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Original Article

Cytotoxic activity Characterization of Scorpion Venom Components on Cancer Cell line

Caractérisation de l'activité Cytotoxique des Composants du Venin de Scorpion sur une lignée Cellulaire Cancéreuse

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ABSTRACT

Introduction: Scorpion venom is a rich source of bioactive peptides and toxins with therapeutic effects against different diseases including cancer. The present study was designed to elucidate the cytotoxic activity of isolated fraction F3 from *Androctonus australs hector* (Aah) scorpion venom and investigate the involved mechanism in colorectal tumor cell line HCT116. **Materials and Methods**: The MTT assay and clonogenic assay were performed to assess cytotoxicity and inhibition of cell growth respectively. Cell apoptosis induction was detected by Hoechst 33258 staining and caspase 3 activity. In addition, ROS (Reactive oxygen species) and RNS (reactive nitrogen species) generation were measured by fluorescent dye (DCFH-DA) and Griess assay. The obtained results revealed a remarkable and concentration-dependent cytotoxic effect of F3 fraction against HCT116 cells (Inhibitory concentration 50 = 13.46 µg/mL) which was in accordance with a significant decrease in colony surviving fractions (SF_{IC50}= 5.43 ± 2.12 %). **Results**: The morphologic alterations confirmed the cell growth inhibition.The Aah venom components displayed nuclear alterations characterized by chromatin condensation, membrane blebbing, cell shrinkage and compartmentalization of the dead cells into apoptotic bodies in accordance with the decrease of cell viability indicating the involvement of apoptosis in this cytotoxicity. Otherwise, the F3 fraction induces the expression of caspase 3, a key protease required for the execution of apoptosis. Enhancement in the intracellular levels of ROS and RNS were also observedin colorectal tumor cells and could be considered as a key early signal to F3 fraction-induced apoptosis. **Conclusion**: Hence F3 fraction could serve as potential therapeutic agents due to their apoptotic effects on cancer cells.

Keywords: Scorpion venom, colorectal tumor cell line, ROS, Cytotoxicity, Apoptosis.

RESUME

Introduction: Le venin de scorpion constitue une riche source de peptides et de toxines bioactives ayant des effets thérapeutiques contre différentes maladies, dont le cancer. La présente étude a été conçue pour étudier l'effet cytotoxique de la fraction F3 isolée du venin de scorpion d'*Androctonus australs hector* (Aah) et son mécanisme d'action au niveau des cellules de l'adénocarcinome colorectale HCT116. **Matériels et Méthodes:** Le test MTT et le test de clonogénécité ont été effectués pour évaluer respectivement la cytotoxicité et l'inhibition de la croissance

cellulaire. L'induction de l'apoptose cellulaire a été détectée par coloration Hoechst 33258 et l'activation de la caspase 3. De plus, la génération de ROS (espèces réactives de l'oxygène) et de RNS (espèces réactives du nitrogène) a été mesurée par des tests fluorescents et colorimétriques. **Résultats:** Les résultats obtenus ont révélé un effet cytotoxique de la fraction F3 sur les cellules HCT116 avec une concentration inhibitrice 50 =13.46 µg/mL associé à l'inhibition de la prolifération cellulaire (fraction de survie $_{IC50}$ = 5.43 ± 2.12 %). Des altérations nucléaires sont observées dans les cellules traitées, caractérisées par une condensation de la chromatine, un rétrécissement cellulaire et une compartimentation des cellules mortes en corps apoptotiques indiquant l'implication de l'apoptose dans cette cytotoxicité. Par ailleurs, la fraction F3 induit l'expression de la caspase 3, une protéase clé requise pour l'exécution de l'apoptose. Cette fraction est responsable d'une production excessive de ROS et de RNS au niveau des cellules tumorales colorectales et serait responsable du déclenchement de l'apoptose. **Conclusion:** Par conséquent, la fraction F3 semble être une ressource naturelle pour l'identification d'agents thérapeutiques nouveaux doués d'activités anticancéreuses.

Mots-clés: Venin de scorpion, Cancer colorectal, ROS, Cytotoxicité, Apoptose.

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1. Introduction

Cancer is the second disease leading to death in the world, and is responsible for an estimated 9.6 million deaths in 2018 [1]. In Algeria, nearly 42000 new cases are registered each year and the highest cause of cancer mortality is breast and colorectal cancers in women and lung and colorectal cancers in men (National plan cancer 2015-2019). Chemotherapy and radiation therapy are two main strategies currently used to treat the disease. However, the effectiveness of chemotherapy is frequently diminished due to the multidrug-resistance phenotype and the induction of severe adverse effects in the patients [2, 3]. Therefore, there is a growing interest in finding new more potent, selective, and better tolerated anticancer remedies [4, 5].

Arthropods venoms are a rich source of pharmacologically active molecules capable of interfering in human cellular physiology; the highlights are venomous arthropods, such as scorpions, bees, wasps, spiders, ants and frogs. The substances found in the venom of these animals present great potential as anti-tumor agents [4, 6]. Despite their dangerous stings that lead in severe cases to patient death, scorpions have become a valuable source of biologically active molecules. Scorpion venoms are highly complex mixtures of peptides, enzymes, mucoproteins, free amino acids, nucleotides, lipids, amines, heterocyclic components, inorganic salts and probably other unknown substances [7-10]. The venom compounds may interact with each other to modulate the function of ion channels, which is usually responsible for the known symptoms of envenoming. In addition to its use as a biological research tool, venom peptides have been identified as a potential agent for identification and cancer treatment [7, 8, 11,12].

Indeed, numerous studies demonstrated the capacity of scorpion venoms and their proteins/small peptides to induce cytotoxicity and cell tumor proliferationand metastasis inhibition in various type of cancer setting through different mechanisms [7, 8, 11, 13]. The venom of *Heterometrus bengalensis* and its protein bengaline induces apoptosis or the autophagy of leukemic cell lines [14-16]. The neopladin 1 and neopladin 2, purified peptides of the scorpion venom *Tityus discrepans* cause the activation of extrinsic pathway of apopototic cell death in SKBR3 breast cancer cells [17]. Chlorotoxin and chlorotoxin-like isolated from the venom of *Leiurus quinquestriatus* and of *Androctonus australis* respectively inhibit the invasion of glioblastoma cells after blockage of the chlorine channel by interaction with metalloprotease-2 [18, 19]. In addition, Mauriporine peptide and the neurotoxin Acra 3, isolated from *Androctonus mauritanecus* and *crassicauda* venoms species leads to necrosis of prostate and murine neuronal tumor cells [20, 21]. Furthermore, other peptides with antitumor activity associated with analgesic (BmK AGAP-SYPU2) or antimicrobial activity have also been isolated (AamAP-1 and AamAP-2, TsAP-1 and TsAP-2) [22-24].

Our study focused on the search of anticarcinogenic therapies from natural sources. Along the same lines as our previous published work where we have demonstrated the effectiveness of F3 fraction isolated from Aah venom on the human adenocarcinoma tumor cell line NCI-H358 [25]. The cytotoxic and cell inhibition proliferation effect of F3 fraction were investigated on one of the most killing cancers in Algeria. Additionally, we explored the mechanism involved in cell death when the colorectal tumor cells were challenged with venom components.

2. Materials and methods

2.1. Chemicals

Thiazolyl blue tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), N-acetylcysteine (NAC),2, 7- dichlorodihydrofluorescein diacetate (DCFDA-H2) and Griess reagent were purchased from Sigma Aldrich. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. Dulbecco's phosphate-buffered saline (PBS 10x) and penicillin/streptomycin (10 000 U/mL) were purchased from Life Technologies. Laemmli sample buffer and nitrocellulose membrane (0.2 µm) were purchased from Biorad Laboratories. Cisplatin and oxaliplatine were provided by Mylan (France). Saturosporine was purchased from Santa Cruz Biotechnologies.

2.2. Scorpion venom and its toxic fractions

F3 fraction isolated from Aah venom by gel filtration through Sephadex G50 column as previously described was provided from Laboratory of Cellular and Molecular Biology, Faculty of Biological Sciences of USTHB [26].

2.3. Cell culture

The human colorectal carcinoma cells (HCT116) wasacquired from American Type Culture Collection (ATCC, Manassas, VA). HCT116 cells were cultured in DMEM medium completed with 10% FBS and 1% penicillin/streptomycin. These cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2. Experiments were performed on cells within 20 passages.

2.4. Inhibition of Cell Viability Assay

The cytotoxicity of F3 fraction on exponentially growing tumor and normal cells were determined using MTT assay as described previously. HCT 116 cells were seeded at 10 000 cells per well in Cellstar 96-well plates and incubated for 24 h. Thereafter, cells were exposed to F3 fraction at different concentration (5–40 µg/mL) in media for 24 h. The medium was removed and replaced with MTT solution (0.5 mg/mL) in media and incubated for an additional one hour. Subsequently, the medium was aspirated, and the purple formazan crystals were dissolved in DMSO. The absorbance due to the dissolved purple formazan was then obtained at 550 nm [27]. Cell viability was expressed as a percent of the control culture value and the IC_{50} was calculated from nonlinear regression using the program GraphPad Prism 5.0 software (GraphPad Software, USA).

2.5. Morphological assessment

Cells were seeded into 6-well plates at the density of 500 000 cells/well and allowed to adhere overnight. After 24 h of treatment with the F3 fraction (IC75, IC50 and IC25), morphological characteristics were observed and the images were captured under an inverted phase contrast microscope (HundWetzlar, Germany) at x20 [25].

2.6. Clonogenic assay

Tumor cells were seeded in 12-well plates (500 cells/well) before treatment with the different concentrations of the F3 fraction. The plates were incubated under standard condition for 8-10 days and stained with crystal violet (0.5% w/v). The Plating Efficiency (PE) represents (Number of formed colony/Number of plated cells) x100. The Surviving Fractions (SF) were calculated as following: (PE of treated cells/PE of control cells) x100 [28].

2.7. Apoptosis assessment

2.7.1. Fluorescence microscopic analysis of cell death

The detection of apoptotic cells including nuclear chromatin condensation and apoptotic bodies was performed with Hoechst 33258 staining [29]. Cells were incubated for 24h with the following concentrations of F3 fraction (IC75= 7.35 µg/mL,

IC50= 13.46 µg/mL and IC25= 25.20 µg/mL) and were pretreated or not with N acetylcystein (NAC). Thereafter, cells were washed with PBS, fixed and stained with Hoechst 33258 (10 µg/mL) for 20 min in obscurity. Nuclear changes were observed under fluorescence microscopy x40 (Zeiss Axioplan, Germany) [25].

2.7.2. Western blot analysis

Western blot analysis was performed as previously described [30]. HCT116 cells were lysed in hot Laemmli buffer and denatured. Protein extracts were loaded on a 15% (50 µg of protein) SDS PAGE gel. Western blotting was performed using primary antibodies raised against caspase 3 (sc-7272) and β-Actin (ab75186). Immunoblotting were analyzed using the appropriate peroxidase-coupled secondary antibodies (GE Healthcare Life Sciences). All blots were revealed by chemiluminescence (ECL, Bio-Rad) and the proteins bands were visualized via enhanced chemiluminescence imaging (PXi, Syngene).

2.8. Intracellular reactive oxygen species (ROS) measurement

HCT116 tumor cells were seeded in black 96-well plates and incubated for 24 h. Cells pretreated or not with the NAC were exposed to different concentrations of F3 fraction for 4h and 24h, Thereafter, media were discarded, and 10 µM DCFH-DA was added [31]. The DCF fluorescence was measured with Victor X2 Multilabel Plate Reader (Perkin Elmer, USA).

2.9. Reactive nitrogen species (RNS) assay

Nitrite production was investigated in the supernatants of cultured cells using NO assay [32]. Media of treated cells with F3 fraction for 24h were collected and centrifuged at 500x *g* for 5 min at 4°. Nitrites were evaluated in supernatants with Griess reagent. The absorbance was read at 540 nm and the nitrite levels were determined after extrapolation to standard $NaNO_2$ and expressed in µM.

2.10. Statistical analysis

All the data presented are mean ± SD. Data analysis were carried out with one-way ANOVA with post hoc analysis by Tukey post hoc using GraphPad Prism version 5.01 for Windows (GraphPad SoftwareInc, San Diego, CA, USA). Differences were considered statistically significant versus respective controls when $p< 0.05$.

3. Results

3.1. F3 fraction induced cytotoxicity and morphological changes

The impact of F3 fraction on colorectal tumor cell viability and proliferation was assessed by the MTT reduction assay. The MTT assay is considered as an indicator of mitochondrial activity and has been usually applied for determination of cell cytotoxicity. F3 fraction was found to reduce cell viability of tumor cells in a potent and dose-dependent manner **(Figure 1a)**. The survival rate of HCT116 after exposure to 5, 10, 20, 30 and 40 µg/mL of F3 fraction was 83.40, 60.89, 36.07, 23.43 and 14.37 percent respectively. Indeed, F3 fraction displayed potent activity with the half inhibitory concentration (IC50) value of 13.46 µg/mL against colorectal cancer cells (HCT116).

The morphological changes in HCT116 cells after treatment for 24h with increasing concentrations of F3 fraction (IC75= 7.35 µg/mL, IC50= 13.46 µg/mL and IC25= 25.20 µg/mL) are represented in **Figure 1b**. Untreated cells showed an elongated shape while treated cells showed cellular shrinkage, round or irregular shape and condensed cytoplasm. Indeed, the highest concentrations F3 fraction IC25 led to detached and round-shaped dead cells in the transformed cell line.

3.2. Effect of F3 fraction on tumor cells proliferation

The effectiveness of cytotoxic fraction F3 (IC75, IC50 and IC25) was tested over a longer treatment period with the clonogenic assay in the two transformed cell lines. A significant reduction in colony formation was observed in treated cells with IC75 and IC50 doses and a complete inhibition of cell proliferation with IC25 concentration for HCT116 in comparison to untreated cells **(Figure 2a)**. In addition, a significant decrease in surviving fraction percentages was noted in treated tumor cells ($p < 0.001$) **(Figure 2b)**.

3.3. Effect of F3 fraction on morphological apoptotic changes of tumor cells

To determine whether the cytotoxicity and growth inhibitory activity of Aah fraction 3 was related to the

induction of apoptosis, morphological assay of cell nucleus was conducted **(Figure 3)**.

In untreated HCT116 control cells was observed a uniformly blue fluorescence stain of nucleus. After incubation with different concentrations of F3 fraction, both tumor cells showed nuclear changes associated with apoptosis such as chromatin condensation, nuclear fragmentation, and apoptotic bodies.

3.4. Effect of F3 fraction on proapoptotic caspase 3 activation

Caspases (cysteine-aspartic proteases) are central components of the intrinsic apoptotic pathway that lead to apoptotic cell death. Western blot analysis of HCT116 cells extracts revealed caspase 3 cleavage products, detected after 24h of F3 fraction treatment **(Figure 4a)**. This product is strongly increased with IC25 as observed in immunoblotting and densitometry analysis compared to proapoptotic molecule staurosporine used as positive control **(Figure 4a and 4b)**. This result indicates the activation of caspase 3 in HCT116.

3.5. Evaluation of oxidative stress in tumor cells treated with F3 fraction

The determination of DCF fluorescence as a measure of intracellular ROS generation in colorectal cells was conducted by using H2DCFDA probe. As shown in **Figure 5**, a remarkable increase in ROS formation was observed on F3 fraction treatment for 4 and 24h in colorectal cancer cell line. It is evident from the histograms that highly significant ROS production was registered with the concentration IC25 at all-timetreatments ($p < 0.001$). F3 fraction enhanced the ROS levels approximately 1 and 2.5 folds for HCT116 cells at both 4 h and 24 h post-treatmentwith respect to control cells.

Interestingly, addition of NAC, a ROS scavenger, one hour prior treatment with venom fraction F3, significantly reduced the above-mentioned venom fraction-induced effects in HCT116 tumor cells, suggesting that F3 fraction probably modulated its cytotoxic action by enhancing cellular ROS.

3.6. Determination of nitrite oxide concentration in culture media

The concentration of nitrite oxide released from treated and control tumor cells were measured by analyzing nitrite level in their culture media. F3 fraction induce enhancement in nitric oxide level in HCT116 cells in a dose–response effect **(Figure 6)**. In HCT116 cells, nitrite level in control was 2.14 µM whereas in treated cells with the highest concentrations of F3 fraction was 4.75 µM for IC50 and 8.31 µM for IC25. In this cell line, increment ofNO at dose of IC75 µg/ml of venom fraction was insignificant as compared to control.

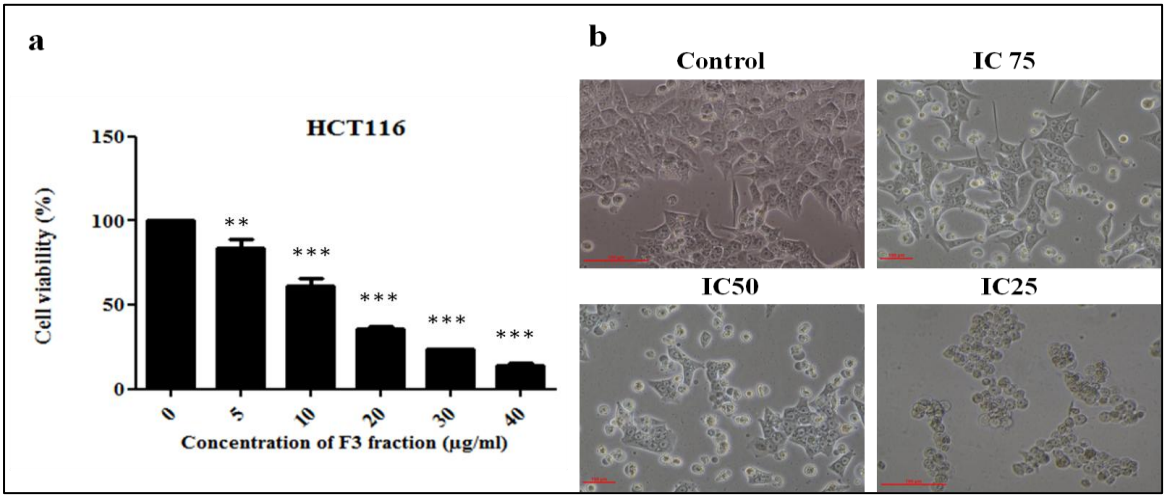

Figure 1: Cell survival rate decrease of HCT116 cells after 24 h of treatment with different concentrations of F3 fraction (5-40 µg/ml), according to MTT reduction assay (a). The data are expressed as the mean ± SD of three independent experiments in triplicate. Significances are shown in comparison to control cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Different concentration of F3 fraction (IC75, IC50 and IC25) induced morphological changes in human colorectal carcinoma cells (b). Cells were photographed under phase-contrast inverted microscopy (x20)

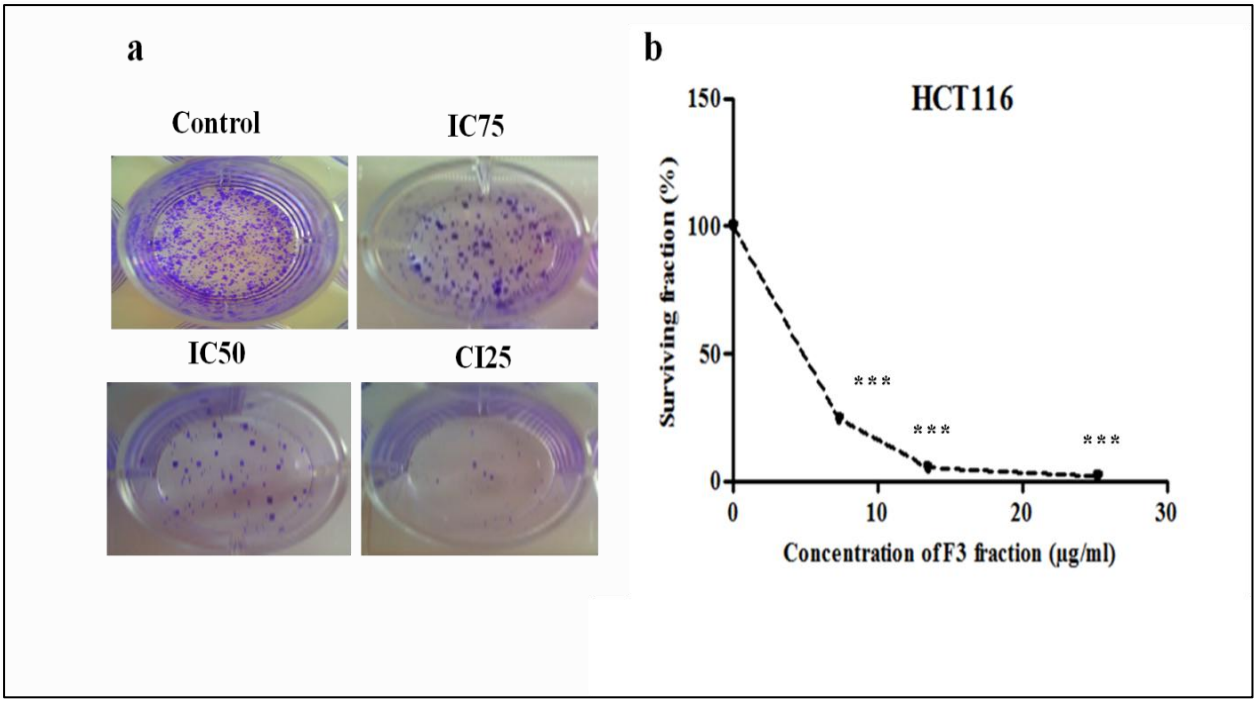

Figure 2: Decrease of colony number (a) and inhibition of surviving fraction (b) after colorectal tumor cells exposure to IC75, IC50 and IC25 concentrations of F3 fraction. The data are expressed as the mean ± SD of three independent experiments in triplicate. Significances are shown in comparison to control cells (***$p < 0.001$)

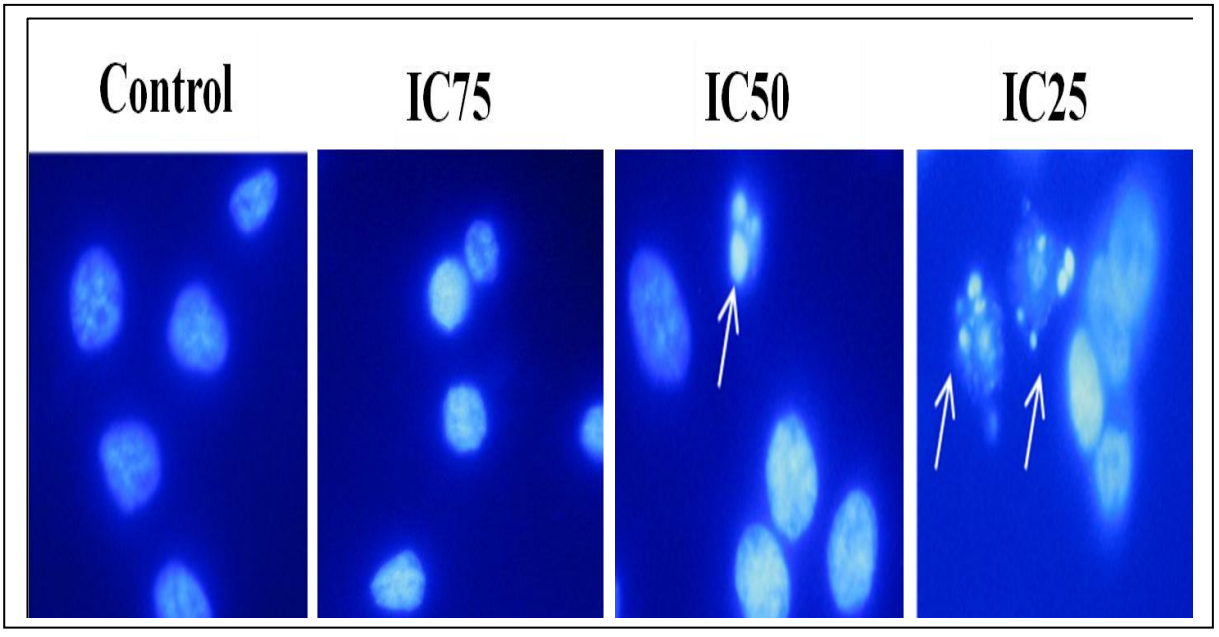

Figure 3: Cells were stained with Hoechst 33258 and visualized by fluorescence microscopy (x40). Control cells appeared to be intact with normal bleu nuclei. While, cells treated with increasing concentrations of F3 fraction exhibited typical features of apoptosis such as chromatin condensation and formation of apoptotic bodies in HCT116 colorectal cell line

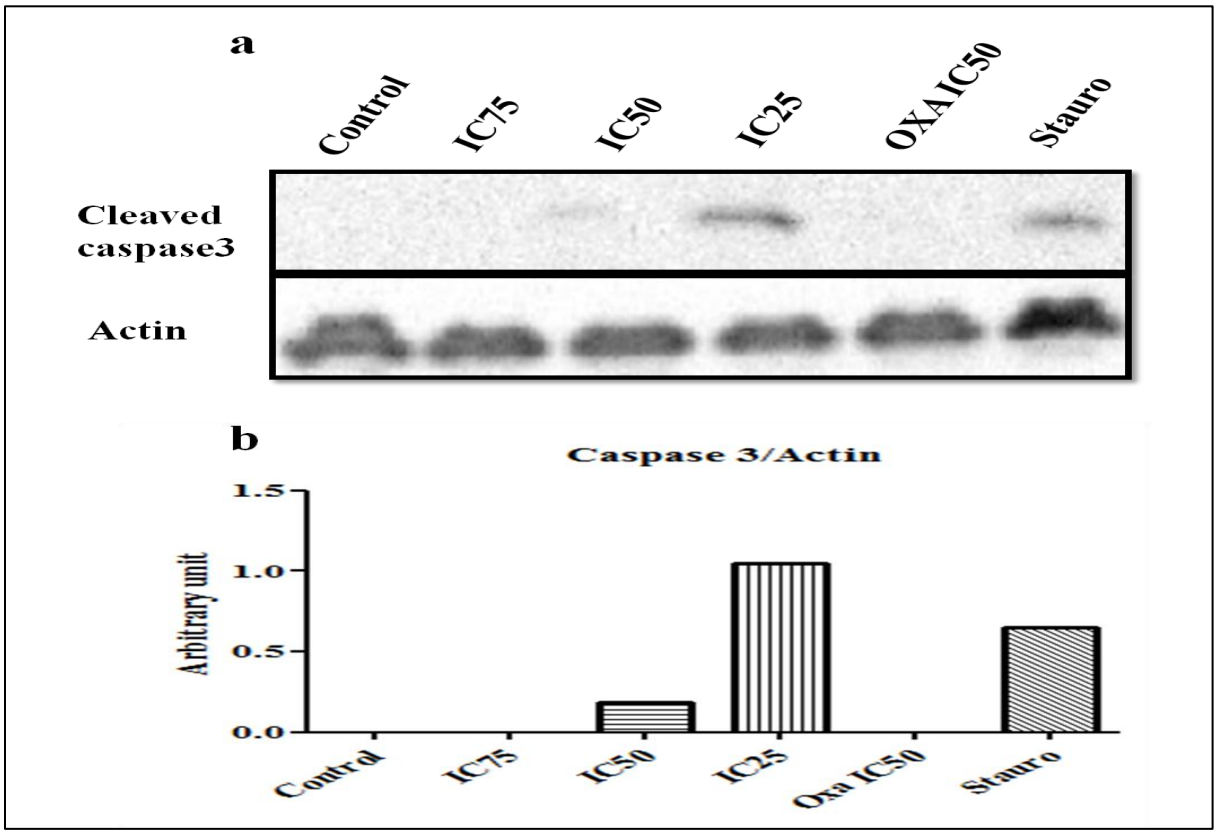

Figure 4: Protein expression of cleaved caspase 3 in HCT116 cells were analyzed by Western blot (a). Evidence that increasing concentration of F3 fraction and apoptotic molecule staurosporine triggered caspase 3 expression unlike chemotherapeutic agent oxaliplatin. The densitometry analysis of protein bands (actin as loading control) on immunoblotting profile reveals a dose dependent expression of caspase 3 in HCT116 (b)

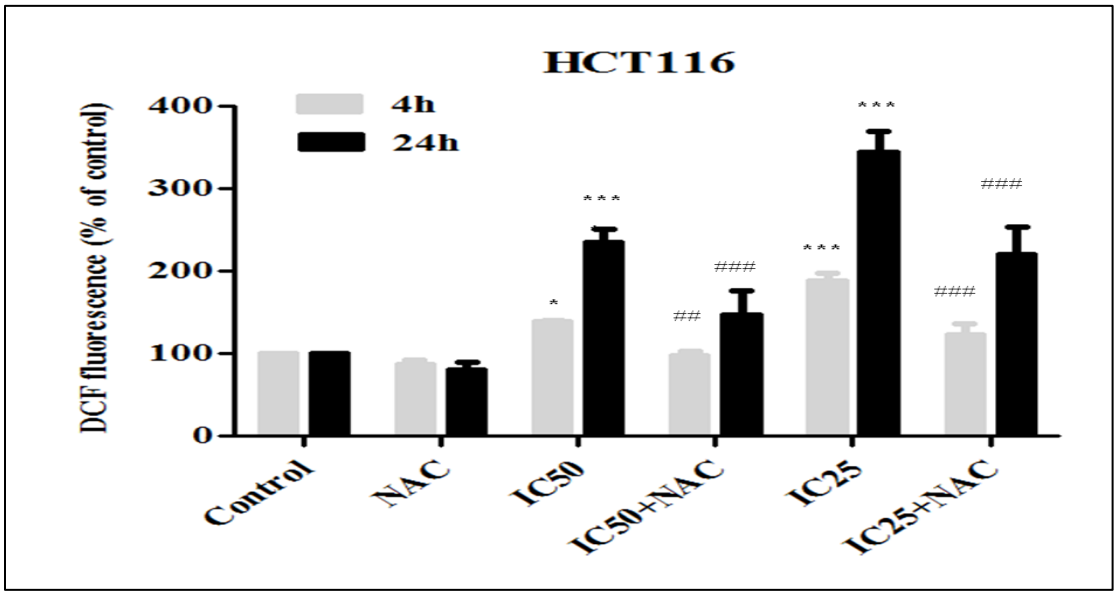

Figure 5: Quantitative analyses of the reactive oxygen species (ROS) generation after scorpion venoms treatment on HCT116 cancer cell lines. A significant increase in ROS formation was observed in colorectal cancer cell line (*p < 0.05; ***p < 0.001; vs. Control) and statistical differences in NAC pretrated cells compared to F3 fraction groups (##p < 0.01; ***p < 0.001)

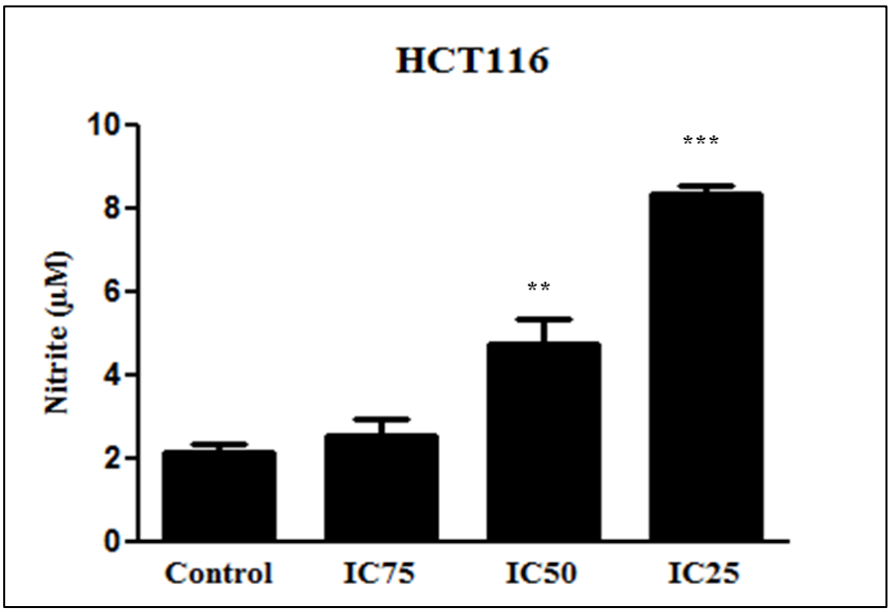

Figure 6: Nitric oxide production was determined by nitrite measurement in the cells supernatant. Increased level of NO in a concentration-dependent manner was noted in HCT116 cells media. The results were expressed as the mean ± SD of three independent experiments carried out in triplicate. The indicated significances are shown in comparison to control (**p < 0.01; ***p < 0.001)

4. Discussion

Scorpion venom contains numerous compounds, which may be adapted for cancer therapy. The major components of venom are neurotoxins peptides and non disulphide bridged peptides (NDBPs) which act on ionic channel blocking, disruption of the cell membrane integrity and damage to internal cell organelles. These properties make good prospects for studies on drugs and adjuvants in cancer treatment [12, 33]. This study is an attempt to elucidate the mechanism of cytotoxicity induced by peptidic fraction isolated from North African scorpion Aah on human colorectal carcinoma cells.

The induction of cytotoxic effect is a key mechanism capable of inhibiting the uncontrolled cell growth that is a hallmark of cancer development [34]. In order to establish an appropriate dose of F3 fraction, the cytotoxicity was measured and IC50 values were determined colorectal cancer cell lines. The current study demonstrated that F3 fraction significantly reduced HCT116 cells viability in dose dependent manner with an IC50 = 13.46 µg/mL. F3 Aah fraction seems more potent on colorectal carcinoma cell line than non-small cancer cell lung (IC50= 27.05 µg/mL) without significantly affecting human normal lung fibroblast (MRC5) [25]. The variation in response to F3 Aah fraction on growth of the two cancer types after 24 h exposure might be a result of the complex composition of the venom and/or the targets expressed on cancer cells [25].

The cytotoxicity was confirmed via the morphological changes observed under inverted microscope. Several studies revealed the selectivity of different species of scorpion venom and their peptides in tumor cells as Cuban *Rhopalurus junceus* towards Hela and A549 cells and Mauriporine from *Androctonus mauritanicus* against prostate tumor cells (PC3) [20, 35]. Also, *Androctonus australis* venom possess the ability to reduce the volume of Ehrlich ascites carcinoma

bearing mice and to inhibit proliferation of early stage hepatocarcinoma induced *in vivo* by Fumonisin B1 mycotoxin through its non-toxic fraction 1 (F1) [13, 36].

The obtained results revealed the proliferation cell inhibition of HCT116 after treatment with Aah F3 fraction in dose dependent manner as demonstrated with reduction of cell surviving fractions with

prominant effect against colorectal tumor cells. A similar pattern of inhibition in cell motility and colony formation was observed with Egyptian scorpion venom *Androctonus australis* confirming that the action of venom was not restricted to one type of cell line, but may display a wide range of anticancer properties, as evidenced by the phenotypic changes observed in the cell lines analyzed in the present study [36, 37]. Therefore, based on these findings and published works, it appeared that F3 fraction possesses selective cytotoxic property towards colorectal and lung cancer cell lines without affecting non tumorigenic cells [25].

Isaad et al. (2018) previously reported major modifications related to actin dynamics and their involvement in Aah venom-mediated cytotoxicity in human epithelial lung tumor cells (A549) [38]. On another hand, the actin cytoskeleton rearrangement needed several proteins of the cytoskeleton such as actin and keratins to mediate the morphological modifications of the apoptotic cells and the regulation of apoptotic signaling [39].

Apoptosis is a well-regulated and porgrammed death process involved in physiological and pathological conditions. Defective apoptosis has been recognized as a fundamental factor in the development and progression of cancer. Restore of appropriately induce apoptosis may establish antitumor therapy based on triggering selective death of cancer cells [40].

The results of scorpion F3 fraction-induced apoptosis in colorectal tumor cells were morphologically observed through fluorescent staining Hoechst 33258. Nuclear damage and margination were clearly seen in treated HCT116 cells in comparison with the control cells. Severe apoptotic bodies could be clearly visualized from the photographs with high fraction concentration (IC25). This mode of cell death was reported to be induced by different scorpion venoms and their components in several cancer types [7]. Using the comet assay and FACS, Das Gupta et al. (2010) have shown that the venom of *Heterometrus bengalensis Koch* and its protein, Bengaline can lead to apoptosis by DNA fragmentation in U937 and K562 cell lines [14, 16]. Similarly, venoms of three species of *Androctonus crassicauda*, *Leiurus quinquestriatus* and *Odontobuthus bidentatus* are able to induce DNA breakage and apoptosis in the ileocecal adenocarcinoma (HCT-8), breast mammary glands (MDA-MB-231) and hepatic carcinoma (HepG2) cell lines respectively [34, 41].

In fact, this feature was stated previously for the isolated proteins from *Tityus discrepans* Neopladine 1 and 2 which induce extrinsic apoptosis as FasL and BcL-2 are expressed and the effect was found proportional to time when tested on SKBR3 cells [17, 42]. In the same way, Bengalin protein selectively induces apoptosis on U937 leukemic cells through mitochondrial pathway and inhibition of heat shock proteins (HSP 70 and 90) [14].

Caspases are a family of protease enzymes playing essential roles in programmed cell death including apoptosis [43]. Our results showed that caspase 3 was dose-dependent overexpressed, suggesting the activation of this catalytic enzyme and DNA fragmentation. Dezianian et al. (2020) showed in recent study that the *Hottentotta schach* scorpion venom extracts could inhibit the growth of MCF-7 cells by inducing the apoptosis through caspase 3 activation [44]. Furthermore, other studies demonstrated that the activity of this protease enzyme expression were upregulated after *Odontobuthus doriae* or *Androctonus crassicauda* scorpion venom breast tumor cells treatment. They also conclude that scorpion venoms activate the expression of caspase 3 enzyme and the inhibition of DNA synthesis to cause selective cell apoptosis in MCF7 cells [44, 45]. Caspase 3 and P21 activities were also increased in Human Kyse-510 cell line [46].

Generation of RONS (Reactive oxygen nitrogen species) within living cells is due to the mitochondrial electron transport system, NADH oxidase, cytochrome P450, xanthine oxidoreductase (XOR) and nitric oxide synthase (NOS) which all play a role in various crucial developmental processes, such as cell differentiation and cell signaling pathways. Recently, RONS have gained enormous attention due to their widespread application in the diverse fields of biology and medicine including cancer treatment [47, 48].

Treatment of cancer cells with venom creates a stressful environment. Once treated with scorpion venoms, cells undergo further elevation of cytotoxicity. These stressful events in the tumor cells results in triggering of the generation of free radicals

and hence reactive oxygen species formation begins [34, 41]. In the present study, a significant extent of ROS and nitrite production under the influence of F3 fraction were detected in HCT116 tumor cells when stained with the specific dye for ROS and Griess assay for RNS (nitrite). Our results are in agreement with the *in vivo* studies establishing that Aah venom as ROS (in term of H_2O_2) and NO inducer in lung tissues of the envenomed mice [49, 50]. In addition, ROS is also known to promote DNA mutations, lipid peroxidation, and protein oxidation [40]. Alikhani et al. (2020) reported the involvment of RONS in DNA damage induction in HepG2 hepatic tumor cells after incubation with *Odontobuthus bidentatus* leading to apoptotic cell death [41].

Conclusion

In summary, this study demonstratedthat F3 fraction Aah venom has cytotoxic effects, inducing apoptosis and inhibiting the colorectal tumor cells growth by inducing RONS generation and oxidative stress. Thus, further studies are needed to elucidate its mechanism of action and develop new apoptosis-inducing compounds from natural origin.

Conflicts of interest

The authors declare that they have no conflicts of interest

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