



Synergistic effect of *Rhopalurus junceus* scorpion venom combined with conventional cytostatics in cervical cancer cell line HeLa

[Efecto sinérgico de la combinación del veneno de escorpión *Rhopalurus junceus* con citostáticos convencionales en la línea celular tumoral HeLa]

Arianna Yglesias-Rivera¹, Hermis Rodríguez-Sánchez², Alexis Díaz-García^{1*}

¹Research Department, Laboratories of Biopharmaceuticals and Chemistries Productions (LABIOFAM), Havana, Cuba.

²Microbiology Department, Tropical Medicine Institute "Pedro Kouri", Havana, Cuba.

*E-mail: alediaz@ipk.sld.cu

Abstract

Context: Venom from endemic scorpion of Cuba, *Rhopalurus junceus*, decreases the viability of epithelial cancer cells and has negligible cytotoxic effect on normal cells. Conventional chemotherapy induces unspecific cytotoxic effect against cancer and normal cells. Pharmacological interaction of this scorpion venom with conventional cytostatics is unknown.

Aims: To evaluate the cytotoxic effect of combined treatment of *Rhopalurus junceus* scorpion venom and conventional cytostatics in the cervical cancer and kidney normal cell line HeLa and Vero, respectively.

Methods: Both cell lines were treated alone with different concentrations of 5-fluorouracil (0.5, 5, 50, 500 and 5000 μ M), doxorubicin (0.005, 0.05, 0.5, 5 and 50 μ M) and cisplatin (3.13, 6.25, 12.5, 25 and 50 μ M) or combined with $1/2$ IC₅₀ in HeLa (0.5 mg/mL) of scorpion venom. Cell viability was determined by MTT assay. The combination index and dose reduction index at different concentration levels were generated by CompuSyn software.

Results: *Rhopalurus junceus* scorpion venom exerted synergic effect in HeLa cancer cell line when combined with 5-fluorouracil, cisplatin and doxorubicin at 0.5 - 500 μ M, 3.13 - 25 μ M and 0.005 - 5 μ M, respectively. Meanwhile, higher concentration levels of cytostatics combined with scorpion venom induced antagonist effects. Besides, 5-fluorouracil, cisplatin and doxorubicin as single treatment and combined with scorpion venom did not showed significant differences respect to cell viability in Vero cells.

Conclusions: *Rhopalurus junceus* scorpion venom is able to potentiate selectively the cytotoxicity, at low concentrations of chemotherapy drugs, against the cervical cancer cell line HeLa.

Keywords: chemotherapy drugs; cytotoxicity; HeLa; *Rhopalurus junceus* scorpion venom; synergism.

Resumen

Contexto: El veneno del escorpión endémico de Cuba *Rhopalurus junceus* disminuye la viabilidad de células tumorales epiteliales y no afecta las células normales. La quimioterapia convencional induce efecto citotóxico no específico sobre las células tumorales y normales. Las interacciones farmacológicas del veneno de escorpión con citostáticos convencionales no han sido estudiadas.

Objetivos: Evaluar el efecto citotóxico de los citostáticos, solos y combinados con el veneno de escorpión *Rhopalurus junceus*, en las células tumorales y normales, HeLa y Vero, respectivamente.

Métodos: Las células se trataron con los citostáticos solos a diferentes concentraciones de 5-fluorouracilo (0,5; 5; 50; 500 y 5000 μ M), doxorubicina (0,005; 0,05; 0,5; 5 y 50 μ M) y cisplatino (3,13; 6,25; 12,5; 25 y 50 μ M) y combinados con la $1/2$ CI₅₀ (0,5 mg/mL) en HeLa del veneno de escorpión. La viabilidad celular se determinó mediante el ensayo de MTT. El índice de combinación e índice de reducción de la dosis, se determinaron mediante el software CompuSyn.

Resultados: El tratamiento combinado del veneno de escorpión con 5-fluorouracilo, cisplatino y doxorubicina a 0,5 - 500 μ M, 3,13 - 25 μ M y 0,005 - 5 μ M, respectivamente, ejerció un efecto sinérgico sobre la citotoxicidad de HeLa. Mientras que la mayor concentración de los citostáticos, combinado con el veneno indujo efectos antagónicos. En células Vero no se obtuvieron diferencias significativas, entre el efecto citotóxico del tratamiento individual de los citostáticos 5-fluorouracilo, cisplatino y doxorubicina o combinados con el veneno, respectivamente.

Conclusiones: El veneno de escorpión *Rhopalurus junceus* potencia selectivamente la citotoxicidad a bajas concentraciones de los citostáticos, en la línea tumoral cérvico-uterina HeLa.

Palabras Clave: citotoxicidad; HeLa; quimioterapia; sinergismo; veneno de escorpión *Rhopalurus junceus*.

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INTRODUCTION

Cervical cancer is the second most common cancer in women from developing countries and is a leading cause of cancer-related death in women worldwide (Ferlay et al., 2014). Traditionally, chemotherapy has only been an option for recurrent cervical cancer or as an auxiliary treatment to enhance sensitivity to radiotherapy in concurrent chemoradiotherapy (CCRT) (Banzai et al., 2011). The most common chemotherapy drugs used for CCRT are 5-fluorouracil (5-FU) and cisplatin (CDDP). Besides, it has been reported that combining CDDP and 5-FU treatments with concurrent radiotherapy in patients with locally advanced cervical carcinoma, is more efficient in comparison to cisplatin monotherapy with concurrent radiotherapy (Nedovic et al., 2012). Cisplatin is generally the most active agent and may be used as first-line single agent chemotherapy for recurrent or metastatic cervical cancer (Long et al., 2005). Meanwhile, 5-FU is widely used in the treatment of cervical, gastrointestinal, breast, and lung cancers (Ahmed and Jamil, 2011). 5-FU and CDDP treatment comprise different side effects, such as: diarrhea, myelotoxicity, nephrotoxicity, upper gastrointestinal toxicity and myelosuppression (Li et al., 2013). Other chemotherapy drug that is used for treatment of cervical cancer is doxorubicin (DOX). However, increased risk of bleeding and infection, loss of appetite, cardiac damage and heart failure are inevitable side effects (Wong et al., 2013). Local recurrence and distant metastasis are still common post-treatment manifestations of CCRT in patients with advanced cervical cancer. Once post-treatment failure occurs, prognosis becomes worse: the 1-year survival rates of patients with such failures are less than 20% (Li et al., 2016). Moreover, side effects produced can greatly influence the patient's quality of life (Lorusso et al., 2014). Due to conventional chemotherapy do not differentiate between tumor and host cells, research efforts have been focused on finding new agents that target tumor tissue (Ahmed and Jamil, 2011). Besides, natural products with low or no toxicity on normal cells and high potency against cancer cells are usually investigated in combined

treatment with conventional chemotherapy to enhance cytotoxicity against cancer cells while reducing damage on normal counterpart (Karimabad et al., 2017).

The scorpion *Rhopalurus junceus* (*R. junceus*) is an endemic species from Cuba belonging to *Buthidae* family. Previous report has shown that venom from this scorpion exerts selective cytotoxic effect against cancer cell lines from epithelial origin without affecting normal cells (Díaz-García et al., 2013). The anticancer effect of scorpion venom includes apoptosis induction observed in the cervical cancer cell line HeLa (Díaz-García et al., 2013) and the metastatic breast cancer cell line, MDA-MB-231 (Díaz-García et al., 2017). Despite these previous reports, there are no evidences about the effect and the type of pharmacological interaction in combined treatment of this scorpion venom with chemotherapy drugs against cancer and normal cells. The aim of the present investigation is the evaluation of combined effect of *R. junceus* scorpion venom with conventional anticancer drugs in the cervical cancer cell line HeLa and the kidney normal cell line, Vero.

MATERIAL AND METHODS

Reagents

Fetal bovine serum (FBS) was purchased from Hyclone. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent, dimethyl sulfoxide (DMSO), doxorubicin (DOX, D1515), cisplatin (CDDP, C2210000) and 5-fluorouracil (5-FU, F6627) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Venom source

Adult *Rhopalurus junceus* scorpions, collected in Cienfuegos province, were kept in captivity for at least 1 month before venom extraction. Recent experiments demonstrated that lesser time of venom collection influence the venom composition and biological activity (unpublished results). Scorpions were maintained under Bioterium conditions, in individual plastic cages at 22.8°C and 76% relative humidity in laboratories belonging to The

Entrepreneurial Group of Biopharmaceuticals and Chemistries Productions (LABIOFAM). Water was given ad libitum using a cotton disc and animals were fed once a week. Venom from scorpions kept alive was obtained by electrical stimulation. This method has been recognized to cause fewer traumas, do not damage animal integrity and permits the obtaining best yields compared to other methods (Oukkache et al. 2013; Yaqoob et al. 2016; Tobassum et al. 2018). The obtaining, bioterium condition, management of scorpion colonies and collection of venom have been approved by the Ministry of Science, Technology and Environment of Cuba (CITMA 20/2016). Venom was dissolved in distilled water, centrifuged at 15 000 rpm for 15 min and supernatant was filtered by 0.2 µm syringe filter and stored at -20°C until used. The protein content was calculated by the method of Lowry modified (Herrera et al., 1999).

Cell lines and culture

The cell lines used in the experiments were HeLa (human cervix adenocarcinoma ATCC CCL-2™) and Vero (normal African green monkey kidney ATCC CRL-1586™). The HeLa cells at passage seven were routinely maintained in Eagle's Minimum Essential Medium in Earle's BSS with non-essential amino acids, 90% (w/v) containing L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Meanwhile, Vero cells at passage five were routinely maintained in Dulbecco's modified Eagle's medium, 90% (w/v) containing L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 10% heat inactivated fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Both cell lines were maintained in a humidified atmosphere containing 95% air/5% CO₂ at 37°C. The growth medium was replaced every three days. At day seven, the cells were trypsinized (Sigma-Aldrich, St. Louis, MO, USA) and seeded at a density of 2×10⁵ cells/mL in 96 multiwell flat bottom plates (Costar Corning, Rochester, NY, USA) in 50 µL of medium/well and incubated overnight to recovery and cell ad-

hesion in a humidified atmosphere of 5% (v/v) CO₂ at 37°C for 24 h.

In vitro cell viability assay (MTT assay)

The effect of individual or combined treatment of conventional cytostatics: 5-fluouracil (5-FU), cisplatin (CDDP) and doxorubicin (DOX), on HeLa and Vero cell viability was determined by the MTT assay (Mosmann, 1983). After 24 h in a humidified atmosphere of 5% (v/v) CO₂ at 37°C, 50 µL of each drug alone or combined simultaneously at non-constant ratio with scorpion venom were applied to the wells. In HeLa and Vero cells different drug concentrations were used for 5-FU (0.5, 5, 50, 500 and 5000 µM), CDDP (3.13, 6.25, 12.5, 25 and 50 µM) and DOX (0.005, 0.05, 0.5, 5 and 50 µM). These concentrations have previously been used in other studies with the same cancer cell line HeLa (Ahmed and Jamil, 2011; Khazaei et al., 2017). For combined treatments, a unique concentration of *R. junceus* scorpion venom of 0.5 mg/mL was employed, which represent the 1/2IC₅₀ value from HeLa (Díaz-García et al., 2013). Cells with culture medium and without treatment were used as untreated growth control. Three wells were included in each concentration evaluated for individual and combined treatment. After treatment for 72 h, 10 µL of 5 mg/mL of sterile MTT was added per well and cultivated for additional 3 h. The supernatant was carefully removed, 150 µL DMSO was added per well and shaken for 15 min at 37°C. The absorbance was measured at 560 nm with 630 nm as reference using a microplate reader ELISA (MRX Revelation Dynex Technologies, Denkendorf, Germany). Absorbance from untreated cells was considered as 100% of growth and used for viability calculation. The % viability was calculated, using the formula: %viability = A_{560 - 630 nm} of treated cells/A_{560 - 630 nm} of control cells × 100%. The experiments were performed at least three times.

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.

Evaluation of drug interaction

For analysis of drug interactions were determined two parameters that describe the interaction in a given combination: the combination index (CI) and the dose reduction index (DRI). The CI and the DRI were generated automatically using CompuSyn software (version 1.0; ComboSyn, Inc., Paramus, NJ, USA), as previously was described by Chou (2006). The $CI < 1$ indicates synergism, $CI = 1$ or close to 1 indicates additive effects, and $CI > 1$ indicates antagonism. $DRI > 1$ and < 1 indicate favorable and not favorable dose-reduction; $DRI = 1$ indicates no dose-reduction.

The CI and DRI were calculated in relation to the fraction of affected cells (Fa) at any given experimental point. Fa was calculated as follows: $(100 - \% \text{ Viable Cells})/100$.

Statistical analysis

Results are presented as the mean \pm standard error media (SEM). Statistical analysis was performed by two-way ANOVA, post-test: Bonferroni, using GraphPad Prism version 5.01 for Windows, (GraphPad Software, San Diego California, USA). Significant differences were considered for $p < 0.05$.

RESULTS

In normal cell line Vero no statistically significant differences were observed between 5-FU alone or combined with *R. junceus* scorpion venom (Fig. 1A).

However, in HeLa cells, combined treatment, at concentrations of 0.5, 5 and 50 μM induced a significant cell viability reduction ($p < 0.001$), when compared to similar 5-FU concentrations in individual treatment (Fig. 1B). Morphological changes such as rupture of cell monolayer and loss of cytoplasmic content was observed in 5-FU, as single agent, in HeLa (Fig. 1C). However, in combined treatment there was an increased number of rounded cells, the vast majority of cells were floating, cytoplasmic content in culture medium in-

creased and cleared zones were prolonged in wells as evidence of amplified cytotoxic effect (Fig. 1C).

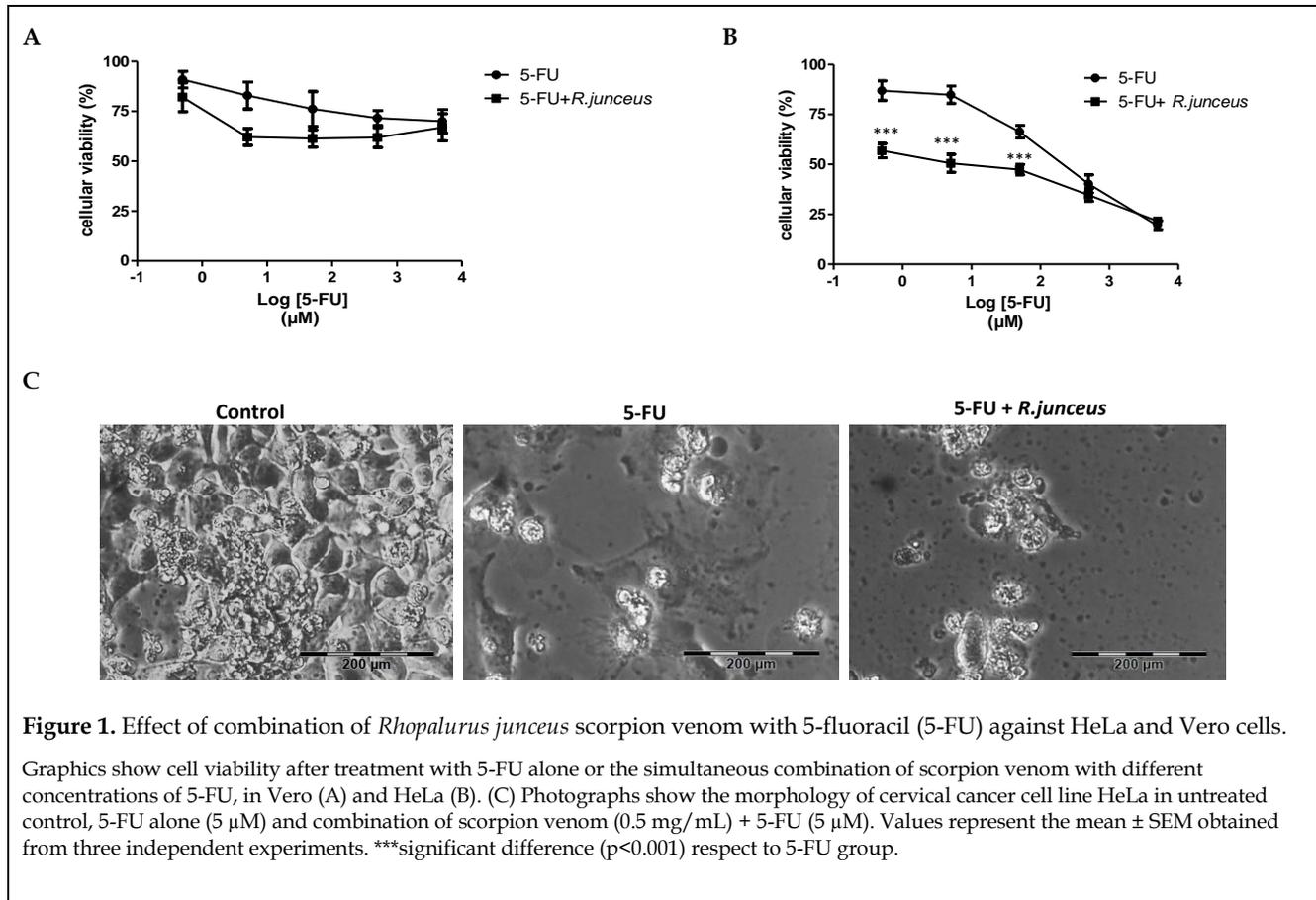
CDDP as single treatment and combined with scorpion venom did not show significant differences respect to cell viability in Vero cells (Fig. 2A).

Meanwhile, combined treatment was more effective than individual treatment at concentrations of 6.25 and 3.12 μM ($p < 0.001$) to reduce cell viability in HeLa (Fig. 2B). The morphological evidences of HeLa cells in culture, respect to CDDP as single treatment and the combination with scorpion venom, were similar to them observed in 5-FU (Fig. 2C). When DOX was used as single agent and combined with scorpion venom, showed similar effect on Vero cells viability (Fig. 3A). However, in HeLa, combined treatment at concentrations of 0.005, 0.05, and 0.5 μM ($p < 0.001$) showed significant differences respect to cell viability compare to DOX as single agent (Fig. 3B). Morphological changes in culture observed for DOX occurred similar to 5-FU and CDDP in HeLa cancer cells (Fig. 3C).

The type of drug interaction between scorpion venom with 5-FU, CDDP and DOX was determined from the CI obtained by the CompuSyn program. *Rhopalurus junceus* scorpion venom exerted synergic effect in HeLa cancer cell line when combined with 5-fluorouracil, cisplatin and doxorubicin at 0.5 - 500 μM , 3.13 - 25 μM and 0.005 - 5 μM , respectively ($CI < 1$) (Table 1).

Depending on the concentration and type of cytostatic used in the combination a slight, moderate or strong synergism was observed. On other hand, the highest concentration in each case showed antagonist effect ($CI > 1$) (Table 1).

In Table 1, the DRI values corresponding to each concentration of 5-FU, CDDP and DOX were also shown. Combined treatment of 5-FU with scorpion venom induced a reduction in cytostatic concentration from 1.92 - 1264.87 times respect to 5-FU as single agent to achieve similar effect against HeLa. Similarly, combination of CDDP with scorpion venom promoted a reduction in a range concentration of 1.33 - 2.95 times. Also, DOX



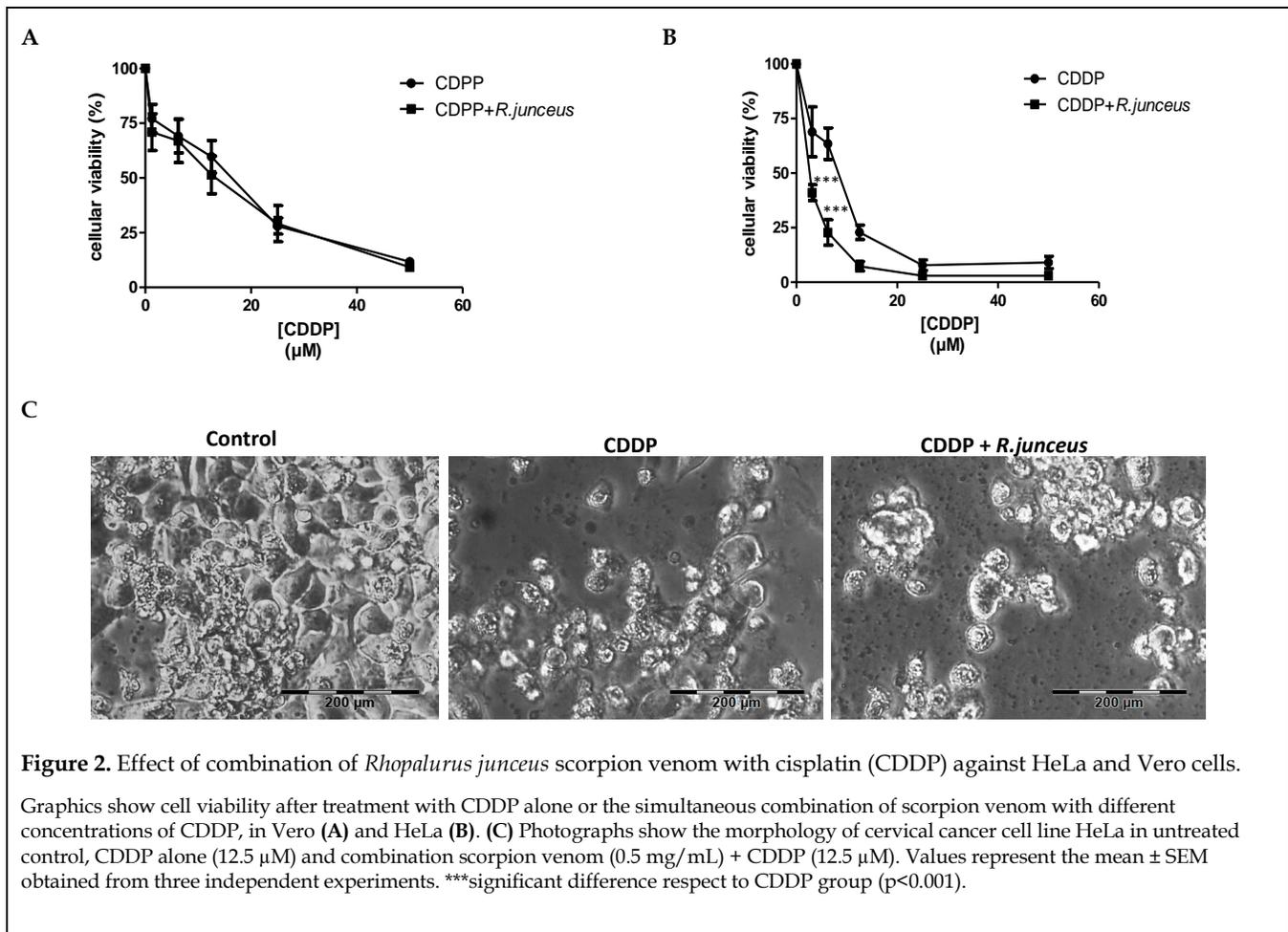
combined with the scorpion venom allowed its concentration to be reduced in a range of 3.17 - 245.48 times, respect to individual treatment, to achieve the same effect. However, at highest concentration of each drug combined with scorpion venom, DRI value indicated no favorable effect in concentration reduction due to antagonist effect (Table 1).

DISCUSSION

Cervical cancer greatly contributes to cancer-associated mortalities worldwide. Despite preliminary responses to chemotherapy and/or surgical interventions, the tumors consistently relapse (Jiang et al., 2018). Due this, several new strategies such as combination of natural products with chemotherapeutic agents are being developed to enhance efficacy (Pinmai et al., 2008). The present work was conducted to evaluate the cytotoxic ef-

fect of combined treatment of *Rhopalurus junceus* scorpion venom and conventional cytostatics on HeLa human cervical cancer cell line.

The exposure of scorpion venom with low concentrations of 5-FU, CDDP and DOX significantly has been more effective than cytotoxic drugs as single agents. The fact that the combination of a natural product with conventional cytostatic potentiates the effect of individual products is consistent with other studies. Vinod et al. (2013) reported that the use of nontoxic natural products such as curcumin, can enhance the efficacy of the chemotherapeutic drugs at non-cytotoxic concentrations. The combination of low concentration of *Aloe vera* crude extract and cisplatin has showed a synergistic effect in growth inhibition compared to the agents applied individually in MCF-7 and HeLa cells (Hussain et al., 2015). Meanwhile, the bee venom may exert anticancer effect on human



ovarian cancer A2780 and has the potential for enhancing the cytotoxic effect of the antitumor agent cisplatin (Alizadehnohi et al., 2012). The combination of flower extract of *Allium atrovio-laceum* with DOX resulting in synergistically enhanced growth inhibitory activity at different dose levels in HeLa, when compared to drug alone (Khazaei et al., 2017).

A additional advantage of the combination with natural products, is the reduction of drug concentration, reducing the potential toxic effect and avoiding the chemoresistance that in many occasions cause an interruption and ineffectiveness of the antitumor therapy (Vinod et al., 2013). In all cases, *R. junceus* induces synergistic effects at low concentration of cytostatic. Besides, the combined treatment and chemotherapy drugs used as single agents do not present differences on normal cell viability. This result is similar to those reported by

Rodríguez et al. (2014) where the simultaneous administration of CDDP with mangiferin do not increased the effect of CDDP on cell viability of CHO-K1 hamster ovary normal cell line. However, simultaneous combined treatments of 5-FU with mangiferin for 72 h induced significant increase of cell death in CHO-K1 cells, which differ from these results where combination of *R. junceus* with 5-FU do not increase cytotoxicity in Vero cells respect to single treatment. Besides, *R. junceus* venom have shown previously, to affect minimally in morphology and viability to normal cell lines, even at the highest concentration used in the study (Díaz-García et al., 2013; 2017). It seems that the presence of scorpion venom sensitizes to cancer and not normal cells to various cytotoxic drugs, which, could represent an advantage respect to development of chemoresistance. It is not far of thinking that *R. junceus* venom, due to results observed here, could overcome potential chemoresistance to

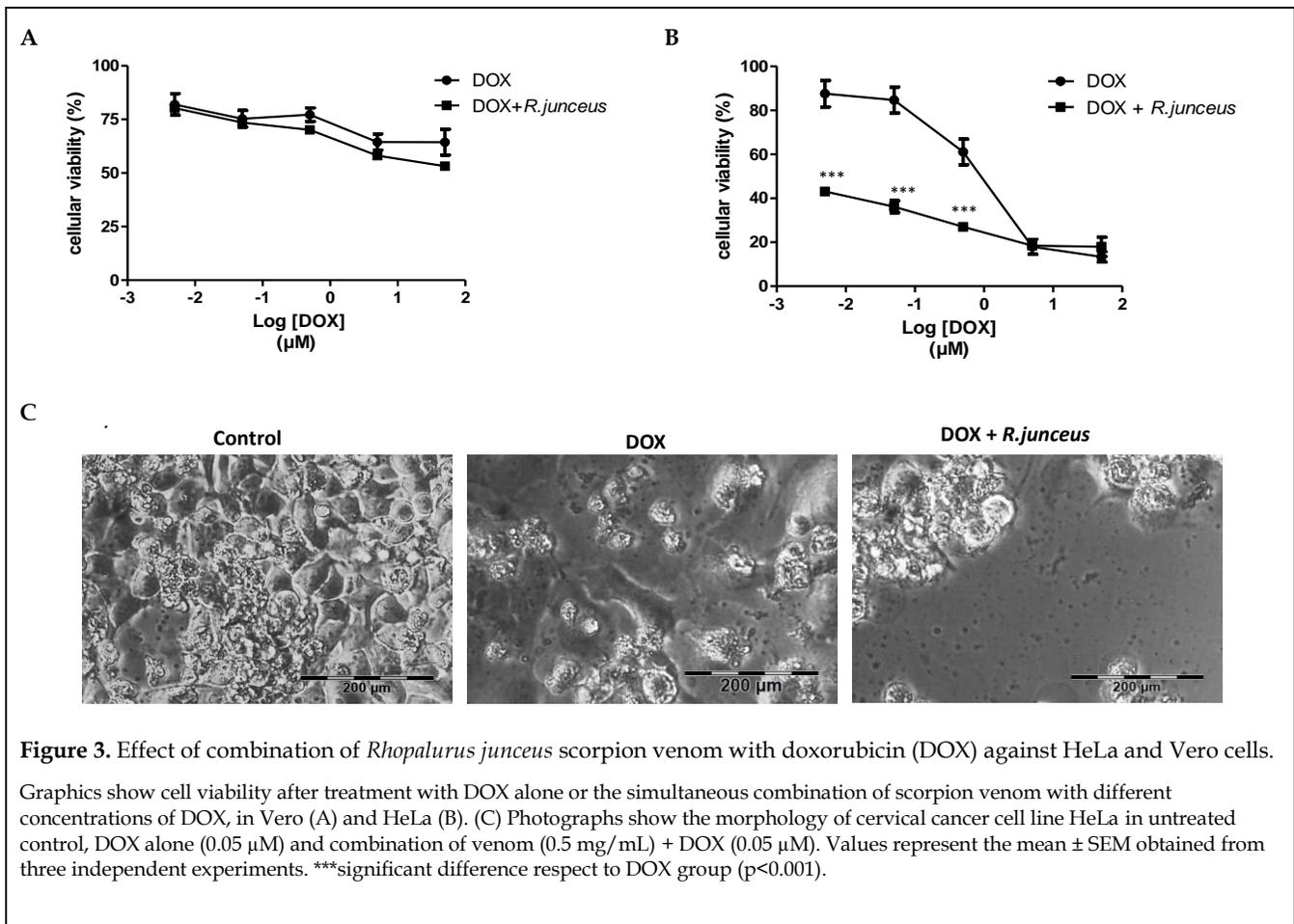


Figure 3. Effect of combination of *Rhopalurus junceus* scorpion venom with doxorubicin (DOX) against HeLa and Vero cells.

Graphics show cell viability after treatment with DOX alone or the simultaneous combination of scorpion venom with different concentrations of DOX, in Vero (A) and HeLa (B). (C) Photographs show the morphology of cervical cancer cell line HeLa in untreated control, DOX alone (0.05 μM) and combination of venom (0.5 mg/mL) + DOX (0.05 μM). Values represent the mean \pm SEM obtained from three independent experiments. ***significant difference respect to DOX group ($p < 0.001$).

chemotherapy drugs. The main agents studied, responsible for overcoming CDDP resistance in cervical cancer, belong to natural products or are natural products-based compounds (Zhu et al., 2016).

The CI obtained, in HeLa cells, for each combination to at non-constant ratio of *R. junceus* venom with 5-FU/CDDP/DOX varies depending on the concentration used, from synergic at low concentrations to antagonist effect at highest concentration. This result is similar to that reported by Aarti Mohan et al. (2014), who indicated that combination of resveratrol and 5-fluorouracil produces antagonist or additive effect depending on concentration. On other hand, Ayyagari et al. (2017) demonstrated that the efficacy of the bithionol (BT)-cisplatin combination depends on concentrations of cisplatin and BT. In cisplatin-sensitive cell lines, BT and cisplatin were mostly antagonistic except when used at low concentrations, where

synergy was observed, which agree with our results. In contrast, in cisplatin-resistant cells, BT-cisplatin combination treatment displayed synergistic effects at most of the drug concentrations ratios (Ayyagari et al., 2017). This suggests that combination effects of two or more compounds are governed by the interaction of their respective concentrations (Tallarida, 2012).

In this study, it was shown that combinations of scorpion venom with 5-FU, CDDP, DOX, respectively, increase the cytotoxic effect in the cervical cancer cell line HeLa, not only in terms of cell viability. Morphological changes characteristic of cytotoxic effect seen under inverted microscope, increased for the combination compared to individual treatment. Previous studies have reported that *R. junceus* scorpion venom induces a significant decrease in cell viability and provokes significant morphological changes like rupture of cell

monolayer (Díaz-García et al., 2013; 2017), similar to that seen in the present study.

The mechanism of action of the combination of scorpion venom with conventional cytostatic is unclear and possibly, multiple compounds in the scorpion venom are involved. However, *R. junceus* scorpion venom has shown to induce apoptotic cell death in HeLa cancer cells by up-regulation of proapoptotic genes p53 and bax and down-regulation of antiapoptotic gen bcl-2 (Díaz-García et al., 2013). Usually, downregulation of Bcl-2 has been associated to enhancement of chemosensitivity to agents such as DOX (Emi et al., 2005). Authors such as Khazaei et al. (2017) have suggested that the downregulation of Bcl-2 expression

by FAA enhances drug sensitivity by modulating the apoptotic signal transduction pathway. This similar action could be suggested for the combination of scorpion venom with CDDP, DOX and 5-FU. The objective of the present study did not include the evaluation of cell death events; however, it is known that both scorpion venom and drugs induce apoptosis. However, in the pictures of the combination, morphological characteristics suggestive of cell death different from apoptosis were observed, which could suggest a faster and more selective cell death at low concentrations of the combination. Additional studies that evaluate these characteristics should go deeper in this aspect.

Table 1. Combination index (CI) grade and dose reduction index (DRI) for each drug concentration combined with *Rhopalurus junceus* scorpion venom.

<i>R. junceus</i> (mg/mL)	Cytostatic (μ M)	CI value	CI grade	DRI
5-FU				
0.5	0.5	0.52	+++ (synergism)	1264.87
	5	0.35	+++ (synergism)	186.92
	50	0.39	+++ (synergism)	19.03
	500	0.85	+ (slight synergism)	1.92
	5000	1.59	--- (antagonism)	0.67
CDDP				
0.5	3.13	0.63	+++ (synergism)	2.95
	6.25	0.64	+++ (synergism)	1.85
	12.5	0.59	+++ (synergism)	1.73
	25	0.76	+ + (moderate synergism)	1.33
	50	1.5	--- (antagonism)	0.67
DOX				
0.5	0.005	0.28	++++ (strong synergism)	245.48
	0.05	0.19	++++ (strong synergism)	45.19
	0.5	0.17	++++ (strong synergism)	11.10
	5	0.35	+++ (synergism)	3.17
	50	2.96	--- (antagonism)	0.34

5-FU: 5-fluorouracil; CDDP: cisplatin; DOX: doxorubicin. The values of CI and DRI were obtained using a Compusyn software 1.0 (n=9, triplicate experiments with at least three replicates for each concentration).

In addition, at high concentrations, antagonism was observed and this means potential interference in the mechanism of action. It is known that cisplatin has more than one mechanism of action coupling either to DNA or proteins (Dasari and Tchounwou, 2014); it is possible that this fact could be responsible for the variability in the type of interaction between scorpion venom and cisplatin. Similar behavior occurs for the remaining drugs used in the study (Khazaei et al., 2017; Díaz-García et al., 2013; Ahmed and Jamil, 2011). *In vitro* combination studies provide an approach to the interaction between compounds. However, due to the living systems are very complex, these results should not be extrapolated directly and therefore should be evaluated in *in vivo* models to identify whether the behavior observed in the present study can be achieved.

CONCLUSIONS

Overall, these findings indicate that *R. junceus* scorpion venom is able to potentiate the cytotoxicity, against the cervical cancer cell line HeLa, at low concentrations of chemotherapy drugs 5-FU, CDDP and DOX.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Yglesias-Rivera A	Rodríguez-Sánchez H	Díaz-García A
Concepts or ideas	x	x	x
Design	x		x
Definition of intellectual content	x		x
Literature search	x		x
Experimental studies	x	x	
Data acquisition	x	x	
Data analysis	x		x
Statistical analysis	x		
Manuscript preparation	x		
Manuscript editing			x
Manuscript review	x	x	x

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