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Anticancer and Cytotoxic Effect of Verotoxin 1 on Colon Cancer Cell Line

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Verotoxin-1 (VT-1) or Shiga-like toxin 1 (Stx-1) is produced by enterohemorrhagic *Escherichia coli* (EHEC) and is an AB5 holotoxin with a strong inhibitor of protein synthesis. VT-1 is a type 2 ribosome-inactivating protein (RIP) that has been shown to have cytotoxic and anticancer potential by inducing necrosis, apoptosis, and cell cycle arrest, making it a promising antitumor candidate. Here, we tested the cytotoxicity of VT-1 on CaCo2 and NCM425 cell lines and the results showed that VT-1 was more potent on CaCo2. Morphological changes were also evaluated on the cellular level and the results showed that VT-1 caused a decrease in viable cell count, altered cell membrane permeability, and an increase in total nuclear intensity. On the other hand, VT-1 displayed a lesser impact on mitochondrial membrane potential (MMP) and cytochrome c release. On the expression of caspases 3 and 9, VT-1 exhibited an insignificant effect on both which alongside the mitochondrial membrane potential (MMP) and cytochrome c results, might indicate that CaCo2 suffered from the necrosis process as a mechanism of cell death after exposure to VT-1.

Keywords: **Verotoxin 1, colon cancer, cytotoxicity, necrosis, apoptosis**

Introduction

Verotoxin 1 also known as VT-1 is a key virulence factor in the pathophysiology of hemorrhagic colitis, with potentially fatal extraintestinal effects as in hemolytic uremic syndrome and central nervous system sequelae are produced by verotoxin-producing *Escherichia coli* (VTEC) [1]. Verotoxins are AB5-structured multifunctional toxins [2].

They are secreted into the intestine following human VTEC infections, travel through the bloodstream, and attack the kidney endothelial cells [3, 4]. Verotoxins proteins are AB5 proteins made up of a sole enzymatic component of 32 kDa A which is non-covalently linked to a pentamer of B components (7.7 kDa). This B component pentamer interacts with the globotriaosylceramide (Gb3) neutral glycosphingolipid (GSL) receptor found on gut and kidney endothelial and epithelial cells [5].

The confirmation of the carbohydrate moiety exposed on the cell surface is highly influenced by the structure of the fatty acid component of Gb3 embedded in the plasma membrane, which plays a key role in Gb3's activity as a VTs receptor [6]. In contrast, the high affinity of VTs for Gb3 is most likely due to the existence of maybe three or at least two Gb3 binding sites per subunit B monomer [7].

Verotoxin has been utilized as a medication to treat malignant tumors. They are ribosomal inactivating proteins that promote cell toxicity, programmed cell death, and cell cycle arrest in malignant human cells [8]. Due to their cytotoxicity, they could be regarded as an anticancer therapy [9].

Apoptosis can be induced by verotoxins in a variety of

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malignancies [10]. Although [11] mentioned that necrosis, apoptosis, or a mix of the two processes can be triggered as a result of VT-1 exposure.

Bowel cancer is one of the leading causes of cancer death around the world. It is the second most frequent cancer diagnosed in females and the third most frequent cancer diagnosed in males [12]. In 2018, around 1.8 million new instances of colonic cancer were diagnosed [13].

The objective of our study was to focus on the anticancer and cytotoxic activity of Verotoxin 1 on the CaCo2 cell line and to detect the induction of apoptosis or necrosis as a cell death mechanism.

Materials and Methods

Cell line maintenance and MTT assay

A complete RPMI-1640 medium was used to suspend CaCo2 and NCM425 cells, then they were propagated in flasks for 24 h at 37℃ in a humidified environment supplemented with 5% of CO_2 [14]. Five concentrations (3.1, 6.25, 12.5, 25, and 50 µg/ml) of the VT-1 (Biorbyt Ltd., UK) were prepared after adding 0.5 ml of PBS to the stock solution, and by using the MTT kit (Intron Ltd. kit, China) cytotoxicity of Vt-1 tested on CaCo2 and NCM425 cell lines. Each concentration was cultured on the medium of RPMI1640; the cells were then removed by EDTA/trypsin solution and resuspended in a medium containing 10^{-1} % bovine serum albumin and then plated on a 96-well microtiter plate [15] (All steps were done in triplicate). The MTT assay was used to examine the anticancer potential activity, and the results were read at 490 nm after 24 h. The cell viability was measured by the formula below [16]:

$$\text{Viability (\%)} = \text{Mean OD}_{\text{sample}} / \text{Mean OD}_{\text{blank}} \times 100$$

High-content screening assay

Phenotypic changes of the cells were determined by the High Content Screening (Thermo Fisher, USA) technique at concentrations of 6.25, 12.5, 25, and 50 µg/ml by measuring mitochondrial membrane potential, cytochrome c releasing, membrane permeability, viable cell count, and nuclear intensity. This technique uses fluorescent markers to determine morphological characteristics and molecular reactivity following chemical treatment [17]. The kit contained cytochrome c as a primary antibody, DyLight™ 649 conjugated goat anti-mouse IgG, Hoechst dyes, wash buffer (10× Dulbecco's phosphate buffered saline [PBS]), permeabilization buffer (10× Dulbecco's PBS with 1% Triton® X-100), and a blocking buffer (10×). The distribution and intensity of fluorescence within cells were imaged (n = 5) using an HCS system. The system was linked to a computerized imaging microscope equipped with a Zeiss, 40× (0.75 NA) Plan-Neofluar objective lens. Cells were treated with VT-1 for 24 h followed by the addition of mitochondrial membrane potential (MMP) and the cell permeability dyes and incubated for 30 min at 37℃. Cells were fixed and permeabilized using a standard procedure [18].

Caspase assay

Cells were seeded on 96-well plates at a density of 1×10^4 cells/well and cultivated for 24 h at 37℃, 5% CO_2. Following that, cells were treated for 24 h with verotoxin 1 (25 and 50 µg/ml). Cells treated with Dimethyl Sulfoxide (DMSO) (Thermo Fisher) were considered a negative control. Following incubation, the plate was allowed to equilibrate at room temperature, 100 µl Caspase-Glo® 3 and 9 (Promega, USA) reagents were added to each well, and the plate was shaken gently for 2 min. Subsequently, the plate was incubated at room temperature for 30 min and caspase 3 and 9 activity were determined by measuring the luminescence of each sample at 405 nm using an ELISA microplate reader. Tamoxifen (Dabur Pharma, India) was used as a positive control [19].

Statistical analysis

Data analysis was carried out by ANOVA and Dunnett's multiple comparisons tests. A p-value of < 0.05 was determined to be statistically significant. GraphPad Prism version 8.4.3 was used for all statistical analyses.

Results and Discussion

Cytotoxic activity of Verotoxin 1

The results of cytotoxicity of VT-1 on CaCo2 and NCM425 showed that there is a concentration-dependent cytotoxic effect on both cell lines (Fig. 1). The highest cytotoxic effect recorded was at a 50 µg/ml concentration. Fig. 1 indicates that VT-1 was more toxic to cancer cells (CaCo2) than normal colonic cells (NCM425) when

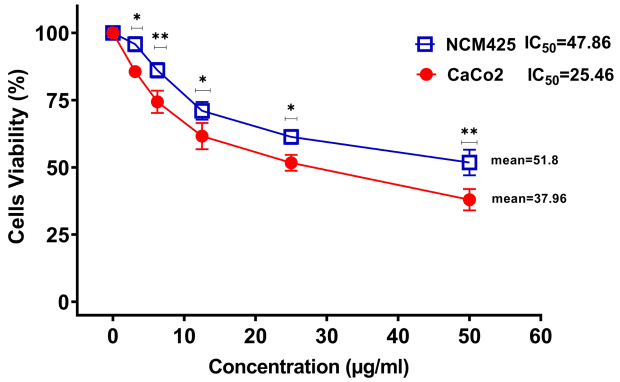

Fig. 1. The cytotoxic effect of verotoxin 1 on CaCo2 and NCM425 cell lines: Normal cell line (NCM425) of colon tissue (in blue) was more tolerant to verotoxin 1 cytotoxicity than CaCo2 (in red) which was more sensitive by approximately 1.8-folds measured by IC_{50} values. Results are presented as mean ± standard deviations, and all treatments were done in triplicate.

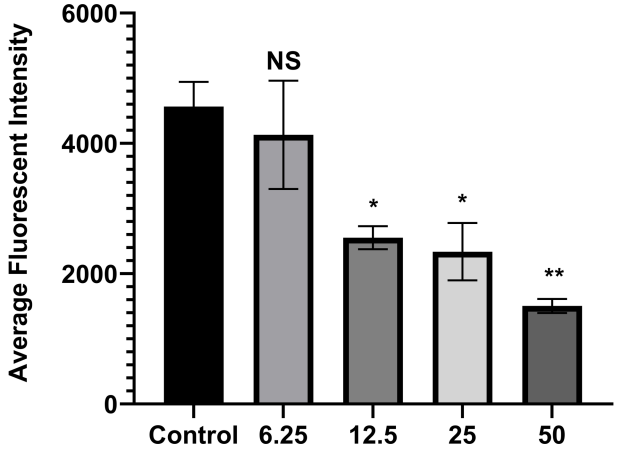

Fig. 2. Verotoxin 1 effect on the viable count of CaCo2 cell line: Viable count of CaCo2 was affected by verotoxin 1 in all tested concentrations (except at 6.25 µg/ml) as evaluated by ANOVA test followed by Dunnett's multiple comparisons tests; results are presented as mean ± standard deviations, and all treatments were done in triplicate.

the results of IC_{50} values were 25.46 and 47.86 µg/ml on CaCo2 and NCM425 respectively.

At all tested concentrations, the effect of VT-1 on CaCo2 and NCM425 was significantly different when estimated by statistical analysis. A similar study demonstrated that VT-1 had a cytotoxic impact on the CaCo2 cell line [20]. [21] also stated in their research that VT-1 induced death in CaCo2 among other mammalian cell types at different concentrations. Furthermore, CaCo2 cells were also killed when treated with the VT-1 B subunit [21].

The NCM425 cell line, on the other hand, was more resistant to VT-1 cytotoxicity. This could be due to Gb3 overexpression, which has been documented in various cancer forms and is mirrored in cultured cell lines from the same cancer type, while Gb3 has a low expression in normal human tissues [9]. Gb3 expression is substantially higher in cancers than in normal or benign colonic cells, according to three previous studies including a total of 141 patients [21–23].

Even though NCM425 was more resistant to VT-1 than CaCo2, the toxin remained cytotoxic. VT-1 cytotoxicity on mammalian cells was earlier documented when [24] indicated that VT can bond with normal colon tissue. Furthermore, VT-1 inhibited protein biosynthesis in adult and infant Human renal microvascular endothelium (HGMVECs) in a concentration-dependent way [25].

High-content screening results

The colonic cell line was subjected to the High Content Screening (HCS) test to better understand the cell-health indicators. HCS provides numerous parameters for measuring a substance's cytotoxicity at the single-cell level [26]. The indications investigated were viable cell count, cell membrane permeability, mitochondrial membrane potential (MMP) alterations, cytochrome c discharge via mitochondria, and nucleus morphological intensity.

The results of viable cell count revealed that VT-1 has a significant effect on the viable count of CaCo2 at all tested concentrations (except at 6.25 µg/ml) in comparison to the untreated cells (Fig. 2). The difference between such viability outcomes and MTT experiments is that in HSC, the findings reflect the influence of past drug exposure on the assessment of morphology and cellular abnormalities in cultures after only a few hours of exposure. In MTT, however, the examined cells were exposed to the toxin for an extended period [27].

A decrease in cell proliferation of glomerular microvascular endothelial cells from humans (HGMVECs) after exposure to VT-1 had been reported [25]. Another study conducted on T47D revealed a drop in viable cell count after treatment with VT extracted from different isolates of VTEC [28].

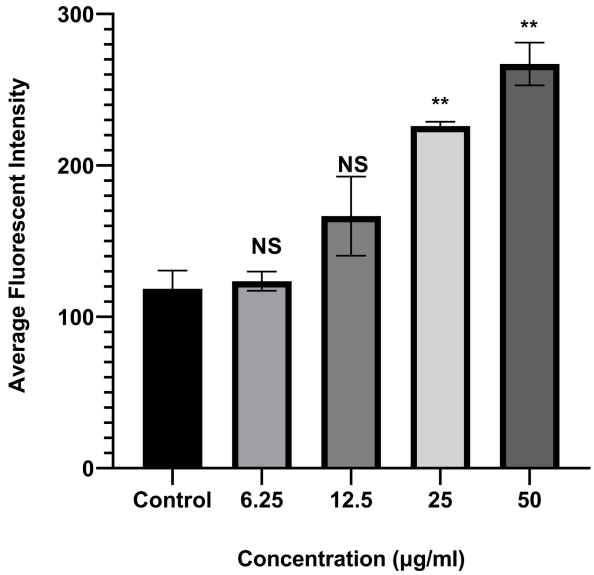

Fig. 3. Verotoxin 1 effect on cell membrane permeability of CaCo2 cell line: Effect of verotoxin 1 observed on cell membrane permeability at 25 and 50 µg/ml as evaluated by ANOVA test followed by Dunnett's multiple comparisons tests; results are presented as mean ± standard deviations, and all treatments were done in triplicate.

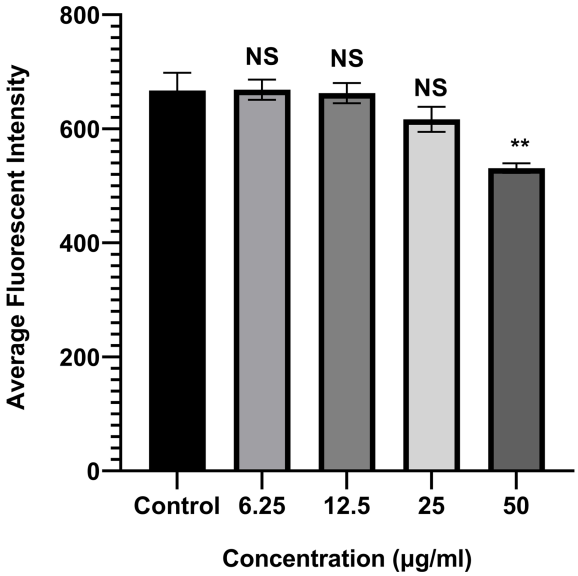

Fig. 4. Verotoxin 1 effect on the mitochondrial membrane permeability of CaCo2 cell line: No impact of verotoxin 1 was observed on mitochondrial membrane potential up to 50 µg/ml which suggests a poor impact that needs a greater dosage to enhance as evaluated by ANOVA test followed by Dunnett's multiple comparisons tests; results are presented as mean ± standard deviations, and all treatments were done in triplicate.

The effect of VT-1 on cell permeability was only at higher concentrations which were statically different from the untreated control group (Fig. 3). On the other hand, a lower concentration of VT-1 showed a weak impact on the cell permeability of colonic cancer cells. These results may indicate that the cell membrane of CaCo2 treated with VT-1 might be suffered from plasma membrane lesions [29] demonstrated that plasma membrane breaks occurred when HEp-2 cells were treated with VT-1 overnight as evidence of a necrotic effect.

The results of MMP and cytochrome c showed that VT-1 was only effective at the highest tested concentration (50 µg/ml) while it had an insignificant impact at all other concentrations (Figs. 4, 5, 7). In this regard [30], mentioned that mitochondrial membrane damage was detected at 3 to 4 hours as a decrease in membrane potential of mitochondrial and a rise in cytochrome c release after exposure of HeLa cells to VT.

In this study, an elevation in total nuclear intensity was also observed by a significant increase in overall nuclear intensity at 25 and 50 µg/ml, as shown in (Figs. 6 and 7), whereas no effect was observed in other treatments in contrast to the control (untreated) group.

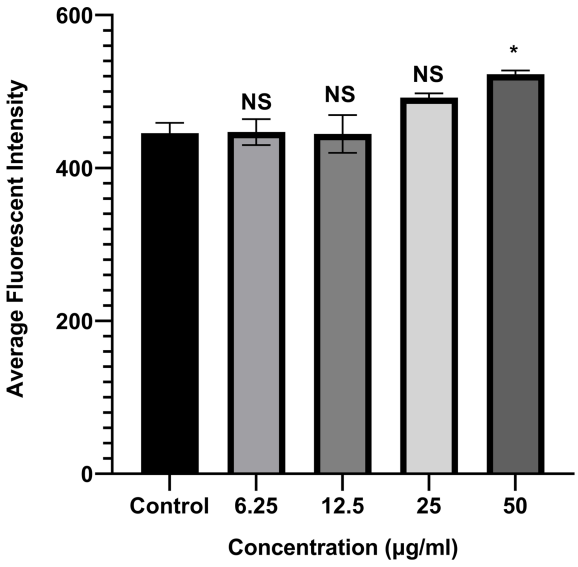

Fig. 5. Verotoxin 1 effect on the cytochrome c releasing of CaCo2 cell line: No impact of verotoxin 1 observed on cytochrome c release up to 50 µg/ml which suggests a weak impact that needs a bigger dosage to boost the effect as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean ± standard deviations, and all treatments were done in triplicate.

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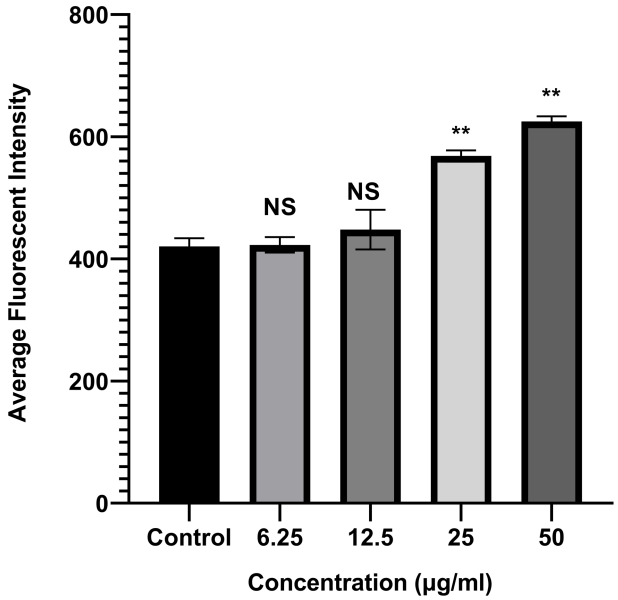

Fig. 6. Verotoxin 1 effect on the total nuclear intensity of CaCo2 cell line: An increase in total nuclear intensity was recorded at 25 and 50 µg/ml which indicates that cells suffered from DNA fragmentation and chromatin condensation. Data were evaluated by ANOVA test followed by Dunnett's multiple comparisons tests; results are presented as mean ± standard deviations, and all treatments were done in triplicate.

Nuclear intensity can increase as harmful substances can induce DNA breakdown and nuclear condensation which are shared features of both apoptosis and necrosis, indicating compromised cell health, whereas nuclear swelling is characteristic of cell-cycle blockers and DNA-damaging agents as carcinogens [31]. In this regard, VT-1 had been shown to cause nucleus fragmentation in most B lymphoblastoid cells following 6 h of treatment [32].

Effect of verotoxin 1 on the expression of caspase 3 and 9

The results of VT-1 impact on caspase expression of CaCo2 cell line indicated that there was a weak impact of VT-1 on both 3 and 9 caspases which was insignificant ($p > 0.05$) when compared to the untreated control group as illustrated in Figs. 8 and 9. This could be attributed to the weak impact of the toxin on mitochondrial membrane permeability and cytochrome c release. When the mitochondrial outer membrane becomes permeable, cytochrome c can be released, which triggers the activation of caspase to bring about the cell's destruction [33]. These results alongside HCS parameters suggest that cells might suffer a necrotic cell death after exposure to VT-1.

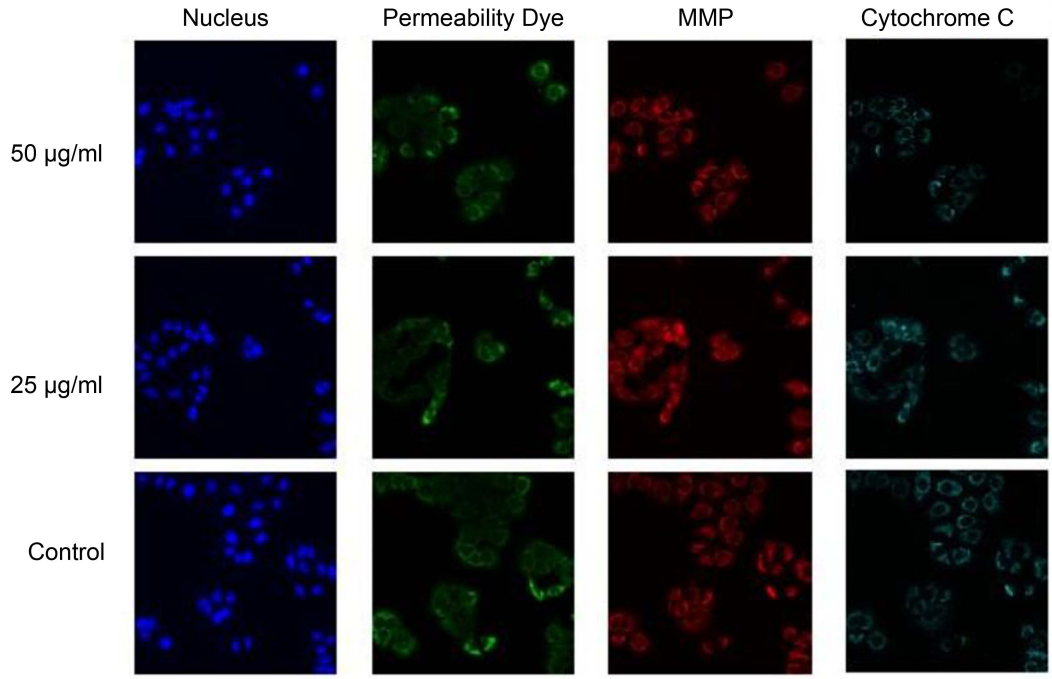

Fig. 7. High content screening and multiparameter cytotoxicity analysis of CaCo2 cell line after verotoxin 1 treatment; MMP: mitochondrial membrane potential.

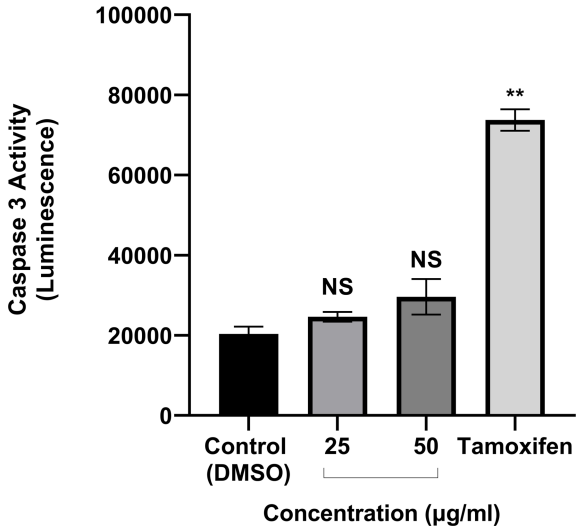

Fig. 8. Verotoxin 1 effect on the caspase-3 expression of Caco2: Activity of caspase 3 was mildly rose but there was a nonsignificant difference ($p < 0.05$) when compared to the negative control (DMSO: Dimethyl Sulfoxide) after treatment with verotoxin 1 as evaluated by ANOVA test followed by Dunnett's multiple comparisons tests; results are presented as mean ± standard deviations, and all treatments were done in triplicate. Tamoxifen was used as a positive control.

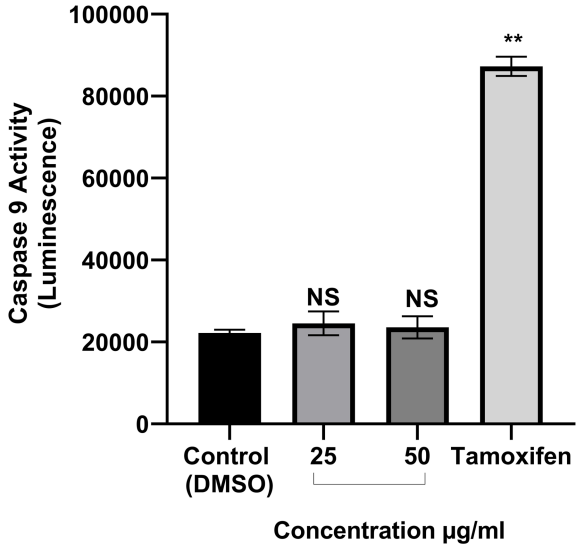

Fig. 9. Verotoxin 1 effect on the caspase-9 expression of Caco2: Activity of caspase 9 was moderately increased, although there was no significant difference ($p < 0.05$) when compared to the negative control (DMSO: Dimethyl Sulfoxide) after treatment with verotoxin 1 as evaluated by ANOVA test followed by Dunnett's multiple comparisons tests; results are presented as mean ± standard deviations, and all treatments were done in triplicate. Tamoxifen was used as a positive control.

Despite that many previous studies proposed that apoptosis is induced as a cell death mechanism after an encounter with VT-1 [34–38], necrotic cell death attributed to VT was reviewed in the study [11] when they declared that it was VT-1 caused necrosis in endothelial cells. They also stated that the cytotoxicity of VT-1 can be attributed to necrosis, apoptosis, or a mix of the two processes. In animal models, necrosis caused by VT has also been seen in brain endothelial cells [39]. Another study by [40] mentioned that VT induced necrotic death in endothelial cells of the kidney while [41] demonstrated a similar effect of VT on monocytic cells.

Furthermore, [42] stated that both necrosis and apoptosis could occur as a result of VT toxicity when some cells of the brain stem and ileum were collected from swine preferred necrosis over apoptosis, whereas others did not as confirm by the development of cytosolic debris and nuclear breaks in necrotic cells.

VT-1 possesses cytotoxic activity that can be employed in cancer treatment. VT-1 is more cytotoxic on cancer than on normal cells. The toxin induces necrosis in the CaCo2 cell line.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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