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RESEACH ARTICLE

CYTOTOXIC EFFECT OF *ECHIS OCELLATUS* VENOM ON PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND HEPATOCELLULAR CARCINOMA ($HEPG_2$) CELLS

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Abstract
Background: "Toxin arsenal" is a term mimicking a handful of harmful substances consisting of bioactive molecules with potentials of therapeutic activity. Hepatocellular carcinoma (HCC), prevalent in sub-Saharan Africa, lacks effective treatments. This study investigates the cytotoxic effect of *Echis ocellatus* venom on Peripheral Blood Mononuclear Cells (PBMC) and Hepatocellular carcinoma cells ($HepG_2$) exploring its immunotherapy and anticancer properties. **Objectives:** To evaluate the cytotoxicity of Northern Nigerian *Echis ocellatus* venom on $HepG_2$ and PBMCs cell lines. **Method:** The (3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay was employed to estimate cell viability and the half-maximal inhibitory concentration (IC_{50}) respectively. The venom was screened using $HepG_2$ and PBMC cell lines. **Result:** Viability was assessed following exposure to a series of concentrations ranging from 0 to 500µg/mL for $HepG_2$ and 0 to 200µg/mL for PBMC showing greater cytotoxicity toward carcinoma cells; with IC_{50} values for $HepG_2$ (14.54 µg/mL) and PBMC (25.70 µg/mL). This suggests the *Echis ocellatus* venom selectively targets cancer cells with reduced effects on healthy immune cells, highlighting its therapeutic potential. **Conclusion:** Nigeria Northern *Echis ocellatus* venom exhibits increased cytotoxic effect on $HepG_2$ compared to PBMC cell lines. The study concludes that the venom shows potential as an anticancer agent against HCC and exhibits immunomodulatory properties. The findings suggest cancer-specific mechanisms like apoptosis, positioning the *Echis ocellatus* venom as a promising candidate for drug development.

Keywords: Northern Nigerian *Echis ocellatus* venom, PBMC, $HepG_2$, immunomodulatory, cancer, Apoptosis, Hepatocellular carcinoma

INTRODUCTION

Saw-scaled or carpet vipers (genus *Echis*) has increased global envenomation mortality. And the specie *Echis ocellatus* is among the rampant snake with higher fatalities than any other African snake species (Ghezellou *et al.,* 2020; Adeyi *et al.,* 2021; Tijani *et al.,* 2024).

Venoms produced by snakes, particularly vipers such as *Echis spp.* (saw-scaled vipers), are complex biochemical cocktails comprised of proteins, peptides, enzymes, and other small molecules which make up the toxins (Offor and Piater, 2024; Serino-Silva *et al.,* 2024). The main toxins responsible for the development of tissue necrosis and ulcerations following envenoming are hematotoxins snake venom metalloproteinases (SVSMP), snake venom serine proteases (SVSP) and myotoxins phospholipase A2 (PLA2) (Qin *et al.,* 2023; Dingwoke *et al*., 2024). These venom components serve as the snake's "toxin arsenal," enabling it to immobilize, kill, and digest its prey through

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modulating ion channels, receptors, coagulation factors and their cellular membranes (Zancolli *et al*,.2022; Misson Mindrebo *et al.,* 2024). Beyond their role in envenomation, many of these molecules exhibit therapeutic potential, including anti-cancer and immunomodulatory properties (Abdallah *et al.,* 2024; Kancha *et al.,* 2024; Salama *et al*., 2024).

Emerging research suggests that venom components may offer a dual function: contributing to the pathological effects of envenomation while presenting a reservoir of bioactive compounds that could inhibit tumor growth or modulate immune responses (Marinho *et al*., 2023). Specifically, these compounds may interfere with mitochondrial metabolism and epigenetic processes, such as methylation, to selectively target cancer cells while minimizing side effects (Sasovsky *et al*., 2024).

Hepatocellular carcinoma (HCC), one of the most prevalent and lethal liver cancers, is particularly common in sub-Saharan Africa due to the high burden of chronic hepatitis infections. Targeting HCC using venom-derived cytotoxins offers a promising approach to cancer therapy. (Ayvazyan *et al*., 2022; Filali *et al.,* 2023). Cell-based assays, such as those assessing cytotoxicity and cell viability, provide a valuable alternative to in vivo models for toxicity testing, aligning with growing ethical considerations surrounding animal welfare (Ahmadi *et al.,* 2022; Ejma-Multański *et al.,* 2023).

This study investigates the cytotoxic effects of *Echis ocellatus* venom on hepatocellular carcinoma cells (HepG2) and peripheral blood mononuclear cells (PBMCs). By evaluating the selective cytotoxicity of the venom, this research explores its potential as an anticancer highlighting its relevance as a natural product with significant therapeutic promise.

MATERIALS AND METHODS

Materials: vacutainer needle (BD biosciences, USA), vacutainer ethylenediaminetetraacetic acid EDTA (Greiner Bio-one, Austria), vacutainer holder (BD biosciences, USA), cryovials (Corning life sciences, USA), falcon tube (BD Bio sciences, USA), cell culture flask (Corning life sciences, USA), sterile serological pipette (Corning life sciences, USA), pipette filler (Eppendorf, Germany), sealant (Dow Corning, USA), hemocytometer (Marienfeld, Germany), cover slip (Sail brand, China, inverted microscope (Am scopeIN300TC,USA) , water bath (cu66, England), Biosafety cabinet (BC-11231BBC86, USA), cold centrifuge (Eppendorf, Germany), CO_2 Incubator (IN-CO80, USA), 96-well plate plastic (Eppendorf, Germany), multichannel pipette (Eppendorf, Germany), pipette (Biohit,Finland), xMArk ELISA plate reader (Biorad, USA).

Reagent: Dulbecco’s Modified Eagle Medium DMEM (Elabscience, China), 10% Fetal bovine Serum FBS (GE Healthcare Life Sciences, USA), 1% SP Penicillin-Streptomycin (Elabscience, China), Ficoll-Paque™ (ICN Biomedical Inc. USA), RPMI-1640 medium (Sigma Aldrich, USA), Trypsin (Thermo Fisher Scientific, USA), Trypan blue (Sigma Aldrich, USA), Phosphate Buffer Saline PBS (Corning life sciences, USA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide MTT (Bio Basic, Canada), dimethyl sulfoxide DMSO (Bio Basic, Canada)

The Study Design

This study was an experimental study design.

Venom source

The lyophilized venom was purchased from the venom bank at Bayero University Kano, Kano State. Venom was obtained from *Echis ocellatus* from North Eastern part of Nigeria.

Subjects Selection

Apparently healthy five (2) male matched 18-40 years in Kaduna State were recruited for the study. A close-ended structured questionnaire was used to know their socio-demographic characteristics among others

Inclusion criteria

- Male participant and between 18 years to 40 years of age
- Does not have any form of aliment

Exclusion criteria

- Does not have blood pressure
- Have not taken any form of drug for the past 2 weeks

- Refuse to sign the consent form

Blood Specimen Collection and Processing

The subject's blood specimen was collected according to WHO guidelines for drawing blood

(Neef *et al.,* 2024). Five milliliters (5 ml) of venous blood was collected from each of the subjects

through the antecubital vein under aseptic condition using sterile vacutainer needle into an ethylenediaminetetraacetic acid EDTA (Greiner Bio-one, Austria) specimen bottles and was processed immediately for use.

Ethical Considerations

Before the study started, the ethical clearance was received from the Ministry of Defence's Health Ethics Council Nigeria (MODHREC) with its approval NHREC/MOD-HREC/15/02/23/C… MOD/SC/HREC/1/121). The HELSINKI declaration's terms were adhered to. Every possible study participant gave their written, informed consent. Benefits, confidentiality, and the ability for participant to withdraw willingly were all covered.

$HepG_2$ Cell Culture and Maintenance

$HepG_2$ cells, representing human hepatocellular carcinoma, were sourced from the Department of Biochemistry, Kaduna State University, Nigeria. The cells were cultured in Dulbecco's Modified Eagle Medium DMEM (Elabscience, China), supplemented with 10% Fetal bovine Serum FBS (GE Healthcare Life Sciences, USA) and 1% Penicillin-Streptomycin SP (Elabscience, China). The culture conditions included a humidified atmosphere maintained at 37°C with 18% Oxygen and 5% Carbon dioxide. Cells were routinely sub cultured twice a week to maintain exponential growth for two weeks (Duff *et al.,* 2021).

Protocol for $HepG_2$ cultivation

- All the reagent was brought to room temperature and every step should be done aseptically in a biosafety cabinet (BC-11231BBC86, USA),
- The $HepG_2$ cryovial was removed from the liquid Nitrogen tank (Praxair,USA) and thawed for 2-3 minutes in water bath (cu-66, England) at 37ºC
- The $HepG_2$ was resuspended with a sterile serological pipette in 1.5ml of DMEM agar, transferred to a tube and sealed
- It was centrifuged at 1000rpm for 5 minutes in cold centrifuge (Eppendorf, Germany), at 4ºC
- The supernatant was decanted into a beaker and the sediment was re-suspended with 10ml fresh DMEM media
- 5ml of the resuspended $HepG_2$ was transferred into the cell culture flask (Corning life sciences, USA) and the flask was properly labelled
- The viability of the cell was checked under the inverted microscope (Am scopeIN300TC,USA) and the flask was incubated in CO_2 incubator (IN-CO80, USA) for 24 hours for mortality

Sub-culturing of the $HepG_2$ cells for media replacement

- The cell culture was observed under the microscope for the attachment of the cells
- The media was poured out from the cell culture flask
- 5ml of DMEM was transferred into the flask each and observed again under the microscope
- It was returned into the incubator for 24 hours

Sub-culturing

- The media was poured out and 3ml of Phosphate Buffer Saline PBS (Corning life sciences, USA) was added
- The PBS was discarded and 0.4ml trypsin (Thermo Fisher Scientific, USA) for 5 minutes
- The trypsin was poured out, 0.4ml trypsin was added again and observed under the microscope for detachment
- 6ml of DMEM each was transferred into a sterile flask
- 2ml of DMEM was used to dislodge the cell and all the mixture are pipetted into the pipette
- 1ml each was added to each flask, labelled properly, observed under the microscope and incubated for 24 hours
- The cell viability was calculated

Cell Viability

Trypan blue (Sigma Aldrich, USA) was used to access the cell viability.

- 10 µL of the suspended cells was mixed with 10 µL of 0.4% trypan blue solution in a microcentrifuge tube.
- It was allowed to stand for approximately 2minutes at room temperature
- The hemocytometer (Marienfeld, Germany) and cover glass (Sail brand, China) were cleaned with alcohol and allowed to dry.
- The cover glass was placed on the hemocytometer and 10 µL of the trypan blue-stained cell solution was placed at the edge of the hemocytometer chamber and capillary action drew the mixture into the chamber
- The hemocytometer chamber was placed under the microscope at 10× magnification
- The hemocytometer chamber has a central grid with 9 squares; the 4 large corner squares was focused for counting of the viable cells
- To calculate the cell density:

n=Number of cells counted per square

N= average number of cells counted

DF= dilution factor

Therefore, Cell density= $(N \times 2 \times 10^4)$ cells/mL

Average cell count = 60cells

Cell density for $HepG_2$ cell cultivated = $60 \times 2 \times 10^4 = 1.2 \times 10^5$ cells/mL

Therefore, to culture 1×10^4 cells/mL pre-well,

The formula C1VI = C2V2;

Where C1= original concentration = 1.2×10^5 cells/mL

V1 = unknown volume= ?

C2 = needed concentration = 1.0×10^4 cells/mL

V2 = needed volume = 6ml

$$V1 = \frac{C2.V2}{C1}$$

$$V1 = \frac{1.0 \times 104 \ \times \ 6}{1.2 \times 105}$$

V1 = 0.5ml of the $HepG_2$ cells will be added to 5.5ml DMEM media to make 6ml of the $HepG_2$ cell culture with concentration of 1.0×10^4 cells/mL for seeding of *Echis ocellatus* venom

PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS) ISOLATION AND CULTURE

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors via density gradient centrifugation using Ficoll-Paque™ (ICN Biomedical Inc. USA) following established protocols (Staab e*t al.,* 2020). Isolated PBMCs were resuspended in RPMI-1640 medium (Sigma Aldrich, USA), enriched with 10% FBS and 1% Penicillin-Streptomycin.

Protocol for PBMC preparation

- 5ml of fresh blood was collected in an EDTA tubes
- The bottles were properly labelled
- The blood sample collected was diluted with an equal volume of sterile PBS (1:1 ratio).
- It was mixed gently to without introducing bubbles.
- 5ml of Ficoll solution was measured into a 15ml falcon tube and the diluted blood mixture was layered gradually into the falcon tube containing the ficoll solution and avoid disturbing the ficoll layer.
- The mixture in the falcon tube was centrifuged at 800rcp for 25 minutes at 4ºC in a cold centrifuge. The centrifuge was programmed to start slowly and end slowly to avoid disturbance of the layered solution.
- The PBMC mixture was harvested by aspirating the buffy coat layer using a pipette, avoiding contamination from other layers.
- The PBMC was transferred into the sterile 15ml falcon tube and was made up to 15ml mark with PBS solution
- The mixture was centrifuged at 150rcp for 10 minutes at 4ºC in a cold centrifuge. The centrifuge was programmed to start slowly and

end slowly to avoid disturbance of the layered solution.

- The supernatant was discarded and pellet was re suspended in RPMI 1640 media solution.
- The mixture was centrifuged at 150rcp for 10 minutes at 4^oC in a cold centrifuge. The centrifuge was programmed to start slowly and end slowly to avoid disturbance of the layered solution.
- Cell viability was calculated

Average cell count = 150cells

Cell density for PBMC cell cultivated = $150 \times 2 \times 10^4 = 3.0 \times 10^6$ cells/mL
Therefore, to culture 1×10^6 cells/mL pre-well,
The formula C1VI = C2V2;
Where C1= original concentration = 3.0×10^6 cells/mL
V1 = unknown volume= ?
C2 = needed concentration = 1.0×10^6 cells/mL
V2 = needed volume = 6ml

$$V1 = \frac{C2.V2}{C1}$$

$$V1 = \frac{1.0 \times 106 \ \times \ 6}{3.0 \times 106}$$

V1 = 2.0ml of the PBMC cells will be added to 4.0ml RPMI-1640 media to make 6ml of the PBMC cell culture with concentration of 1.0×10^6 cells/mL for seeding of *Echis ocellatus* venom

VENOM EXPOSURE PROTOCOL

The serial concentration of *Echis ocellatus* venom (0-500µg/mL) for $HepG_2$ cells and (0-200µg/mL) for PBMCs were exposed to their respective culture media. $HepG_2$ cells were exposed for 48 hours to ensure sufficient interaction between venom components and cellular processes (Huang *et al.,* 2020) and PBMCs, which are more susceptible to immediate metabolic effects, were exposed for 24 hours. (Moellerberndt *et al.,* 2024; Serrano-Belmonte *et al*., 2024).

Preparation of *Echis ocellatus* Venom Dilutions

A. Stock *Echis ocellatus* venom

0.05mg/ml (5000 µg/ml) was prepared from the lyophilized *Echis ocellatus* venom

0.05g of *Echis ocellatus* venom was weighed and dissolved in 1ml of RPMI 1640 media

B. Working solution

400µg in 5ml was prepared from the of stock *Echis ocellatus* venom

Using the formula C1V1= C2V2

C1=5000 µg/ml

V1= ?

C2= 400 µg/ml

V2= 1ml

$$V1 = \frac{C2.V2}{C1}$$

$$V1 = \frac{400 \ \times 1}{5000}$$

V1 = 0.4ml of stock *echis ocellatus* venom

0.08ml of working *Echis ocellatus* venom solution was added to 0.92ml of RPMI 1640 media solution

C. The serial dilutions of *Echis ocellatus* venom started from 200 µg/mL in RPMI 1640 media for PBMC cell culture media

Dilution steps for PBMC

- 200µg/ml: 100µl of 400µg/ml *Echis ocellatus* venom was mixed with 100 µL of PBMC media
- 100 µg/mL: 100 µL of 200 µg/ml *Echis ocellatus* venom was mixed with 100 µL of PBMC media
- 50 µg/mL: 100 µL of 100 µg/ml *Echis ocellatus* venom was mixed with 100 µL of PBMC media
- 25 µg/mL: 100 µL of 50 µg/ml *Echis ocellatus* venom was mixed with 100 µL of PBMC media
- 12.50 µg/mL: 100 µL of 25 µg/ml *Echis ocellatus* venom was mixed with 100 µL of PBMC media
- 0 µg/mL: Control (PBMC medium only, no venom).

D. The serial dilutions of *Echis ocellatus* venom started from 500 µg/mL in RPMI 1640 media for $HepG_2$ cell culture media

500µg in 5ml was prepared from the of stock *Echis ocellatus* venom

Using the formula C1V1= C2V2

C1=5000 µg/ml

V1= ?

C2= 500 µg/ml

V2= 1ml

$$V1 = \frac{C2.V2}{C1}$$

$$V1 = \frac{500 \times 1}{5000}$$

V1 = 0.1ml of stock *Echis ocellatus* venom

0.1ml of working *Echis ocellatus* venom solution was added to 0.9ml of RPMI 1640 media solution

Dilution steps $HepG_2$

- 500µg/ml: 100µl of 250µg/ml *Echis ocellatus* venom was mixed with 100 µL of $HepG_2$ media
- 250 µg/mL: 100 µL of 125 µg/ml *Echis ocellatus* venom was mixed with 100 µL of $HepG_2$ media
- 125 µg/mL: 100 µL of 62.5 µg/ml *Echis ocellatus* venom was mixed with 100 µL of $HepG_2$ media
- 62.5 µg/mL: 100 µL of 31.25 µg/ml *Echis ocellatus* venom was mixed with 100 µL of $HepG_2$ media
- 31.25 µg/mL: 100 µL of 15.625 µg/ml *Echis ocellatus* venom was mixed with 100 µL of $HepG_2$ media
- 0 µg/mL: Control ($HepG_2$ medium only, no venom).

E. The treatment of the cells for PBMC

- In the 96-well plate, the prepared PBMC solution was placed in triplicate into the plate
- 100 µL of each *Echis ocellatus* venom dilution were seeded onto triplicate wells of the 96-well plate containing PBMC solution
- The final concentrations in wells were 0, 12.5, 25, 50, 100, 200 µg/ml

- The plate was incubated at 37°C, 5% CO_2 for 24 hours.

F. The treatment of the cells for $HepG_2$

- In the 96-well plate, the prepared $HepG_2$ solution was placed in triplicate into the plate
- 100 µL of each *Echis ocellatus* venom dilution were seeded onto triplicate wells of the 96-well plate containing PBMC solution
- The final concentrations in wells were 0, 31.25, 62.5, 125, 250, 500 µg/ml

- The plate was incubated at 37°C, 5% CO_2 for 48 hours.

Cytotoxicity Assessment Using MTT Assay

The cytotoxic effects of *Echis ocellatus* venom were quantified using the MTT assay, a colorimetric method that measures metabolic activity as an indicator of cell viability. Briefly, after venom exposure, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide MTT (Bio Basic, Canada) solution was added to each well, and the plates were incubated for 4 hours at 37°C. Metabolically active cells converted the yellow tetrazolium salt to water-insoluble dark blue formazan crystals via the mitochondrial enzyme succinate dehydrogenase. Following incubation, the formazan crystals were dissolved by the addition of 200 µl dimethyl sulfoxide DMSO (Bio Basic, Canada) to yield a colored solution (Nga *et al.,* 2020). and the absorbance was read at 570 nm with a reference wavelength of 630 nm to correct for background interference in an xMArk ELISA plate reader (Biorad, USA). The intensity of the color was directly proportional to the number of viable cells (Alashi *et al*., 2024).

Determination of IC_{50} and Statistical Analysis

Growth inhibition was compared with untreated controls to find the venom concentration which inhibited growth by 50% (IC_{50}). Control values were set at 0% cytotoxicity. Mean IC_{50} is the concentration of agent which reduces cell growth by 50% under the experimental conditions and is the average of at least three independent reproducible statistically significant measurements. This analysis was performed using GraphPad Prism 2022 (San Diego, USA).

Results

Table 1 demonstrates a marked increase in cytotoxicity as the venom concentration rises in $HepG_2$ cells. At a relatively low concentration of 31.25 µg/mL, cytotoxicity is recorded at 39.3%, indicating the venom's significant impact even at minimal exposure. This cytotoxic effect intensifies progressively, peaking at 56.3% when the concentration reaches 500 µg/mL. This pattern suggests that the venom's bioactive components have a strong affinity for $HepG_2$ cell receptors, likely targeting specific molecular pathways that induce programmed cell death (apoptosis) in cancerous cells. Such concentration-dependent behavior indicates a potent therapeutic potential of the venom for targeting hepatocellular carcinoma cells.

Similarly, the data in Table 2 shows that PBMC experience a gradual but less pronounced increase in cytotoxicity with escalating venom concentrations. At 12.5 µg/mL, the cytotoxicity is 7.6%, reflecting the venom's relatively mild impact on this normal, non-malignant cell line. However, as the venom concentration increases to 200 µg/mL, cytotoxicity rises to 23.7%. This moderate effect suggests that the venom components exhibit lower affinity or specificity for normal PBMC receptors compared to malignant cells. The pattern of necrotic cell death observed at higher concentrations in PBMC contrasts with the apoptosis induced in $HepG_2$ cells, further supporting the venom's differential cytotoxic mechanisms based on cell type.

The graphical representations in Figures 1 and 3 depict the correlation between cytotoxicity and venom concentration, demonstrating that cytotoxicity rises in a concentration-dependent manner. For the $HepG_2$ cell line, the venom demonstrates a pronounced increase in cytotoxicity as its concentration rises. This relationship suggests a strong interaction between the venom's bioactive components and the molecular targets within cancer cells, such as specific receptors or signaling pathways. At optimal venom concentrations, the cells undergo a controlled and energy-dependent mechanism that avoids inflammation and is often a desired outcome in cancer therapy.

In contrast, the venom's effect on PBMCs, a representative normal cell line, reveals a less intense cytotoxic response, with necrosis observed at higher concentrations. Necrosis, unlike apoptosis, is a form of uncontrolled cell death typically resulting from severe stress or damage, leading to the release of cellular contents and potential inflammation. The less pronounced cytotoxicity in PBMCs compared to $HepG_2$ cells suggests a lower affinity of venom components for the receptors or pathways in these normal cells. This differential response underscores the venom's selectivity for cancerous cells, a critical factor in developing safe and effective therapeutic agents.

Figures 2 and 4 provide insights into the half-maximal inhibitory concentration (IC_{50}) of *Echis ocellatus* venom required to induce 50% cell death in hepatocellular carcinoma cells ($HepG_2$) and human peripheral blood mononuclear cells (PBMCs), respectively.

In Figure 4, IC_{50} for PBMCs is determined to be 25.72 µg/mL, indicating moderate cytotoxicity and suggesting that the venom has a relatively low potency against this normal cell line. This moderate effect can be attributed to the reduced interaction of venom components with the receptors or molecular pathways in PBMCs. As non-malignant cells, PBMCs typically lack the abnormal or overexpressed targets found in cancer cells, such as specific membrane proteins or intracellular signaling pathways. The reduced affinity of venom bioactive molecules for PBMCs underlines the selective cytotoxic nature of the venom and highlights its safety margin for normal cell populations. In contrast, the figure 2 IC_{50} for $HepG_2$ cells is significantly lower, at 14.54 µg/mL, reflecting the venom's heightened potency against this cancer cell line. The reduced IC_{50} suggests a strong interaction between the venom's bioactive components and molecular targets specific to cancer cells, such as overexpressed growth factor receptors, disrupted signaling pathways, or altered cell cycle regulators. This strong interaction likely triggers pathways leading to programmed cell death (apoptosis), a key mechanism in

the venom's cytotoxic effects on $HepG_2$ cells. The lower IC_{50} value underscores the venom's efficacy in selectively targeting malignant cells while sparing normal ones at similar concentrations.

Echis ocellatus **venom Treatment µg/mL**	**% Cytotoxicity**
0	0
31.25	39.3
62.5	43.9
125	47.1
250	51.7
500	56.3

Table 1: Cytotoxic effect of $HepG_2$ cells on serial concentrations of *Echis ocellatus* venom

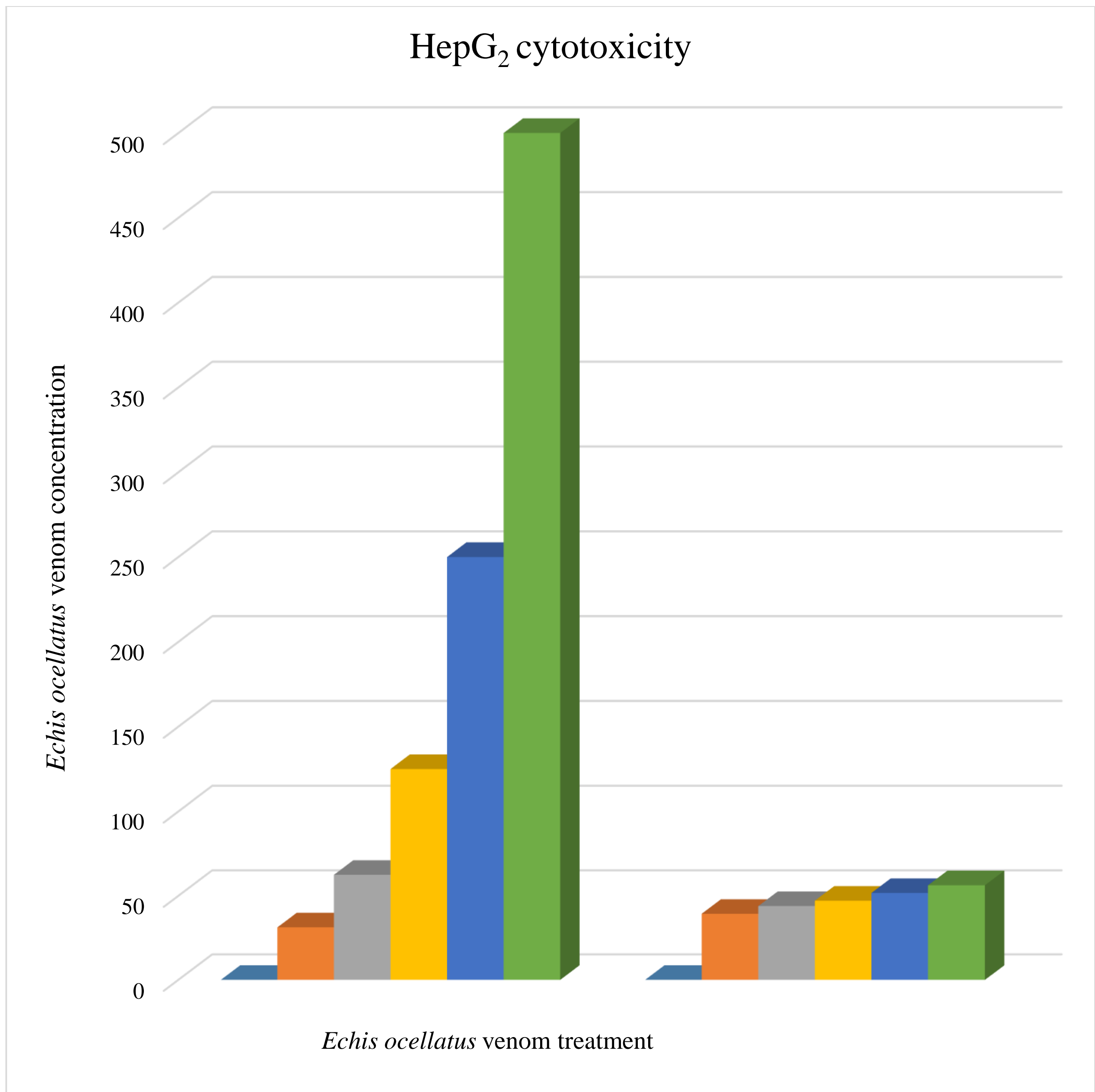

Figure 1: Effect of *Echis ocellatus* venom treatment on $HepG_2$ cells

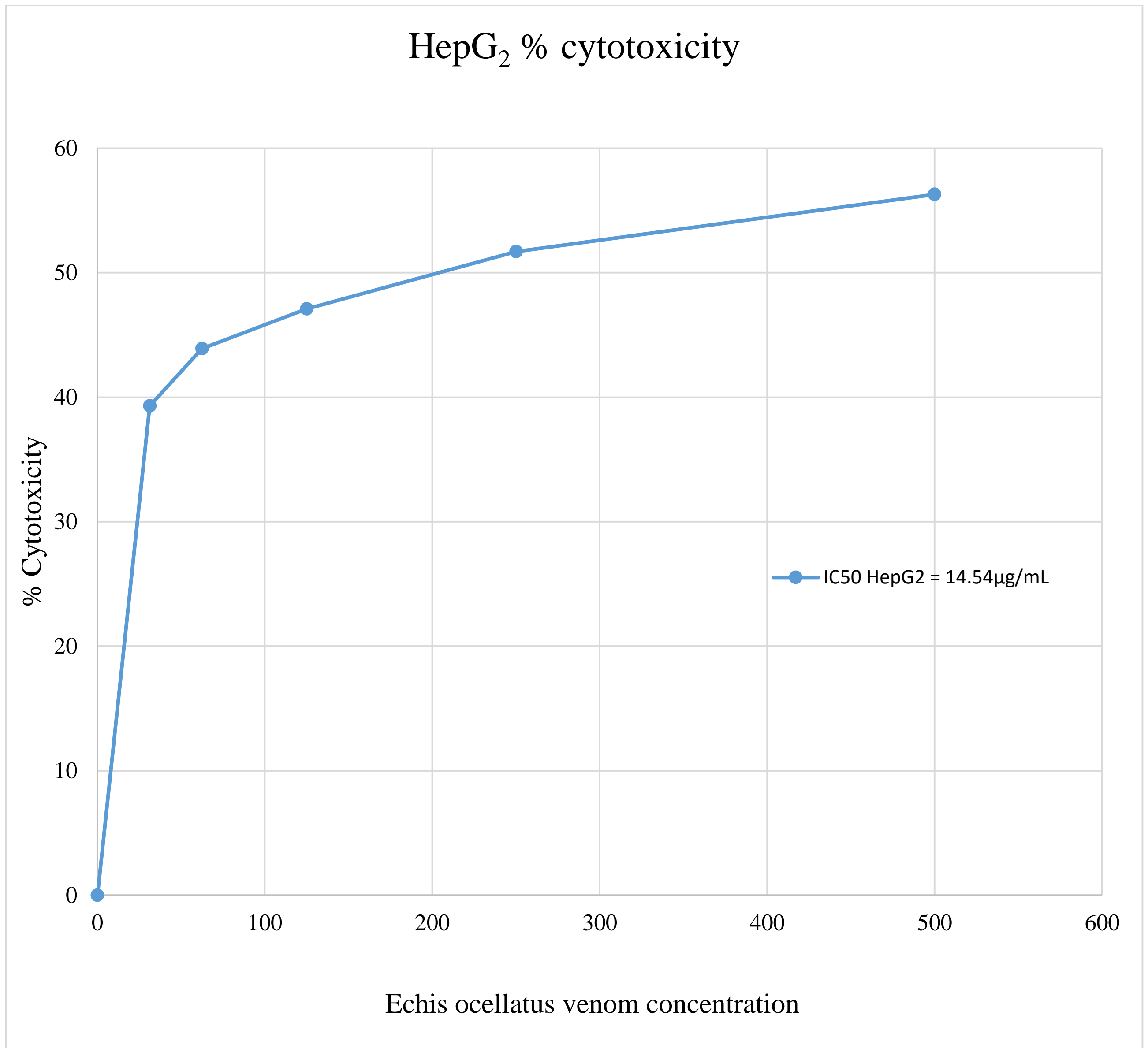

Figure 2: Half inhibitory concentration of $HepG_2$ cells on *Echis ocellatus* venom

***Echis ocellatus* venom Treatment µg/mL**	**% Cytotoxicity**
0	0
12.5	7.64
25	19.33
50	21.91
100	23.71
200	30.28

Table 2: Cytotoxic effect of PBMC cells on serial concentrations of *Echis ocellatus* venom

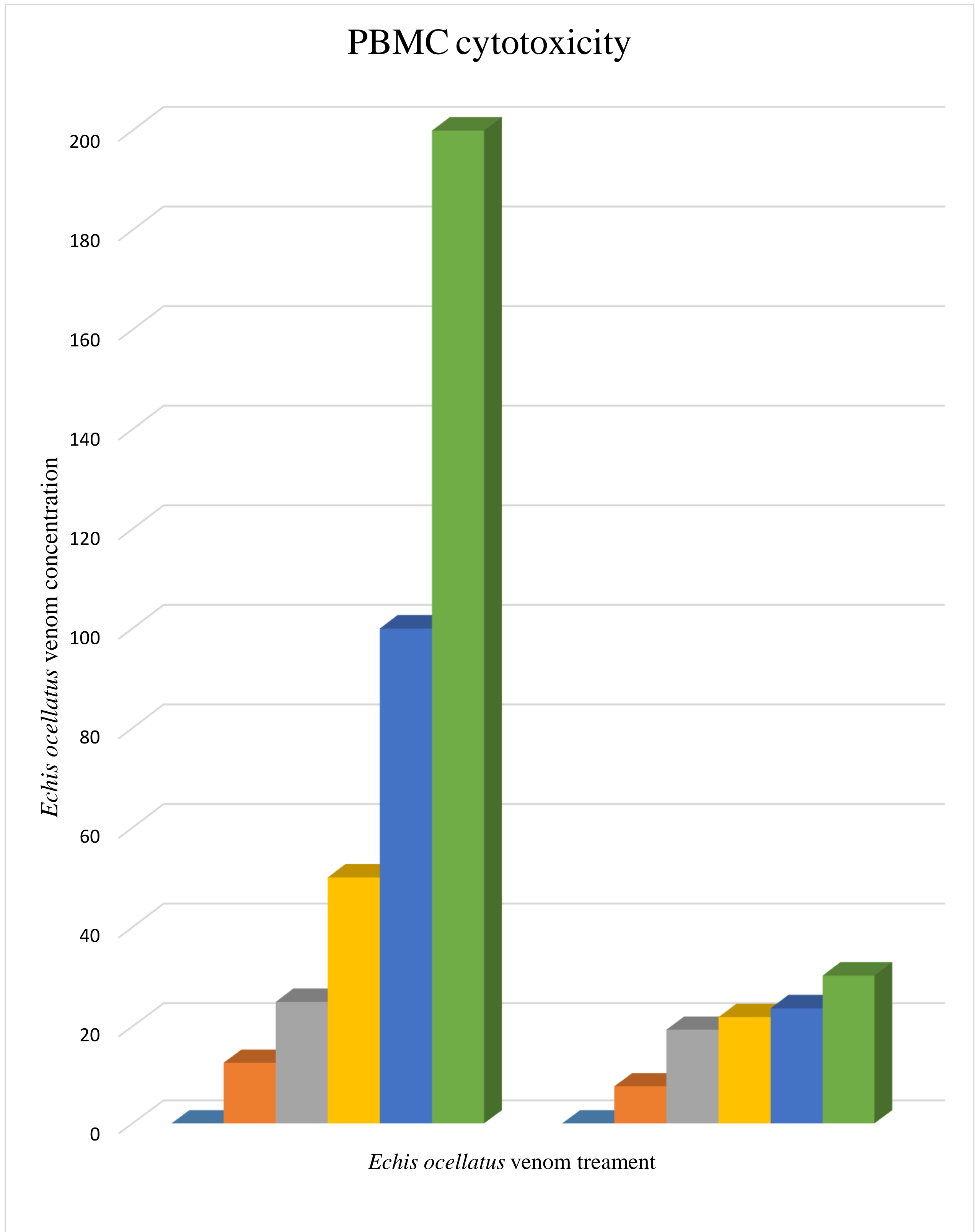

Figure 3: Effect of *Echis ocellatus* venom treatment on PBMC cells

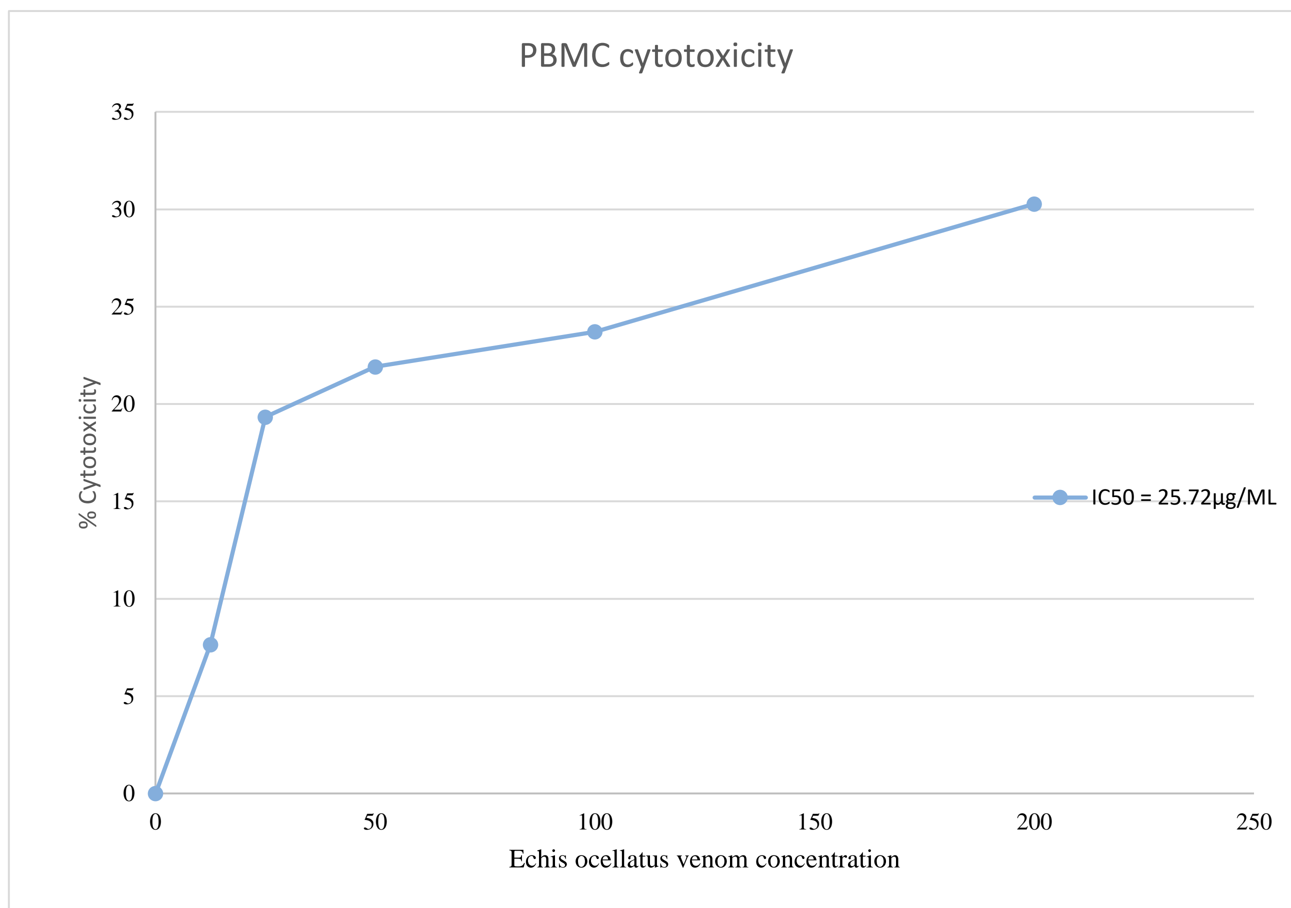

Figure 4: Half inhibitory concentration of PBMC cells on *Echis ocellatus* venom

Discussion

The findings from the data and figures provide a comprehensive understanding of the cytotoxic effects of *Echis ocellatus* venom on hepatocellular carcinoma cells ($HepG_2$) and human peripheral blood mononuclear cells (PBMCs), highlighting its selective toxicity and potential therapeutic applications.

The results demonstrate a significant concentration-dependent cytotoxic response in $HepG_2$ cells (Marković *et al.,* 2024; Avella *et al.,* 2024). As reported in Rodríguez-Vargas *et al.* (2023) at a relatively low venom concentration of 31.25 µg/mL, a substantial cytotoxic effect of 39.3% is observed, which intensifies to 56.3% at the maximum tested concentration of 500 µg/mL. This progressive increase in cytotoxicity underscores the venom's strong affinity for $HepG_2$ cell receptors, likely targeting cancer-specific molecular pathways (Shahbazi *et al.*, 2019).

$HepG_2$ cells are characterized by overexpression of various receptors and signaling molecules associated with cancer, such as growth factor receptors, anti-apoptotic proteins, and cell cycle regulators (Schönthal *et al.,* 2020). The venom's bioactive molecules—potentially proteins, peptides, or enzymes—are hypothesized to bind to these targets, disrupting cellular homeostasis and triggering apoptosis (Bickler 2020; Castro-Amorim *et al.,* 2023). Apoptosis is a desirable outcome in cancer therapy because it is a controlled, non-inflammatory process that eliminates malignant cells without affecting neighboring healthy tissues (Almeida *et al.,* 2020; Bittenbinder *et al*., 2023). The steep dose-dependent increase in cytotoxicity further suggests that the venom's components operate through highly specific and effective mechanisms,

making it a promising candidate for targeting hepatocellular carcinoma (Jimenez-Canale *et al.,* 2024).

The effect of the venom on PBMCs is less pronounced compared to HepG2 cells, with cytotoxicity rising from 7.6% at 12.5 µg/mL to 23.7% at 200 µg/mL (Sadat *et al.,* 2023). This moderate cytotoxicity indicates a lower potency of the venom against normal, non-malignant cells (Lazcano-Pérez *et al.,* 2022). PBMCs, being healthy immune cells, lack the overexpressed or aberrant molecular targets typically found in cancer cells, which likely explains the reduced interaction and cytotoxic effects of the venom (Siigur and Siigur, 2022; Marinho *et al.,* 2023).

The cell death observed in PBMCs at higher concentrations is primarily necrotic, as opposed to apoptotic in HepG2 cells (Moridikia *et al.,* 2018). Necrosis, characterized by membrane rupture and the release of intracellular components, can lead to inflammation and is generally less favorable in therapeutic contexts (Costa *et. al.,* 2022). However, the relatively high IC_{50} value for PBMCs (25.72 µg/mL) compared to $HepG_2$ cells (14.54 µg/mL) demonstrates a therapeutic window, wherein the venom exhibits selective cytotoxicity against cancer cells while sparing normal cells at lower concentrations. This selectivity is crucial for minimizing potential side effects and enhancing the safety profile of venom-derived therapies (Shin *et al.,* 2022).

The dose-dependent cytotoxicity observed in both cell lines reflects a clear structure-activity relationship (Renovato-Martins *et al*., 2022). As venom concentration increases, its bioactive components, such as enzymes (e.g., phospholipases A2 and metalloproteinases), peptides, and toxins, likely engage more extensively with cellular targets. In $HepG_2$ cells, these interactions result in the disruption of cellular processes critical for survival, including mitochondrial function, DNA replication, and cell signaling pathways, ultimately leading to apoptosis (Li *et al.,* 2021). In PBMCs, the lower interaction affinity and necrotic mechanism of cell death indicate that these cells are less vulnerable to venom components under normal conditions (El-Didamony *et al.,* 2022).

The IC_{50} values provide quantitative insights into the venom's cytotoxic potency. For $HepG_2$ cells, the IC_{50} of 14.54 µg/mL signifies potent cytotoxic effects at relatively low concentrations, emphasizing the venom's potential as an anticancer agent. In contrast, the higher IC_{50} of 25.72 µg/mL for PBMCs indicates moderate cytotoxicity and reduced potency, supporting the venom's selectivity for malignant cells over normal ones (Magdy *et al.,* 2023).

This differential IC_{50} highlights the venom's therapeutic promise, as selective cytotoxicity is a cornerstone of effective cancer treatments. The ability to induce apoptosis in $HepG_2$ cells while exerting minimal effects on PBMCs suggests that *Echis ocellatus* venom could serve as a source of bioactive compounds for drug development (Yaacoub *et al.,* 2022). These findings align with the broader principle of targeted therapy, which seeks to exploit differences between malignant and normal cells to achieve therapeutic efficacy with minimal toxicity (Lopes-de-Souza *et al.,* 2023).

Cancer is related as one of non-communicable diseases (NCDs) subsequently related to pathogenicity of oxidative stress and inflammatory damage which is a public health threat with a leading cause of morbidity and mortality worldwide (Zhang *et al*., 2024; Romero-Trejo *et al*., 2024; Iksen *et al*., 2024).

This will need a natural therapy from animal toxin-based drugs; and protein therapy is designed to bind the active site which has been in constant research for the development of new treatment strategies for anticancer treatment (Offor and Piater, 2024; Singh *et al*., 2024).

The component of the snake venom has inflammatory characteristics that can enhance innate immunity that can aid the system to recognize and destroy cancer cells (Mahmoud Shokhba *et al*., 2024). The pronounced cytotoxicity of *Echis ocellatus* venom towards $HepG_2$ cells highlights its potential as a source of anticancer compounds. $HepG_2$ cells, which represent human hepatocellular carcinoma, are known for their aggressive nature by excessive formation of blood vessels and its angiogenesis and inflammation (Chan *et al.,* 2023; Oliveira *et al.,* 2024). The venom's ability to induce cytotoxicity at relatively low concentrations suggests that specific venom components may interfere with key cellular processes in cancer cells, such as apoptosis, cell cycle regulation, or angiogenesis (Guo *et al.,* 2024).

Venom binds to the cancer cell surface receptors (intergrins, glycoproteins, growth factor receptors (Almeida *et al.,* 2024). The proteolysis and or hydrolysis by degradation of extracellular matrix (ECM) is the enzymatic breakdown of venom; thereby inhibition of cell adhesion through disruption of cell-cell and cell-matrix interaction (Olaoba *et al.,* 2020). The signaling pathway modulation comes into play my affecting the pro-inflammatory and anti-inflammatory pathways and finally release of bioactive peptides to stimulate immune response (Ma *et al.,* 2024; Chen *et al.,* 2024).

Venoms affect different cells, cellular structures and immune systems which are components of both the innate and adaptive immune systems can be stimulated or suppressed eliciting cascade of immune responses (Avalo *et. al.,* 2022; Zuliani, 2023). At cellular level, venom initiates immune response to the cellular tissue resulting to inflammation that is formation of pores in cell membranes, structural changes in cell ion channels (Noack and Miossec, 2023).

Certain venom proteins and peptides can stimulate T cells and NK cells which are cytotoxic cells that are sensitive to foreign substances through Antibody-dependent cellular cytotoxicity (ADCC) (Dick and Hart, 2022). This immune activation may increase the production of cytokines such as interferons and interleukins IL, enhancing their ability to recognize and destroy cancer cells (Rattanasrisomporn *et. al.,* 2022; de Castro *et al.,* 2023).

Venom can trigger apoptosis (programmed cell death) by activating T cells, imposing a negative feedback mechanism that constrains the growing T cell population. This autoregulatory pathway can be recognized through various mechanisms, such as activating caspases and mitochondrial pathways (Pohida *et al.,* 2022; Piede *et al.,* 2023). The selective induction of apoptosis in cancer cells without harming normal PBMCs is a key focus of venom-based therapies (Lu *et al.,* 2022; Santos *et al.,* 2024).The selective action on PBMCs could be indicative of an immune-modifying effect, either by enhancing immune cell activity by concentration dependent (Silva *et al*., 2022) implying the induction of apoptosis triggering apoptotic pathways at higher doses (Vanuopadath *et al*. 2020; Almeida *et al*., 2023). The venom's ability to modulate the survival of these cells suggests that it might influence immune signaling pathways, which could lead to applications in managing immune-related disorders (Zhang *et al.,* 2024). Venom-induced cytokine production by PBMCs can create an environment that either inhibits cancer cell proliferation or enhances immune cell activity (Larid *et al.,* 2022). The venom's ability to disrupt membranes and cause cell lysis could also target the tumor microenvironment. This may involve breaking down cancer cell protective barriers or altering the signaling pathways that support tumor growth and immune evasion (Kantapan *et al*., 2021; Suzdaltseva *et al*., 2022; Kim *et al*., 2024).

The data underscore the potential of *Echis ocellatus* venom in cancer therapy, particularly for hepatocellular carcinoma. The venom's bioactive molecules could be further isolated, characterized, and optimized for their anticancer properties. Moreover, the concentration-dependent response and differential cytotoxicity pave the way for precise dosing strategies that maximize efficacy while minimizing adverse effects on healthy cells.

In conclusion, the results reveal that *Echis ocellatus* venom exhibits strong cytotoxic effects against cancer cells, with selective potency that spares normal cells at comparable concentrations. These findings provide a promising foundation for further exploration of venom-based therapies, with the ultimate goal of developing targeted, effective, and safe anticancer treatments.

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Authors' Contributions

We declared that this work was conducted by the authors named in this article. MYG conceived the original idea and produced the theory and final supervisory of the manuscript. AJA gave critical revision of the manuscript for important intellectual content and co-supervised the work. RA performed laboratory works, co-wrote and proof read the manuscript. Fatima Abdu did the analysis and interpretation of data. Jelani Ismail proof read the manuscript.

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