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Montivipera bornmuelleri Venom: Inhibitory Effect on *Staphylococcus epidermidis* and *Escherichia coli* F_1F_0-ATPases and Cytotoxicity on HCT116 Cancer Cell Lines

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Abstract: In this work, we pursued the biological characterization of the venom of *Montivipera bornmuelleri*, a viper from the Lebanese mountains. In relation to its antibacterial potential, the inhibitory effect of this venom on the F_1F_0-ATPase enzymes of Gram-positive *Staphylococcus epidermidis* and Gram-negative *Escherichia coli* bacteria was examined. In order to determine the degree of cytotoxicity of the venom on the HCT116 human colon cancer cell lines, the biological MTT proliferation and cell viability test were implemented. After validation of the enzymatic F_1F_0-ATPase model by the spectrophotometric method, using quercetin as the reference ligand, results revealed that *M. bornmuelleri* venom is able to inhibit the activity of the enzyme of these two bacteria with a concentration of the order of 100–150 µg/mL. In addition, a venom concentration of 10 µg/mL was sufficient to kill the totality of HCT116 cell lines cultivated in vitro. These data show that *M. bornmuelleri* venom is a mixture of diverse molecules presenting activities of interest, and is a potential source to explore in order to discover new drug candidates.

Keywords: *Montivipera bornmuelleri* snake venom; F_1F_0-ATPase; *Staphylococcus epidermidis*; *Escherichia coli*; antibacterial activity; HCT116 cells; anticancer activity

1. Introduction

Montivipera bornmuelleri is a rather small, venomous snake (maximum length of 75 cm) belonging to the viperidae family [1,2]. It is endemic to the Mount Lebanon region, found only at high altitudes (above 1800 m) and listed as endangered by the IUCN due to its limited geographical range [2]. The venom of *M. bornmuelleri* is a mixture of different compounds, with the major being Phospholipase A2 (PLA2), serine-protease, metalloproteinase III [1] and L-amino acid oxidase (LAAO) [3] and certainly other various molecules unexplored to date. This venom, as well as its components, displays a wide range of biological activities. In fact, crude venom of *M. bornmuelleri* possesses antibacterial activity against Gram-positive and Gram-negative bacteria [4], plays an anticancer effect against HaCaT cells [5], induces upregulation of pro-inflammatory cytokines [6], has pro- and anti-coagulant activities, indirect hemolytic activity [7] and a relaxant effect on vascular contractility [8]. As for its characterized components, PLA2 displays antibacterial,

hemolytic, anti-coagulant and pro-inflammatory activities [9] and the purified LAAO from *M. bornmuelleri* venom shows also an anti-bacterial activity [3].

Since the discovery of penicillin in 1928 by Alexander Fleming [10], many bacteria have developed resistance to one or more classes of antibiotics, rendering the treatment of bacterial infections increasingly difficult [11]. In fact, 25,000 patients die annually in the EU due to infection by multidrug-resistant (MDR) bacteria, indicating that mortality rates are very high [10]. Therefore, the need for novel antibacterial agents has emerged, especially those based on new bacterial biological processes [11].

Staphylococcus epidermidis, a Gram-positive staphylococcus found on the human skin and mucosal surfaces [12], capable of forming biofilms and causing nosocomial infections [13] and *Escherichia coli*, a Gram-negative bacillus, a part of the mammalian gut microbiome, but also possessing pathological strains [14], both developed a resistance to antibiotics [13,15] and both possess an F_1F_0-ATPase [16,17]. This membrane-associated enzyme found in mitochondria, bacteria and thylakoids [18] consists of a membrane-bound F_0 sector, a proton channel, and a soluble F_1 sector. The soluble portion is responsible for both the hydrolysis and formation of ATP. Both activities are achieved by a rotary mechanism of the F_0 and F_1 sectors [19]. Its important role in cell life and death makes it an interesting target for new antimicrobial drugs [20].

Cancer has a high incidence and mortality, and both are increasing rapidly worldwide. It is important to note that cancer has a very high and negative impact on public health, as it is considered the second cause of death (21%) among diseases that are not transmissible and not caused by infectious agents. Among the numerous human cancers, colon cancer is a major cause of death (9.2% mortality rate, after lung cancer) and has a very high incidence (10.2%) in both males and females, preceded by lung and female breast cancer [21,22]. Some of the current therapies used to treat this type of cancer include radical surgery, considered to be the most effective method of treatment, and adjuvant chemotherapy (Palliative chemotherapy, anti-EGFR therapy and anti-angiogenic therapy) [23]. However, it is known that treatments such as chemotherapy have significant side effects and influence the quality of life of patients. In fact, many patients express heavy concerns towards the known side effects of chemotherapy, such as loss of hair (50% of patients), constant fatigue (42% of patients) and disturbance of work duties (39% of patients) [24]. Some chemotherapeutic agents may even cause cardiotoxicity and other cardiac side effects [25]. As these types of treatments continue to prove unsatisfactory and even dangerous, it is important to continue screening for new anticancer medication and improved therapies to treat colon cancer. Since then, the search for a natural extract that has a cytotoxic effect on the human colon cancer cell lines will undoubtedly contribute to the discovery of new molecules which could possibly be explored as drug candidates for this type of cancer.

This work aims to assess *M. bornmuelleri* venom's ability to inhibit two bacterial F_1F_0-ATPases, those of *S. epidermidis* and *E. coli*, and its cytotoxic effect on the HCT116 colorectal cancer cell lines.

2. Materials and Methods

2.1. Reagents and Chemicals

Extraction of the venom from *Montivipera bornmuelleri* snake was carried out by Dr. Riyad Sadek (American University of Beirut). The venom sample was stored at −20 °C until use.

Adenosine 5′-triphosphate disodium salt hydrate ATP (99%), ammonium molybdate tetrahydrate (99%), dimethyl sulfoxide DMSO (99%), ethylenediaminetetraacetic acid disodium salt dehydrate EDTA (99%), potassium phosphate monobasic KH_2PO_4, Trizma base (99%) and quercetin (95%) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). L-Ascorbic acid (99%) was provided by HiMedia (Mumbai, India) and Glycerol was obtained from Loba Chemie (Mumbai, India). Magnesium chloride $MgCl_2$ was supplied by Acros organics (Trenton, NJ, USA). Hydrochloric acid HCl and sulfuric acid H_2SO_4 (96.3%) were provided by VWR chemicals.

2.2. Bacterial Cultures

Staphylococcus epdiermidis (ATCC® 14990™) and *Escherichia coli* (ATCC® 25922™) were obtained from the American type culture collection ATCC (Manassas, VA, USA). Both bacterial strains are nonpathogenic and thus classified as biosafety level 1 (BSL-1). Nutrient broth and agar were purchased from CONDA (Madrid, Spain).

S. epidermidis: 50 µL from frozen stock tubes are inoculated on nutrient agar (NA) plates. In order to prepare the pre-cultures, 8–10 colonies of the bacteria from NA culture plates are inoculated in sterile tubes containing 10 mL TSB. Both plates and tubes are incubated for 48 h with partial aeration at standard conditions and 37 °C, and finally stored at 5 °C until use.

E. coli: 2–3 colonies of the bacteria from NA culture plates are inoculated in 5 mL of nutrient medium (TSB) and incubated at 37 °C for 18 h, with slightly open caps. In order to determine the number of bacteria, a UV-VIS spectrophotometer is used to measure the optical density (OD) of the obtained pre-cultures at 620 nm. This value corresponds to the number of bacteria $\times 10^9$ (CFU/mL).

2.3. Preparation of the Bacterial Sample

In order to study the membrane-bound F_1F_0-ATPase, it must first be isolated from bacterial cells. The protocol used followed the method by Issa et al. [26] with some modifications. A volume of 500 µL of *S. epidermidis* bacterial suspension was centrifuged at 10,000× *g* and 4 °C for 10 min. Supernatant was removed and the pellet is resuspended in 500 µL of Tris-HCL (pH 8.5) then sonicated on ice 6 times for 30 s each, and 10 s of rest between each cycle. The suspension was again centrifuged at 10,000× *g* and 4 °C for 10 min and the supernatant removed. The obtained pellet contains the F_1F_0-ATPase enzyme.

Similarly, 500 µL of *E. coli* bacterial suspension was centrifuged at 10,000× *g* and 4 °C for 15 min. 400 µL of a Tris-HCL buffer (50 mM; pH 8.5) containing 4% (*v*/*v*) of EDTA was used to resuspend the pellet after the supernatant was removed, followed by incubation for 10 min at room temperature. A volume of 100 µL of 4% (*v*/*v*) glycerol was added to the bacterial suspension. Next, a freeze-thaw cycle is performed on the suspension, which is then sonicated 3 times on ice for 1 min each. Finally, the suspension was subjected to another centrifugation at 10,000× *g* and 4 °C for 15 min and the supernatant was removed. The obtained pellet contains the F_1F_0-ATPase enzyme and was washed twice with Tris-HCL buffer (50 mM; pH 8.5) to remove glycerol and EDTA.

2.4. Phosphate Quantification Protocol

The hydrolysis of ATP by F_1F_0-ATPase leads to the formation of ADP and inorganic phosphate (Pi). The latter is used to quantify the enzymatic reaction. A linear regression showing absorbance values as a function of standard Pi solution concentrations ranging from 10 to 50 µM is used to deduce the concentration of Pi. Standard Pi solutions are prepared in tris-HCL buffer (50 mM, pH 8.5) and their concentrations are quantified using the method of Lowry et al. [27]. In each tube containing 1000 µL of the different Pi concentrations, and one tube containing 1000 µL of Tris-HCL buffer serving as a negative control, 100 µL of a 1% ammonium molybdate solution is added, and the contents are thoroughly mixed, allowing the formation of a phosphomolybdic acid complex. After adding 100 µL of a 1% ascorbic acid solution to each tube and incubating for 10 min at room temperature, a blue molybdous compound is formed. Both ammonium molybdate and ascorbic acid solutions are prepared in 0.25 N of H_2SO_4. A spectrophotometer is used to measure the optical density of each tube at 700 nm. The assays for each Pi concentration were carried out in triplicate.

2.5. Study of the Inhibition of the Membrane-Bound S. epidermidis and E. coli ATP Synthases by Quercetin

After obtaining a pellet containing the isolated F_1F_0-ATPase enzyme as previously described in Section 2.3, inhibitory assays were carried out using quercetin, a natural

inhibitor of the enzyme, at different volumes, for both *S. epidermidis* and *E. coli*. The pellet was first resuspended in Tris-HCL buffer (50 mM; pH 8.5) and $MgCl_2$ (15 mM) and incubated at 37 °C for 10 min. Then, to each sample a different volume of quercetin solution was added, so that different concentrations were obtained (1–500 µM), as well as 100 µM of ATP to start the reaction. The concentration of ATP used was saturating for the enzyme. Next, each tube was incubated at 37 °C for 40 min, and 1% (*v*/*v*) of SDS was added to each sample in order to stop the reaction. The samples were then centrifuged at 10,000× *g* for 10 min and finally the pellet was removed and the supernatant, containing the Pi released by the enzymatic reaction, was tested as described in Section 2.4 to study the inhibition of the enzyme. Two control tubes were prepared, one lacking the inhibitor quercetin to obtain the maximum activity of the enzyme, and one lacking both the inhibitor and ATP, which was used as a blank. All tests were performed in triplicate.

Inhibitory concentration (IC_{50}) value of quercetin was calculated using GraphPad Prism (GraphPad Software, San Diego, CA, USA). This was achieved by using a curve of the enzymatic activity (%) as a function of log of different concentrations of quercetin (µM).

2.6. Study of the Potential Inhibitory Effect of M. bornmuelleri Venom on the Membrane-Bound S. epidermidis and E. coli F_1F_0-ATPases

The pellet containing the isolated F_1F_0-ATPase as described in Section 2.3 is tested with *M. bornmuelleri* venom as described in Section 2.4 to study its effect on the enzyme. The same protocol was applied on both *S. epidermidis* and *E. coli* membrane-bound F_1F_0-ATPase: To each tube containing the membrane-bound enzyme different concentrations of *M. bornmuelleri* venom (0–200 µg/mL) are added, prepared in Tris-HCL buffer (50 mM; pH 8.5), $MgCl_2$ (15 mM), Tris-HCL buffer (50 mM; pH 8.5) and lastly ATP (100 µM, a saturating concentration) to start the reaction, and for a final volume of 1 mL for each tube. Samples are then incubated at 37 °C for 40 min, and 1% (*v*/*v*) of SDS is added to each sample in order to stop the reaction. Lastly, the tubes are centrifuged at 10,000× *g* and 4 °C for 10 min. Two blanks, one containing Tris-HCL $MgCl_2$ and ATP and the other without ATP were prepared. A positive control tube, containing the enzyme, Tris-HCL, $MgCl_2$, and ATP was prepared to determine the maximum activity of the enzyme. All assays were carried out in triplicate.

For both bacteria, curves of the enzymatic activity (%) as a function of different concentrations of the venom (µg/mL) were established.

2.7. HCT116 Cell Culture

To the medium used for HCT116 Cell line culture 1% Penicillin Streptomycin (100 $U \cdot mL^{-1}$) and 10% heat-inactivated fetal bovine serum (FBS) were added in order to amplify it. DMEM acquired at Sigma Aldrich (Gibco Dulbecco's modified Eagle medium) was used to culture the HCT116 cell line at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air.

2.8. Cellular Viability Assay

The MTT test for cellular viability [28] was performed as follows: 96-well plates were used to seed HCT116 cells at a density of 10^4 cells/well, when they reached 60–80% confluency the cells were treated for 24 h and experiments were carried out with different concentrations of the venom extract. The medium was then discarded and to each well 100 µL of MTT solution was added. Finally, the absorbance was measured by ELISA READER at 570 nm. All tests were carried out in triplicate.

2.9. Statistical Analysis

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and represented as mean ± SD of at least 3 independent experiments performed in triplicate for each condition. One-way ANOVA tests were performed. Differences were considered significant at $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***) and $p < 0.0001$(****).

3. Results

3.1. Validation of the Phosphate Dosage Method

The F_1F_0-ATPase enzyme catalyzes the hydrolysis of ATP, which results in inorganic phosphate (Pi) that can be measured in order to assess enzymatic activity. This measurement must be linear in order to use it for quantitative analysis on assays. Therefore, we used the colorimetric method described by Lowry et al. 1945 [27] with some optimizations elaborated previously by our team [29]. Experiments were performed with solutions of low concentrations of P_i of 10, 20, 30, 40 and 50 µM.

Results represented in Figure 1 show a positive linear correlation between the optic density (OD) and the P_i concentration ($R^2 > 0.9954$). In addition, the relative standard deviation (RSD%) is less than 3%, indicating that the measurements performed are highly repeatable; and it is important to add that all Pi solutions were assayed in triplicate. Thus, we determined that this linear regression is suitable for the analysis of *S. epidermidis* and *E. coli* membrane-bound F_1F_0-ATPases' activity by way of quantifying the Pi produced during the catalytic reaction.

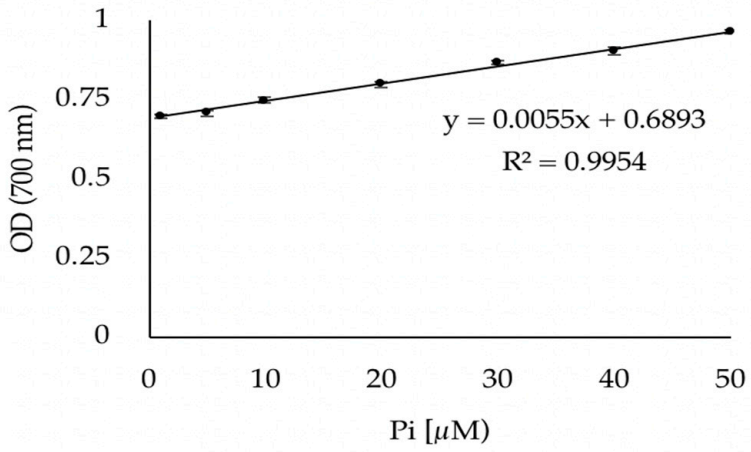

Figure 1. Linearity of the P_i dosage. Standard P_i solutions of 10 µM to 50 µM prepared in tris-HCl buffer (pH = 8.5; 50 mM). Incubation time: 10 min. λ = 700 nm. Error bars show the standard deviation obtained from experiments carried out in triplicate.

3.2. Screening of the Reference Inhibitor Quercetin on S. epidermidis and E. coli F_1F_0-ATPase Enzymes

The natural quercetin was chosen as reference inhibitor of the two bacteria F_1F_0-ATPases in order to validate the experimental model. The dose–response curves representing the enzymatic activity (%) of *S. epidermidis* and *E. coli* F_1F_0-ATPases as a function of log of reference inhibitor concentrations are represented in Figure 2a,b, respectively. The corresponding IC_{50} values obtained are 24.2 ± 2.2 µM for *S. epidermidis* and 30.0 ± 1.5 µM for *E. coli*. These values are in good agreement with those reported in literature [30] which validate the developed method. Good repeatability was obtained on assays with RSD < 5% for both bacteria.

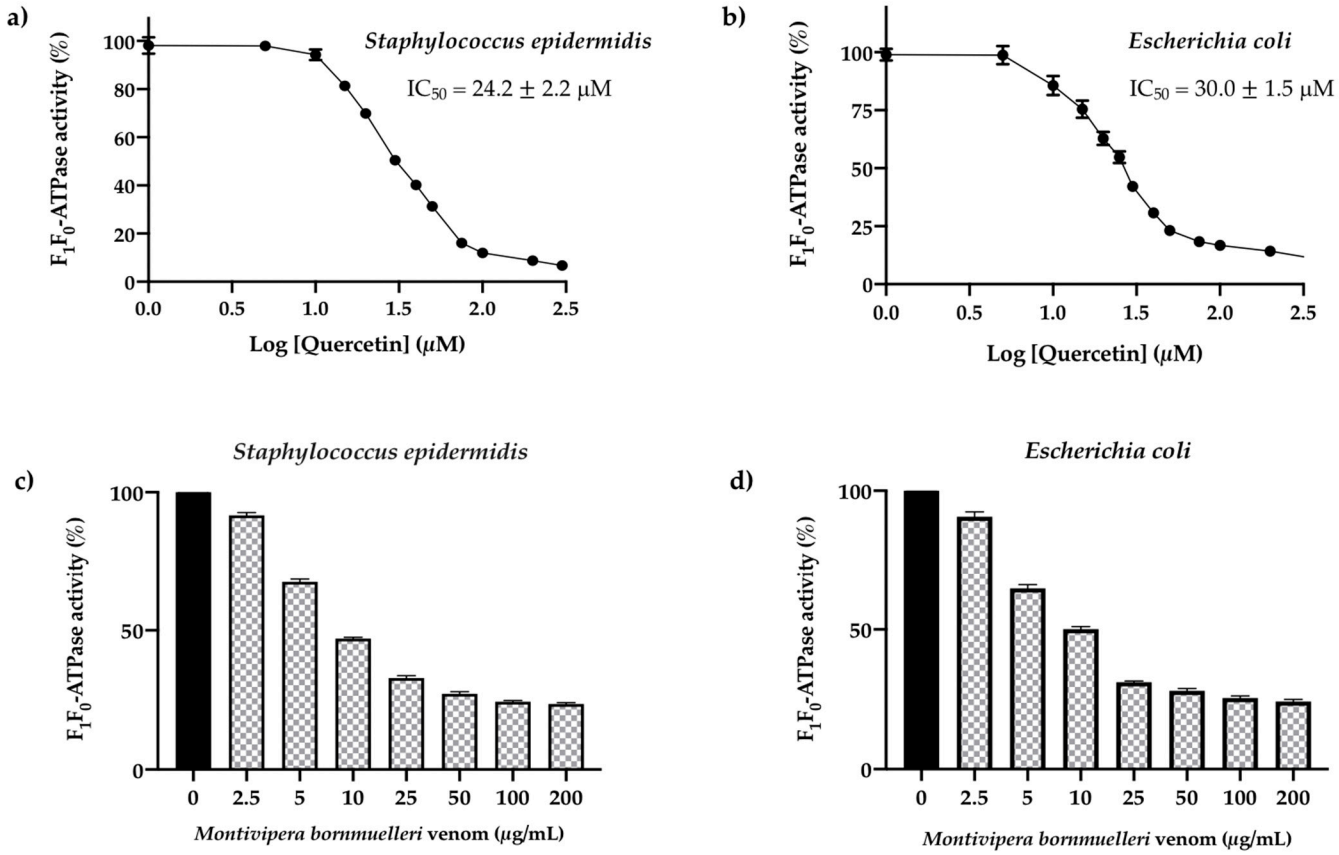

Figure 2. Effect of the *M. bornmuelleri* venom on the F_1F_0-ATPase enzymatic activities of *S. epidermidis* and *E. coli* bacteria strains. The IC_{50} of the quercetin—used as an inhibitory reference ligand—on the enzymes is shown. (**a**) Dose-response curve showing the F_1F_0-ATPase activity (%) of *S. epidermidis* as a function of the log of the concentration of quercetin. (**b**) Dose-response curve showing the F_1F_0-ATPase activity (%) of *E. coli* as a function of the log of the concentration of quercetin. (**c**) Histogram representing the effect of *M. bornmuelleri* venom (µg/mL) on the activity (%) of membrane-bound *S. epidermidis* F_1F_0-ATPase. (**d**) Histogram representing the effect of *M. bornmuelleri* venom (µg/mL) on the activity (%) of membrane-bound *E. coli* F_1F_0-ATPase. For the four graphs represented here, the error bars show the standard deviation obtained from experiments carried out in triplicate. Data are expressed as mean ± SD (n = 3). Quercetin was used as a positive control. One-way ANOVA test: ns (not significant), when compared with the control. The *M. bornmuelleri* venom exerts a dose dependent inhibition on ATPase activity.

3.3. Effect of the M. bornmuelleri Venom on the Membrane S. epidermidis and E. coli. F_1F_0-ATPase Enzymes

The *M. bornmuelleri* venom test on the membrane *S. epidermidis* and *E. coli* F_1F_0-ATPase enzymes shows that this extract is a potentially active inhibitor on those enzymes. In fact, seven concentrations in µg/mL of *M. bornmulleri* venom were tested (2.5, 5, 10, 25, 50, 100, 200). When the snake venom concentration increases, the enzyme activity for the two strains of bacteria Gram-positive *S. epidermidis* and Gram-negative *E. coli* decreases and reaches a plateau with a minimum enzymatic activity of approximately 20% (maximum inhibition of 80%) (Figure 2c,d).

3.4. Effect of the M. bornmulleri Venom on HCT116 Cancer Cell Lines

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was the method used to elucidate the cytotoxic activity of crude *M. bornmuelleri* venom on human colon cancer HCT116 cells. These latter were exposed to a range of snake venom concentrations (0.5, 1, 2.5, 5, 10, 50 and 50 µg/mL) for 24 h and the obtained results were represented as the percentage of cell viability as a function of concentration of *M.*

bronumelleri venom (µg/mL). Untreated cells with 100 % viability were used as a control to compare with HCT116 cells.

The data showed that the *M. bornmuelleri* venom inhibits the total cell viability of HCT116 cells with a low concentration of 10 µg/mL of the *M. bornmuelleri* venom. In fact, this concentration was able to induce a significant decrease in cell viability (95% of viability) in comparison to the control (Figure 3a). The results reveal the strong cytotoxic effect of the Lebanese snake *M. bornmuelleri* venom on HCT116 cells with an IC_{50} of 6.9 µg/mL calculated by Prism software (Figure 3b).

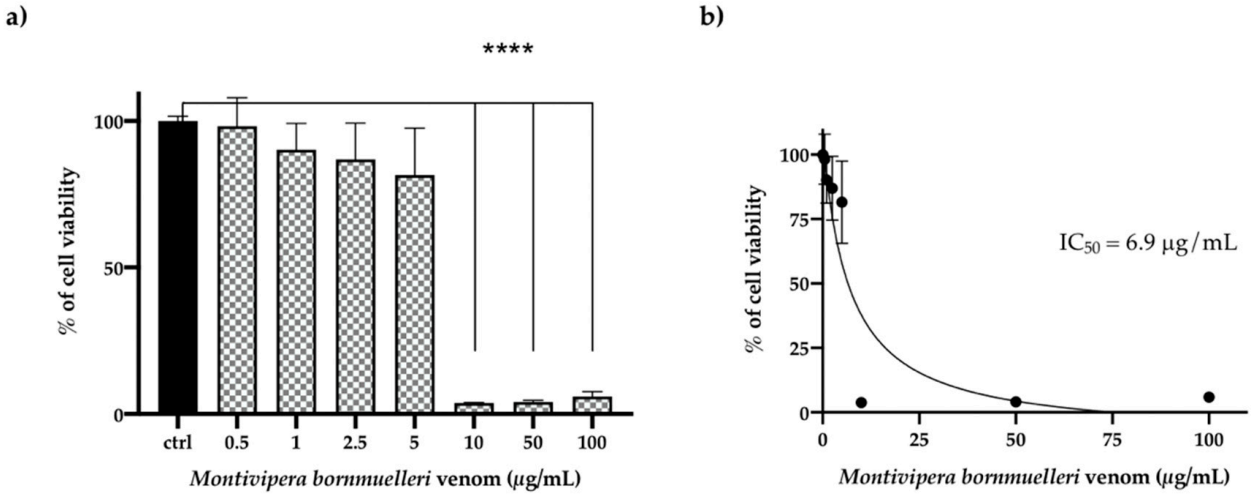

Figure 3. Cell viability of HCT116 colon cancer cells was measured by MTT assay after treatment with increased concentrations of *M. bornmuelleri* venom. (**a**) Results are expressed as mean ± SD of three independent experiments ($n = 3$). One-way ANOVA test: ns (no significant), **** $p < 0.0001$ when compared with the control. At 10, 50 and 100 µg/mL, the *M. bornmuelleri* venom showed a high cytotoxic activity against HCT116 cancers cells. (**b**) Curves for MTT assay showing IC_{50} values and the % of cell viability in function of concentrations *M. bornmuelleri* venom.

4. Discussion

Montivipera bornmuelleri is among three viper species found in Lebanon that inhabits mountainous regions [2]. Our previous studies aimed to characterize this snake and its venom in order to prove its important therapeutic applications. Indeed, an ecological study found that the snake prefers rocky regions with few thorny cushion plants and its maximum size is around 75 cm [1,2]. Its venom was analyzed and it was shown that it contains mainly three enzymes: PLA2, Metalloprotease III and serine protease, which possess many biological activities. In fact, the venom displayed both pro- and anti-coagulant activities depending on its concentration, as well as indirect hemolytic activity, possibly due to the presence of PLA2 [7]. It was shown by in vivo experiments on mice that it induced an upregulation of pro-inflammatory cytokines (INF-γ, TNF-α, IL-1β, IL-4 and IL-17), while also downregulating the anti-inflammatory cytokine IL-10 [6]. In addition, the *M. bornmulleri* venom exhibits antibacterial effect against both Gram-positive and Gram-negative bacteria, as well as antifungal activity [4]. Moreover, given the resistance to antibiotics of a multitude of bacteria and—now more than ever—the need to discover new antibacterial agents [31], such as those capable of acting on the F_1F_0-ATPase enzymes which are essential for the pathogenic microorganisms survival, we were interested in testing the venom effect of *M. bornmuelleri* on the F_1F_0-ATPases of bacteria from both Gram-positive (*S. epidermidis*) and Gram-negative (*E. coli*) genera. In agreement with our previous results [4], we found here that the venom inhibited the activity of the F_1F_0-ATPase enzyme presented in the membranes of these two bacteria. This membrane-bound enzyme plays an important role in cell life and death by both synthesizing and hydrolyzing ATP. Since ATP deprivation is linked to cell death [20], *M. bornmuelleri* venom having the ability to inhibit the activity of this enzyme translates to its ability as an effective antimicrobial

agent. Thus, we showed that this crude venom managed to reduce the enzymatic activity to a minimum of 20% at concentrations of 100 to 150 µg/mL. However, this inhibitory effect was only assayed on isolated F_1F_0-ATPase of both bacteria, and not on total cells. The ability of the venom to inhibit the ATPase of Gram-negative bacteria, which are more resistant to antibiotics than Gram-positive ones due to the presence of an outer membrane that must be passed by drugs to attain their target [31], with the same level of inhibition (maximum of 80%) as Gram-positive bacteria, confers it a broad spectrum of activity and an important property. Indeed, it can be used against both types of bacteria instead of acting against only one, and this expands its potential application. Another potential benefit for this activity is the fact that the transfer of antibiotic resistance genes is possible between Gram-positive and Gram-negative bacteria [32] or between the same types of bacteria, such as *S. epidermidis* which is able to transfer these genes to *S. aureus*, and thus spread antibiotic resistance [13], so an antibacterial agent that is not susceptible to resistance by the various bacteria proves to be even more useful. As for compounds responsible for the inhibition of the bacterial enzyme, many aromatic derivatives from various plants have been found to exhibit this type of activity, such as resveratrol, piceatannol and quercetin [30]; however, similar compounds have not been characterized in *M. bornmuelleri* venom to date. Instead, we suggest that compounds found in the venom and known for their antibacterial effect, such as PLA2 and LAAO [4], are inhibiting the bacterial F_1F_0-ATPase. Indeed, PLA2, an enzyme purified from *Protobothrops mucrosquamatus* snake venom is able to control both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*) bacterial strains [33]. Metalloproteinase isolated from *Agkistrodon halys*, also inhibited Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Proteus vulgaris*) bacteria [34]. LAAO, purified from *Bothriechis schlegelii* venom, also exerts an inhibitory effect against both Gram-positive (*S. aureus*) and Gram-negative (*Acinetobacter baumannii*) bacteria [35]. All of these enzymes are found in *M. bronmuelleri* venom and could be responsible for its antibacterial activity, which is in accordance with these findings in the literature [2].

In terms of cytotoxicity, our previous studies showed that the *M. bornmuelleri* venom has a specific cytotoxicity against B16 skin melanoma cells and 3-MCA-induced murine fibrosarcoma cell lines, which were more sensitive to the venom when overexpressing ovalbumin. However, it did not manage to reduce tumor size in vivo [36]. It has been shown that the venom possesses a vasorelaxant effect on K^+ depolarized smooth muscle and inhibits the contraction of smooth muscle tissue induced by $CaCl_2$, phenylephrine and AngI [8], but did not have a strong cytotoxicity towards human erythrocytes [3]. Here, different concentrations of the crude venom of *M. bornmuelleri* were tested against human colon cancer cell line HCT116, and it was found that a low concentration of 10 µg/mL was able to totally reduce cancer cell viability (by 95%) compared to untreated control cells and with an IC_{50} value of 6.9 µg/mL. This is in agreement with the literature, where the snake venom toxin from *Vipera lebetina turanica* was also tested on HCT116 colon cancer cells and reduced their viability with an IC_{50} value of 1.14 µg/mL. This cell growth inhibition was achieved by inducing apoptosis in the treated cells through an increase in ROS generation, upregulation of death receptors DR4 and DR5, cleavage of caspases-3, 8 and 9 and activation of the JNK pathway [37]. *M. bornmuelleri* venom could act through similar pathways and it would be beneficial to elucidate its mechanism of action in future studies. In addition, crude venom from the Malaysian *Cryptelytrops purpureomaculatus* demonstrated cytotoxic activity in SW480 and SW620 colon cancer cell lines with EC_{50} values of 29.43 µg/mL and 23.19 µg/mL respectively, which is in good agreement with our results. In this study, LAAO was also isolated from the venom and decreased the viability of the SW480 and SW620 colon cancer cell lines with lower EC_{50} values of 13.56 µg/mL and 13.17 µg/mL respectively through apoptosis [38]. The purification of a single component from *M. bornmuelleri* venom in order to test it on HCT116 cells could similarly prove to possess an enhanced cytotoxic effect compared to the crude venom. However, another study in which Malaysian mangrove pit viper *Trimeresurus purpureomaculatus* venom was assayed on HT-29 colon cancer cell lines, showed that viability was reduced with a low IC_{50} value of 0.42 µg/mL,

suggesting that high cytotoxicity could result from synergistic interactions between various compounds present in the venom instead of only one component. Indeed, the different fractions obtained by HPLC had demonstrated each variable's cytotoxic activity [39]. A viable concern for the use of snake venom in anticancer therapy is the potential cytotoxicity of its components against normal cells, in other words, its lack of selectivity for cancer cells. Indeed, PLA2 found in the venom is known to act on membrane phospholipids, and LAAO acts by producing H_2O_2, which is directly correlated with the deterioration of cell membranes. However such effects have been found to be most potent against cancer cells in particular, since these latter possess asymmetric plasma membranes [40]. One of the previous studies carried out on skin cancer cells demonstrated that the crude venom of *M. bornmuelleri* caused the least damage to HaCaT cells (non-tumorigenic), reducing their viability to 70.63% as opposed to 44% for A5 cells and 21.33% for II4 cells, both tumorigenic, using the same venom concentration of 60 µg/mL, further proving the venom's selective cytotoxicity against cancer, and not normal, cells [5]. Since colon cancer is a major cause of cancer-related death and its incidence and mortality are increasing quickly [22], and its treatment faces the problem of the resistance of this disease to chemotherapy [41]—which is also unsatisfactory since it has many adverse side effects and lowers the quality of life of patients [24]—finding new treatments for this life-threatening disease is an important step that must be taken to improve public health, and natural sources such as snake venoms, and specifically *M. bornmuelleri* venom, as we showed in this study, prove to be of special interest in terms of discovering such therapeutic agents.

Author Contributions: Conceptualization, H.N. and Z.F.; methodology, M.K., C.M., M.R. and R.S.; software, C.M. and Z.F.; formal analysis, H.N. and Z.F.; investigation, Z.F., H.N. and J.-M.S.; resources, Z.F.; writing—original draft preparation, M.K.; writing—review and editing, H.N. and Z.F.; supervision, Z.F.; project administration, Z.F.; funding acquisition, Z.F. All authors have read and agreed to the published version of the manuscript.

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