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In Vitro Study of Anti-cancer Properties of Egyptian Scorpion (*Leiurus quinquestriatus*) Venom on Triple Negative Human Breast Cancer Cell Line MDA-MB-231

Mahmoud Abd El-Atti[1], Nagwa El-Badri[2], and Jihad El-Qassas[1*]

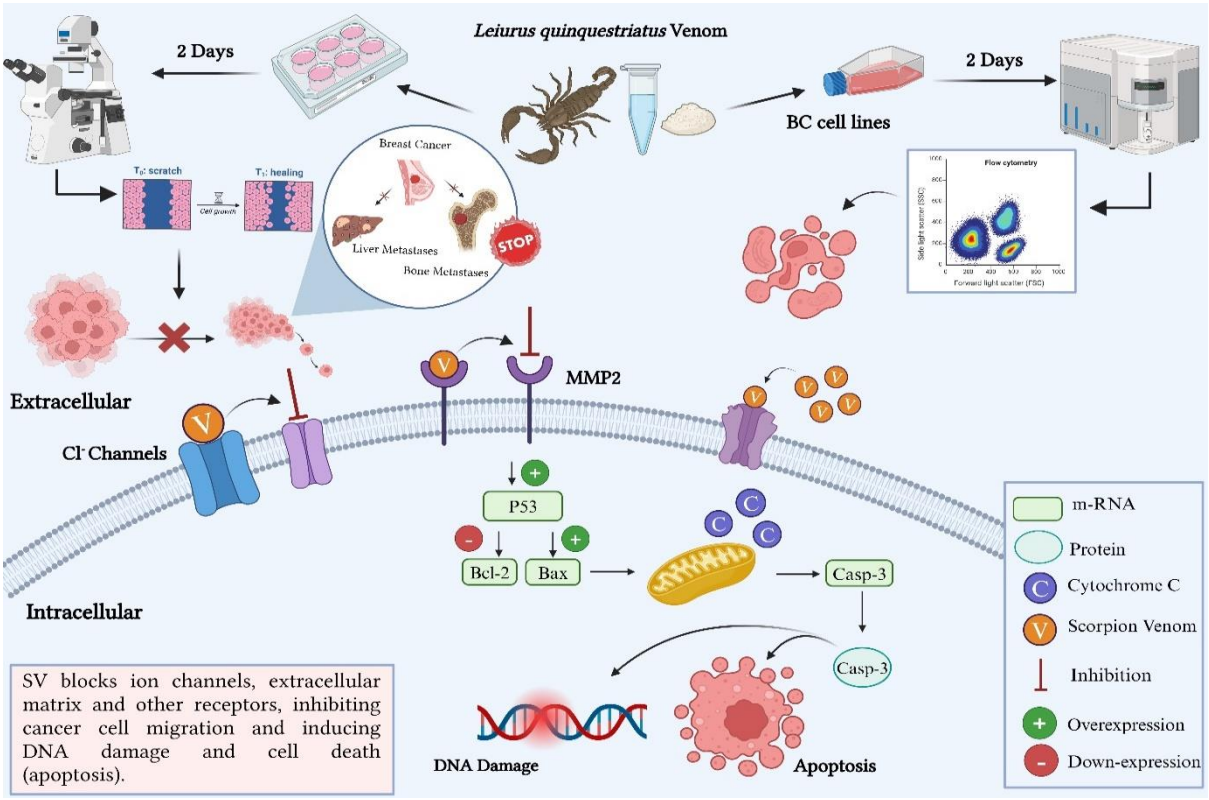
[1]Department of Zoology, Faculty of Science, Zagazig University, Zagazig 44519, Egypt
[2]Center of Excellence for Stem Cells and Regenerative Medicine, Zewail City of Science and Technology, 6th of October City, Giza 12578, Egypt.

Corresponding author: E-mail: jihadelqassas@gmail.com

ABSTRACT: **Despite advances in therapy of breast cancer, it still remains a significant health concern. During the past recent years, various research studies have encompassed that novel therapeutic approaches are urgently needed. Scorpion venom has emerged as a promising anti-cancer agent. This is achieved primarily via scorpion venom-derived peptides revealed high and selective affinity for ionic channels over-expressed in many cancer cells. In particular, This study refer to the anti-cancer properties of *L. quinquestriatus* venom against human breast cancer cells MDA-MB-231. Cell viability was assessed using MTT assay. Apoptosis induction was evaluated using Annexin V/PI staining kit. Wound healing assay was employed to analyze cell migration. The MTT assay results demonstrated that *Lq* venom decreased the cell viability and had a selectively cytotoxicity against cancer cells, owing to its possible anti-cancer activity. The Annexin V/PI staining revealed a substantial increase in the percentage of apoptotic cells upon *Lq* venom treatment, and the wound healing assay showed a marked reduction by (~70%) in the migration ability after *Lq* venom treatment in comparison with non-treated cells. Significant decrease in human breast cancer cell viability (~ 60%) was observed after 48 hours. of treatment with *Lq* venom compared with non-treated cells ($P < 0.0001$). These present findings provide evidence that *Lq* venom possesses potent anti-cancer properties and may be useful in the development of breast cancer treatment strategies.**

KEYWORDS: ***Leiurus quinquestriatus*** **venom, Cell viability, Apoptosis, Cell migration, Triple Negative Human Breast Cancer Cell Line (MDA-MB-231) (TNBC).**

Graphical Abstract



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1. INTRODUCTION

Breast cancer (BC) is one of the leading causes of cancer-related mortality worldwide **(Arnold, Melina *et al.*, 2022)**. In 2021, it was estimated that 25% of all newly diagnosed cancers among women are breast cancer **(Sung *et al.*, 2021)**. Despite better therapeutic approaches and early detection methods, the incidence of breast cancer is on the rise, particularly among young women worldwide **(De Silva *et al.*, 2022)**. Traditional treatment of cancer including surgery, chemotherapy, immunotherapy and radiotherapy have better efficacy but cause severe side effects **(Himmat *et al.*, 2020)**. This created the need to identify potentially efficacious compounds using natural product such as plant extracts or animal products venoms that specifically target cancer cells and spares normal cells **(Andersen *et al.*, 2015; Purohit *et al.*, 2024; Qu *et al.*, 2024)**. The use of scorpion venom for cancer treatment has been the focus of several laboratories in recent years, as a selective and non-toxic therapy with minimum damage to the normal adjacent cells **(Mikaelian *et al.*, 2020)**. *Leiurus quinquestriatus* (*L. quinquestriatus*) is the largest genus in the most dangerous Buthidae family and inhabits various regions worldwide. *L. quinquestriatus* is yellow, about 80 mm long and lives in Egypt, especially in Upper Egypt and Sinai **(El-Hennawy, 2014)**. *Lq* venom is a powerful mixture of neurotoxins **(Borneman *et al.*, 1993)**. A high incidence of *L. quinquestriatus* stings was reported in Egypt **(Ismail *et al.*, 2022)**. In a related study, it has been shown that whole venom of *L. quinquestriatus* collected from Saudi Arabia induced a selective and high cytotoxic effect against cancer cells without affecting normal cells **(Al-Asmari *et al.*, 2016)**. Interestingly, *L. quinquestriatus* attracted a lot of attention after chlorotoxin peptide (36- amino acids, CTX) isolation **(DeBin *et al.*, 1993)**. CTX is the most functional peptide identified in scorpion venom **(Boltman *et al.*, 2023)**. Recent studies showed that chlorotoxin (CTX or ClTx) has an effective inhibitory effect on various MMPs in glioblastoma **(Wiranowska, 2024)**, pancreatic cancer **(El-Ghlban *et al.*, 2014)**, breast cancer **(Zuo *et al.*, 2019)**, and cytotoxic effects on tumor cell lines inducing cell death **(Elrayess *et al.*, 2021)**, especially in breast cancer cell lines (MDA-MB-231, and MCF-7) **(Teleb *et al.*, 2022)**. More recently, the efficacy of loading *L. quinquestriatus* on nanoparticles has improved its utility in various biomedical applications **(El-Sheikh *et al.*, 2022),** and using CTX peptides in cancer diagnostic and therapeutic approaches **(Boltman *et al.*, 2023)**. The underlying molecular mechanisms of the anti-tumor effects of *Lq* venom are still under investigation, especially of *Lq* venom on breast cancer. The current study aims to investigate the anti-cancer effects of *Lq* venom in MDA-MB-231 breast cancer cells on cell viability, apoptosis, and cell migration.

2. MATERIAL AND METHODS

2.1. Scorpions and Venom Collection

100 *Leiurus quinquestriatus* scorpions were collected from Aswan, and Luxor by professional hunters in July 2022. Each scorpion was kept in a clear plastic box in the Invertebrate Lab., Zoology Department, Faculty of Science, Zagazig University, Zagazig, Egypt. Scorpions were fed on mealworms daily. The crude venom was collected in a caliber Eppendorf tube by using the electrical stimulation method (12 V, 3ms) of the scorpion telson **(Abd El-Atti *et al.*, 2020)**. 1µL of venom was dissolved in distilled water and centrifuged at 5000 rpm for 10 min, then immediately freeze-dried (Thermo Fisher Scientific-Lyophilizer, Labconco freeze-drying system, USA) and stored at −20℃ until use. The lyophilized samples were dissolved in phosphate-buffered saline, filtered by using a 0.22µm sterile membrane and stored at −20℃ until use.

2.2. Determination of Protein Concentration (µg/mL)

The protein concentration and purity of the *Lq* venom sample was determined spectrophotometrically using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA) at a wavelength of 260–280nm. Further dilutions were made as required for the experiments **(Kampo *et al.*, 2019)**.

2.3. Human Breast Cancer Cell Culture

Human Breast cancer cell line MDA-MB-231 (ATCC®, Manassas, VA, USA) was cultured with a combination designated as complete culture medium containing Dulbecco's Modified Eagle's medium (GIBCO, GERMANY), supplemented with 10% fetal bovine serum (GIBCO, GERMANY), 2mM L-glutamine (Corning, USA), 1% antibiotic-antimycotic penicillin (100µg/mL), and 100µg/mL streptomycin (PAN BIOTECH™, USA). Cells were incubated at 37℃ under 5% CO_2/90% humidity air under standard culture conditions **(Sedky *et al.*, 2018)**.

2.4. IC_{50} and *In vitro* cell viability assay (MTT assay)

MDA-MB-231 and HSF cell line ($3x10^3$/well) were seeded in 100µL of medium/well in 96-well culture plates, and incubated overnight at 37˚C. After incubation, serial dilutions of *Lq* venom were dissolved in DMEM to give a final concentration of 80, 100, 200µg/mL and added in wells of treated cells. Cells with culture medium only (without scorpion venom) an equal amount of PBS was added and used as non-treated cells. After treatment for 48 hours, 10µL of 5µg/mL of sterile MTT solution (Cat no. 32030, SERVA, Germany) was added per well and then incubated for 3 hours. The supernatant was removed and 100µL of dimethyl sulfoxide (Belgium) was added into each well and incubated for 10 min. with shaking using the orbital shaker in dark conditions to dissolve the insoluble formazan crystals. The absorbance was measured with a microplate reader at 570nm. The percentage of cell viability was expressed using the formula: % viability = A 570 of treated cells/A570 of negative non-treated cells ×100%. The median inhibitory concentration (IC_{50}) value was determined. The experiment was repeated three times **(Guo *et al.*, 2022)**.

2.5. Cell Viability Assay Using Trypan Blue Dye (Survival Assay)

After 48 hours. of treatment with *Lq* venom, both treated and non-treated cells were counted via a trypan blue dye exclusion assay in quadruplicate. Once the cells were dispersed, 10µL of cell suspension was transferred to a microcentrifuge tube. 10µL of trypan blue (PAN BIOTECH™, USA) was added and mixed gently with a pipette; 10µL of cell/trypan blue suspension was then loaded into a cell counting chamber and placed under a Leica DMi8 inverted microscopy (Leica Microsystems, Wetzlar, Germany) for counting **(Wu, J. *et al.*, 2019)**.

2.6. Apoptosis Analysis Using Flow Cytometry

Cell apoptosis was analyzed by flow cytometry using FITC-labelled-Annexin V and PI Apoptosis Detection Kit (eBioscience™, Thermo Fisher Scientific, Vienna, Austria, CA, USA) as the manufacturer's protocol **(Sedky *et al.*, 2018)**.

2.7. Wound Healing Assay (Scratch Assay)

Wound healing assay was performed in 6-culture well plates. MDA-MB-231 cells (9×10^5) were seeded and cultured at ~80–90% confluence, then washed twice using phosphate-buffered saline (PBS). The scratch was done using a sterile pipette tip and immediately photographed. This time point was designated as 0 hour. The cells were cultured for 48 hours. in the serum-free medium (SF) containing 200µg/mL of *Lq* venom. The control cells were treated with equivalent volumes of PBS. After 48 hours, the migration of cells from the edge of the scratch towards the center was monitored microscopically by using a Leica DMi8 inverted microscopy (Leica

Microsystems, Wetzlar, Germany) at magnification 10x. The width of the scratch was measured at 0, 24, and 48 hours. respectively, and analyzed using Image J software NIH, USA **(Al-Asmari *et al.*, 2016)**.

2.8. Statistical Analysis

All the experiments were conducted in triplicate, and statistical analyses of the obtained data were represented using GraphPad Prism 9 software, version 4.0 In-state computer program (GraphPad, San Diego, CA, USA). The results were reported as mean ± standard deviation (SD) of the mean. *P* values were calculated by t-test from the mean values of the indicated data. **P* < 0.05 was considered statistically significant **(Sedky *et al.*, 2018)**.

2.9. Ethical Approval

The Research Ethics Committee of Zagazig University (ZU-IACUC) Zagazig, Egypt approved present study (Research protocol No., ZU-IACUC/1/F/80/2021).

3. RESULTS

3.1. *Lq* venom inhibited MDA-MB-231 cell viability.

Lq venom induced a significant decrease in cell viability in breast cancer MDA-MB-231 cells in a dose-dependent manner (**P*<0.05), while no significant effect on viability was observed on HSF cells in all examined concentrations (Fig. 1). The IC_{50} value (200µg/mL) of *Lq* venom was the highest effect (**P* < 0.05) on MDA-MB-231 cells compared with non-treated MDA-MB-231 cells. In addition, 200µg/mL of *Lq* venom didn't inhibit the HSF viability, but markedly increased HSF viability (Fig. 1).

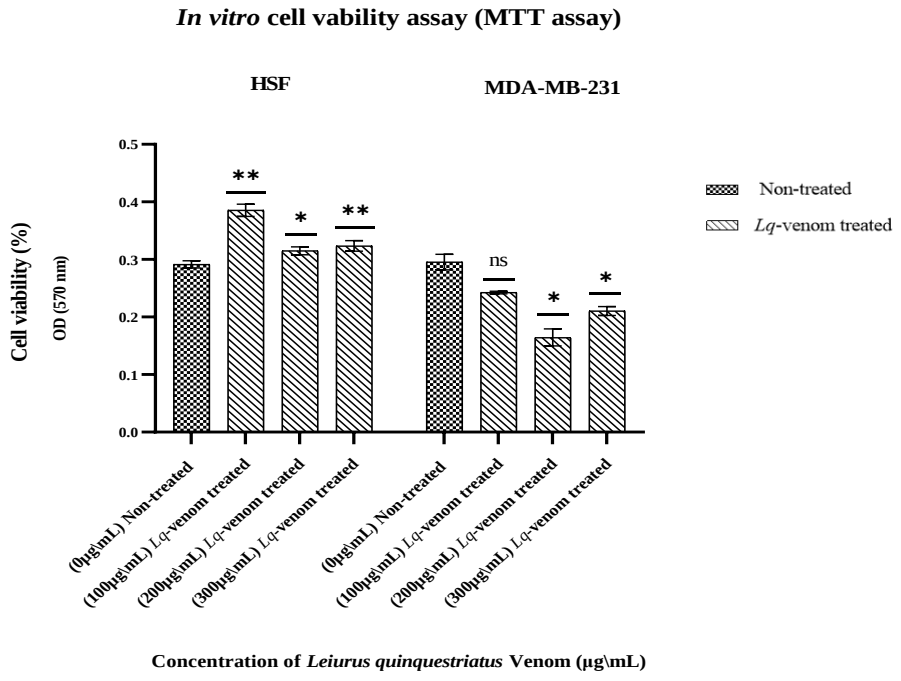


Figure 1. Effect of *Lq* venom treatment against MDA-MB-231 and HSF cells. Representative graph of cell viability after *Lq* venom treatment, showing differential effect of *Lq* venom, in breast cancer cells MDA-MB-231 and HSF, after 48 hours of treatment at different concentrations.

Data was statistically significant at (**P* < 0.05) and (***P* < 0.01). Data are presented as the mean ± SD of three independent experiments. *Lq*: *Leiurus quinquestriatus* scorpion venom, IC_{50}: half maximal inhibitory concentration.

3.2. *Lq* venom decreased the MDA-MB-231 surviving cells count.

The inhibitory activity of *Lq* venom on cell viability was examined and the surviving cells were counted using Trypan blue dye. Fig. 2 shows a significant decrease in human breast cancer MDA-MB-231 cell viability by ~60% after 48 hours. of treatment with *Lq* venom, compared with non-treated cells (****P* < 0.0001). Surviving cells count decreased significantly from 2510000 to 505000 cells after the treatment with *Lq* venom Fig. 2.

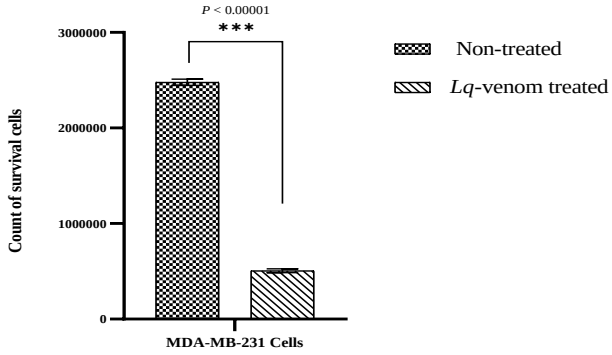
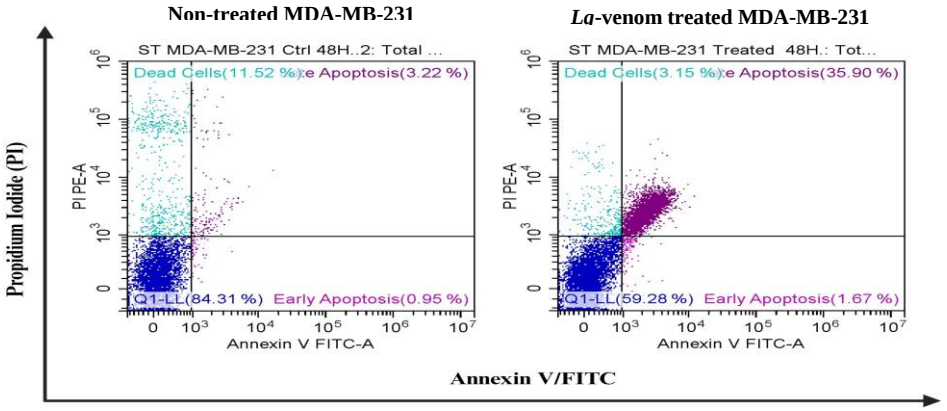


Figure 2. Representative graph of cell counting after 48 hours of *Lq* venom treatment using Trypan blue dye exclusion.

3.3. *Lq* venom induced apoptosis of MDA-MB-231 human breast cancer cell line.

Results showed that after treatment with *Lq* venom at 200µg/mL, the apoptosis percentage of MDA-MB-231 cells increased from 3.625% (non-treated cells) to 38.21% (***$P < 0.0001$), (Fig. 3A and B). Annexin V-FITC stained early apoptotic cells increased significantly in *Lq*-venom treated MDA-MB-231 cells from 3% to 38% (***$P < 0.0001$). Similarly, propidium iodide stained late apoptotic cells increased significantly in *Lq*-venom treated MDA-MB-231 cells from 11% to 41% (***$P < 0.0001$).

(A)



(B)

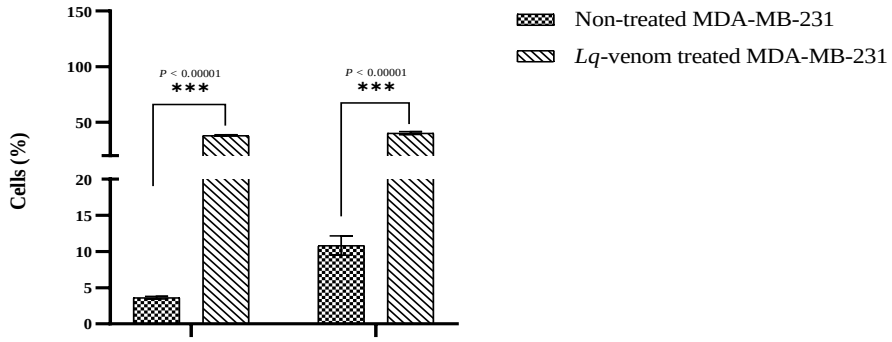


Figure 3. *Lq* venom **Annexin V-FITC** **Propidium Iodide (PI)** induced apoptosis. **(A)** Apoptosis of treated breast cancer cells compared with non-treated cells. **(B)** The percentage of apoptotic cells at the indicated times with the total number of cells.

Data was statistically significant at ***$P < 0.0001$. Data are presented as the mean ± SD of three independent experiments.

3.4. *Lq* venom ameliorated the migration ability of MDA-MB-231 human breast cancer cells.

MDA-MB-231 human breast cancer cell line was treated with 200µg/mL *Lq* venom for 48 hours. and measured using wound-healing assay. The results revealed that the motility of treated MDA-MB-231 cells with 200µg/mL *Lq* venom decreased significantly, and a complete halt in cell motility was observed following 24 hours. of incubation. In addition, when the treatment exceed to 48 hours. the capacity of cell migration decreased, and the inhibition rate was 70% (***$P < 0.0001$) (Fig. 4 A and B).

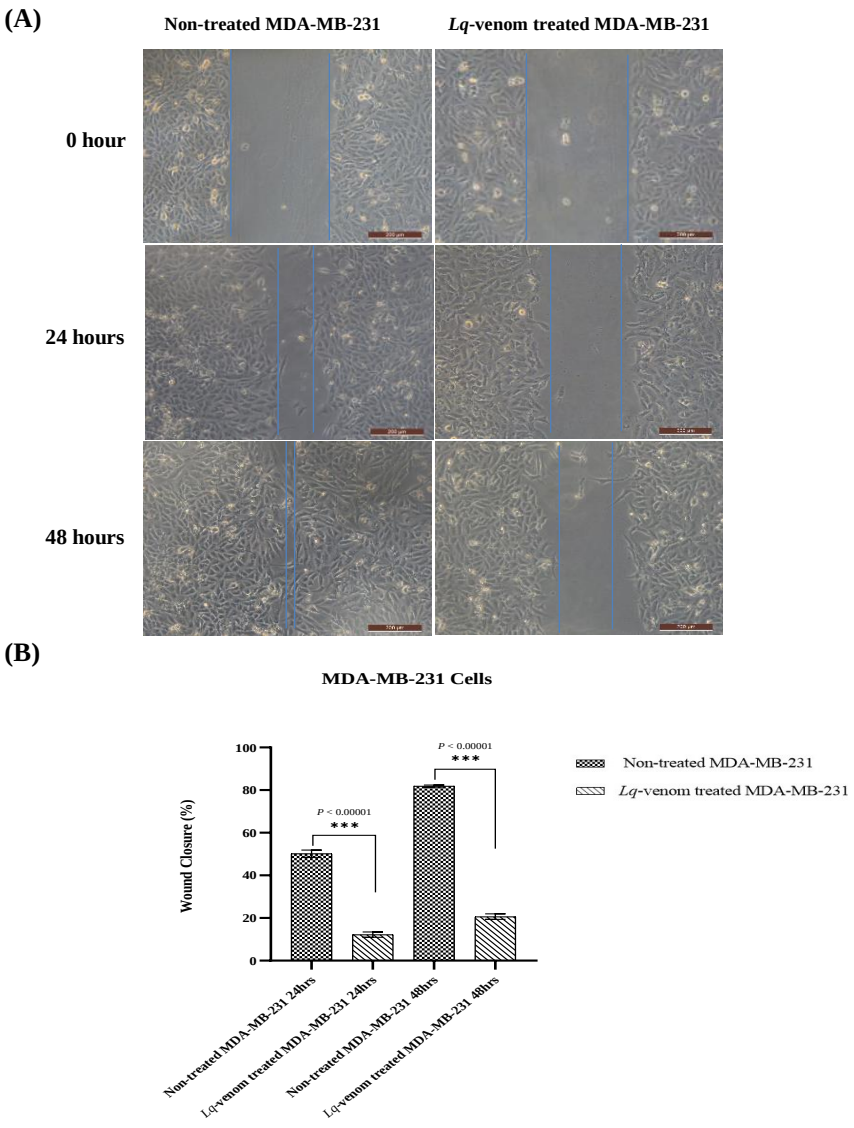


Figure 4. Inhibitory effect of *Lq* venom on cell migration of MDA-MB-231 human breast cancer cell line. **(A)** Representative images of cell migration in MDA-MB-231 cells after treatment with 200µg/mL of *Lq* venom for 48 hours. compared to control cells. **(B)** Graph showing the percentage of decrease in cell migration of human breast cancer cell line after treatment.

Data was statistically significant at ***$P < 0.0001$. Data represented as the mean ± SD of three independent experiments.

4. DISCUSSION

Breast cancer is one of the fastest spreading malignant diseases due to its various forms, aggressive tumor biology and the lack of safe and effective therapies **(De Silva *et al.*, 2022)**. By 2025, the number of women who still be affected by breast cancer each year is estimated to be over one million **(Arnold, M. *et al.*, 2022)**. Currently, chemotherapy remains the standard method for treatment **(Miroshnychenko *et al.*, 2023),** however, the incidence and mortality rate of BC continue to increase rapidly due to high rates of recurrence and metastasis **(Himmat *et al.*, 2020)**.

Natural products from plant and animal origin have long been utilized for therapeutic purposes, and more than 70% of the human disorders are treated using these natural products and their related drugs **(Liu *et al.*, 2021)**. While scorpion venom is harmful, and its stings can cause severe health hazards such as cardiac and respiratory failure **(Zoccal *et al.*, 2016),** the venom contains numerous beneficial components, which are currently utilized in the pharmaceutical industry **(Ahmadi *et al.*, 2020)**. Biochemically, scorpion venom is an enriched in hundreds to several thousands of bioactive proteins and peptides, which have various biological effects **(Uzair *et al.*, 2018)**. In cancer, scorpion venom was found to act as a potential anti-cancer agent in various types such as glioma **(Wu, S. *et al.*, 2018)**, breast adenocarcinoma **(Kampo *et al.*, 2019)**, prostate cancer **(BenAissa *et al.*, 2020)**, pancreatic cancer **(Salama *et al.*, 2021)**, and leukemia **(Salimi *et al.*, 2022)**. Targeting cell death, apoptotic pathways, and migration in cancer cells are important factors that are widely used in cancer research and represent an important approach for cancer treatment and anti-cancer drug development **(Islam *et al.*, 2014)**.

The present study showed marked reduction in cell viability in human breast cancer cell line MDA-MB-231 without toxic effect on normal HSF. These findings support that *L. quinquestriatus* venom has a selective cytotoxic activity against cancer cells, in particular, breast cancer cell line MDA-MB-231 **(El-Fiky *et al.*, 2019)**. Previous studies have demonstrated that scorpion venom can inhibit cancer growth and proliferation **(Zhang *et al.*, 2009)**, induce apoptosis **(Al-Asmari *et al.*, 2018)**, and cell cycle arrest **(Li, B. *et al.*, 2018)**, as well as inhibit metastasis **(Qin *et al.*, 2014) of** both *in vitro*, and *in vivo* **(Salem *et al.*, 2016)**.

The tumor suppressor gene p53 plays an important role in response to different cell damage **(Mirzayans *et al.*, 2012)**, and is crucial in the p53-dependent pathway, involving Bax (apoptotic gene), and Bcl-2 (anti-apoptotic gene). Apoptotic proteins like Bax accumulate 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)**, which can activate the caspase-3 **(Li, Y. *et al.*, 2013)**, and directly trigger cell death and apoptosis of cancer cells. Our data show apoptosis induction after treatment with *Lq* venom in MDA-MB-231 cells. The selective cytotoxic effect on cell survival after treatment with *L. quinquestriatus* venom, and increased apoptotic cells support the involvement of the intrinsic apoptotic pathway or the mitochondrial pathway pro-apoptotic potential of the crude venom of *Lq* **(Salama *et al.*, 2021)**.

Similarly, an inhibitory effect of *Lq* venom was observed in the cell migration up to 70% reflects on a potential therapeutic effect. Tumor metastasis require the destruction of the extracellular matrix (ECM) including mesenchymal collagen and the endothelial basement membrane **(Winkler *et al.*, 2020)**. The anticancer mechanism of crude venom of *L. quinquestriatus* scorpion is mediated by blockage of Cl^{-1} ion channels and inhibiting invasion and migration of cancer cells through inhibition of extracellular matrix proteins **(Farkas *et al.*, 2023)** as matrix metalloproteinases (MMPs) that are the main ECM degradation enzyme and have important roles in tissue development, remodeling, and wound healing **(Nabeshima *et al.*, 2002)**. Additionally, focal adhesion kinase (FAK) are critical genes for the focal assembly and contraction of cells, which facilitates cell motility and invasion into the ECM. The activation of FAK is necessary to achieve adhesion, followed by cell motility in various directions and enhancement of cell migration and invasion **(Schlaepfer *et al.*, 2004)**. Recent studies revealed that the inhibition of MMPs and FAK suppresses cell

migration **(Karlsen *et al.*, 2011)**. Similarly, the inhibition of cell migration may be due to the lack of active FAK, and MMPs following treatment with *Lq* venom.

In conclusion, the present study provide evidence to support the significance of *Lq* venom to be used as a potential anti-cancer agent selectively targeting cancer cells in comparison. We demonstrated that *Lq* venom acts as an anti-cancer agent by its selective cytotoxicity on cancer cells without toxic effect on normal HSF cells. The venom also induced late apoptosis and decreased cell migration of human breast cancer cell line MDA-MB-231. *Lq* venom may thus represent a valuable therapeutic source of molecules to be used as new anti-cancer drugs and for further investigation of treatment strategies of breast cancer. Additional studies are merited to determine the important proteins and peptides in this venom, which account for the anti-cancer toxic effects, and the mechanism by which these effects are achieved.

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