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Montivipera bornmuelleri venom selectively exhibits high cytotoxic effects on keratinocytes cancer cell lines

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ABSTRACT

Context: The Viperidae family venom is a rich source of bioactive compounds such as many proteases, which cause tissue necrosis and affect mostly the vascular system. However, the venom exhibits therapeutic potentials and has contributed to the development of some medical drugs. Specifically, the *Montivipera bornmuelleri* venom has shown to exhibit antibacterial, pro-inflammatory and antifungal activities.
Objective: This work evaluates the cytotoxic effect of the *M. bornmuelleri* venom on human-derived keratinocytes including the non-tumorigenic HaCaT, the benign A5 and the low-grade malignant II4 cells.
Materials and methods: The toxicity of different venom concentrations (0.9, 1.87, 3.75, 7.5, 15, 30 and 60 μg/mL) and their effect on the viability of the cells lines were assessed using the Lactate Dehydrogenase (LDH) activity and the Trypan blue tests after 24 h of incubation.
Results: The venom was able to reduce the viability of all cell lines in a dose dependent manner with the HaCat cells being the least affected. For example, the 60 μg/mL dose induced a more significant decrease the viability of A5 (44%) and II4 (21.33%) keratinocytes as compared to HaCaT cells (70.63%). Also, this venom showed a higher cytotoxic activity on the A5 (52.45%) and II4 (98.67%) cells as compared to HaCaT cells (30.14%) with an IC_{50} estimated at 10 μg/mL on II4 and at 60 μg/mL on benign A5.
Discussion and conclusion: Those differential cytotoxic effects of the *M. bornmuelleri* venom pave the road for more advanced studies which might unravel the potential anticancer effects of this venom.

1. Introduction

Cancer arises from normal cells affected by a number of mutations, which are mostly genetic caused by errors during replication or reproduction in DNA genes and which are not repaired by the usual processes (Elmore, 2007; Lahtz and Pfeifer, 2011).

Skin cancer is considered as one of the most common cancer types affecting people all over the world according to reports written by the American Cancer Society and the Skin Cancer Foundation (Andrade et al., 2012; D'Orazio et al., 2013). Skin cancer is a multi-stage process that begins with a random mutation. Subsequently, several mutations accumulate altering the chromosomal genes and inducing the progression of cancer from the non-invasive stage to the invasive one (DiGiovanni, 1992; De Gruijl et al., 2001). Among skin carcinomas, basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (SCC) can be distinguished. Basal cell carcinomas are the most common (70% of skin cancers) and are highly related to intense and repeated exposure to sunlight. They are also less serious because of their slow evolution and local development which means they do not metastasize. On the other hand, cutaneous squamous cell carcinomas are more rare (20% of skin cancers). They sometimes develop on so-called precancerous lesions forming actinic keratosis (Foster et al., 2008; World Health Organization, 2009; Narayanan et al., 2010).

Despite the development of several modes of anticancer therapies, skin cancer cure is still facing many obstacles. It is for

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this reason that considerable research has been carried on in the hope of discovering anticancer agents that are highly specific against both proliferative and non-proliferative tumorigenic cells, with no major effect on healthy tissues (Sharquie and Noaimi, 2012).

One of the best models used in skin cancer researches involves the HaCaT and the related keratinocytes skin cancer cells. Keratinocyte cells originate from the basal layer and are involved in the secretion of cytokeratin (Montagner et al., 2013). The HaCaT cells are spontaneously immortalized cells obtained originally from the normal human adult skin keratinocytes by mutating the p53 tumor suppressor gene using ultra-violet radiation. HaCaT cells remain nontumorigenic for an extended number of passages in culture (around 320 passages). Although they represent an early stage in skin carcinogenesis, HaCaT cells can be approximated to the normal keratinocytes, due to many reasons. Firstly, they are able to maintain a stable number of chromosomes as well as the ability to differentiate. However, they can still proliferate *in vitro* and hence are used as a prototype to study epidermal cells differentiation (Boukamp et al., 1998; Mueller et al., 2001). HaCaT cells are also capable of inducing epidermal differentiation when transfected with candidate genes that control homeostasis (Schoop et al., 1999). Starting with the HaCaT cell line, the benign cell line (A5) can be obtained via a transfection of the oncogene Ras under appropriate culture conditions and at very high temperatures. Out of (A5) the low grade malignant (II4) can be obtained via additional genetic aberrations (Fusenig and Boukamp, 1998).

Werner (1938) described the Lebanese *Montivipera bornmuelleri* snake (Viperidae family) for the first time and it was later shown that this snake venom has considerable biological effects including pro-inflammatory and antibacterial effects against *Staphylococcus aureus and Morganella morgana* strains (Hraoui-Bloquet et al., 2012; Accary et al., 2013). This venom was shown to contain metalloproteases III, serine protease and phospholipase A2 (Accary et al., 2014) which is known to have a wide spectrum of biological activities including neurotoxicity, mytotoxicity, cardiotoxicity, cytotoxicity among many other effects (Shimuta et al., 2009; Khunsap et al., 2011).

This study investigates the cytotoxic effect of the *M. bornmuelleri* snake venom on the cancer keratinocytes cells (low-grade malignant II4 cell line) as well as on the benign A5 cells lines compared to HaCaT cell line (used as control cells).

2. Materials and methods

Dulbecco Modified Eagle Media (DMEM) and Fetal Bovine Serum (FBS), Trypsin-EDTA are obtained from Gibco-BRL, Paisley, Scotland. Penicillin-streptomycin, L-glutamine and Trypan Blue Exclusion Dye are obtained from Sigma-Aldrich Chemie, Steteiheim, Germany. CytoTox 96 Non-radioactive Cytotoxicity and Phosphate Buffer Saline (PBS) are obtained from Roche Applied Sciences, Mannheim, Germany.

2.1. Cell lines and culture

All cell line clones (HaCaT, A5 and II4) were kindly provided by Dr. Marwan Sabban (American University of Beirut). The culture medium consisted of 2 mM alcium in Dulbecco's Modified Eagle's Medium (DMEM) to which 10% heat-inactivated fetal bovine serum (FBS), 1% of 100 μg/mL penicillin-streptomycin, and 1% of 2 mM L-Glutamine were added. The cells were maintained at optimal conditions in a humidified incubator providing 95% air, 5% CO_2 and a temperature of 37 °C. Passaging of the cells was performed upon attaining 80% confluency via Trypsin-EDTA acknowledged treatment. The adherent cells were first washed with 5 mL Phosphate Buffer Saline PBS (0.05%) in order to remove all the factors that inhibit trypsin activation (serum, Ca^{2+}, Mg^{2+}). Trypsin-EDTA (5 mL) with a ratio of (0.025%): (0.05%) were then added and cells were incubated for up to 7 min. Flasks were tapped gently until the cells came off the plastic completely. Following the cells detachment, an equal volume of growth medium was added to the culture flask to inactivate trypsin. Cells were then re-suspended and transferred into falcon tubes for centrifugation (5 min, 4 °C, and 1200 rpm). Finally, the pellet was re-suspended in fresh medium into new cell culture flasks in a ratio of 1:3 for future experimental use.

2.2. Cell viability measurement

Cells were seeded at a density of 10×10^3 cells in a volume of 1 mL media/well in a 24 well plate and were incubated to grow for 24 h providing identical experimental conditions. Then, old media was replaced with the same volume of fresh media containing the different snake venom concentrations (0.9, 1.87, 3.75, 7.5, 15, 30 and 60 μg/mL). All the tests were performed in triplicates along with a negative control consisting of PBS alone and a positive control consisting of 1 mL TritonX-100 (2%). After further incubation for 24 h under the same conditions, cell viability was assessed using the Trypan Blue that stains cells with damaged membranes in blue. Living and dead cells were counted using a hemocytometer and calculated using the following formula:

$$\text{Cells/mL} = \text{Number of cells in 4 squares}/4 \times \text{ dilution factor} \times \text{volume of suspension} \times 10^4$$

2.3. Cell cytotoxicity assay

Lactate Dehydrogenase (LDH) cytotoxic detection kit (CytoTox 96 Non-radioactive Cytotoxicity Assay Promega Corp., Madison, WI) was used to measure the lactate dehydrogenase released from the cytosol of lysed cells into the culture supernatants. Briefly, cells were seeded into 96-well microtiter plates (Thermo Labsystems) at different concentrations (10×10^3, 1×10^3, 5×10^3, and 2.5×10^3 cells/well) till an optimum density of $5 \times 10^3 \text{cells}\cdot\text{well}^{-1}$ was determined. A volume of 200 μL/well was added and cells were incubated for 24 h to allow attachment. At the end of the incubation point, cells were treated with different concentrations of the snake venom. Cytotoxicity of the venom on HaCaT, A5 and II4 cell lines was monitored after incubation of 24 h. Released LDH from damaged cells converted the tetrazolium salt (INT) into a red formazan product. The amount of color formed correlates directly with the number of lysed cells. Finally, the product was measured colorimetrically at 490 nm and 620 nm using an ELISA microplate reader (Multiskan Ascent, Thermo Labsystems) (Harakeh et al., 2006).

The% of cytotoxicity was calculated as follows:

$$\% \text{ of Cytotoxicity} = \frac{\text{Experimental value(average of the triplicate)} - \text{low control(average)}}{\text{High control(average)} - \text{low control(average)}}$$

Where:

Experimental value: absorbance of cells treated with different concentrations of *Montivipera bornmuelleri* venom.

Low control: Absorbance of untreated cells

High control: Absorbance of Triton-X 100 treated cells

2.4. Statistical analysis

All the triplicate experimental values attained were conveyed as the mean ± standard error means (SEM). Experimental data were then analyzed using Graph Pad Prism 6 statistical software

(GraphPad Software Inc., San Diego, USA) whereby the statistical significance was assessed via one way AnnovaDunnet test for comparison with control, and two way Annova Bonferroni Test for comparisons among cell lines with *P values <0.05 considered as statistically significant.

3. Results

3.1. Viability of non-tumorigenic HaCaT at 24 h after administration of Montivipera bornmuelleri venom

At a concentration of 0.9 μg/mL venom, 94.04% of the cells were viable. As concentrations increased from 1.87 to 3.75 and then to 7.5 μg/mL of venom, a slight decrease in the percentage viability was detected which remained almost constant (92.62%, 88.95%, and 85.13% respectively). Further increase in the concentrations of venom to 15, 30, and 60 μg/mL induced a significant change from 81.43%, 73.41%, to 70.63% viability correspondingly in the HaCaT non-tumorigenic control (Fig. 1).

3.2. Viability of benign A5 at 24 h M. bornmuelleri venom treatment

The venom's dose-associated effect was also monitored in the A5 benign tumorigenic keratinocytes. The investigated concentrations significantly induced a dose dependent decrease in the percentage cell viability. For instance, an increase in the concentrations from 0.9 to 1.87, 3.75, and 7.5 μg/mL decreased the percentage of cell viability from 94.33%, to 87.94%, 84%, and 75% respectively. The upsurge in the concentrations of venom to15, 30, and 60 μg μg/mL induced a further significant decrease in the viability to 66.24%, 53.44%, and 44% respectively in the A5 benign tumorigenic cell line (Fig. 2).

3.3. Viability of low grade malignant II4 at 24 h after administration of M. bornmuelleri venom

Although 85.67% and 74.2% of cells remained viable when treated with 0.9 μg/mL and 1.87 μg/mL of *M. bornmuelleri* venom, viability of cells at concentrations ranging from 3.75 μg/mL, 7.5 μg/mL, and 15 μg/mL decreased significantly to 65.38%, 60.37%, and 54.33% of cells respectively. Moreover, higher concentrations of 30 and 60 μg/mL caused a higher statistically significant decrease in the viability of the studied cell line whereby only 37.03% and 21.33% of cells remained viable correspondingly (Fig. 3).

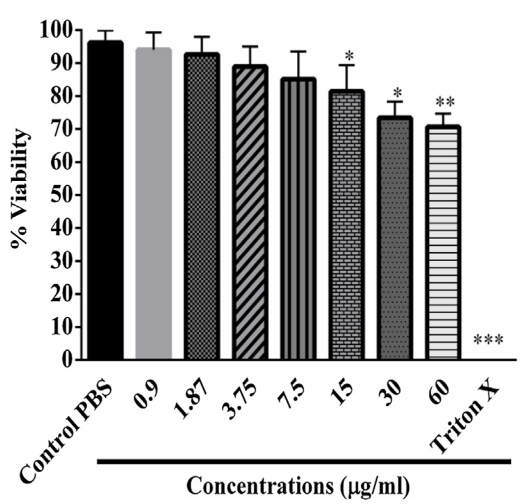

Fig. 1. Effect of *M. bormmuelleri* Venom on the Viability of HaCaT Cells at 24 h Measured by Trypan Blue. Significance of Each Group is Statistically Determined as Follows:*p < 0.05, ** p < 0.01 and *** p < 0.001as Compared to the Control (PBS). Independent Experiments were Performed in Triplicates Where the Number of Replications is 3. Error Bars Signify the Standard Error Mean (SEM) of the Results.

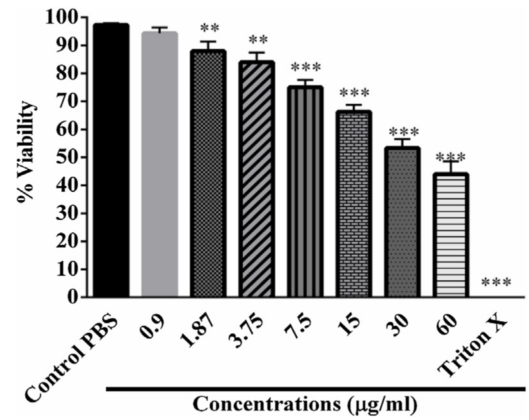

Fig. 2. Effect of *M. bormmuelleri* Venom on the Viability of the A5 Benign Tumorigenic Cells at 24 h Measured by Trypan Blue. Significance of Each Group is Statistically Determined as Follows:*p < 0.05, ** p < 0.01 and *** p < 0.001 as Compared to the Control (PBS). Independent Experiments were performed in Triplicates. Error Bars Signify the Standard Error (SE) of the Results.

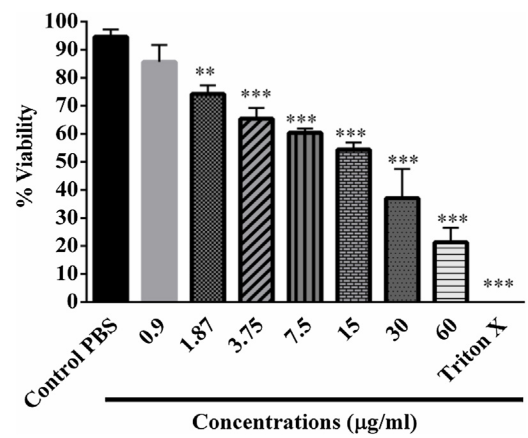

Fig. 3. Effect of *M. bormmuelleri* Snake Venom on the Viability of Low Grade Malignant II4 Cells at 24 h as Measured by the Trypan Blue Assay. Statistical Significance of Each Group Compared to the PBS (Untreated Control) is Determined as Follow:*p < 0.05, ** p < 0.01 and *** p < 0.001. The Values are Expressed as Triplicates in 3 Independent Experiments. Error Bars Represent the Standard Error (SE) of the Results.

3.4. Effect on the three considered cell lines after the administration of M. bornmuelleri venom treatment

M. bornmuelleri snake venom, resulted in highly different features between each of the three considered cell lines. In fact, low concentrations of venom, which showed very little effect on the viability of the normal skin cells HaCaT, revealed lethal effects on the human skin cancer cells. At even lower concentrations (1.87 μg/mL), the treatment with the considered snake venom exhibited an obvious grade of statistical significance upon comparison of the malignant II4 cells with the reduction in the percent viability ascribed to the HaCaT cell line (Fig. 4).

3.5. Evaluation of M. bornmuelleri venom cytotoxicity on non-tumorigenic HaCaT after incubation for 24 h

The optimal cell density for the HaCaT skin keratinocytes was found to be about 5×10^3 cells/well according to previous

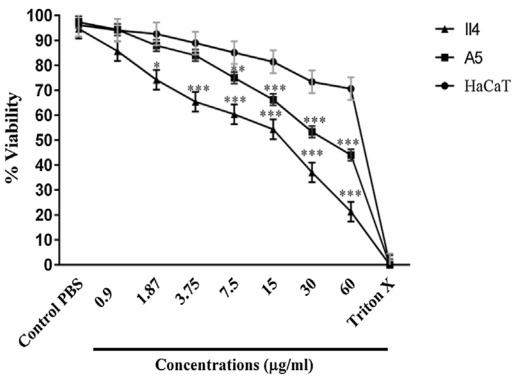

Fig. 4. Comparison of *M. bornmuelleri* Venom Effect on Cell Viability Among Different Skin Cancer Stages Representative Keratinocytes after 24 h Treatment. *M. bornmuelleri* Snake Venom Effects on Tumorigenic A5 and II4 were Compared in Reference to Results of Treatment on Control Non-Tumorigenic HaCaT Cell Line. Significance is Determined as Follow:*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.Viability of Cells is Determined by Trypan Blue. The Values are Expressed as Triplicates in 3 Independent Experiments. Error Bars Represent the Standard Error (SE) of the Results.

experiments done on the HaCaT keratinocytes model. Hence cells were seeded in a volume of 200 µL (Roche Applied Sciences, Mannheim, Germany).

The different concentrations of *M. bornmuelleri* venom (0.9, 1.87. 3.75, 7.5, 15, 30, and 60 µg/mL) were used in order to study their cytotoxic effect on the control HaCaT Cell line. The venom caused a dose-dependent cell death starting at 3.75 µg/mL when the cytotoxicity was 4.54% as compared to the control (*$p < 0.05$) reaching a peak at 60 µg/mL when the cytotoxicity was 30.14% with a $p < 0.001$ as compared to the control (Fig. 5).

3.6. Evaluation of M. bornmuelleri *cytotoxic effects on benign A5 cells after incubation for 24 h*

Cytotoxicity of *M. bornmuelleri* venom on benign A5 cell line was then assayed using the LDH colorimetric test. Cytotoxicity of the venom exhibited a significant concentration-dependent effect on the benign A5 starting with the concentrations of 1.87 µg/mL (10.66%,) reaching a peak of 52.45% at 60 µg/mL (Fig. 6). The IC_{50} on A5 was determined at 60 µg/mL of venom.

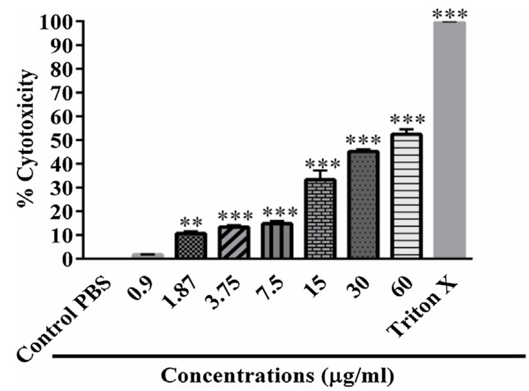

Fig. 6. Cytotoxic Effect of *M. bornmuelleri* Venom on Benign A5 Cell Line as Measured by LDH Cytotoxic Assay. Triton-x was Used as a Positive Control. The Values are Expressed as Triplicates of 3 Independent Experiments. Statistical Significance was Determined as Follows *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.7. Evaluation of M. bornmuelleri *cytotoxic effects on low grade malignant II4 cells after incubation for 24 h*

The cytotoxic effect of *Montivipera bornmuelleri* venom was finally assayed on the low grade malignant II4 cell line using the LDH test. The venom caused a highly significant effect on the malignant II4 cells which was also dose dependent. For instance, an increase in the concentrations from 0.9, 1.87, 3.75, 7.5, 15, 30, to 60 µg/mL induced a significant increase (with $p < 0.001$ in all cases) in percentage cell death (13.91, 24.49, 34.20, 39.51, 45.67, 62.31, to 76.97% respectively) (Fig. 7). The IC_{50} on II4 was estimated at 10 µg/mL.

3.8. Comparison of M. bornmuelleri *snake venom cytotoxicity on different keratinocytes related cell lines after 24 h*

Upon comparing the percentage of cell death attributed to the A5 and the II4 keratinocytes with the one ascribed to the HaCaT keratinocytes, considered as control, statistically significant differences were noticed when using the different concentrations of the *M. bornmuelleri* venom. Concentrations that were nontoxic on the HaCaT cell line exhibited higher toxicity on cancerous keratinocytes such as A5 and II4. For example, at the lowest concentration (0.9 µg/mL), the treatment was found to induce a significantly

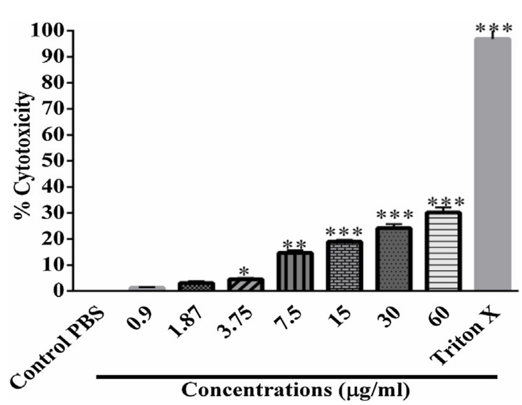

Fig. 5. Cytotoxicity of *M. bornmuelleri* Venom on Non-Tumorigenic HaCaT at 24 h Measured by LDH Cytotoxicity Test. Statistical Significance of Each Group Compared to the Control (PBS) is Determined as Follow:*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. The Values are Repeated in 3 Independent Experiments with N = 3. Error Bars are Representatives of the Standard Error (SE) of the Results.

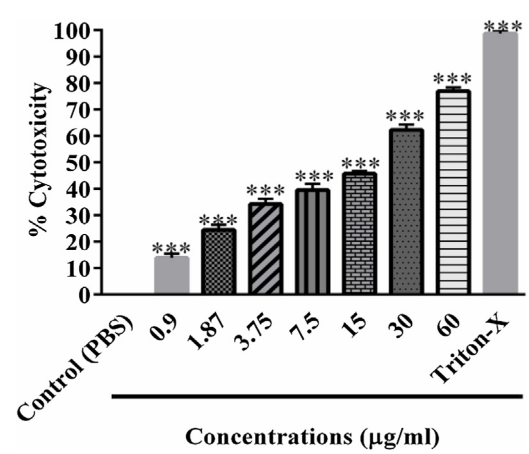

Fig. 7. Cytotoxic Effect of *M. bornmuelleri* Venom on Low Grade Malignant II4 at Different Concentrations Using LDH Cytotoxic Assay. Absorbance was Measured at 490 nm. The Values are Expressed as Triplicates of 3 Independent Experiments. Statistical Significance of Each Group was Compared to Control (PBS) and Determined by Dunnett Test; *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$.

higher cell death percentage on malignant II4 but not on benign A5 cells when compared to HaCaT. Also, at a concentration of 15 μg/mL, snake venom exhibited a trivial cell death percentage in the non-tumorigenic HaCaT cells (19.04%) in contrast to a significant cytotoxic effect on the benign A5 (around 35.00%) and on the malignant II4 (around 45.00%) (Fig. 8).

4. Discussion

Among cancer treatments, radio and chemotherapies are the most popular to induce apoptosis by causing DNA damages to the cells but without discriminating between normal and malignant ones (Wilson et al., 2009; Yalcinet al., 2014). Hence was the urge to discover new therapeutic drugs that could destroy carcinogenic cells while preserving the normal ones intact. Therefore, scientists focused on the snake venoms as novel cancer therapeutics since they are composed of various agents known to have many biological (Hraoui-Bloquet et al., 2012; Accary et al., 2013) and potential ant-tumor activities (Zhou et al., 2000; Nalbantsoyet al., 2012).

In this study, the cytotoxic effect of the Lebanese *M. bornmuelleri* snake venom was evaluated on the immortalized HaCaT cell line, the benign A5 cell line, and the malignant II4 cell line. A significant decrease in the cell viability percentage was noticed when increasing the concentrations of the snake venom but to different extent with the different cell lines. In fact, the HaCaT cell viability was around 70.63% when treated with 60 μg/mL, the highest concentration of snake venom used in this project. A more relevant reduction in the percentage cell viability was noticed after the treatment of the benign A5 cell line with the same concentration of the snake venom whereby 44% remained living while the low grade II4 keratinocytes showed an even lower decrease in the viability of cells reaching a percentage of 21.33% at the same concentration of 60 μg/mL.

The LDH cytotoxicity test was performed in order to evaluate the cytotoxic effect of the studied snake venom on the three cell lines and the results confirmed the ones obtained using the trypan blue assay. The effect of this venom was mainly found cytotoxic on the low grade malignant II4 cell line where the percentage cell death exhibited an obvious upsurge reaching 98.67% cell death at 60 μg/mL of venom).

Therefore, we show here that, for the same concentration, the benign and malignant cells were mostly affected by the *M. bornmuelleri* snake venom as compared to the HaCaT keratinocytes. The correlation of these results with previous investigations in the literature cannot be really performed since this is still among the very first studies investigating the *M. bornmuelleri* venom's effect on human-derived skin cancer cell lines. However, these effects might be attributed to one or more components of the venom which can selectively target specific steps of apoptosis and/or cellular proliferation.

In conclusion, the selective cytotoxic effects of the *M. bornmuelleri* venom on the studied cell keratinocytes cancer cell lines open the horizon for more advanced studies involving the identification and characterization of the bioactive proteins found in this snake venom as well as the determination of the possible mechanism of cell death. In vivo studies can be also performed to investigate the high and exceptional anticancer potentials of this venom.

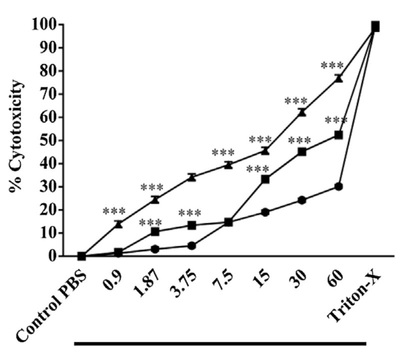

Fig. 8. Comparison of *M. bormmuelleri* Venom Cytotoxicity on Different Keratinocytes related cell lines after 24 h. *M. bormmuelleri* Snake Venom Effects on Tumorigenic A5 and II4 were Compared in Reference to Results of Treatment on Control Non-Tumorigenic HaCaT Cell Line. Significance is Determined Using Bonferroni as Follow:*p < 0.05, ** p < 0.01 and *** p < 0.001. Error Bars Represent the Standard Error (SE) of the Results.

Conflict of interest

No conflict of interest about this work.

Ethical statement

All experimental procedures were carried out with strict adherence to the ethical guidelines for the study of experimental pain in conscious animals.

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