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Results: Electrophoretic analysis demonstrated similarities and differences among experimental groups. Treated groups showed significant reduction of HeLa cell viability and morphological changes respect to untreated cells, but this effect is maintained regardless of the sex of this scorpion.

Conclusion: Despite of differences in the electrophoretic profile among experimental groups, the cytotoxic effect does not change in the HeLa tumor line.

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I. Introduction

Cancer is among the leading causes of death worldwide and has a major impact in both developed and underdeveloped countries (Siegel et al., 2020). Specifically, cervical cancer is one of the main public health problem affecting middle-aged women, particularly in developing countries (Arbyn et al., 2020). Conventional antitumor therapies used in clinical practice are surgery, radiotherapy and chemotherapy (Somayeh et al., 2017). These treatments are effective only to some extent, as they are not applicable in all cases and the undesirable side effects often make them impractical (Topcul and Cetin, 2014). For this reason, the antitumor potentialities of natural products such as scorpion venoms have been evaluated (Moradi et al., 2018; Desales-Salazar et al., 2020).

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The scorpion venom is a highly complex and heterogeneous mixture of compounds, mainly proteins and peptides (Ahmadi et al., 2020). Around 2000 scorpion species have been described and only 30 species of the *Buthidae* family are considered dangerous to humans (Desales-Salazar et al., 2020). In a study carried out with two scorpions from *Buthidae* family: *Androctonus finitimus* and *Hottentota tumulus*, it was shown that the quantity and quality of extracted venom were associated with temperature, diet and the extraction method (Tobassum et al., 2018). Besides, venom composition can be influenced by different factors like sex, geographical location, age, time intervals of extraction and others (Pucca et al., 2014). For example, males and females of *Tityus nororientalis* scorpions produce venoms with different composition and activity (De Sousa et al., 2010).

Rhopalurus junceus (R. junceus) belongs to *Buthidae* family and is an endemic scorpion from Cuba. Preclinical studies have shown that *R. junceus* scorpion venom decreases the viability of tumor cells of epithelial origin and has no cytotoxic effect on normal cells (Díaz-García et al., 2013). In a proteomic comparative analysis of male and female *R. junceus* scorpion venom, from their 200 components just 63 were common and the most abundant component appeared in both sexes (Rodríguez-Ravelo et al., 2015). Previous studies by our group, using a mixture of venom of female and male scorpions in the same proportion and similar laboratory conditions have demonstrated their cytotoxic and apoptotic effect against cancer cells (Díaz-García et al., 2013; Díaz-García et al., 2015; Díaz-García et al., 2017; Yglesias-Rivera et al., 2019). Considering the described scenario, the objective of the present study was determine if there are differences among *R. junceus* scorpion venoms from female and males individually and mixture.

II. Methods

a) Scorpion Venom source

Female and male adults of *Rhopalurus junceus* scorpions, collected in Isla de la Juventud (Cuba), were kept in captivity for at least one month before venom extraction by electrical stimulation. Scorpions were maintained under Bioterium conditions in individual

plastic containers at 23 ±1°C temperature, 60 ± 10 % relative humidity and 12:12 h light-dark cycle, in the laboratories belonging to the Entrepreneurial Group of Biopharmaceuticals and Chemicals Productions (LABIOFAM). Bioterium conditions about management of scorpion colonies and collection of venom have been approved by the Ministry of Science, Technology and Environment of Cuba (CITMA 20/2016). Three groups of scorpions containing 50 female (G1), 50 male (G2) or a mixture of 25 females with 25 males (G3) were used. 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 Lowry Modified Method (Herrera *et al.*, 1999).

b) SDS-PAGE and determination of molecular weight (MW)

Electrophoretic analysis of each pooled venom was carried out according to the previous method (Díaz-García *et al.,* 2015) with 4% stacking gel and 16% separating gel under non-reduced and reduced (2-mercaptoethanol, 95°C, 10 min) conditions using an electrophoresis chamber (Biorad). All samples were dissolved in a sample buffer (50mM Tris–HCl, pH 6.8, 0.1M DTT, 10% glycerol, 2% SDS, and 0.1% bromophenol blue). In each well, 50 µg of venom was applied and a protein MW marker was used. The run conditions were 120 V to free current for two hours. The gels were stained with Coomassie Brilliant Blue G-250 and were subsequently rinsed with Methanol: Acetic acid: Water (45:10:45). The gels were photographed and analyzed using ImageJ 1.46 software.

c) Cell line and culture

HeLa (cervix adenocarcinoma ATCC CCL-2™) cell line was maintained in minimum essential medium (MEM). Vero (normal African green monkey kidney ATCC CRL-1586™) cell line was maintained in Dulbecco's modified Eagle's medium. The mediums of both cell lines were supplemented with 2 mM of glutamine and non-essential amino acids, 10% of fetal bovine serum (SFB) and penicillin-streptomycin 100 UI/mL -100 µg/mL. The cells were grown in a humidified atmosphere, 5% CO_2 at 37°C.

d) In vitro cell viability assay (MTT assay)

The effect of scorpion venom on cell viability was determined by the MTT Assay (Mosmann, 1983). HeLa cells ($1x10^4$/well) and Vero cells ($1x10^4$/well) were plated in 50µl of medium/well in 96-well culture plates (Costar Corning, Rochester, NY) and incubated overnight in a humidified atmosphere of 5% (v/v) CO_2 at 37°C. After incubation, 50µl of venom was dissolved in medium at final concentration of 0.0625, 0.125, 0.25, 0.5 and 1mg/mL and was added in five well for every concentration. Cells without scorpion venom were used as untreated control. After 72h of incubation, 10µl of 5mg/mL of sterile MTT was added per well and incubated for another 3h. The supernatant was carefully removed, 150µl DMSO was added per well and incubated for 15min at 37°C. The absorbance was determined in 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 percentage of viability, using the formula: %viability= $A_{560\text{-}630nm}$ of treated cells/ $A_{560\text{-}630nm}$ of control cells x 100%. The IC_{50} values (venom concentration that causes 50% reduction of the cell) from cancer cells were determined. The experiments were performed three times by triplicate.

e) Phase-contrast microscopy

After treatments, cells were washed with PBS and morphological changes in culture were then observed under microscope IX-71 (Olympus Corporation, Tokyo, Japan). Images were captured using the camera DP-72 (Olympus Corporation, Tokyo, Japan) and 10X objectives.

f) Statistical analysis

Kruskal-Wallis non-parametric test and Dunn's multiple comparison tests was used to compare different assays. Two-way Anova and Bonferroni post-test were performed to analyze differences in MW, protein band intensity and cell viability. The IC*50* value was determined by interpolation of tendency line from linear regression curve. GraphPad Prism version 5.01 for Windows, (GraphPad Software, San Diego California, USA) for $p<0.05$ was used for all analysis.

III. Results

There was no significant difference among total protein concentration of female (G1), male (G2) and the mixture of both sexes (G3) of *R. junceus* scorpion venom in our experimental conditions (Figure 1). The values of Mean ± SD for G1, G2 and G3 were 8.3 ± 1.1 mg/mL, 8.5 ± 2.9 mg/mL and 8.2 ± 1.6 mg/mL, respectively. However, the electrophoretic analysis of protein content from G1, G2 and G3 under non-reduced and reduced conditions demonstrated similarities and differences among experimental groups (Figure 2).

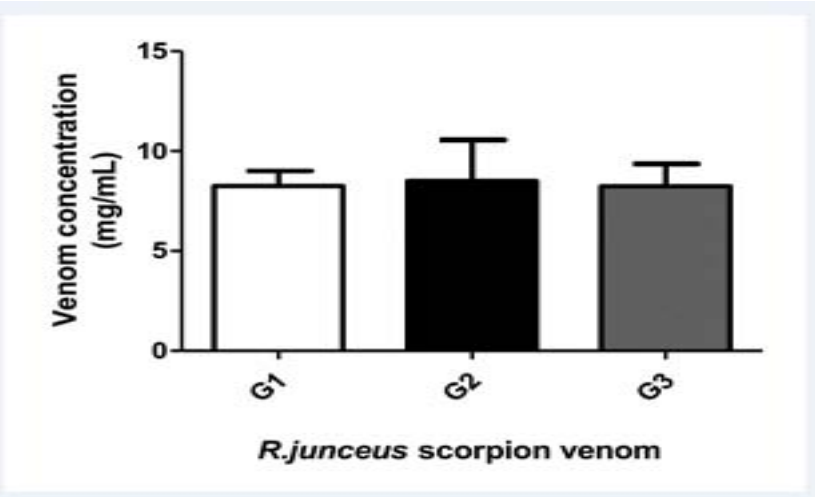

Figure 1: Comparison of protein concentration in male, female and the mixture of sexes in *R. junceus* scorpion venom. The graphic represents the mean ± SD of total protein concentration values of three independent experiments with female (G1), male (G2) and female + male (G3) *R. junceus* scorpion venom. Data were analyzed using Kruskal-Wallis followed by Dunn test.

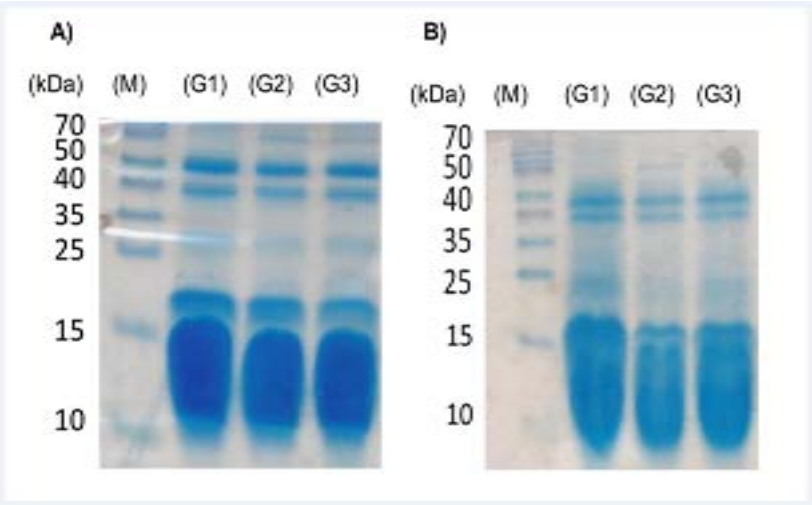

Figure 2: SDS-PAGE analysis of male, female and the mixture of both sexes R. junceus scorpion venom. A) Non-reduced SDS-PAGE. B) Reduced SDS-PAGE. G1: female, G2: male, G3: female + male *R. junceus* scorpion venom. Separating gel 16%, stacking gel 4%. Molecular weight protein marker from 10 kDa-175 kDa was used

Six bands were observed in non-reduced (Figure 2A) and reduced (Figure 2B) conditions of SDS-PAGE, in the venom of female and male scorpions. While in the case of the venom obtained from the mixture of scorpions of both sexes, six bands were observed under non-reduced conditions and seven under reduced conditions. The comparison of molecular weight (MW) and protein band intensity among the groups of *R. junceus* scorpion are presented in Table 1. Non-reduced electrophoresis conditions showed five similarities in the MW of (55, 45, 39, 28 and 19 kDa) from G1 and G2 scorpion groups. However, in these conditions was observed a difference between them. A band of 11kDa was displayed only in G2 and 12kDa band only in G1 and G3. The molecular weights observed in non-reduced conditions of the mixture of both sexes were: 52, 45, 39, 28, 19 and 12 kDa. Electrophoresis under reduced conditions displayed two resemblances (at 44 and 11 kDa) and four differences between the protein MW of G1 and G2 groups. An 11kDa coincident band was observed in all groups. There were no statistically significant differences between the molecular weight of the bands in both electrophoretic conditions for G1 and G2. The appearance of 47kDa band in G3 group was the only statistically significant difference in MW respect to G1 and G2 groups.

Regarding to intensity values, statistically significant differences were found between both G1 and G2 groups for the bands at 12 and 11 kDa ($p < 0.001$); for G3 group in the bands at 52 ($p < 0.05$) and 12 kDa ($p < 0.001$) concerning to G1 and G2 groups. All these results were based in electrophoretic analysis under non-reduced conditions. In reduced conditions, the

intensity values for the 11 kDa band obtained for G3 demonstrated a statistically significant difference respect to G1 ($p < 0.05$) and G2 groups ($p < 0.001$).

Table 1: Comparison of MW and intensity of protein band in female (G1), male (G2) and female + male (G3) of *R. junceus* scorpion venom

SDS-PAGE	G1		G2		G3	
	MW (kDa	Band Intensity	MW (kDa	Band Intensity	MW (kDa)	Band Intensity
non-reduced conditions	55	585	55	1105	52	$1762^{a'b'}$
	45	1813	45	2060	45	1642
	39	1090	39	1201	39	991
	28	759	28	1178	28	695
	19	2055	19	2714	19	2883
	12	$21540^{b'''c'''}$	11	$26950^{a'''c'''}$	12	$24290^{a'''b'''}$
reduced conditions	66	656	65	729	64	1011
	50	922	49	1295	52	816
	44	412	44	363	$47^{a'''b'''}$	448
	23	1374	25	1465	42	674
	17	2858	18	923	24	1266
	11	$10926^{b'''c'}$	11	$15010^{a'''c'''}$	19	957
					11	$7479^{a'b'''}$

Legend: Data were analyzed using two-way ANOVA followed by Bonferroni test: a': $p<0.05$ (G1), a''': $p<0.001$ (G1); b': $p<0.05$ (G2), b''': $p<0.001$ (G2) and c': $p<0.05$ (G3), c''': $p<0.001$ (G3).

Viability was significantly reduced on the HeLa tumor cell line treated respect to untreated control after 72h for 0.25 mg/mL ($p<0.05$), 0.5 mg/mL ($p<0.001$) and 1 mg/mL ($p<0.001$) for G2 group. The same results were observed for G1 and G3 at 0.5 mg/mL ($p<0.01$) and 1 mg/mL ($p<0.001$) (Figure 3A). The effect on the Vero cell line resulted in no significant difference in cell viability between treated and untreated cells for all groups and concentrations studied (Figure 3B). However, no statistically significant differences were observed for the percentages of cells viability among all groups and concentrations of *R. junceus* scorpion venom evaluated in HeLa and Vero cell lines.

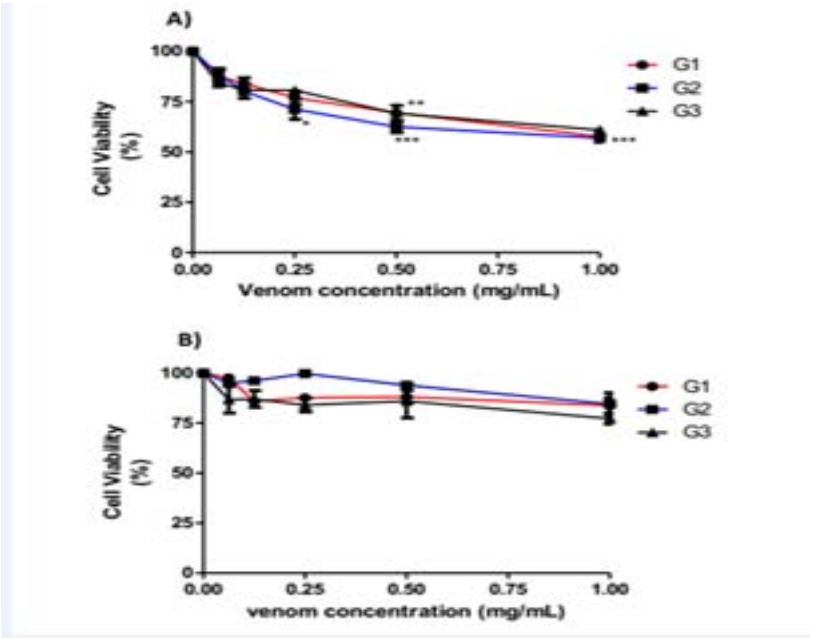

Figure 3: Cytotoxicity of G1, G2 and G3 of *R. junceus* scorpion venom on the HeLa and the Vero cell lines. The cells HeLa (A) and Vero (B) were incubated for 72h with scorpion venom at concentrations of 0.063-1mg/ml. Cell viability percentages were compared respect to untreated control group by Kruskal-Wallis non-parametric test and Dunn's multiple comparison test. Significant differences *$p<0.05$, **$p<0.01$,***$p<0.001$. Comparison of cell viability among G1, G2 and G3 groups for each concentration were analyzed by two-way Anova and Bonferroni post-test.

The IC_{50} values found for HeLa cells were not significantly different among G1 (1.13mg/mL), G2 (1.034 mg/mL) and G3 (1.175 mg/mL) groups. In Vero cell line, no cytotoxic effect was observed in all concentration tested and the theoretical IC_{50} values were higher than 1 mg/mL: 3.1, 3.5 and 2.6 mg/mL for G1, G2 and G3, respectively (Table 2).

Table 2: The IC_{50} values on the HeLa and the Vero cell lines exposed to G1, G2 and G3 of *R. junceus* scorpion venom. Values represent the mean ± SD derived from three independent experiments. Data showed as >1 means No effect.

Samples of *R.junceus* scorpion venom	IC_{50} (mg/mL)	
	HeLa	Vero
G1	1.13 ± 0.004	> 1
G2	1.034 ± 0.262	> 1
G3	1.175 ± 0.128	> 1

The morphological changes induced at 1 mg/mL of G1, G2 and G3 of *R. junceus* scorpion venom are shown in the Figure 4. All of them induced a loss of membrane integrity on HeLa cells; meanwhile Vero cells were not affected by scorpion venom from all studied group.

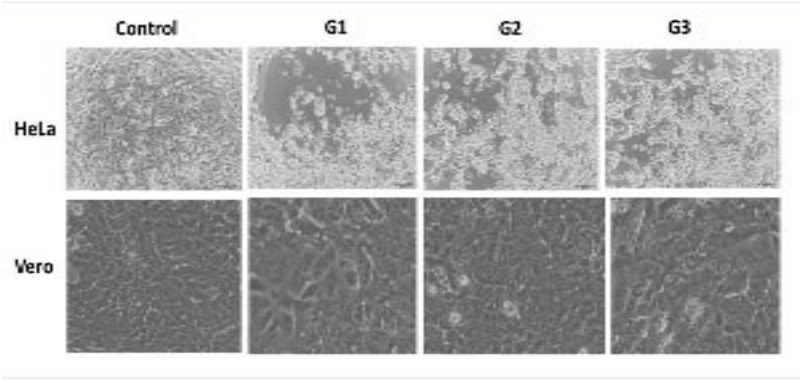

Figure 4: Morphology of HeLa and Vero treated with G1, G2 and G3 of *R. junceus* scorpion venom. A representative image of the effect of G1, G2 and G3 of *R. junceus* scorpion venom at 1 mg/mL on the treated monolayer for 72 hours. Cell controls showed 100% of viability. The images were captured using the DP-72 camera (Olympus Corporation, Tokyo, Japan). The experiments were performed in triplicate and repeated 3 times.

IV. Discussion

Scorpion venoms are very effective in the treatment of several diseases. Hence, during many years scorpion venom had been used in traditional medicine in many countries (Gomes et al. 2010, Tobassum et al., 2018) for its properties as analgesic, anticancer, anti-inflammatory and antimicrobial (Almaaytah and Albalas 2014, Harrison et al. 2016). Studies about venom recovery in *Androctonus finitimus* and *Hottentota tumulus* scorpions kept in the laboratory, demonstrated better yield and quantity by electrical method than manual method. Also, it was revealed the influence of diet and temperature on venom production (Tobassum et al., 2018). However, there are others parameters affecting venom consistency such as sex, geographical location, age and time intervals for extraction (Pucca et al., 2014). Regarding this last parameters, it has been reported that extended periods of Cuban *R. junceus* venom collection was positively correlated with the regeneration of venom composition and the increase of cytotoxic effect against A549 lung cancer cells (Díaz-García et al., 2019). On the other hand, 200 individual molecular masses were identified in male and female *R. junceus* scorpion venom from which 63 are identical in both sexes (Rodríguez-Ravelo et al., 2015).

The present study, demonstrated that total protein concentration was similar among female, male and the mixture of both sexes of *R. junceus* scorpion venom. Moreover, the numbers of bands in all sex studied groups were identical from both electrophoretic conditions, with no statistically significant differences in the molecular weight in female and male scorpions. Nevertheless, unique bands were observed in each one of the sexes. Our results are similar with other achieved

with poisonous animals how *Cerastes cerastes* snake, where specific bands were found in male (42 and 39 kDa) and female (46 and 44kDa) venoms (Sarhan et al, 2017). However, in the study with *Cerastes cerastes* snake only was shown the MW in the electrophoretic analysis. While we also determined, the intensity of each one of the bands obtained under both electrophoretic conditions. Statistically significant differences were observed between both sexes (G1 and G2) for the intensity of the bands to 12 kDa (under non-reduced conditions) and 11 kDa (under non-reduced and reduced conditions).Previous proteomic studies with *R.junceus* scorpion venom disagree to current study where the scorpion venom was kept in captivity. That study evaluated by HPLC and mass spectrometry, the venom of females and males scorpions kept in its natural medium. As results, the relative abundance of identical components was different among the genders (Rodríguez-Ravelo et al., 2015). We have already reported the majority band below 14 kDa in the electrophoretic profile (Díaz-García et al., 2015). Several authors that work with scorpion venom have reported the presence of the mixture peptides in a diffuse area conformed by small molecules lower than 14 kDa, due to the little resolution power of SDS-PAGE technique, these peptides cannot be observed separated (Hernández-Betancourt *et al.,* 2009).

This is the first study which compares the electrophoretic profiles of this venom from different sexes and the combination of both sexes (same proportion) from scorpion maintained under conditions of captivity. Previous studies with no same proportion have been done (Díaz-García et al., 2013; Díaz-García et al., 2017; Díaz-García et al., 2019). One of the important contributions of this study was the demonstration that morphological changes and loss of viability on the HeLa tumoral cell line; and the lack of cytotoxicity on the Vero cell line induced by *R.junceus* scorpion venom was independently of gender. This biological effect could be possible because the most abundant components are present in both sexes (Rodríguez-Ravelo et al., 2015). Also, the bands of the most intensity in all sex studied groups corresponded to proteins with low MW that they are the main responsible for their therapeutic potentialities. However, next studies are needed to compare this biological effect of venom from scorpions with different sexes with other tumor cells.

V. Conclusions

In our experimental conditions, there were similarities in the protein concentration and some differences in the electrophoretic profile of female, male and the combination of both sexes of *Rhopalurus junceus* scorpion venom. However, the cytotoxic effect of *Rhopalurus junceus* scorpion venom is maintained regardless of the sex of the scorpions on the HeLa tumor cell line.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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