RESEARCH REPORT

Rhopalurus junceus scorpion venom induces apoptosis in the triple negative human breast cancer cell line MDA-MB-231

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ABSTRACT

Rhopalurus junceus scorpion venom has demonstrated high cytotoxic activity in epithelial cancer cells. In the present study, the effect of scorpion venom on cell viability and apoptosis was evaluated in the MDA-MB-231 human breast carcinoma cell line. Cell viability was analyzed using MTT assay. The cell death event was examined trough end-point RT-PCR to identify the expression of apoptosis-related genes, fluorescent microscopy and mitochondrial membrane potential ($\Delta\Psi m$) alteration. The results demonstrated that scorpion venom induced apoptosis in MDA-MB-231 cells in a time-dependent manner. Besides, scorpion venom treatment also resulted in p53, bax, noxa, puma, caspase 3 and p21 over-expression, while the expression of bcl-2 and bcl-xl was down-regulated. Apoptosis was associated with depolarization of $\Delta\Psi m$. The overall effect indicates that the selective cytotoxic effect of the scorpion venom is associated with its apoptosis-inducing effect through the mitochondrial pathway. Therefore, *R. junceus* scorpion venom may be an interesting natural extract for further investigation in breast cancer treatment strategies.

KEYWORDS: *Rhopalurus junceus*, scorpion venom, breast cancer, mitochondria, apoptosis

INTRODUCTION

Breast cancer is one of the most common cancers among women aged between 40–55 years, and is the leading cause of death in women globally (Jemal et al, 2011). The common treatments include surgery, chemotherapy, immunotherapy or radiotherapy; however they usually induce adverse effects (Williams et al, 2014). Additionally, standard hormonal therapies for estrogen receptor-positive (ER+) breast cancers act as an estrogen antagonist on breast tissue (Bush, 2007). They are also used as adjuvant therapy for breast cancer to reduce the risk of recurrence. However, there are a growing number of breast cancer subtypes that do not respond to all these treatments due to intrinsic and acquired resistance (Chen et al, 2011). This represents a continuing problem that compromises the effectiveness of anti-cancer therapy (Luqmani, 2008). Therefore, there is a growing interest in finding more effective and selective anti-cancer remedies.

The use of scorpion venom for cancer treatment has been the focus of several laboratories in recent years (Gomes et al, 2011). The potential of these natural extracts as selective and non-toxic anticancer treatment has been scientifically demonstrated (Das Gupta et al, 2007; D'Suze et al, 2010). The scorpion *Rhopalurus junceus* (*R. junceus*) is an endemic species from Cuba. Venom from this scorpion is considered a popular Cuban traditional medicine for cancer treatment. Recently, we demonstrated that *R. junceus* scorpion venom induces a selective and cytotoxic effect against a panel of epithelial cancer cells without affecting normal cells (Díaz-García et al, 2013). Among them, breast cancer cell lines became susceptible to scorpion venom treatment; however, until now there are no experimental evidences about the scorpion venom treatment-related cell death in triple negative-breast cancer cells (TNBC).

MATERIAL AND METHODS

Reagents

Dulbecco's modified Eagle's medium was 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-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) reagent and remainder chemicals and reagents were obtained from Sigma (St Louis, MO).

Venom source

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

In vitro cell viability assay (MTT assay)

MDA-MB-231 cells and Vero cells were seeded in 96-well plates ($5x10^3$ cell/well) in 50µl as previously (Díaz-García A et al, 2013). Briefly, serial dilutions of scorpion venom were dissolved in DMEM to give a final concentration of 0.12; 0.25; 0.5; 0.75 and 1mg/ml. Untreated cells represent

100% of cell growth and were used as negative control. After treatment for 72hr, 10µl of 5mg/ml of sterile MTT was added per well and cultivated for 3hr (Mosmann T, 1983). The supernatant was removed and 150µl DMSO was added per well. The absorbance was measured with a microplate reader (ELISA MRX Revelation Dynex Technologies 560nm with 630nm as reference). Percentage of cell viability was expressed using the formula: %viability = A560 of treated cells/A560 of negative control cells x100%. The median inhibitory concentration (IC_{50}) value was obtained. The experiment was repeated three times.

Morphological assessment and measurement of apoptotic cells

To study the cell death event in MDA-MB-231, the cells ($1x10^5$/well) were grown in 24 well-culture plates overnight and treated with ½IC_{50} of scorpion venom during 48hr. At this period, 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. Besides, 400 cells were analyzed and counted in each of three independent experiments using fluorescence microscopy IX-71 (Olympus, Japan) at 480nm and 520nm filters.

Analysis of mitochondrial membrane potential (ΔΨm)

MDA-MB-231 cells were grown on 24 well-culture plates ($1x10^5$/well) overnight and treated with ½IC_{50} of scorpion venom at 37°C during 48hr. Mitochondrial permeability transition was determined by staining the cells with 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethyl- benzimidazolylcarbocyanin iodide (JC-1) in the dark. JC-1 is a fluorescent dye that is incorporated into mitochondria in a ΔΨm-dependent manner. After treatment, the culture medium was replaced with a new medium containing JC-1 (1µM) for 30min at 37°C in the dark. For fluorescence microscope observations, the cells were washed twice

with PBS and the new culture medium was added. From each well three field (600 cells) were analyzed and variation of ΔΨm was observed and photographed by using the fluorescence microscope IX-71 (Olympus, Japan) at 480nm and 520nm fluorescence channels. The experiments were repeated three times.

RNA isolation and end-point reverse transcription (RT-PCR) analysis of apoptotic-related genes in MDA-MB-231 cells

MDA-MB-231cells ($1x10^5$/well) seeded on 24-well plates were cultured for 24hr. The concentration of scorpion venom used with fresh medium was $½IC_{50}$ and triplicate cell culture wells were exposed including vehicle (control cells). Treated and control cell cultures were incubated for a further 0hr, 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). Experiments were repeated two times. 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). β-actin amplification was performed as a control gene. The primers sequences, PCR conditions, characteristics and size were previously published (Frión-Herrera et al, 2015). Amplified PCR products were subjected to electrophoresis at 70V in 1.5% (w/v) agarose gel for 90min.

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).

Statistical analysis

The IC_{50} values were determined by interpolation of tendency line from linear regression curve. Apoptotic and non-apoptotic cells in DAPI staining were compared using Mann-Whitney U test. Band intensity of each gene from scorpion venom-treated and non-treated cells was 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 $p<0.05$.

RESULT

The effect of scorpion venom induced a significant decrease in cell viability and rupture of cell monolayer in breast cancer cells in a concentration-dependent manner (Figure 1A), as evidenced in cell culture seen under inverted microscope (Figure 1B), while Vero cells were affected minimally in morphology and viability at the highest concentration used in the study (Figure 1A, 1B). The IC_{50} value confirms this behavior where scorpion venom was found to be 0.75±0.15mg/ml in MDA-MB-231 cells.

Effect of scorpion venom on morphological apoptotic changes of MDA-MB-231 Cells

To determine whether the growth inhibitory activity of scorpion venom was related to the induction of apoptosis, morphological assay of cell death was investigated using the DAPI staining. DAPI revealed the changes associated with apoptosis in MDA-MB-231 cells treated with the scorpion venom (Figure 2A, white arrows). In untreated control cells was observed a uniformly blue fluorescence stain of

nucleus. The morphological changes associated with apoptosis such as chromatin condensation, nuclear fragmentation, and apoptotic bodies are evident upon treatment and was identified in cells treated with venom (Figure 2A, white arrows), which confirms the morphological evidence of apoptosis. Morphological analysis of cell culture revealed that ½IC_{50} of scorpion venom induces a significant proportion (24% apoptotic cells; $p<0.01$) to die by apoptosis compared to control cells (2% apoptotic cells) (Figure 2B).

Effects of scorpion venom on the mitochondrial membrane potential (ΔΨm) in MDA-MB-231 cells

Mitochondrial membrane potential can be evaluated by staining with JC-1 dye. We found that after treatment with the scorpion venom, the mitochondrial membrane potential decreased in breast cancer cells as evidenced by the decrease in the fluorescence compared to the untreated cells (Figure 3). This characteristic was corroborated using a K+ ionophore, valinomycin which causes mitochondrial inner membrane destabilization and reduction of membrane potential.

Effect of scorpion venom on apoptosis-related genes in MDA-MB-231 cells

The constitutive levels of apoptotic-related genes and the time course of scorpion venom effect, on these genes in MDA-MB-231 cells, were studied by RT-PCR. As shown in Figure 4, scorpion venom treatment induced a time-dependent variation of level expression in apoptotic-related genes (Figure 4A). The variation in pro-apoptotic genes analyzed (p53, bax, noxa, puma, caspase 3) is related to significant over-expression ($p<0.05$), while a significant down-expression ($p<0.05$) was observed in anti-apoptotic genes (bcl-2, bcl-xl) in all cases compared to untreated control (Figure 4B).

DISCUSSION

The use of scorpion venom for cancer treatment has been the focus of researchers in recent years (Gomes et al, 2011). In particular, we have shown in a previous report that *R. junceus* scorpion venom has a noteworthy anticancer effect against a panel of epithelial cancer cells including breast cancer cell lines (Díaz-García et al, 2013). Our present findings support that the scorpion venom has a notable anticancer activity against human breast cancer cells MDA-MB-231, while it has a minimal effect on normal cells. This distinct effect is comparable to other few scorpion venoms that have shown a detectable difference in their effect on normal and cancer cells (Wang and Ji, 2005; D'Suze et al, 2010). A comparison of the effects of anticancer agents on tumor and normal cells is the necessary first steps in the screening studies because it can shows if the compound can, in fact, discriminate between abnormal from normal cell behavior, predicting the potential toxicity of the compound.

Targeting the cell cycle and apoptotic pathways in cancer cells is an important approach for cancer treatment and anti-cancer drug development (Danial and Korsmeyer, 2004). The results of scorpion venom-induced apoptosis in MDA-MB-231 cells were morphologically observed through fluorescent staining DAPI. The scorpion venom-treated group showed shrunken and marginated nuclei in cancer cells in contrast to the normal and large nucleus in the untreated cells. Besides was verified the formation of apoptotic nuclei, chromatin condensation, apoptotic bodies and cell membrane blebbing providing evidences of the apoptotic potential of the natural extract.

At molecular level, the effect of the scorpion venom induced high level expression of p53 gene. Besides, the scorpion venom was able to induce high level expression of $p21^{WAF1}$. This observation due to scorpion venom treatment points to the p53-dependent apoptosis induction and cell cycle arrest due

to concomitant increase of $p21^{WAF1}$ gene. MDA-MB-231 cells usually accede to apoptosis through p53 induction (Wang et al, 2013). The tumor suppressor gene p53 plays an important role in response to different cell damage (Mirzayans et al, 2012) and is crucial in p53-dependent pathway, leading to cell cycle arrest and apoptosis (Ouyang et al, 2012). In p53-dependent apoptosis, this protein is responsible for increasing the $p21^{WAF1}$ tumor suppressor gene (Mirzayans et al, 2012; Ouyang et al, 2012). The $P21^{WAF1}$ protein can decrease the activity of cyclin-dependent kinases (CDKs), by binding to active CDK-cyclin complexes and inhibits their kinase activities resulting in growth arrest and apoptosis (Pérez-Sayáns et al, 2013).

In the present study, several pro-apoptotic genes like bax, noxa and puma showed a significantly high level expression while anti-apoptotic genes like bcl-2 and bcl-xL were down-expressed. All these genes belong to Bcl-2 family which has a key role in controlled balance of apoptosis in mitochondria (Renault et al, 2013). The over-expression of apoptotic-related genes at protein level provokes an accumulation of pro-apoptotic proteins on the mitochondrial outer membrane resulting in increased mitochondrial membrane permeability. This in turn, causes the release of cytochrome c into the cytoplasm (Strasser et al, 2011) where it binds to the cytosolic protein Apaf-1 to facilitate the formation of apoptosomes, which can recruit and activate the caspase-9 (Li et al, 2013). The complex apoptosome-Caspase-9 directly activates the catalytic enzyme caspase-3 or caspase-7 and triggers the DNA fragmentation (Cotter, 2009). Our result showed that caspase 3 gene was time-dependent over-expressed, suggesting the activation of this catalytic enzyme. Several studies indicated that the ratio of bax/bcl-2 is a key factor for mitochondrial apoptosis induction (Xiong et al, 2014). In fact, this feature was stated previously for this scorpion venom in the cervical cancer cell line Hela (Díaz-García et al, 2013) and was confirmed in this study.

To settle the relationship between scorpion venom effect and mitochondrial apoptosis we used the fluorescent mitochondrial marker JC-1 to identify the variation of ΔΨm. Our findings confirmed the decrease in ΔΨm as evidenced from the decreased of JC-1 fluorescence intensity when MDA-MB-231 cells were treated with the scorpion venom. These results add strong evidences that the scorpion venom-induced apoptosis might be through the intrinsic pathway.

Apoptotic cell death, against breast cancer cells, has been reported in other scorpion species. *Tityus discrepans* venom against SKBR3 (D'Suze et al, 2010), *Androctonus crassicauda* (Zargan et al, 2011a) and *Odontobuthus doriae* (Zargan et al, 2011b) venom against MCF-7 are some examples of these facts. However, in our case the effect of *R. junceus* scorpion venom was observed against TNBC which normally is not responsive to standard treatment. Thus, the susceptibility of MDA-MB-231 suggests that the *R. junceus* scorpion venom may be an interesting natural extract for further investigation into treatment strategies of non-responsive breast cancers.

CONCLUSIONS

Our results show that *Rhopalurus junceus* scorpion venom, i) inhibits the growth of triple negative breast cancer cells MDA-MB-231, and ii) regulates the expression of apoptosis-related genes, inducing apoptosis through the mitochondrial-apoptotic pathway.

COMPETING INTERESTS

None declared.

REFERENCES

Bush N. 2007. Advances in hormonal therapy for breast cancer. Semin Oncol Nurs, 23, 46-54.

Cotter T. 2009. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer, 9, 501-507.

Chen X, Sneed K and Zhou S. 2011. Pharmacokinetic profiles of anticancer herbal medicines in humans and the clinical implications. Curr Med Chem, 18, 3190-3210.

D'Suze G, Rosales A, Salazar V and Sevcik C. 2010. Apoptogenic peptides from Tytius discrepans scorpion venom acting against SKBR3 breast cancer cell line. Toxicon, 56, 1495-1505.

Danial N and Korsmeyer S. 2004. Cell death: critical control points. Cell Prolif, 116, 205-219.

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. Leuk Res, 31, 817-823.

Díaz-García A, Morier-Díaz L, Frión-Herrera Y et al. 2013. In vitro anticancer effect of venom from Cuban scorpion Rhopalurus junceus against a panel of human cancer cell lines. J Venom Res, 4, 5-12.

Frión-Herrera Y, Díaz-García A, Ruiz-Fuentes J, Rodríguez-Sánchez H and Sforcin J. 2015. Brazilian green propolis induced apoptosis in human lung cancer A549 cells through mitochondrial-mediated pathway. J Pharm Pharmacol, 67, 1448-1456.

Gomes A, Bhattacharjee P, Mishra R, Biswas A, Dasgupta S and Giri B. 2011. Anticancer potencial of animals venom and toxins. Indian J Exp Bio, 48, 93-103.

Herrera Y, Heras N and Cardoso D. 1999. Adaptación a microplacas y validación de la técnica de Lowry. VacciMonitor, 3, 7-11.

Jemal A, Bray F, Center M, Ferlay J and Ward E. 2011. Forman D: Global cancer statistics. CA Cancer J Clin, 61, 69-90.

Li Y, Li D, Yuan S et al. 2013. Embelin-induced MCF-7 breast cancer cell apoptosis and blockade of MCF-7 cells in the G2/M phase via the mitochondrial pathway. Oncol Lett, 5, 1005-1009.

Luqmani Y. 2008. Mechanisms of drug resistance in cancer chemotherapy. Med Princ Pract, 14, 35-48.

Mirzayans R, Andrais B, Scott A and Murray D. 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, 55-63.

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, 487-498.

Pérez-Sayáns M, Suárez-Peñaranda J, Gayoso-Diz P, Barros-Angueira F, Gándara-Rey J and García-García A. 2013. The role of p21Waf1/CIP1 as a Cip/Kip type cell-cycle regulator in oral squamous cell carcinoma (Review). Med Oral Patol Oral Cir Bucal, 1, e219-225.

Renault T, Teijido O, Antonsson B, Dejean L and Manon S. 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, 64-67.

Strasser A, Cory S and Adams J. 2011. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. EMBO J 30, 3667-3683.

Wang C, Gao C, Chen Y, Yin J, Wang P and Lv X. 2013. Expression pattern of the apoptosis-stimulating protein of p53 family in p53+ human breast cancer cell lines. Cancer Cell Int, 13, 116.

Wang W and Ji Y. 2005. Scorpion venom induces glioma cell apoptosis in vitro and inhibits glioma tumor growth in vivo. J Neurooncol, 73, 1-7.

Williams N, Pigott K, Brew-Graves C and Keshtgar M. 2014. Intraoperative radiotherapy for breast cancer. Gland Surg, 3, 109-119.

Xiong S, Mu T, Wang G and Jiang X. 2014. Mitochondria-mediated apoptosis in mammals. Protein Cell, 5, 737-749.

Zargan J, Sajad M, Umar S, Naime M, Ali S and Khan H. 2011a. Scorpion (Androctonus crassicauda) venom limits growth of transformed cells (SH-SY5Y and MCF-7) by cytotoxicity and cell cycle arrest. Exp Mol Pathol, 91, 447-454.

Zargan J, Umar S, Sajad M, Naime M, Ali S and Khan H. 2011b. Scorpion venom (Odontobuthus doriae) induces apoptosis by depolarization of mitochondria and reduces S-phase population in human breast cancer cells (MCF-7). Toxicol In Vitro, 25, 1748-1756.

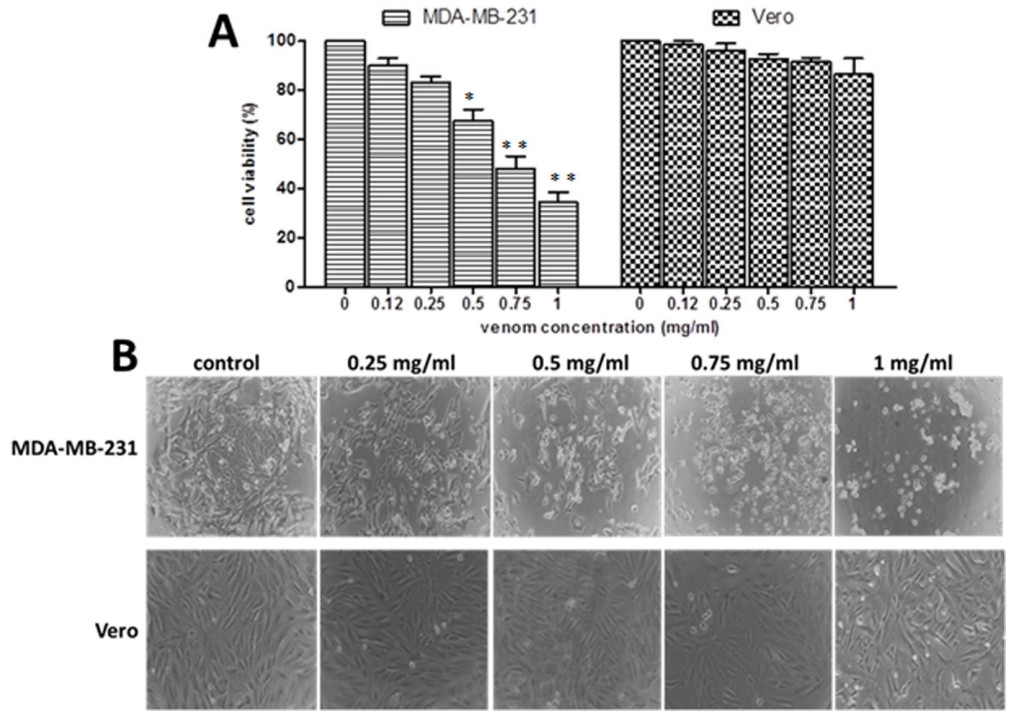

Figure 1. Effect of *R. junceus* scorpion venom against MDA-MB-231 and Vero cells. **A.** Graphics of cell viability after scorpion venom treatment. **B.** Micrographs showing differential effect of *R. junceus* venom, in breast cancer cells MDA-MB-231 and normal kidney cells Vero, after 72hr of treatment at different concentration. Values represent the mean±SEM obtained from three independent experiments. *significant difference (p<0.05); **significant difference (p<0.01).

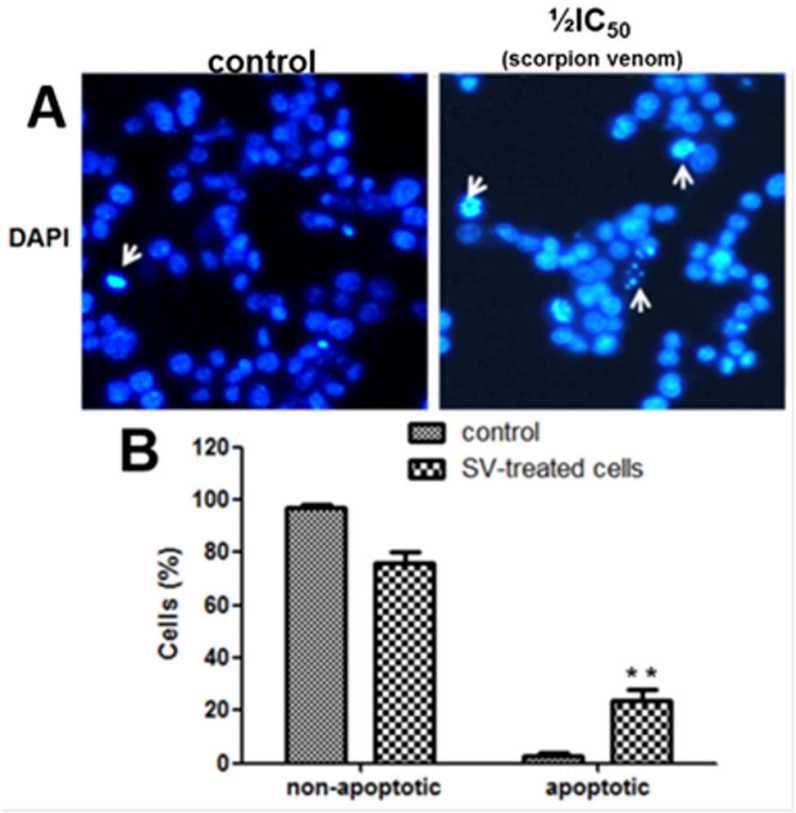

Figure 2. Evidences of apoptosis in MDA-MB-231 cells. **A.** Morphological changes of nucleus in MDA-MB-231 cells after 48hr of scorpion venom treatment using DAPI stain in untreated control and scorpion venom-treated cells. White arrows in DAPI staining indicate chromatin condensation and apoptotic bodies. **B.** Graphic showing percentage of apoptotic cells in untreated control and scorpion venom-treated cells. **significant difference (p<0.01).

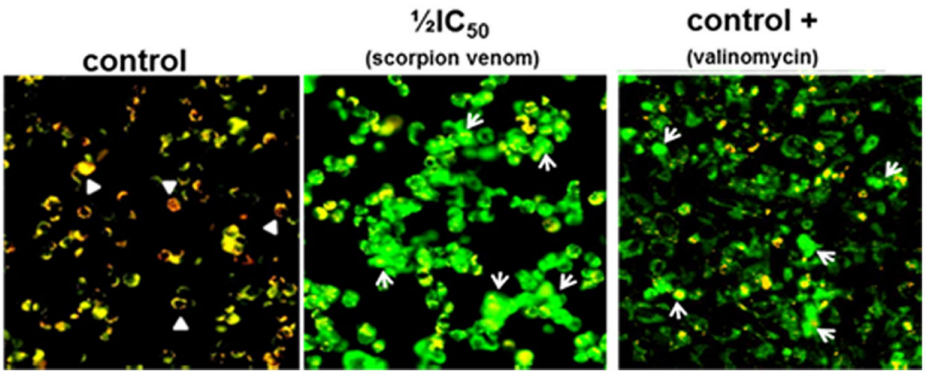

Figure 3. Fluorescent micrographs of mitochondrial membrane potential in MDA-MB-231 cells obtained at 48hr of *R. junceus* venom treatment. Arrow head indicates orange-yellow fluorescence for intact ΔΨm. White arrows indicate bright green fluorescence in ΔΨm decrease. The picture is representative of one field from three fields analyzed on each treatment.

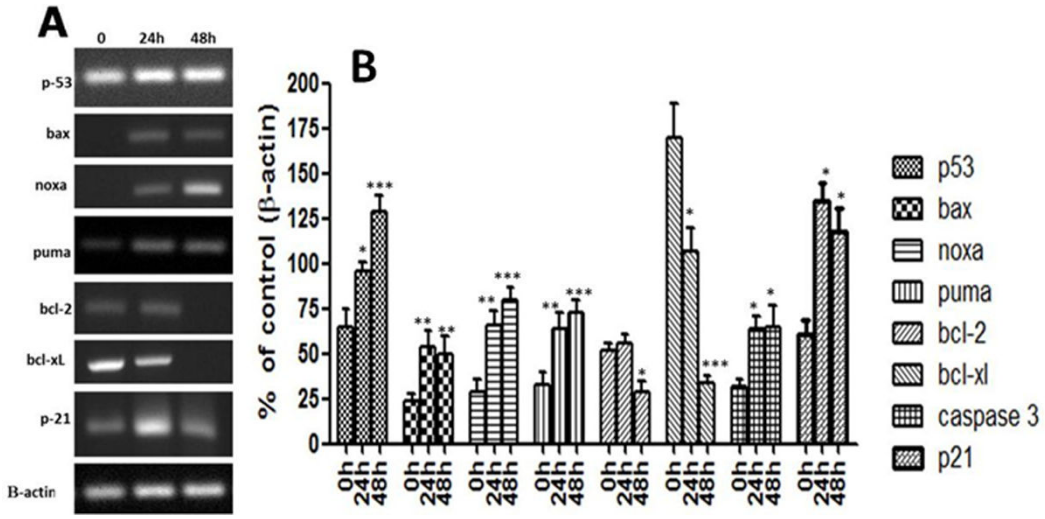

Figure 4. Detection of expression levels of apoptotic-related genes in MDA-MB-231 cells. **A.** Images of RT-PCR products for each analyzed gene at each selected time. **B.** Graphic with relative signal intensities normalized to β-actin gene expression level. Values represent the mean±SEM obtained from three independent experiments. The p values were obtained comparing the control group at 0hr versus treatments at 24hr and 48hr. Significant differences *p<0.05, ** p<0.01, ***p<0.001.