REVISTA CUBANA DE CIENCIAS BIOLÓGICAS
http://www.rccb.uh.cu

ARTÍCULO ORIGINAL

Actividad citotóxica y antiproliferativa de la apitoxina de *Apis mellifera* (Hymenoptera, Apidae) contra células tumorales cervicales

Cytotoxic and antiproliferative activity of apitoxin from Apis mellifera (Hymenoptera, Apidae) against cervical tumoral cells

Erlyn Tereza Valcarcel Soriano [1], Luis Francisco Morier Diaz [2], Idania Rodeiro Guerra [3], Lai Heng-Hung Ricardo [4], Sonia Resik Aguirre [4], Gloria del Carmen del Barrio Alonso [5], Liena de Regla Ponce Rey [5*]

1 Provincial Center for Hygiene, Epidemiology and Microbiology of Las Tunas Lucas Ortiz Street, number 13, Máximo Gómez corner, Las Tunas, Las Tunas, Cuba

2 Laboratory of Cell Cultures. Institute of Tropical Medicine ¨Pedro Kourí¨. Novia del Mediodía Avenue, KM 6 1/2, La Lisa Municipality, Havana, Cuba.

3 Institute of Marine Research. Loma 35, Alturas del Vedado, Plaza de la Revolución, Havana, Cuba

4 National Reference Laboratory for Enterovirus. Institute of Tropical Medicine ¨Pedro Kourí¨. Novia del Mediodía Avenue, KM 6 1/2, La Lisa Municipality, Havana, Cuba

5 Virology Laboratory. Department of Microbiology and Virology. Faculty of Biology, University of Havana. Calle 25 No. 455 between J and I, Vedado. Havana. Cuba.

*Autor para correspondencia: lponce@fbio.uh.cu

RESUMEN

El veneno de abeja o apitoxina es un producto natural utilizado tradicionalmente para tratar diversas enfermedades. Consiste en una mezcla compleja de sustancias bioactivas con actividades antimicrobianas, antivirales, citotóxicas y antiproliferativas. En el presente estudio se evaluaron las actividades citotóxica y antiproliferativa de la apitoxina obtenida de la especie *Apis mellifera* contra líneas celulares de carcinoma cervical. La citotoxicidad se evaluó mediante el método del MTT. Las células tumorales (SiHa, HeLa) y no tumorales (Vero CCL81, Vero E6) se trataron con diferentes concentraciones de la apitoxina (100; 80; 50; 30; 20; 10; 5; 2,5 y 1 µg/mL) durante 48 h y 72 h. La concentración citotóxica media (CC_{50}) se determinó mediante análisis de regresión. Se realizó un ensayo clonogénico para evaluar el efecto de la apitoxina en la formación de colonias celulares durante 72 h. Se contaron los clones celulares y se determinó la eficiencia clonogénica (EC). Se observaron efectos citotóxicos (lisis celular, desprendimiento de la monocapa) a concentraciones superiores a 20 µg/mL y toxicidad selectiva contra las células SiHa pero no contra las células HeLa cuando se trataron monocapas completamente confluentes durante 48 h. Se observó selectividad contra las células HeLa cuando se trataron monocapas de células semiconfluentes durante 72 h. La proliferación se inhibió a partir de una concentración de 10 µg/mL. El veneno de abeja muestra efectos citotóxicos y antiproliferativos contra las células tumorales cervicales, sin embargo, es necesario realizar más estudios para mejorar su selectividad e incorporarlo como candidato terapéutico alternativo o complementario contra el cáncer.

Palabras clave: veneno de abeja; apitoxina; tumoral; citotoxicidad; antiproliferativo; clonogénico.

Recibido: 2024-05-15

Aceptado: 2024-08-20

ABSTRACT

Bee venom or apitoxin is a natural product traditionally used to treat several diseases and pathologies. It is a complex mixture of bioactive substances with antimicrobial, antiviral, cytotoxic and antiproliferative activities. In the present study the cytotoxic and antiproliferative activities of the apitoxin obtained from species Apis mellifera were evaluated against cervical carcinoma cell lines. Cytotoxicity was evaluated by MTT cell viability assay. Tumoral (SiHa, HeLa) and non-tumoral (Vero CCL81, Vero E6) cells were treated with different concentrations of the apitoxin (100; 80; 50; 30; 20; 10; 5; 2.5 and 1 µg/mL) during 48 h and 72 h. The media cytotoxic concentration (CC_{50}) was determined by regression analysis. A clonogenic assay was performed in order to evaluate the effect of apitoxin on cell colony formation. After allowing adhesion to plate substrate, cells were treated with different concentrations of apitoxin during 72 h. Cell clones were counted and clonogenic efficiency (CE) was determined. Cytotoxic effects (cell lysis, monolayer detachment) were observed at concentrations above 20 µg/mL. Apitoxin showed selective toxicity against SiHa cells but not against HeLa cells when completely confluent monolayer were treated during 48 h. Selectivity against HeLa cells was observed when semi-confluent cell monolayers were treated after during 72 h. Proliferation of both tumoral cells was inhibited from 10 µg/mL concentration. Bee venom shows cytotoxic and antiproliferative effects against cervical tumoral cells, however, more studies need to be carried out to improve its selectivity and to incorporate it as an alternative or complementary therapeutic candidate against cancer.

Keywords*: bee venom; apitoxin; tumoral; cytotoxicity; antiproliferative; clonogenic .*

INTRODUCTION

Every year around 20 million people are diagnosed with cancer worldwide and 10 million die from the disease (Chhikara and Parang, 2023). Cervical cancer is one of the most prevalent cancers among women and in 2020 a total of 604 127 cases was diagnosed for an incidence of 6.5% of all cancer cases affecting women worldwide (Sung *et al*., 2021). The annual number of global new cases of this type of cancer is predicted to increase by 2030 from 570,000 to 700,000 while the number of deaths from 311 000 to 400 000 (Uddin *et al*., 2023). At present, chemotherapy remains the main option in combination with surgical treatment in cases where the latter is possible. However, resistance to chemotherapeutic drugs (Bukowski *et al*., 2020), serious side effects or the impossibility of administering them due to the deterioration of the health of affected patients, are recurrent challenges (Drury. *et al.,* 2022; Śliwa-Tytko *et al.,*2022).

In the last decades researches have focused on the search and development of new antitumoral drugs from natural sources such as plants, animals and microorganisms (Ajayi *et al.,* 2023; Barzkar *et* al., 2024; Librizzi *et al.,* 2024). Toxins from animal venoms have gained attention among the studied natural substances as a source of biologically active compounds with multiple therapeutic potentials (Pascoal *et al*., 2019; Fischer and Riedl, 2022). Such is the case of bee venom or apitoxin, whose cytotoxic, antiproliferative and antitumor effects has been reported in numerous studies (Lim *et al*., 2019; Hwang *et al*., 2022; Rocha *et al.,* 2022).

In Cuba, cancer is the second leading cause of death, and in 2022, 711 women were diagnosed with cervical cancer. Traditional treatments imply surgery and chemotherapy with the subsequent occurrence of side -effects and diminution of life quality. Novel specific medications are needed against these tumours (Sánchez Ed. 2023) as well as appropriate drug delivery systems. Considering animal toxins potentiality in this field, the present work aims to evaluate the *in vitro* cytotoxic and antiproliferative activity of *Apis mellifera* venom against cervical carcinoma SiHa and HeLa cells.

MATERIALS AND METHODS

Bee venom (apitoxin)

The bee venom or apitoxin was kindly provided by the Cuban Beekeeping Company (APICUBA, Havana, Cuba). The material consisted in a lyophilized form of the bee venom obtained from Cuban bee specie *Apis mellifera*.

Cell lines

Tumoral cells (SiHa, ATCC® HTB-35 TM and HeLa, ATCC® CCL-2 TM) and non-tumoral cells (Vero ATCC® CCL-81™ and Vero E6, ATCC® CCL- 81™) used in the present study belong to the Cell Culture Laboratory of the Institute of Tropical Medicine “Pedro Kourí”. They were originally acquired from the American Type Culture Collection (ATCC, USA). Cells were grown in Minimum Essential Medium (MEM) supplemented with 10 % of Inactivated Fetal Bovine Serum (IFBS), 1 % solution of non-essential amino acids and 2 mM of glutamine (GibcoTM). Cells were incubated in humidified atmosphere at 37°C and 5 % of CO_2.

Cytotoxicity assays by MTT method

Cytotoxicity of apitoxin was evaluated by the MTT (SIGMA®) cell viability method (Mosmann, 1983). For this purpose, a stock solution of the lyophilized apitoxin was prepared with non-supplemented cell culture medium at a final concentration of 100 µg/mL. Cells were seeded in 96-well plates at a concentration of $2.5*10^4$ cells/mL and incubated at 37°C, 5% of CO_2. Two independent assays were carried out, the first one with completely confluent cell monolayers and a second one with 50-60% confluent cell monolayers, the latter in order to additionally evaluate the effect of the apitoxin on cell multiplication.

Growth medium was removed and 100 µL of apitoxin were added at different concentrations (100; 80; 50; 30; 20; 10; 5; 2.5 and 1 µg/mL). For cell controls 100 µL of non-supplemented culture medium were added. Apitoxin semiconfluent treated cells were incubated during 72 h while apitoxin totally confluent treated cells were incubated during 48 h, both at 37°C, 5% of CO_2. Subsequently effect of apitoxin on cell viability was measured. For this, 10 µL of a MTT solution (5 mg/mL) was added to cells and plates were incubated at 37ºC, 5% of CO_2 protected from light for 4 hours. Subsequently, media was removed and formazan salts were dissolved with 100 µL of isopropanol. Finally, the absorbance was read at 520 nm with a reference filter at 630 nm, in an multiwell plate reader spectrophotometer (Dynex Technologies®, Unite State) with the Dynex Revelation 4.02 integrated program.

The percentage of cell viability associated with each concentration of the apitoxin was calculated as follows: OD_{50} CC(t)/ DO_{50} CC(c) *100.

OD_{50} CC(t): Mean absorbance value of treated cell cultures.

OD_{50} CC(c): Mean absorbance value of cell controls considered as 100 % cell viability.

Calculation of the Selective Index

To determine the presence or absence of specific toxicity of the apitoxin on tumoral cell lines, the Selective Index (SI) was calculated using the following formula:

$$\mathbf{SI} = \frac{\text{CC50 non tumoral cells}}{\text{CC50 tumoral cells}}$$

An SI value greater than 1 indicates that the product is more cytotoxic to tumoral cells than to non-tumoral cells while less than 1 means the cytotoxic effect on non-tumoral cells exceeds the effect on tumoral cells (Churampi, 2016).

Antiproliferative activity by clonogenic assay

The clonogenic assay was performed to evaluate the antiproliferative activity of apitoxin. For this $3*10^3$ cells/mL were seeded in 24-well plates with 10 % IFBS supplemented medium and incubated at 37°C, 5 % of CO_2 for four hours to allow cell adhesion to plate substrate. After this time 1 mL of apitoxin (50, 30, 20, 10 and 5 µg/mL) was added while 1 mL of media was added to cell controls. Then plates were incubated for 72 hours at 37°C and 5 % CO_2 (Piloto-Ferrer, 2017). Later, cells were fixed and stained with a 1 % solution of violet crystal in 4 % paraformaldehyde for 30 minutes. The plates were washed under running water and allowed to dry. Cell clones were counted using a Miotic® inverted microscope. Only clones with more than fifty cells were considered according to Piloto-Ferrer, 2017. Clonogenic Efficiency (CE) for each cell line was determined by the percentage of cells that formed colonies with respect to the cells control.

$$CE = \frac{\text{No. of cell colonies of the treatments}}{\text{No. of cell colonies of controls}} * 100$$

Statistical analysis

The cytotoxicity of the apitoxin was expressed as the mean of the cytotoxic concentrations (CC_{50}) with the corresponding standard deviations. This value was determined by linear regression analysis using Microsoft Office Excel 2013 program and considering a regression coefficient value (R^2) greater than 0.85. In order to compare the results obtained for the different cell lines, the Kruskal-Wallis nonparametric test was performed, with a significance level of $p<0.05$. The statistical program used was Statistica version 8.

RESULTS AND DISCUSSION

Cytotoxicity of apitoxin in tumoral and non-tumoral cell lines

The cytotoxicity assay showed a dose-dependent effect, since the higher the concentration of the apitoxin, the lower the percentage of viable cells and conversely for the lower concentrations (Fig.1).

When treated with concentrations of apitoxin higher than 20 µg/mL, Vero and Vero E6 cells, in contrast to non-treated cells, showed cell monolayer detachment, loss of the characteristic epithelial morphology and lysis (Fig. 2). The media cytotoxic concentrations values were 22.00 ± 0.26 µg/mL and 22.22 ± 1.95 µg/

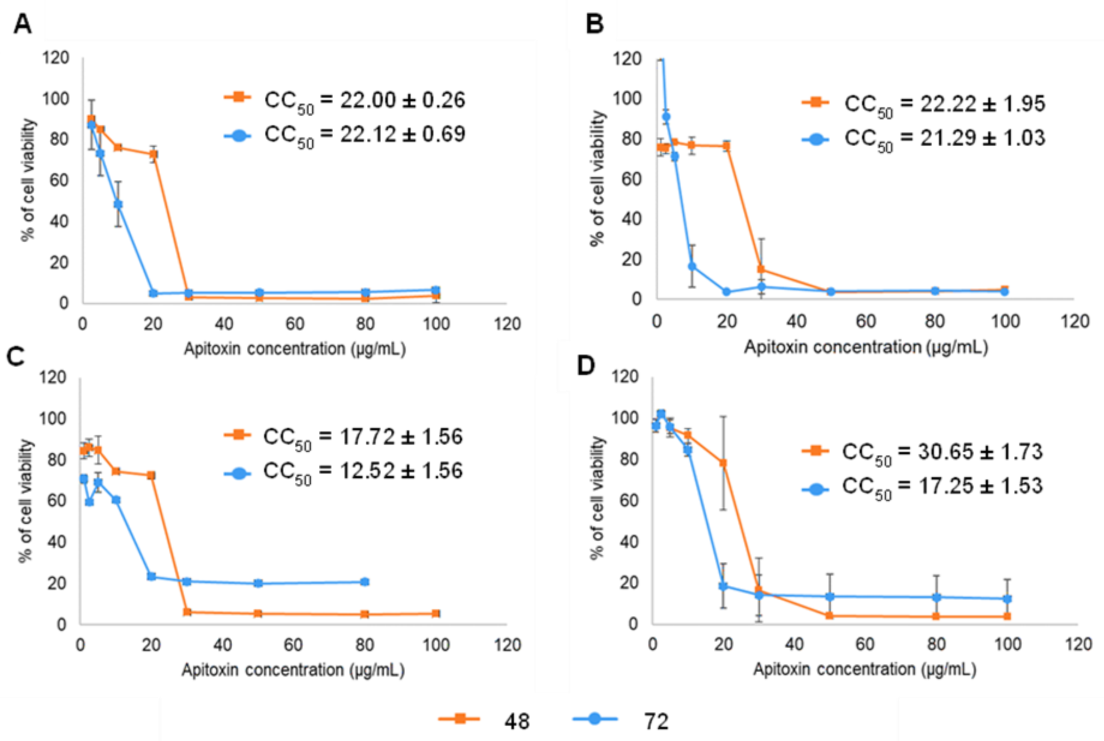

Figure 1. Effect of apitoxin on viability of non-tumoral Vero (**A**) and Vero E6 (**B**) cells and tumoral SiHa (**C**) and HeLa (**D**) cells by MTT method. Orange curves: completely confluent cell monolayers treated during 48 h, blue curves: semi-confluent cell monolayers treated during 72 h. Data are the mean ± standard deviation (n = 3).

mL respectively. Cell damage caused by the apitoxin in SiHa and HeLa cells consisted in a remarkable cell retraction and lysis at concentrations above 30 µg/mL with CC_{50} values of 30.65 ± 1.73 µg/mL and 17.72 ± 1.56 µg/mL respectively (Fig. 2). For tumoral cells, a longer time of apitoxin-exposition (72 h) of semi-confluent cell monolayers, showed lower media cytotoxic concentrations values (SiHa: 12.52 ± 1.56 and HeLa: 17.25 ± 1.53) and therefore higher toxicity.

The selective index (SI) values were determined between tumoral and non-tumoral cells treated with apitoxin during 48 and 72 hours respectively in the next combinations: Vero/SiHa, Vero/HeLa, Vero E6/SiHa and Vero E6/HeLa (Table I). After 48 h of treatment, apitoxin showed selective activity against SiHa tumoral cells respect to the non-tumoral cells due to SI values greater than 1 while not selectivity was observed for HeLa cells. After 72 h of treatment, besides the toxicity effects already described, apitoxin inhibited cell multiplication in both tumoral cell lines and showed selectivity for HeLa cells compare to non-tumoral cells. Significant statistical differences were found in CC_{50} values among tumoral and non-tumoral cell lines when treated during 48 h and 72 h, while no differences were found for Vero E6 and SiHa at 72 h.

As shown in figure 3, when semi-confluent cells monolayers were treated with different concentrations of apitoxin, cell multiplication was affected compared to untreated cells, which showed a complete confluence after 72 h of treatment, treatments with apitoxin caused a decrease in cell viability and multiplication. For concentrations above 20 µg/mL, changes in morphology and cell death were observed while cells treated with the 5 µg/mL survived

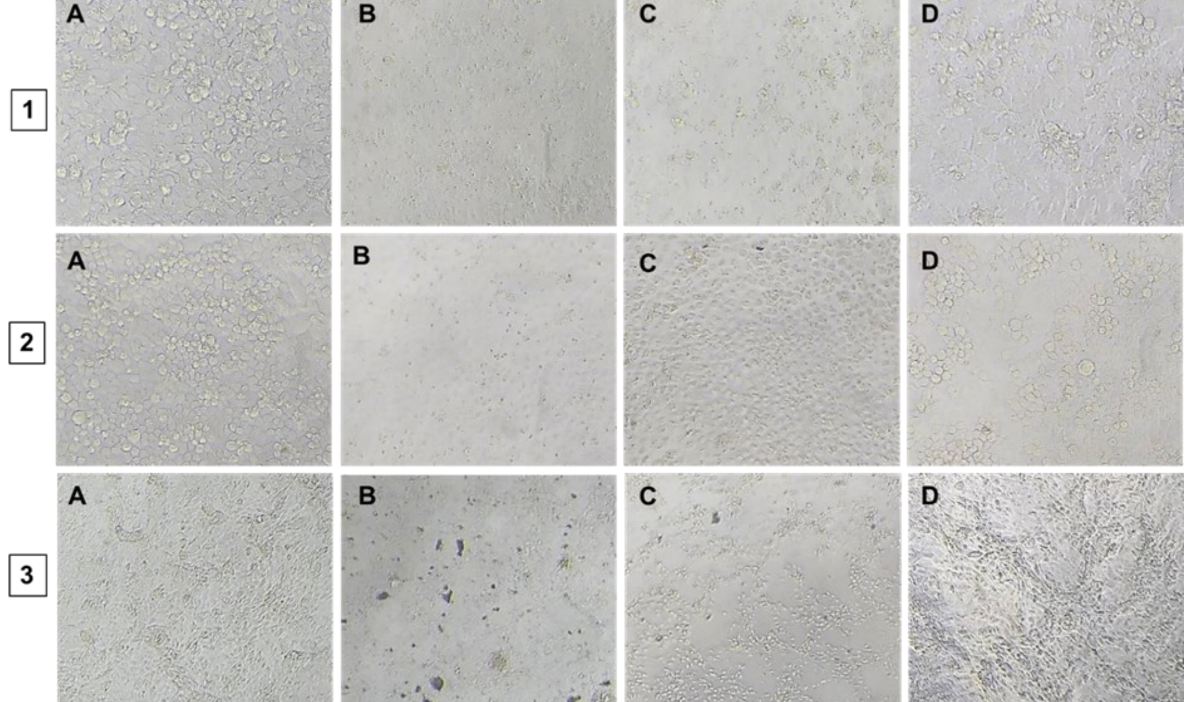

Figure 2. Effect of apitoxin on morphology and viability of HeLa (**1**), SiHA (**2**) and Vero (**3**) cells. **A**. Non-treated cells. **B**. Cells treated with 100 µg/mL of apitoxin. **C**. Cells treated with 30 µg/mL of apitoxin. **D**. Cells treated with 2.5 µg/mL of apitoxin. Magnification 40X.

Table I. Selective Index (SI) values determined between tumoral and non-tumoral cells treated with apitoxin during 48 and 72 hours on complete and semi-confluent cell monolayers respectively.

Selective Index (48h / 72h)		
Cells	SiHa	HeLa
Vero	1.24 / 1.77	0.71 / 1.28
Vero E6	1.25 / 1.70	0.72 / 1.23

and multiplied similarly to untreated cells (Fig. 3). Cytotoxicity of apitoxin at concentrations above 20 µg/mL was confirmed by the absence of formazan salts formation as an indicator of mitochondrial activity in viable cells.

Clonogenic assay

In the present study, a decrease in the number of tumoral and non-tumoral colonies cells was observed after 72 hours of treatment with apitoxin compared to the untreated cells. Colonies formation was inhibited at 20 µg/mL while at higher concentrations cells were no viable.

Vero E6 cells showed a similar number of cell clones compared to untreated cells, when treated with 5 µg/mL of apitoxin, while treatment with 10 µg/mL inhibited 38.24% of colony formation in relation to non-treated cells. Vero cells colonies formation was not inhibited at both apitoxin concentrations. In the case of tumoral cells, treatment with 10 µg/mL apitoxin inhibited HeLa and SiHa proliferation by 39.76 % and 36 %, respectively (Fig. 4). Statistical differences in the number of cellular colonies were found at this concentration between non-tumoral Vero cells and tumoral SiHa and HeLa cells respectively, as well as for combinations Vero-Vero E6 and SiHa-HeLa. At 20 µg/mL, independent stained cells and small clusters of less than 50 cells were observed, indicating loss of the ability to proliferate.

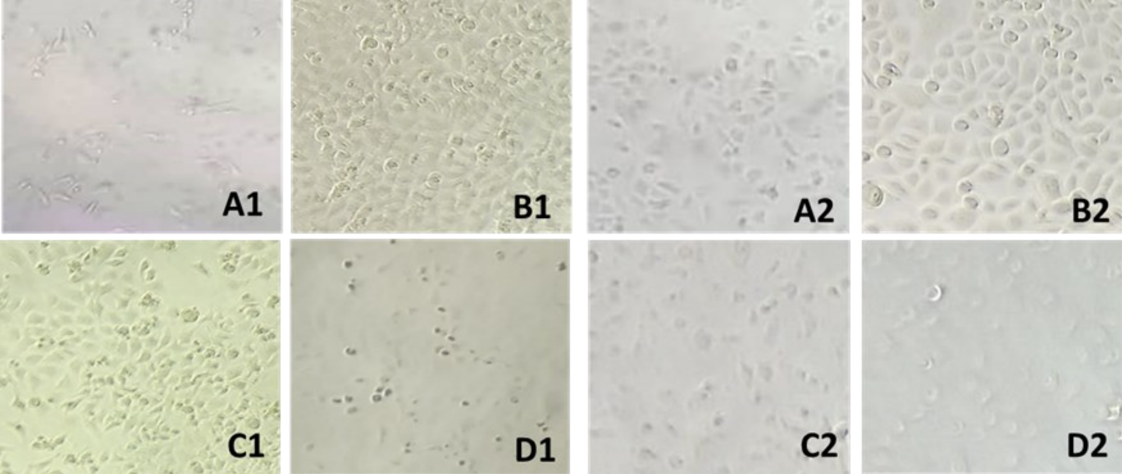

Figure 3. Effect of apitoxin on multiplication of semi-confluent monolayers of HeLa (**1**) and SiHa (**2**) cells after 72 hours of treatment. **A**. Semi-confluent cells monolayer before treatment. **B**. Non-treated cells monolayer completely confluent after 72 hours. **C**. Cells monolayer treated with 10 µg/mL (HeLa) and 20 µg/mL (SiHa). **D**. Cell monolayer treated with 50 µg/mL. Magnification 40X.

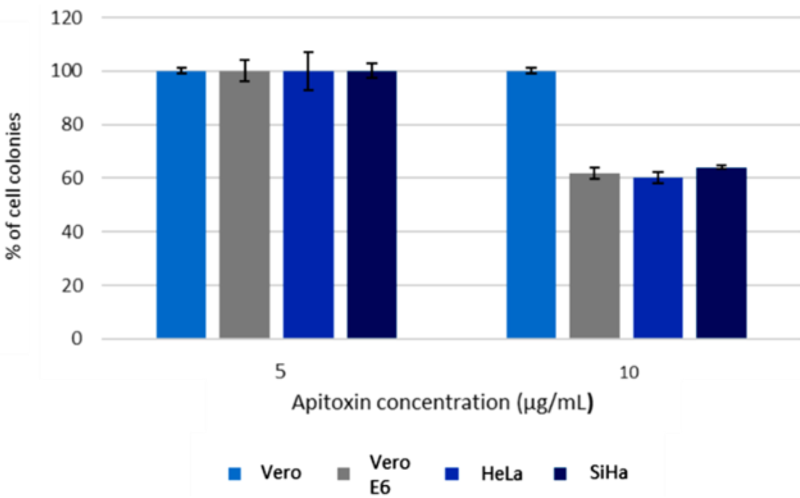

Figure 4. Effect of apitoxin on Vero, Vero E6, HeLa and SiHa cellular proliferation when treated with non-cytotoxic concentrations. Data is presented as the percent of colonies of treated cells related to the untreated cells. Values represent the mean of three independent experiments and their standard deviation.

DISCUSSION

Cervical cancer is among the most common diseases affecting women worldwide, especially in developing countries. So far around of 200 HPV genotypes have been identified and the number of those that contribute to cervical cancer has increased. Currently, cervical cancer treatment implies methods such as surgery, radiotherapy, and chemotherapy. However, patients suffer remarkable side effects and can face drug resistance and therefore cancer recurrence (Sisin *et al.,* 2023).

Natural products exert a wide versatility in terms of biological activities against infectious and non-infectious diseases. Their application in cancer therapy is supported by several *in vitro* and *in vivo* studies (Wang *et al.*, 2024a; Wang *et al.*, 2024b; Qin *et al.*, 2024). Venoms obtained from different organisms have been widely used in traditional medicine (Díaz-Gómez *et al*. 2024). Among them, the venom from bee *Apis mellifera,* is one of the most extensively studied (Gajski *et al.,* 2024). This species was introduced in Cuba in the 17th century (Benítez *et al.*, 2014) and is the main honey bee producer in the country (Alvarez-Suarez *et al.*, 2018).

Apitoxin or bee venom represents a natural defense of bees and consist in a complex mixture that includes peptides such as melittin and mast cell degranulating peptide, proteins, biogenic amines (histamine, dopamine, noradrenalin), phospholipids, sugars, pheromones, minerals as well as enzymes such as phospholipase A2 and B and hyaluronidase (Pucca *et al.,* 2019; Pascoal *et al.,* 2019; Gajski *et al.,* 2024).

Apitoxin peptides target ion channels, receptors and form pores in the lipid membranes of cells (Hung *et al.,* 2017). Although the therapeutic use of bee venom is not widely accepted due to the secondary effects, the purified components show promising activities (Aufschnaiter *et al.*, 2020). Variables such as geographic region, environmental conditions and the bee species from which the venom is extracted determine its composition and therefore influence their toxicity effects (Pucca *et al*., 2019). Apitoxin cytotoxicity has been reported for tumoral and non-tumoral cell lines (El Mehdi *et al.,* 2021; Viana *et al.*, 2021). Considering the importance of cervical cancer in women population and the antitumoral potentialities of bee venom, in this study, both cytotoxic and antiproliferative activities of apitoxin, obtained from Cuban bee species, *Apis mellifera,* were

evaluated against tumoral uterine and cervical adenocarcinoma cells.

Toxicity exhibited by apitoxin against the cells employed in this study was verified through the decrease in viability, changes in morphology and cellular death. These effects can be explained by a set of mechanisms well described by other authors for apitoxin's components such as melittin and phospholipase A2, such as disruption of biological membranes via the formation of pores and the hemolytic activity (Hung *et al.*, 2017). In addition, it has been reported that apitoxin triggers apoptosis mechanisms mediated by an increase in reactive oxygen species. Additionally, melittin inhibits calmodulin protein binding activity which causes an increase in intracellular Ca^{2+}, attenuation of the mitochondrial membrane potential and subsequent release of cytochrome C and caspase-3 proteins and DNA fragmentation (Chaisakul *et al*., 2016).

The morphological changes observed in the present study in apitoxin-treated cells are very similar with those described by Zarrinnahad *et al.* 2018. These authors reported that at a concentration of 4 µg/mL melittin caused cell shrinkage, condensation of the cytoplasm, disordered cell structure and significant loss of morphology of HeLa cells. Another study showed a remarkable cytotoxicity of apitoxin in HeLa cells and others cervical cancer cell lines such as Caski and C33A. It was suggested that cytotoxicity was mediated by antiviral mechanisms of action such as the suppression of Human papilloma virus proteins expression (Kim D. *et al.*, 2020). An *in vivo* study in model mice demonstrated that bee venom has a significant inhibitory effect on the growth of cervical tumors (Lee H. L. *et al.*, 2015). In relation to SiHa cells, there are few studies about the effect of animal toxins (Pinotti *et al.*, 2018). This work represents a preliminary approach to the cytotoxic and anti-proliferative activity of bee venom on these tumoral cells.

Some studies have shown a selective cytotoxic effect against tumoral cells but not against no-tumoral cells (Viana *et al*. 2021), while others have demonstrated a general toxicity on both types (Lee Y.J. *et al.*, 2007). These differences are suggested to be related from a molecular perspective with the positive charges of melittin that that might preferentially favor the binding to cancer cells membranes, highly negative charged compared to non-tumoral cells (Riedl *et al.*, 2011; Jamasbi *et al*., 2015).

In the present study apitoxin showed selectivity against SiHa cells but not HeLa cells, compared to non-tumoral cells, when completely confluent cells were treated during 48 h. However, selectivity was observed for HeLa cells when semi-confluent (50-60%) monolayers were treated with apitoxin during 72. This result suggests inhibition of cell proliferation and a selective effect influenced by the number of treated cells and the time of exposure to the product.

Lack of selectivity represents a disadvantage for pharmacological uses of apitoxin components, however, there are alternatives to increase the specificity against tumor cells. One example is the chemical modification of melittin that guarantees its effect in response to the acidic pH characteristic of tumor microenvironments and then promotes targeted action as well as enhanced biocompatibility (Luo *et al*., 2018). Another novel approach is the incorporation of melittin into nanoliposomes. This formulation showed efficient activity against hepatocellular carcinoma and at a high dose showed less toxicity in mouse liver tissue than free melittin (Mao *et al.*, 2017). Approaches such as alginate-based nanomaterials to enhance the therapeutic potentials of bee products have been explored as well (Al-Hatamleh *et al.*, 2022). Melittin conjugates with tumor -targeting proteins or compounds have been formulated and functionalized based on cervical cancer microenvironment characteristics as the overexpression of glutathione and the folate receptor. These conjugates may be potential anticancer drug candidates with fewer adverse effects and greater specificity (Sahsuvar *et al.,*2023).

The clonogenic assay is considered one of the most accurate methods to determine the effects of a product on cell proliferation due to it measures the ability of a cell to divide and form a colony (Kabakov y Gabai, 2018). In the present study apitoxin significantly inhibited the proliferation of tumoral cells and non-tumoral Vero E6 cells at concentration of 10 µg/mL. Interestingly, Vero cells proliferation was not affected at this concentration compared to Vero E6. Vero cell line, which contains chromosomal anomalies and are interferon-deficient is a continuously culturable cell

lines that does not enter senescence. All of these make it extensively used in virus isolation and the study of antiviral drugs (Sène et al., 2022). On the other hand, Vero E6 is a Vero-derived cell line that exhibit contact inhibition in culture and therefore grow to a lower density. Genetic differences among the Vero sublines have been reported and it is suggested that those influence Genetic differences between Vero sublines have been described and it is suggested that these influence their biological responses (Konishi et al., 2022).

The antiproliferative activity of apitoxin is suggested by other authors to be determined by a set of mechanisms related to the regulation of apoptosis or cell death. Cells treated with apitoxin showed an increase in the expression of the cell death receptors DR3 and DR6, a decrease in the expression of the antiapoptotic protein Bcl-2 and an increase in the expression of proapoptotic proteins (Lee H. L. *et al.,* 2015). Apitoxin and melittin antiproliferative activity in in B16F10 melanoma cells was verified at a concentration of 5 μg/mL (Lim et al., 2019). Other studies report that apitoxin and melittin induced cell death in aggressive breast cancer cells (Duffy et al., 2020), reduce cell viability in acute lymphoblastic leukemia (CCRF-CEM) and chronic myelogenous leukemia (K-562) cell lines (Ceremuga *et al.,* 2020) and mediated cell membrane changes within one minute of exposure in AGS, COLO205, and HCT-15 cell lines (Soliman *et al.,* 2019). Melittin-induced effects such as suppression of the matrix metalloproteinase-2 expression in non-small cell lung cancer (Sisakht *et al.,* 2017), induction of death receptors and inhibition of the JAK2/STAT3 pathway, were describe in ovarian cancer cells (Jo et al., 2012), HeLa (Zarrinnahad et al., 2018) and pancreatic ductal adenocarcinoma cells (Wang et al., 2018).

More studies are required to determine the mechanisms involved in the antiproliferative activity of apitoxin on the tumoral cells observed in our work. Although the cytotoxicity and antiproliferative assays remains the gold standard methodology for the evaluation of the sensitivity of cells to radiation and various anticancer drugs (Kabakov and Gabai, 2018), does not inform about the mechanisms by which apitoxin causes death or loss of the proliferative capacity of the cells. The advantage of this method is the possibility of precisely quantifying the number of metabolically active cells that survive treatment with the product of interest to be evaluated, as well as determining cell proliferation at the population level or the number of total viable cells in a well (Olmedo et al., 2016; Kim D. H. et al., 2020). Taking into account that tumoral and no-tumoral cells have noticeably different metabolism, using additional and complementary methods is recommended (Galluzzi et al., 2009) in order to evaluate the tumoral-specific cytotoxicity and antiproliferative activity of apitoxin. While the MTT assays allows determination of the percentage of metabolically active cells, the clonogenic assay informs how many of the viable cells are capable to proliferate. In the present study, even though viable cells were observed in the presence of apitoxin, the absence of clones may be due to a negative regulation of mitosis (Yao et al., 2020; Haque et al., 2023).

In the present study was confirmed that apitoxin induce cell death and inhibits cell proliferation of cervical carcinoma cells. Despite unspecific cytotoxicity against both non-tumoral and tumoral cells, some selectivity was observed for SiHa and HeLa cells. The ability to effectively inhibit cell proliferation, makes it a substance of interest for future *in vitro* and in vivo studies, including the purification and evaluation of its components. In Cuba, hundreds of cases of cervical cancer are reported annually (Sánchez Ed. 2023). This, together with the limitations presented by conventional anticancer therapies (Tolossa *et al.,* 2021), motivates the search and development of alternative or complementary antitumoral candidates

Acknowledgements

The authors are very grateful to the Cuban Beekeeping Company, Havana, Cuba for providing the apitoxin for the *in vitro* experimental studies.

Statements: All authors give their consent for the submission and publication of the manuscript. The manuscript has not been submitted to simultaneous review by another journal.

Funding: The authors did not receive funds for the execution of this work.

Conflict of interest: The authors declare that there are no conflicts of interest.

RFERENCE

Al-Hatamleh, M.A.I., Alshaer, W., Hatmal, M.M., Lambuk, L., et al. (2022). Applications of Alginate-Based Nanomaterials in Enhancing the Therapeutic Effects of Bee Products. Frontiers in molecular biosciences, 9, 865833. https://doi.org/10.3389/fmolb.2022.865833.

Alvarez-Suarez, J.M., Giampieri, F., Brenciani, A., Mazzoni, L., et al. (2018). *Apis mellifera* vs Melipona beecheii Cuban polifloral honeys: A comparison based on their physicochemical parameters, chemical composition and biological properties LWT - Food Science and Technology. 87: 272-279. https://doi.org/10.1016/j.lwt.2017.08.079.

atcc.org [internet]. ATCC: The Global Bioresourse Center. c2022 [cited 2022 marzo 10]. https://www.atcc.org/search#q.

Aufschnaiter, A., Kohler, V., Khalifa, S., Abd El-Wahed, A. et al. (2020). Apitoxin and Its Components against Cancer, Neurodegeneration and Rheumatoid Arthritis: Limitations and Possibilities. Toxins (Basel).12(2):66. doi: 10.3390/toxins12020066. PMID: 31973181; PMCID: PMC7076873.

Barzkar, N., Sukhikh, S., Babich O. (2024). Study of marine microorganism metabolites: new resources for bioactive natural products. Front. Microbiol. 14:1285902.

Benítez Álvarez, L., Artiles, A., Morales, A., Perez, V., Espinosa-López, G. (2014). Consanguinidad de *Apis mellifera* en el apiario del centro de investigaciones apícolas, Cuba. Apiciencia. XVI. 56-61.

Bukowski, K., Kciuk, M., & R. Kontek. (2020). Mechanisms of Multidrug Resistance in Cancer Chemotherapy. Int J Mol Sci. 21 (9):3233.

Ceremuga, M., Stela, M., Janik, E., Gorniak, L.*et al.* (2020). Melittin-A Natural Peptide from Bee Venom Which Induces Apoptosis in Human Leukaemia Cells. Biomolecules. ;10(2):247. doi: 10.3390/biom10020247. PMID: 32041197; PMCID: PMC7072249.

Chaisakul, J., Hodgson, W.C., Kuruppu, S., & N. Prasongsook (2016). Effects of Animal Venoms and Toxins on Hallmarks of Cancer. J Cancer.; 7(11): 1571-1578. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4964142/

Chhikara, B. & K. Parang (2023). Global Cancer Statistics 2022: the trends projection analysis. Chemical Biology Letters; 10(1): 451.

Churampi, D. (2016) Evaluación de la acción antiproliferativa del extractoacuoso de Physalis peruviana L. (Aguaymanto) en cultivos celulares de linfocitos humanos y leucemia mieloide crónica (K562). Tesis de Diploma, Universidad Nacional Mayor de San Marcos, Perú.

Díaz-Gómez, J.L., Martín-Estal, I., Rivera-Aboytes, E., Gaxiola-Muñíz, R.A., et al. (2024). Biomedical applications of synthetic peptides derived from venom of animal origin: A systematic review. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 170, 116015. https://doi.org/10.1016/j.biopha.2023.116015.

Drury, Z., Ly, T. & J.Abraham (2022). Nephrotoxic Effects of Chemotherapeutic Agents. Clin J Oncol Nurs. 26(2):219-223.

Duffy C., Sorolla, A., Wang, E., Golden, E. *et al.* (2020). Honeybee venom and melittin suppress growth factor receptor activation in HER2-enriched and triple-negative breast cancer, NPJ Precision Oncology 4 (1): 1–16.

El Mehdi, I., Falcão, S.I., Harandou, M., Boujraf, S., *et al.* (2021). Chemical, Cytotoxic, and Anti-Inflammatory Assessment of Honey Bee Venom from *Apis mellifera intermissa*. Antibiotics 10 (1514): 1-16. https://www.mdpi.com/2079-6382/10/12/1514

Fischer, T. & R. Riedl (2022). Paracelsus' legacy in the faunal realm: Drugs deriving from animal toxins. Drug Discovery Today, Volume 27, Number 2 February.

Gajski, G., Leonova, E. & N. Sjakste (2024). Bee Venom: Composition and Anticancer Properties. Toxins, 16(3), 117. https://doi.org/10.3390/toxins16030117.

Galluzzi, L., Aaronson, S.A., Abrams, J., Alnemri, E.S. *et al.* (2009). Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. 16: 1093–1107.

Haque, S., Hussain, A., Joshi, H., Sharma, U., *et al.* (2023). Melittin: a possible regulator of cancer proliferation in preclinical cell culture and animal models. Journal of cancer research and clinical oncology, 149(19), 17709–17726. https://doi.org/10.1007/s00432-023-05458-8.

Hung, A., Kuyucak, S., Schroeder, C.I. & Q. Kaas (2017). Modelling the interactions between animal venom peptides and membrane proteins. Neuropharmacology, 127: 20–31.

Hwang, Y N., Kwon, I.S., Na, H.H., Park, J.S. *et al.* (2022). Dual Cytotoxic Responses Induced by Treatment of A549 Human Lung Cancer Cells with Sweet Bee Venom in a Dose-Dependent Manner. Journal of Pharmacopuncture. 25(4):390-395

Jamasbi, E., Mularski, A. & F. Separovic (2015). Model membrane and cell studies of antimicrobial activity of melittin analogues. Curr. Top. Med. Chem., 16: 40–45.

Jo, M., Park, M.H., Kollipara, P.S., An, B.J. *et al.* (2012). Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway, Toxicology and Applied Pharmacology 258 (1): 72–81.

Kabakov, A.E. & V.L. Gabai (2018). Chaperones: Methods and Protocols, Methods in Molecular Biology. Vol 1709. Humana New York.

Kim, D.H., Lee, H.W., Park, H.W., Lee, H.W. *et al.* (2020). Bee venom inhibits the proliferation and migration of cervical-cancer cells in an HPV E6/E7-dependent manner. BMB Rep.; 53(8): 419 -424. https://www.bmbreports.org/journal/view.html?volume=53&number=8&spage=419

Konishi, K., Yamaji, T., Sakuma, C., Kasai, F., *et al.* (2022). Whole-Genome Sequencing of Vero E6 (VERO C1008) and Comparative Analysis of Four Vero Cell Sublines. Front. Genet. 13:801382. doi: 10.3389/fgene.2022.801382.

Lee, H.L., Park, S.H., Kim, T.M., Jung, Y.Y. *et al.* (2015). Bee venom inhibits growth of human cervical tumors in mice. Oncotarget 6(9): 7280-7292. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4466684/

Lee, Y.J., Kang, S.J., Kim, B.M., Kim, Y.J. *et al.* (2007). Cytotoxicity of honeybee (*Apis mellifera*) venom in normal human lymphocytes and HL-60 cells. Chem. Biol. Interact. 169: 189–197.

Lim, H.N., Baek, S.B. & H.J. Jung. (2019). Bee Venom and Its Peptide Component Melittin Suppress Growth and Migration of Melanoma Cells via Inhibition of PI3K/AKT/mTOR and MAPK Pathways. Molecules 24 (929): 1-13. https://www.mdpi.com/1420-3049/24/5/929

Luo, L., Wu, W., Sun, D., Dai, H. B. *et al*. (2018). Acid-Activated Melittin for Targeted and Safe Antitumor Therapy. Bioconjug Chem; 29: 2936−2944. https://pubs.acs.org/doi/10.1021/acs.bioconjchem.8b00352

Mao, J., Liu, S., Ai, M. Wang, Z. *et al*. (2017). A novel melittin nano-liposome exerted excellent anti-hepatocellular carcinoma efficacy with better biological safety. J. Hematol. Oncol.; 2017.10 (71): 1-4. https://jhoonline.biomedcentral.com/articles/10.1186/s13045-022-01277-5

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55-63.

Olmedo, D.A., Marrone, N.S., Espinosa, A.F., Guerra, C.P. *et al.* (2016). Evaluación de la actividad antiproliferativa de extractos metanólicos de plantas de la familia *leguminosae*. Revista Cubana de Plantas Medicinales; 21(3): 272-283. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S1028-47962016000300003

Pascoal, A., Estevinho, M.M., Choupina, A.B., Sousa-Pimenta, M., & L. M. Estevinho (2019). An overview of the bioactive compounds, therapeutic properties and toxic effects of apitoxin. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 134, 110864. https://doi.org/10.1016/j.fct.2019.110864.

Pinotti, S., Oliveira, V. A., Pupo, A. V., Henrique, L. *et al*. (2018). Crotoxin from Crotalus durissus terrificus venom: *in vitro* cytotoxic activity of a heterodimeric phospholipase A2 on human cancer-derived cell lines. Toxicon; 6003: 1-28. https://www.sciencedirect.com/science/article/abs/pii/S0041010118307141?via%3Dihu

Pucca, M.B., Cerni, F.A., Oliveira, I.S., Jenkins, T.P. *et al*. (2019). Bee Updated: Current Knowledge on Bee Venom and Bee Envenoming Therapy. Front. Immunol. 10(2090): 1-15. https://www.frontiersin.org/articles/10.3389/fimmu.2019.02090/full

Qin, P., Li, Q., Zu, Q., Dong, R., & Qi, Y. (2024). Natural products targeting autophagy and apoptosis in NSCLC: a novel therapeutic strategy. Frontiers in oncology, 14, 1379698. https://doi.org/10.3389/fonc.2024.1379698.

Riedl, S., Zweytick, D. & K. Lohner (2011). Membrane-active host defense peptides—Challenges and perspectives for the development of novel anticancer drugs. Chem. Phys. Lipids 164: 766–781.

Rocha, M.M., Dariva, I., Comelli, G., Sanches, G. *et al*. (2022). A new therapeutic approach for bone metastasis in colorectal cancer: intratumoral melittin. J Venom Anim Toxins incl Trop Dis, 28: e20210067

Sahsuvar, S., Guner, R., Gok, O., Can, O. (2023). Investigation of novel cervical cancer-targeting and redox-responsive melittin conjugates. Scientific reports, 13:18225. https://doi.org/10.1038/s41598-023-45537-x

Sánchez, G. Ed. (2023). Anuario estadístico de salud. Dirección de registros médicos y estadísticas de salud de la República de Cuba. La Habana. 210 pp.

Sène, M.A, Xia, Y. & Kamen A.A. (2022). Overview of recent advances in Vero cells genomic characterization and engineering for high-throughput vaccine manufacturing. Clin Transl Disc.; 2:e40. https://doi.org/10.1002/ctd2.40.

Sisakht, M., Mashcan,i B., Bazi, A., Ostadi, H.*et al*. (2017). Bee venom induces apoptosis and suppresses matrix metaloprotease-2 expressions in human gioblastoma cell. Rev. Bras. Farmacogn.27: 324–328.

Sisin, N.N.T., Kong, A.R., Edinur, H.A., Jamil, N.I.N., & Che Mat, N.F. (2023). Silencing E6/E7 Oncoproteins in SiHa Cells Treated with siRNAs and Oroxylum indicum Extracts Induced Apoptosis by Upregulating p53/pRb Pathways. Applied biochemistry and biotechnology, 10.1007/s12010-023-04762-w. Advance online publication. https://doi.org/10.1007/s12010-023-04762-w.

Śliwa-Tytko, P., Kaczmarska, A., Lejman M., Zawitkowska, J. (2022). Neurotoxicity Associated with Treatment of Acute Lymphoblastic Leukemia Chemotherapy and Immunotherapy. Int J Mol Sci.23(10):5515.

Soliman, C., Eastwood, S., Truong, V.K., Ramsland, P.A. *et al.* (2019). The membrane effects of melittin on gastric and colorectal cancer. PLoS One. 14(10) doi: 10.1371/journal.pone.0224028. PMID: 31622415; PMCID: PMC6797111.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M. *et al*. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. Clin. Cancer Investig. J. 71: 209–249. https://acsjournals.onlinelibrary.wiley.com/doi/10.3322/caac.21660

Tolossa, D., Muzazu, S.G.Y, Digamo, K., Tayamika, M. *et al*. (2021). New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Medicine. 9: 1 –10

Uddin, A.F.M.K., Sumon, M.A., Pervin, S., Sharmin, F. (2023). Cervical Cancer in Bangladesh. South Asian J Cancer. 12 (1):36 –38.

Viana, G.A., Freitas, C.I.A., de Almeida, J.G.L., de Medeiros, G.V.D. *et al.* (2021) Antioxidant, genotoxic, antigenotoxic, and antineoplastic activities of apitoxin produced by *Apis mellifera* in Northeast, Brazil. Cienc Rural. 51 (4): 1-9.

Wang, J., Guo, Z., Shen, M., Xie, Q., & Xiang, H. (2024). Potential application mechanism of traditional Chinese medicine in treating immune checkpoint inhibitor-induced colitis. Frontiers in immunology, 15, 1366489. https://doi.org/10.3389/fimmu.2024.1366489

Wang, X., Li, H., Lu, X., Wen, C. *et al*. (2018). Melittin-induced long noncoding RNA NONHSAT105177 inhibits proliferation and migration of pancreatic ductal adenocarcinoma, Cell Death & Disease 9 (10): 1–11.

Wang, Y., Zeng, Y., Yang, W., Wang, X., & Jiang, J. (2024). Targeting CD8+ T cells with natural products for tumor therapy: Revealing insights into the mechanisms. Phytomedicine: international journal of phytotherapy and phytopharmacology, 129, 155608. Advance online publication. https://doi.org/10.1016/j.phymed.2024.155608

Yao, J., Zhang, Z., Li, S., Li, B., & Wang, X.H. (2020). Melittin inhibits proliferation, migration and invasion of bladder cancer cells by regulating key genes based on bioinformatics and experimental assays. Journal of cellular and molecular medicine, 24(1), 655–670. https://doi.org/10.1111/jcmm.14775.

Zarrinnahad, H., A. Mahmoodzadeh, M. P. Hamidi, M. Mahdavi *et al.* (2018). Apoptotic Effect of Melittin Purified from Iranian Honey Bee Venom on Human Cervical Cancer HeLa Cell Line. Int. J. Pept. Res. Ther.; 24:563–570 https://link.springer.com/article/10.1007/s10989-017-9641-1.