma cell apoptosis in vivo and inhibits glioma tumor growth in vitro. J Neurooncol, 73, 1–1.

Wu YL, Mehew JW, Heckman CA et al. 2001. Negative regulation of bcl-2 expression by p53 in hematopoietic cells. Oncogene, 20, 240–251.

Xiao GY. 1990. Advance of research on antitumor activity of scorpion venom. Chin J Cancer biother, 6, 161–163.

Zargan J, Sajad M, Umar S et al. 2011. Scorpion (*Odontobuthus doriae*) venom induces apoptosis and inhibits DNA synthesis in human neuroblastoma cells. Mol Cell Biochem, 248, 173–381.