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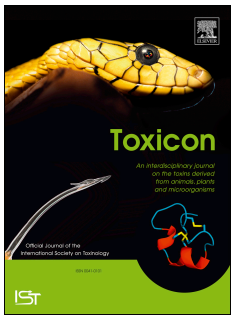

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Purification of Nasulysin-1: a new toxin from *Porthidium nasutum* snake venom that specifically induces apoptosis in leukemia cell model through caspase-3 and apoptosis-inducing factor activation

Angelica Rocio Bonilla-Porras[1], Leidy Johana Vargas[2], Marlene Jimenez-Del-Rio[1], Vitelbina Nuñez[2,3], Carlos Velez-Pardo[1*]

[1]Neuroscience Research Group, Medical Research Institute, Faculty of Medicine, University of Antioquia, Calle 70 N° 52-21, and Calle 62 #52-59, Building 1, Room 412, SIU, P. O. Box 1226, Medellín, Colombia

[2] Ofidism and Scorpionism Program, University of Antioquia, Calle 70 N° 52-21, and Calle 62 #52-59, Building 1, Room 631, SIU, P. O. Box 1226, Medellin, Colombia

[3]School of Microbiology, University of Antioquia, Calle 70 N° 52-21, P. O. Box 1226, Medellin, Colombia

**Address for correspondence*: Dr. Carlos Velez-Pardo, Neuroscience Research Group, Medical Research Institute, Faculty of Medicine, University of Antioquia, Calle 70 N° 52-21, and Calle 62 #52-59, Building 1, Room 412, SIU, P. O. Box 1226. Medellin, Colombia
E-mail: calberto.velez@udea.edu.co, phone 574.219.64.57, Fax 574.219.64.44

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Abstract

Nasulysin-1, a new zinc-metalloproteinase from the snake venom of the hognose pit viper *Porthidium nasutum*, was purified to homogeneity using molecular exclusion chromatography and high performance liquid chromatography on a reverse phase column. The molecular mass of the purified enzyme was 25,900 kDa and pI 4.1, as determined by 1D and 2D polyacrylamide gel electrophoresis. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of the N-terminal amino acid sequence ($_1$FSPRYIELVVVADHGMFKKYNSNLNTIR$_{28}$; $_1$TASLANLEVWSK$_{12}$; $_1$DLLPR$_6$) of the purified nasulysin-1, shows close structural homology with other snake venom metalloproteinases isolated from different snake venoms. The purified nasulysin-1 showed specific apoptosis-inducing activity in Jurkat and K562 cells, a T-cell acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (AML) cell model, respectively, without affecting the viability of human lymphocyte cells. After 48 h treatment, nasulysin-1 (20 µg/mL) induced loss of the mitochondrial membrane potential ($\Delta\Psi_m$), activated the apoptosis-inducing factor (AIF), activated the protease caspase-3, and induced chromatin condensation and DNA fragmentation, all hallmarks of apoptosis. These results strongly suggest that nasulysin-1 selectively induces apoptosis to eliminate leukemia cells. Thus, these data warrant further investigation into the use of the metalloproteinase protein, nasulysin-1 as a potential therapeutic agent for treating leukemia.

1. Introduction

Acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML) are hematologic disorders characterized by uncontrolled cell production of lymphoblast and myeloblast cells, respectively, in the bone marrow. At present, however, little is known about its causes. According to recent data by the American Cancer Society, about 12,910 new cases and about 2,590 deaths occurred in the United States during 2015 from ALL/CML (http://www.cancer.org/cancer/leukemia). Despite progressive improvements in the efficacy of treatment (**Ju et al., 2014; Jamison et al., 2016**) and increased knowledge about the biologic features of leukemia cells (**Durinck et al., 2015**), a subset of patients relapse or remain refractory to chemotherapy and anti-kinase treatments (**Locatelli et al., 2012; Frey and Luger, 2015; Lim et al., 2015; Muraoka et al., 2016**). Therefore, ALL and CML are incurable diseases in an important fraction of pediatric (~10%) and adult (~30%) patients (**August et al., 2013**). One of the major reasons for this outcome might be cellular evasion of apoptosis (**Hanahan and Weinberg, 2011**) -a regulated form of cell death. Apoptosis is characterized by morphological and biochemical changes such as the reduction of cellular and nuclear volume, loss of plasma membrane, dissipation of the mitochondrial membrane potential ($\Delta\Psi m$), loss of the mitochondrial membrane potential (MMP), massive activation of caspases (e.g., caspase-3), activation of the apoptosis-inducing factor (AIF), chromatin condensation, and DNA fragmentation (**Kroemer et al., 2009; Galluzzi et al., 2012**). Therefore, a rational strategy would be to stimulate ALL/ CML cells to trigger apoptosis.

Porthidium nasutum (*P. nasutum*, hognose pit vipers) are found from northwestern Mexico throughout Central America to Panama and the Caribbean lowlands, and further from northern Colombia to Ecuador (http://www.iucnredlist.org/details/64344/0). Interestingly, snake venoms are complex mixtures of proteins, mainly with enzymatic activities capable to kill different tumor and leukemia cell lines by apoptosis (**Vyas et al., 2013; Calderon et al., 2014**). Snake venoms therefore represent a potential anticancer source (**Utkin, 2015**). Recent proteomic analysis of the *P. nasutum* venom from Costa Rica has shown SVMPs as its major component (52.1%, expressed as percentages content) followed by moderate amounts of phospholipases A2 (PLA2, 11.6%), C-type lectin/lectin-like (CTL, 10.4%), disintegrins (DIS, 9.9%), serine proteinases (SP, 9.6%), and low/scant amounts of L-amino acid oxidases (LAO, 3%), bradykinin-potentiating peptides (BPP, 1.9%), cystein-rich secretory proteins (CRISP, 1.3%), and nucleotidases (NUCL, 0.2%) (**Lomonte et al., 2012**). Recently, an acidic phospholipase A_2 has shown antibacterial activity from Colombian *P. nasutum* snake venom (**Vargas et al., 2012**). However, whether *P. nasutum* venom is capable to induce cell death in ALL/ CML cells is not yet established.

Snake venom metalloproteinases (SVMPs) are monozinc endopeptidases classified according to their functional domain organization as P-I class metalloproteinases (20-30 kDa), composed of one metalloproteinase domain; P-II class metalloproteinases (30-60 kDa), composed of a metalloproteinase and a disintegrin domain; and P-III class metalloproteinases (60-100 kDa), composed of a metalloproteinase, a disintegrin, two lectin-like and cysteine-rich domains (**Escalante et al., 2011)**. Although the functional activities of SVMPs are mainly associated with hemorrhage or the disruption of the homeostatic system (i.e., proteolysis of fibrinogen, fibrin, and capillary basement), SVMPs

are also able to provoke apoptosis in cell lines, such as human umbilical-vein endothelial cells, endothelial cells, and rat smooth muscle cells (**Takeda et al., 2012**). However, no data are available to establish whether SVMPs can specifically induce apoptosis in leukemia cells.

To gain insight into the biological activities of Colombian *P. nasutum* venom, the first aim of this study was to determine the *in vitro* effect of various concentrations of crude *P. nasutum* snake venom and isolated venom fractions on Jurkat (clone E6-1) and K562 cell lines, as models of human T cell ALL and human CML, respectively. Analysis of cell nuclear morphology by fluorescence microscopy and evaluation of $\Delta\Psi$m by flow cytometry revealed that crude *P. nasutum* snake venom induces both apoptosis and necrosis – characterized by cytoplasmic swelling, rupture of the plasma membrane, and mild clumping of nuclear chromatin, while the fractions F I-VI and sub-fractions SF IV.1-9 induced only apoptosis in Jurkat and K562 cells. Thus, the second aim of this study was to further isolate a small SVMP (MW ~26 kDa), named hereafter nasulysin-1, from *P. nasutum* venom, and evaluate its capacity to provoke apoptotic cell death in Jurkat/K562 cells and normal lymphocytes, as control non-leukemic cells. To further characterize the molecular mechanism of apoptotic cell death in the cells treated with nasulysin-1, AIF and Casp3 activation were quantified by immunocytochemistry. Our findings suggest that nasulysin-1 specifically induces apoptosis in leukemia cell lines. Accordingly, the current effort is directed towards biologically driven therapies (**Saedi et al., 2014**) and nasulysin-1 might be a useful metalloproteinase protein to investigate as a potential therapy to treat leukemia.

2. Materials and methods

2.1 Reagents

3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, cat # D-273) was obtained from Invitrogen Molecular Probes (Eugene, OR, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Venom

Venom was obtained by manual extraction of 45 *P. nasutum* specimens from the state of Antioquia located in northwest Colombia. The vipers are maintained in captivity at the Universidad de Antioquia's Serpentarium (Medellin, Colombia). Venoms were centrifuged at 800 *g* for 15 min, and supernatants lyophilized and stored at −20 °C.

2.3 Purification of the venom of *P. nasutum* by molecular exclusion chromatography and HPLC.

The crude venom from *P. nasutum* (150 mg) was fractionated by molecular exclusion chromatography using a HiPrep 26/60 Sephacryl S-200 HR column (16 mm, Pharmacia Biotech, cat# 17-9511-01). The sample was separated using 0.5 mM sodium phosphate buffer (pH 7.2) at a flow rate of 1 mL/min. The fractions obtained were dialyzed, lyophilized and stored at -20 °C. The fraction IV (2 mg) dissolved in 200 µL 0.1% trifluoroacetic acid (TFA) was further fractionated by reverse- phase high performance liquid chromatography (RP-HPLC) using a Pinacle DB C18 column (250 mm x 4.6 mm, 5 µm particle size, 140 Å pore size, RESTEK Corporation, cat·# 9414575) eluted with a 0-

66% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) and developed on a Shimadzu Prominence system at a flow rate of 0.7 mL/min. Absorbance was monitored at 215 nm. The fractions were manually collected and dried under vacuum in a Eppendorf vacufuge concentrator until total evaporation of the eluents. Samples were then stored at 4 °C to evaluate their pro-apoptotic activity.

2.4 One-dimensional (1D) and two-dimensional (2D) Electrophoresis.

The crude venom, the fractions (F I-VI) and sub-fraction IV.5 (SF IV.5) obtained from the venom of *P. nasutum* were analyzed by denaturing (1D) SDS-PAGE electrophoresis (5 min at 90 °C) in reducing conditions (2 mL of 1 mM DTT). Samples (20 μg) were separated on a 12% polyacrylamide gel at 120 V for 1 h and 30 min. For 2D electrophoresis, a total of 250 μg of protein was loaded onto 7-cm DryStrip strips with a non-linear pH of 4-7 (GE Healthcare Life Sciences, cat # 17-6001-14) and allowed to hydrate passively for 12 h. The first dimension (isoelectric focusing) was conducted at 20 ˚C with a 50 mA current per strip, using the Ettan IPGphor 3 system (GE Healthcare Life Sciences, cat # 11003364) until 12,000 V/h was reached. The strips were equilibrated by incubating them in buffer I (7 mM Urea, 4mM de thiourea, 4% CHAPS, 1 mM DTT and 1% ampholytes) for 20 min, followed by incubation in buffer II (6 M urea, 2% SDS, 375 mM Tris–HCl pH 8.8, 20% glycerol, 25 mg/mL of iodoacetamide) for 20 min. After equilibration, the strips were placed on 12.5% acrylamide/bis-acrylamide gels, and the second dimension was conducted using the MiniPROTEAN system (Biorad, cat # 1658025, Hercules, CA) at 100 V for 120 min. The gels obtained from 1D and 2D were fixed for 1 h in a solution of 50% methanol and 10% acetic acid, and subsequently stained with CBB R250 and/or silver nitrate.

160 **2.5 Protein preparation and analysis by LC-MS/MS**

161 The protein spot of interest from sub-fraction IV.5 (SF IV.5), visualized by CBB R250
162 staining, was excised from blue native gel and in-gel digested with trypsin was performed
163 according to laboratory standardized procedures. The peptides were taken up in 10 µL of
164 0.05% TFA and 4 µL was loaded onto 5 mm × 300 µm i.d. trapping column on the
165 nanoLC-MS/MS Ultimate 3000 system (Dionex, Amsterdam, The Netherlands), which was
166 interfaced to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific,
167 Bremen, Germany) via robotic nanoflow ion source TriVersa (Advion BioSciences Ltd.,
168 Ithaca, NY) equipped with a LC coupler, according to **Junqueira et al., (2008).** The
169 acquired spectra were contrasted first with the NCBI protein library without species
170 restriction using v.2.2.0 software MASCOT (Matrix Science Ltd, London) under the
171 following parameters: 5 ppm and accuracy of mass 0.5 kDa for precursor and fragments,
172 specific enzyme: trypsin, two missing cuts, variable modifications: propionamide oxidized
173 methionine and cysteine. All pairings with more than two peptides and protein result
174 (score) above 100 were evaluated manually. Predicting novo sequence was made by
175 sequence similarity in the databases, the search was developed using the software and the
176 MS PepNovo BLAST tool, according to **Waridel et al (2007).**

177

178 **2.6 Isolation of lymphocytes.**

179 Peripheral blood lymphocytes were obtained from the venous blood of healthy adult males
180 (30-40 years old) by gradient centrifugation (lymphocyte separation medium, density:
181 1.007 G/M; Bio-Whittaker Inc., Walkersville, MD, USA) and cultured as described
182 elsewhere (**Avila-Gomez et al., 2010**).

183

184 **2.7 Leukemia cancer cell culture**

185 Jurkat clone E6-1 (ATCC® Catalog No. TIB-152™) and K562 (ATCC® Catalog No.
186 CCL-243™) were cultured according to supplier's indications. Cells at 1×10^6 cells/mL
187 (passage 5-10) were exposed to crude/fractions/sub-fractions of *P. nasutum* venom.

188

189 **2.8 Experiments with leukemia cell line and lymphocyte cells.**

190 **2.8.1** *Morphological assessment of cell death by fluorescence microscopy using acridine*
191 *orange/ethidium bromide (AO/EB) double staining.*

192 The cancer cell suspension (1 mL, final volume) was exposed to increasing concentration
193 of crude, fractions and sub-fractions from *P. nasutum* snake venom concentrations (10, 20,
194 50, 100, 200, 400 μg/ml) freshly prepared in RPMI-1640 medium for 24 h at 37 °C.
195 Lymphocyte cells and cancer cell suspension were treated with sub-fraction IV.5 (SF IV.5,
196 20 μg/ml) snake venom for 48 h at 37 °C. The cells were then used for fluorescence
197 microscopy analysis as detailed elsewhere (**Bonilla-Porras et al., 2014**).

198

199 **2.8.2 Analysis of Mitochondrial Membrane Potential ($\Delta\Psi_m$) by Flow Cytometry and**
200 **Fluorescent Microscopy.**

201 Jurkat and K-562 cell lines and lymphocyte cells were treated as described above. Cells
202 (1×10^5 cells/mL) were then incubated with 10 nM cationic lipophilic $DiOC_6(3)$ for 20 min
203 at RT in the dark. Cells were then analyzed using a flow cytometer Beckman Coulter Epics
204 XL, according to **Bonilla-Porras et al., 2014**. The assessment was repeated 3 times in
205 independent experiments.

206

2.8.3 Immunocytochemistry detection of caspase-3 and apoptosis-inducing factor (AIF).

Untreated or treated leukemia and lymphocyte cells were subjected to immunocytochemistry assays. The Santa Cruz Biotechnology (SCB) supplier protocol goat ABC staining System (cat # sc-2023) was followed for the immunocytochemistry using primary goat polyclonal antibodies for caspase-3 (cat # sc-22171) and AIF (cat # sc-9417). The cells were immune-stained and diaminobenzidine positive (DAB^+) cells were quantified blind to the experimenter. Cells with nuclear DAB^+ staining were counted as cells with active caspase-3 or AIF proteins, indicators of apoptotic cell death.

2.9 Photomicrography.

The light microscopy and fluorescent photomicrographs shown in the figures were taken using a Zeiss (Axiostart 50) microscope equipped with a Canon PowerShot G5 digital camera.

2.10 Statistical Analysis.

The aforementioned parameters were quantified by counting a minimum of 500 total cells blind to the experimental setting and viewer. The experiments were performed in 3 independent settings. Data are means ± S.D. of three independent experiments. One-way analyses of variance ANOVA with Bonferroni or Games-Howell post-hoc comparison were calculated with SPSS 18 software. A p-value of < 0.05 was considered significant.

3. Results

3.1 Crude *P. nasutum* snake venom induces both apoptosis and necrosis in Jurkat and K562 cells.

As a first approach, leukemia cells were exposed to crude *P. nasutum* snake venom. As shown in **Fig. 1**, crude venom induced cell death in Jurkat **(Fig. 1A)** and K562 **(Fig. 1C)** in a concentration dependent manner (**Fig. 1B and 1D**), according to nuclei morphological changes such as chromatin condensation, DNA fragmentation, and mild clumping of nuclear chromatin detected by conventional AO/EB technique, and loss of mitochondrial membrane potential detected by flow cytometry. Noticeably, apoptosis (~30%) and necrosis (~60%) morphology of cell death were observed at higher concentrations (e.g., 100-400 µg/mL). Remarkably, the K562 cell line was significantly more sensitive to the crude venom than Jurkat cells. For example, 100 µg/mL crude venom induced 30% apoptotic nuclei morphology and 43% necrotic nuclei morphology in Jurkat cells, whereas the same concentration of venom induced 100% necrosis in K562 cells. These observations prompted us to further examine the effect of *P. nasutum* venom on leukemia cells.

3. 2 Purification of nasulysin-1 from *P. nasutum.*

Crude snake venom from *P. nasutum* fractionated by column chromatography on Sephacryl S-200 resulted in six fractions named FI, FII, FIII, FIV, FV and FVI (**Fig. 2A**). The proteins migrated as single bands corresponding to molecular masses ranging from ~72 kDa to ~12 kDa during SDS/PAGE (7.5% polyacrylamide) (**Fig. 2B).** The pro-apoptotic activity of the fractions (20-300 µg/mL) were assayed by monitoring the nuclear morphology of Jurkat and K562 cells with AO/EB staining and $\Delta\Psi_m$ with $DiOC_6(3)$ flow

cytometry, respectively. Although all the fractions tested induced apoptosis in both of the leukemia cell lines (data not shown), fraction IV (**Fig. 2C and D**) was selected for further experiments. It was chosen because it displayed a thick single band with a molecular weight of ~26 kDa, consistent with a low molecular weight protein such as a metalloproteinase (**Fig. 2B)**, as described by **Lomonte et al** (**2012**).

Further purification of fraction IV by HPLC yielded nine peaks corresponding to sub-fractions SF IV.1-9 (**Fig. 3A**). Clearly, the pro-apoptotic activity of the sub-fraction SF IV.5 induced >25% apoptosis morphology in both Jurkat (**Fig. 3B)** and K562 (**Fig. 3C)** cells. Importantly, the electrophoretic profile of sub-fraction SF IV.5 showed a thick single band under non-reducing conditions, and, in a bidimentional electrophoretic profile, it showed a MW of 25,900 kDa and pI 4.1 (**Fig. 3D**).

3.3 Nasulysin-1 shares homology with other SVMPs from *Bothrops spp*.

The nasulysin-1 (SF IV.5) submitted to LC-MS/MS analysis identified three peptides $_{1}$FSPRYIELVVVADHGMFKKYNSNLNTIR$_{28}$; $_{1}$TASLANLEVWSK$_{12}$; $_{1}$DLLPR$_{6}$. These peptides shared important similarity with other SVMPs, according to a BLAST sequence analysis (**Fig. 4**).

3.4 Nasulysin-1 selectively induces apoptosis in leukemia cells.

Next, we evaluated the cytotoxic effect of nasulysin-1 on the leukemic Jurkat and K562 cell lines, as well as on normal human lymphocytes, as non-leukemic control cells. As shown in **Fig. 5**, Jurkat and K562 but not lymphocytes, showed signs of cell death by apoptosis when

exposed to nasulysin-1 (20 μg/mL), according to AO/EB staining and $\Delta\Psi_m$ analysis (**Fig. 5A**). Notably, K562 cells were significantly more sensitive to nasulysin-1 than Jurkat cells. Most importantly, lymphocytes did not show signs of apoptosis or necrosis as measured by the methods employed (**Fig. 5B**).

Since Jurkat and K562 cells exposed to nasulysin-1 displayed the typical morphological features of apoptosis, i.e. chromatin condensation, an indication of the apoptosis-inducing factor (AIF) activity (e.g. **Fig. 6A, inset**), and nuclei fragmentation, as indication of the protease caspase-3 (Cas-3) activity (e.g. **Fig. 6A, *asterisk***), we further investigated the involvement of AIF and Cas-3 in nasulysin-1-induced apoptosis. As shown in **Fig. 6B**, nasulysin-1 (20 μg/mL) induced the activation of Cas-3 (i.e., DAB^+ nuclei) and AIF in both cell lines, as indicated by the presence of DAB^+ immunocytochemistry staining in the nucleus (**Fig. 6C**). No statistically significant difference between nasulysin-1 treated and untreated lymphocytes was identified when comparing AIF and Cas-3 activity (**Fig. 6C**).

4. Discussion

In this study, we describe the purification of a toxin from *P. nasutum* snake venom that specifically induced apoptosis in ALL and CML cell models. The fractionation of the *P. nasutum* venom was carried out by two chromatography steps involving molecular exclusion chromatography on a Sephacryl S-200 HR column and reverse-phase HPLC chromatography; and LC-MS/MS analysis. The purified protein, named nasulysin-1 (SF IV.5), showed a homogenous single band of 25,900 kDa (~26 kDa) with a pI 4.1, according to 1D and 2D gel polyacrylamide gel electrophoresis. The LC-MS/MS analysis identified three peptides $_{1}$FSPRYIELVVVADHGMFKKYNSNLNTIR$_{28}$; $_{1}$TASLANLEVWSK$_{12}$; $_{1}$DLLPR$_{6}$ that showed sequence homology with other SVMPs (**Bernardes et al., 2008; Ferreira et al., 2009; Gomes et al., 2011**). This observation indicates that SVMPs are highly conserved through several genera and species of vipers. Interestingly, BmooMPα1, BaP1, Leuc-A and BleucMP toxins from *B. moojeni* (**Bernardes et al., 2008**)**, *B. asper*** **(Watanabe et al., 2003)** and *B. leucurus* (**Ferreira et al., 2009; Gomes et al., 2011**) are homomeric proteins composed of 22-23 kDa polypeptides resembling nasulysin-1. The findings reported here suggest that nasulysin-1 can be considered a P-I class metalloproteinase since it showed high amino acid sequence homology and molecular weight similarity with other SVMPs (**Figure 4**). However, to confirm this, analysis of the complete nasulysin-1 protein amino acid sequence is required.

We report for the first time that nasulysin-1 specifically induces apoptosis in Jurkat and K562 cell models of T-cell ALL and CML, respectively, by both caspase-dependent i.e., Cas-3 dependent, and caspase-independent i.e., AIF-dependent, mechanisms. Several

observations support our assumption. First, when Jurkat and K562 cells were exposed to nasulysin-1, we observed morphological and biochemical changes typical of apoptosis such as loss of $\Delta\Psi_m$, activation of the protease caspase-3 and of the flavoprotein AIF. Indeed, loss of $\Delta\Psi_{m,}$ caspase-3 and AIF activation have been consistently used as classic markers of this type of regulated cell death process (**Galluzzi et al., 2012**). However, how exactly nasulysin-1 provokes apoptosis in Jurkat/K562 cells is not fully established. Since most of the SVMPs PI class metalloproteins operate through degrading extracellular membrane proteins, cellular membrane proteins and interacting with specific receptors (e.g., **Okamoto et al., 2014; Calderon et al., 2014**), it is reasonable to think that nasulysin-1 might trigger cell death through an extrinsic apoptotic pathway in leukemia cells (**Zaman et al., 2014**). However, other mechanisms such as cellular internalization of toxin and direct damage of mitochondria should also be considered (**Gasanov et al., 2014**). Interestingly, lymphocytes treated with nasulysin-1 showed no signs of apoptosis when compared with naïve cells. To our knowledge, this is the first report demonstrating the selective cytotoxic action of a small molecular weight SVMP, specifically from *P. nasutum*. This cell-type specific cytotoxic action displayed by nasulysin-1 is critical if this SVMP were to be developed as an anti-leukemic agent. Taken together, these results suggest nasulysin-1 as a promising anticancer drug.

Here, we showed that sub-fraction IV.5 (SF IV.5) induced apoptosis in Jurkat/K562 cells. However, we also observed that other sub-fractions (e.g., SF IV.9) induced apoptosis. This observation suggests that, except nasulysin-1, other yet uncharacterized SVMPs might be present in the fraction IV from *P. nasutum* venom (**Moura-da-Silva et al., 2011)**.

Effectively, analysis of SF IV.9 by MALDI-TOF technique showed high homology to other SVMPs (e.g., KSHDNAQLLTNTDFDGPTIGLAYVGTMCDPK, m/z 3386.6, z:1, UniProtKB Q8QG89.1). This observation implies that either the sub-fractions (e.g., SF IV.1-4, .6-9) represent different SVMP proteins or represent partial fragments of a bigger SVMP with pro-apoptotic activity. Further study is now in progress to identify the remaining proteins, mainly from sub-fraction SF IV. 2 and 8 which induced between 40-50% apoptosis in leukemia cells. We hypothesize that such proteins might also belong to SVMPs.

Our results show that crude venom of *P. nasutum* induces both apoptosis and necrosis in leukemia cells. This is compatible with the notion that *P. nasutum* snake venom might be an important source of potentially biologically active agents not only against leukemia cells, as demonstrated in this work, but also against other cancer cells. This hypothesis is re-enforced by the fact that a proteomic analysis has demonstrated that *P. nasutum* venom contains other important proteins such as *L-amino oxidase* (LAO) with potential pro-apoptotic activity (**Costa et al., 2014**). Interestingly, Apoxin-I, a LAO purified from *Crotalus atrox*, induced apoptosis in human promyelocytic leukemia HL-60, U337 and K562 (**Torii et al., 1997**). However, no data on toxicity specificity was shown. Therefore, further investigation is warranted to establish whether other SVMPs, LAO and/or PLA_2 from *P. nasatum* might specifically induce apoptosis in leukemia cells.

Conclusions.

Nasulysin-1 is a SVMP isolated from *P. nasutum* that showed toxic specificity on leukemia cells. These findings open perspectives for future investigations into the use of nasulysin-1

as a model therapeutic agent against leukemia. Furthermore, since the crude venom, other fractions and sub-fractions from the venom showed anti-leukemia activity, the *P. nasutum* venom provides an important biological source to investigate anticancer drugs.

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Figure Legends.

Figure 1. Crude venom from *P. nassutum* induces nuclei fragmentation/ chromatin condensation and dissipation of mitochondrial membrane potential in leukemic cells as indicative of apoptosis or necrosis.

Jurkat (A and B) and K562 (C and D) cells were either left untreated or treated with increasing concentrations (10-400 μg/mL) of crude venom (CV) for 24 h. Nuclear morphological changes were evaluated using AO/EB staining and ΔΨm was evaluated using $DiOC_6(3)$. The circles represent the percentages of total apoptosis (early and late) and loss of ΔΨm produced by each treatment. Insets in A and C showed nuclear fragmentation typical of apoptosis, and cytoplasmic swelling, rupture of plasma membrane, and highly chromatin condensation (yellow-red fluorescence) typical of necrosis. The ANOVA revealed significant differences among the groups, $p< 0.001$. The Bonferroni comparison test showed significant differences in a percentage of induction of apoptosis, necrosis between each treatment vs. untreated group, $^{**}p< 0.0001$. NOR= normal, NEC= necrosis, APO= apoptosis. Magnification 400x

Figure 2. Purification of crude venom into six fractions: Fraction IV induces apoptosis in leukemia cells.

(**A**) Size-exclusion chromatography of crude venom from *Portidium nasutum* (150 mg) on Sephacryl S-200 HR, in 0.5 mM phosphate buffer (pH 7.2) at a flow rate 1 mL/min; 0.5 mL/tube, yielded six fractions (I-VI). Fraction IV is shown as black under the curve; (**B**) SDS-PAGE 12.5% (w/v) profile analysis of (20 μg) fraction (I-VI) stained with silver nitrate; (**C and D**) Fraction IV induces apoptosis in both Jurkat and K562 cells. Jurkat (C) and K562 (D) cells were either left untreated or treated with increasing concentrations (20-300 μg/mL) of fraction IV for 24 h. Nuclear morphological changes were evaluated using AO/EB staining and ΔΨm was evaluated using $DiOC_6(3)$. Bars represent normal (NOR), total apoptosis (early and late APO), necrosis (NEC), and loss of $\Delta\Psi_m$ produced by each treatment. The ANOVA showed significant differences among the groups, $p< 0.001$. The Bonferroni comparison test showed significant differences in the percentage of induction of apoptosis/necrosis between each treatment vs. untreated group, $^{**}p< 0.0001$.

Figure 3. Purification of fraction IV from *P. nasutum* venom by RP-HPLC.

(**A**) Fraction IV (2 mg) dissolved in 200 μL 0.1% trifluoroacetic acid (TFA) was further fractionated by reverse- phase high performance liquid chromatography (RP-HPLC) using a Pinacle DB C18 column (250 mm x 4.6 mm, 5 μm particle size, 140 Å pore size, RESTEK Corporation, cat·# 9414575) eluted with a 0- 66% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) and developed on a Shimadzu Prominence system at a flow rate of 0.7 mL/min. Absorbance was monitored at 215 nm. Circles represent sub-fractions (SF IV. 1-9); (**B and C**) SF IV. 1-9 induce apoptosis in both Jurkat and K562 cells. Jurkat (**B**)

and K562 (**C**) cells were either left untreated or treated with (20 μg/mL) SF IV.1- SF IV.9 for 24 h. Nuclear morphological changes were evaluated using AO/EB staining and ΔΨm was evaluated using $DiOC_6(3)$. Bars represent normal (NOR), total apoptosis (early and late APO), necrosis (NEC), and loss of $\Delta\Psi_m$ produced by each treatment. **(D**) 2D gel electrophoresis of sub-fraction SF IV.5 (red circle in A) shows a band of 25.9 kDa molecular weight, and isoelectric point of 4.1.

Figure 4. (A) Nasulysin-1 partial sequence alignment with some SVMP metalloproteinase homologous sequence obtained from Uniprot protein data. Access codes: *Bothrops moojeni*, BmooMPα-I (P85314); *Bothrops leucurus*, Leuc-A (P84907); *Bothrops asper*, BaP1 (P83512). The percentage of similarity (%) between amino acid sequence is shown in *blue*. (**B**) Deduced cDNA sequence of nasulysin-1 based on the sequence of *Bothrops moojeni* (BmooMPα-I) published by **Bernardes et al (2008)**. Amino acid residues directed sequenced from the protein and corresponding nucleotides are shown in *red.*

Figure 5. Nasulysin-1 specifically induces apoptosis in leukemia cells. (A) Jurkat, K562 and human peripheral blood lymphocytes were cells were either left untreated or treated with (20 μg/mL) SF IV.5 for 48 h. Nuclear morphological changes were evaluated using AO/EB staining and ΔΨm was evaluated using $DiOC_6(3)$. Circles represent total apoptosis (early and late APO), and loss of $\Delta\Psi_m$ produced by SF IV.5; (**B**) Percentage of the nuclear morphological changes and dissipation of ΔΨm evaluated by AO/EB staining and $DiOC_6(3)$ technique, respectively, in Jurkat, K562 and lymphocytes treated with 20 μg/mL SF IV.5 for 48 h. Noticeably, SF IV.5 induces apoptosis in both cells lines but not in

lymphocytes. The ANOVA revealed significant differences among the groups, $p< 0.001$. Except for the lymphocytes, the Bonferroni comparison test showed significant differences in percentage of apoptosis induction between each treatment vs. untreated group, $^{**}p< 0.0001$.

Figure 6. Nasulysin-1 induces simultaneous morphological changes in the nucleus and activation of the apoptosis-inducing factor (AIF) and caspase-3 in leukemia cells.

(A) Jurkat cells were exposed to (20 μg/mL) SF IV.5 for 48 h. Nuclear morphological changes were evaluated using AO/EB staining. Figure shows normal, condensed (**inset**) and fragmented (**asterisk**) nucleus, typical of apoptosis; (**B**) Jurkat, K562 and human peripheral blood lymphocytes cells were either left untreated or treated with (20 μg/mL) SF IV.5 for 48 h. After this time, cells were stained with anti-caspase-3 (Cas-3) and anti-AIF (AIF) according to the procedure described in *Materials and Methods*. Notice that Cas-3 and AIF positive nuclei (dark brown color) reflect their nuclear translocation/ activation and appear to correlate with the apoptotic nuclear morphology shown in (A); (**C**) Percentage of the nuclear morphological changes evaluated by AO/EB staining in Jurkat, K562 and lymphocyte cells treated with 20 μg/mL SF IV.5. The ANOVA showed significant differences among the groups, $p< 0.001$. Except for the lymphocytes, the Bonferroni comparison test revealed significant differences in the percentage of apoptotic cells between treatment and untreated groups, $^{**}p< 0.0001$. Magnification A (2900x, inset 3750x); magnification B (800x).

Figure 1

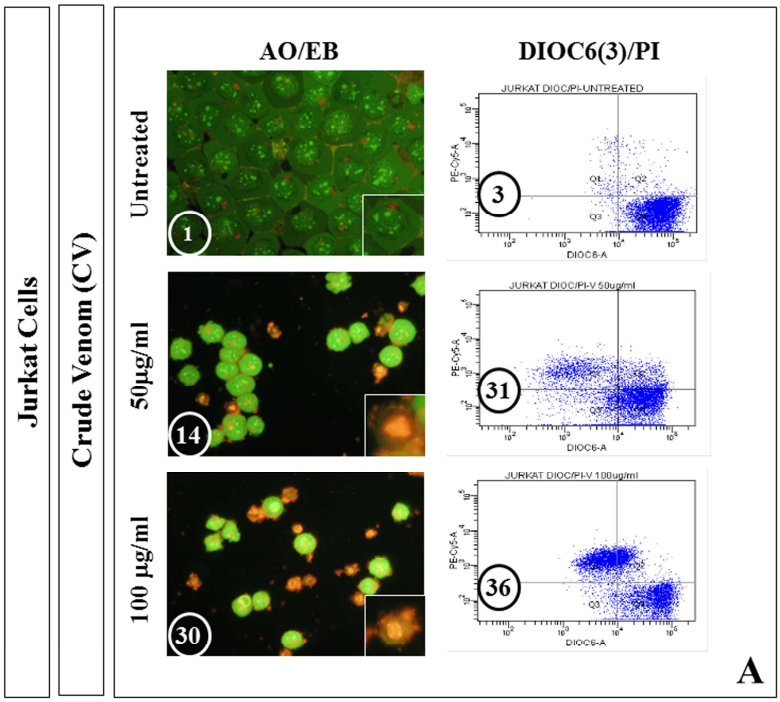


Figure 1

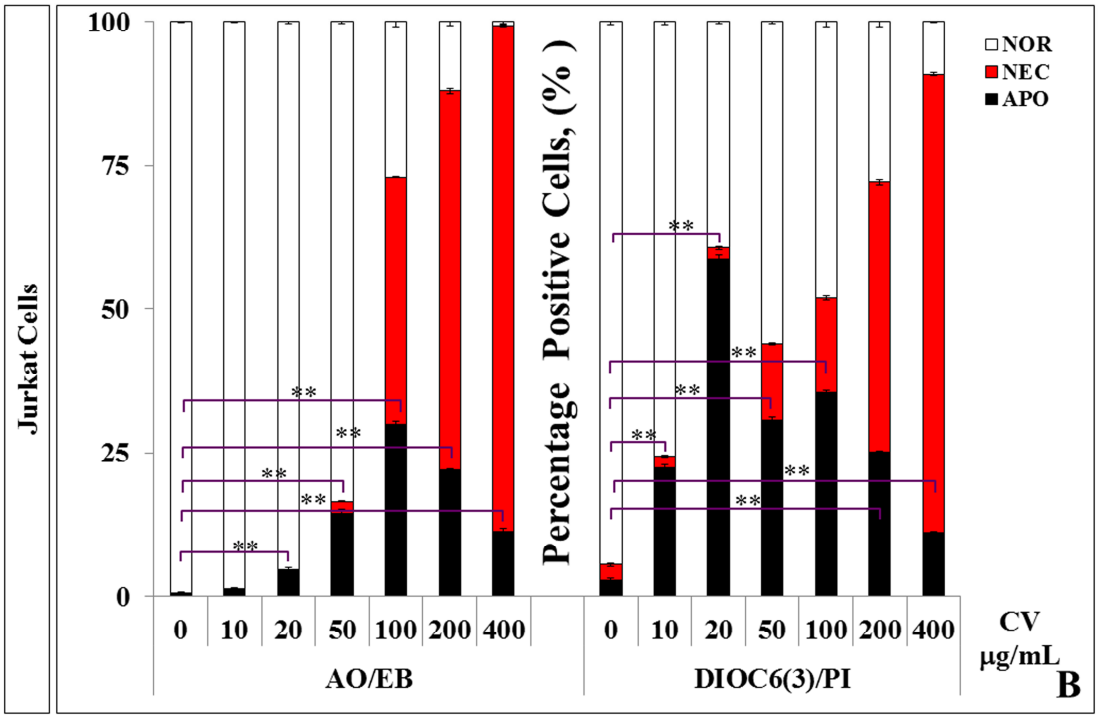


Figure 1

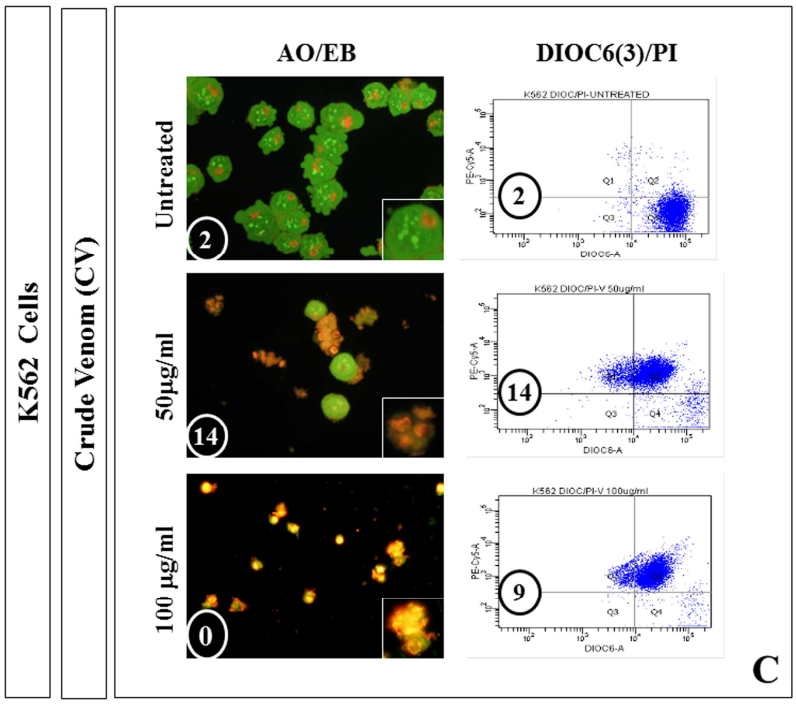
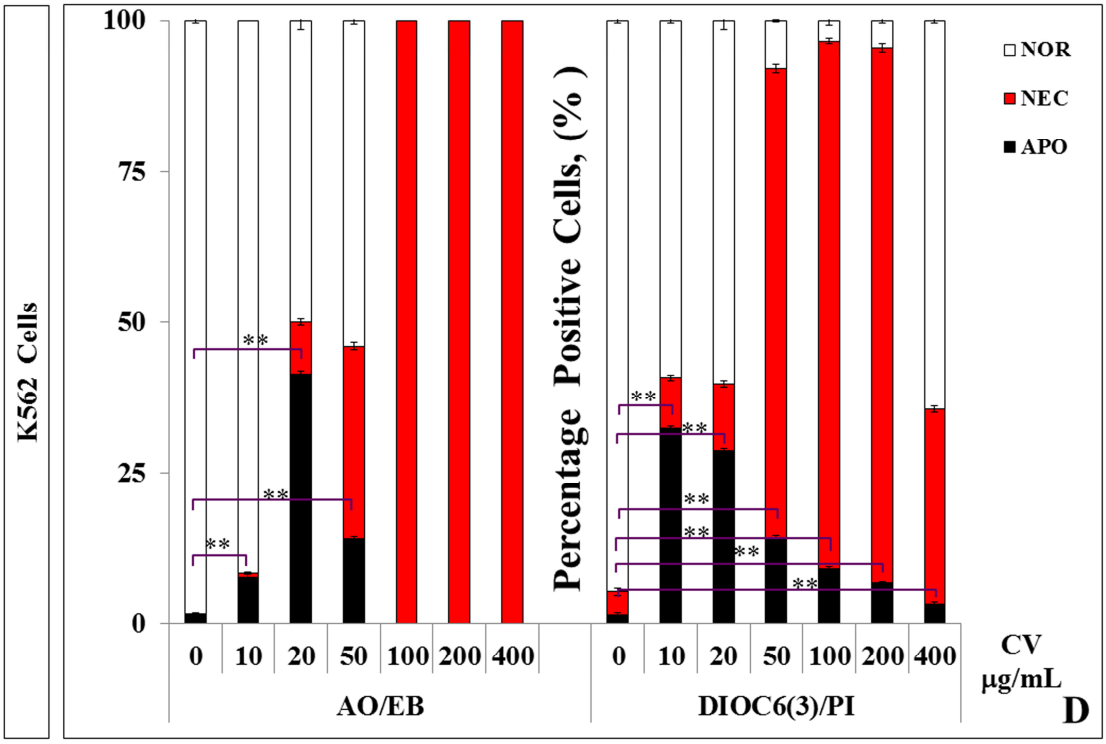


Figure 1



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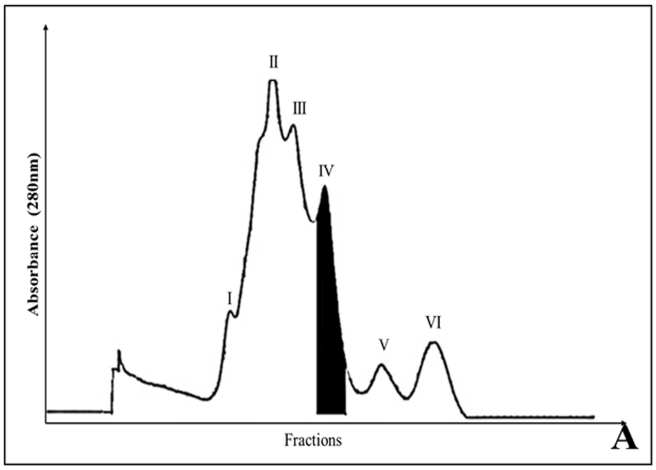

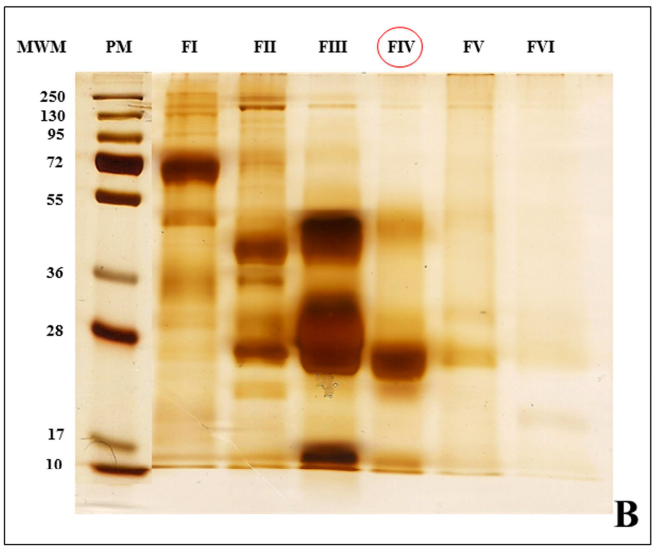

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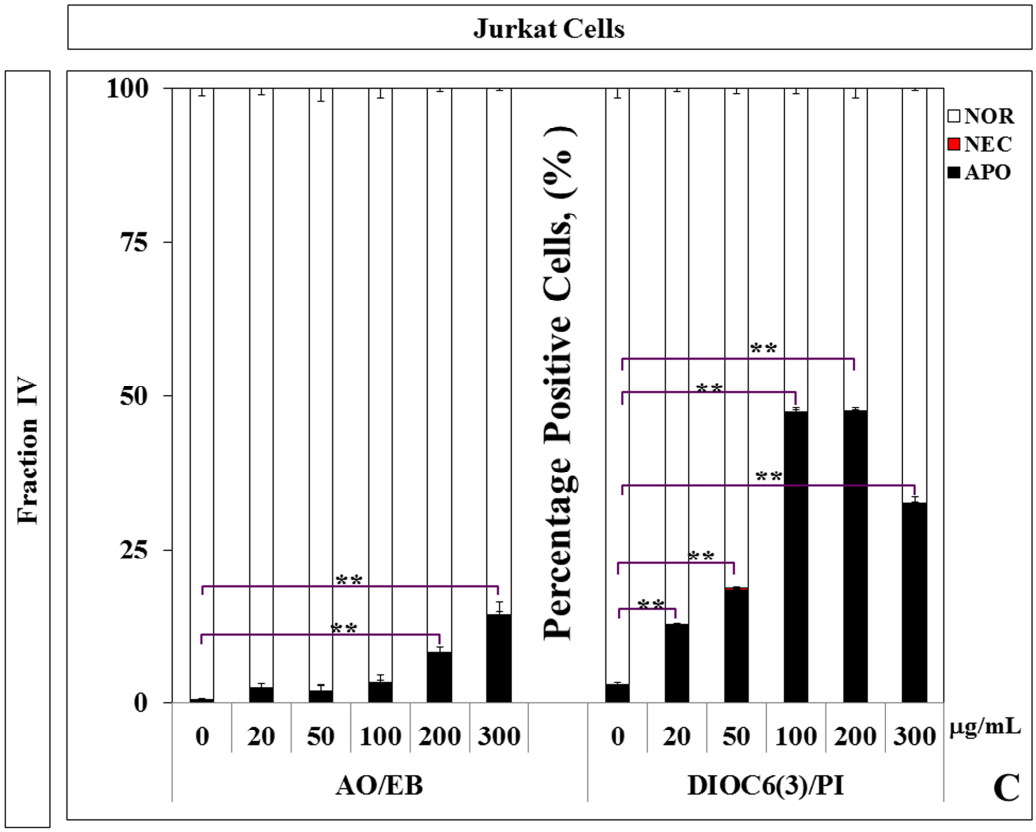

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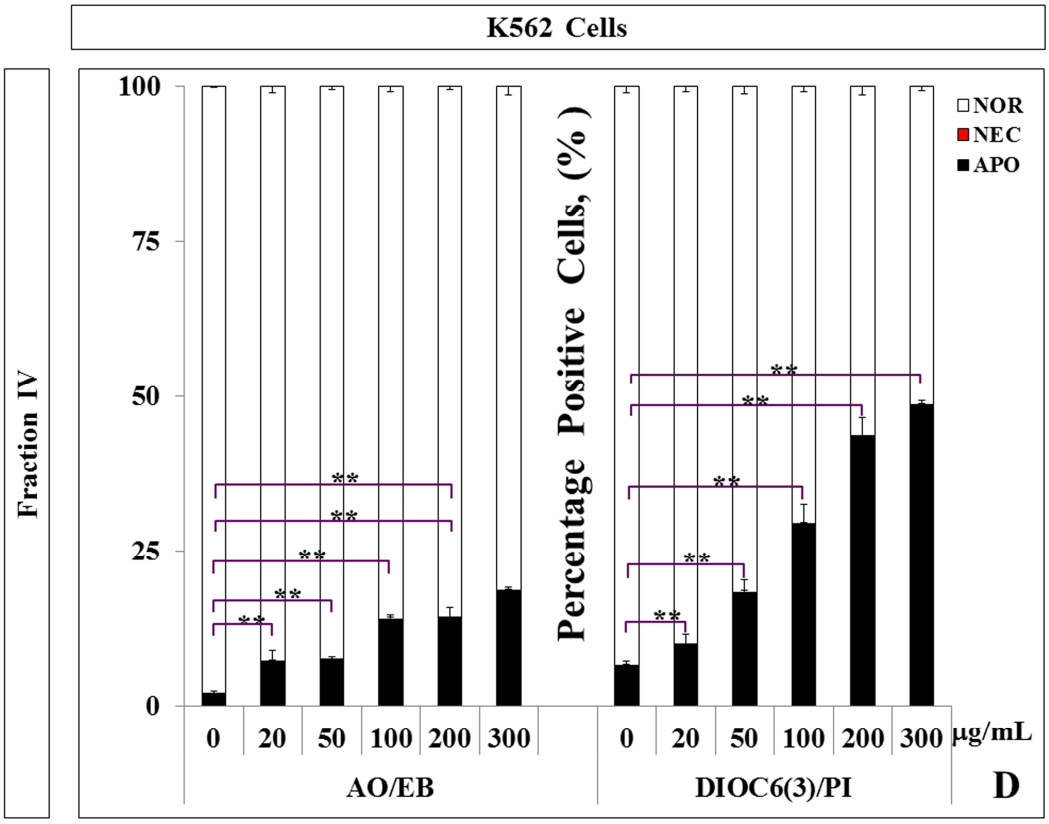

K562 Cells
Fraction IV
Percentage Positive Cells, (%)
100
75
50
25
0
NOR
NEC
APO
**
0
20
50
100
200
300
µg/mL
AO/EB
DIOC6(3)/PI
D

Figure 3

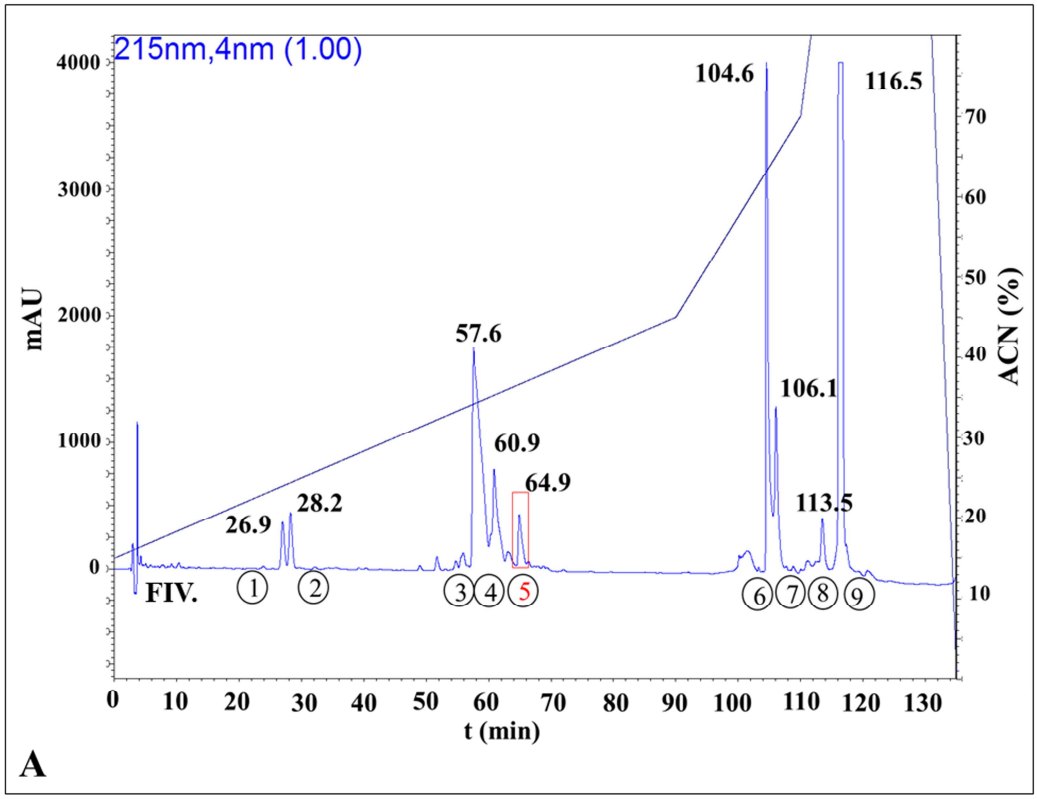
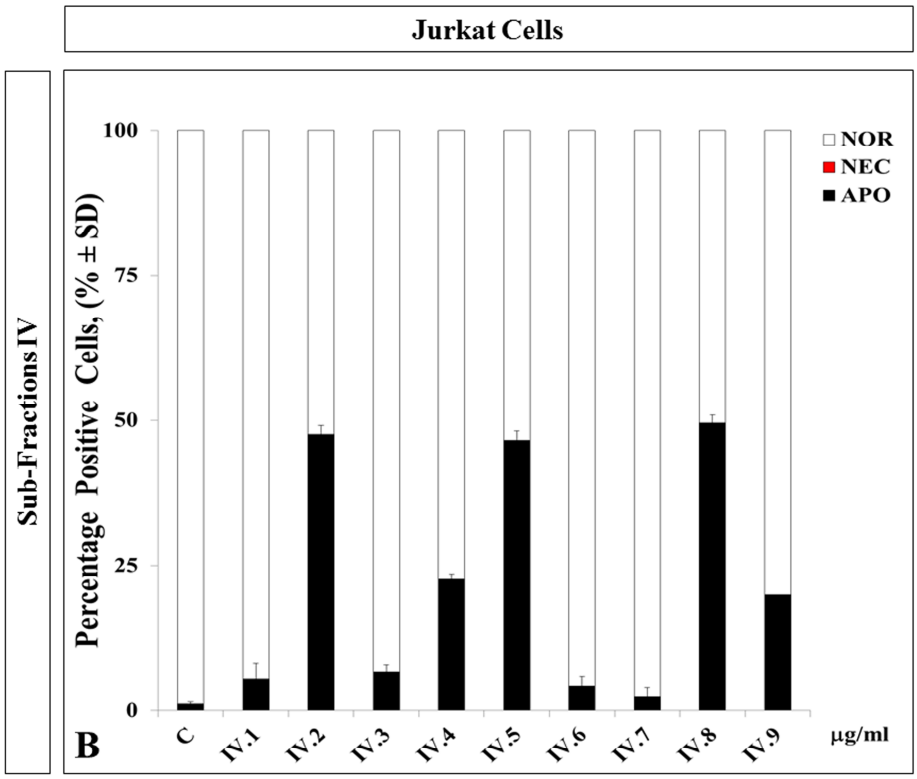


Figure 3



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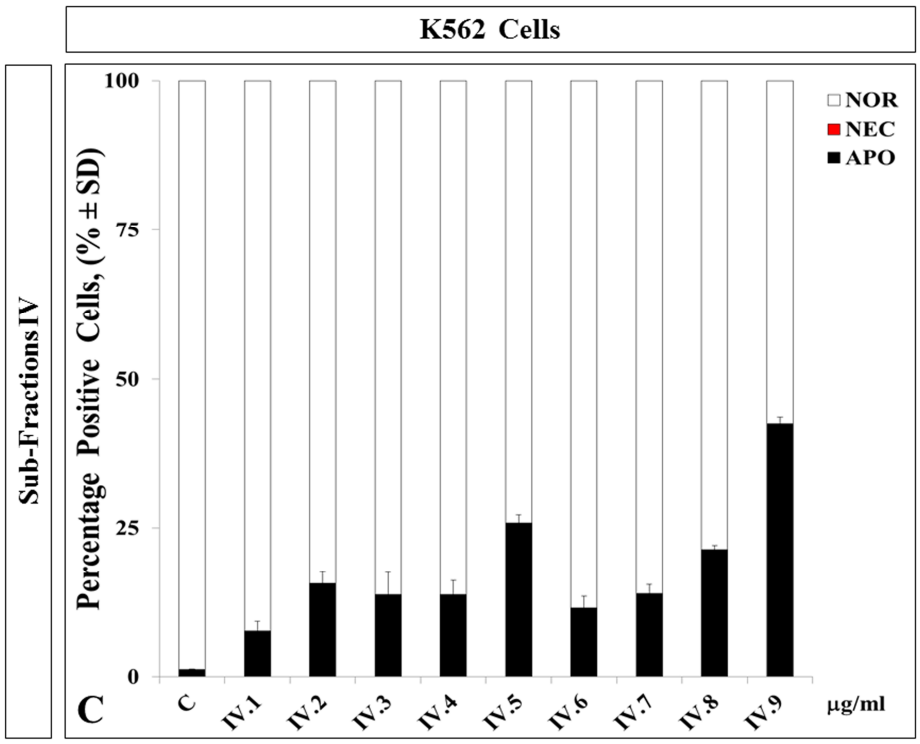


Figure 3

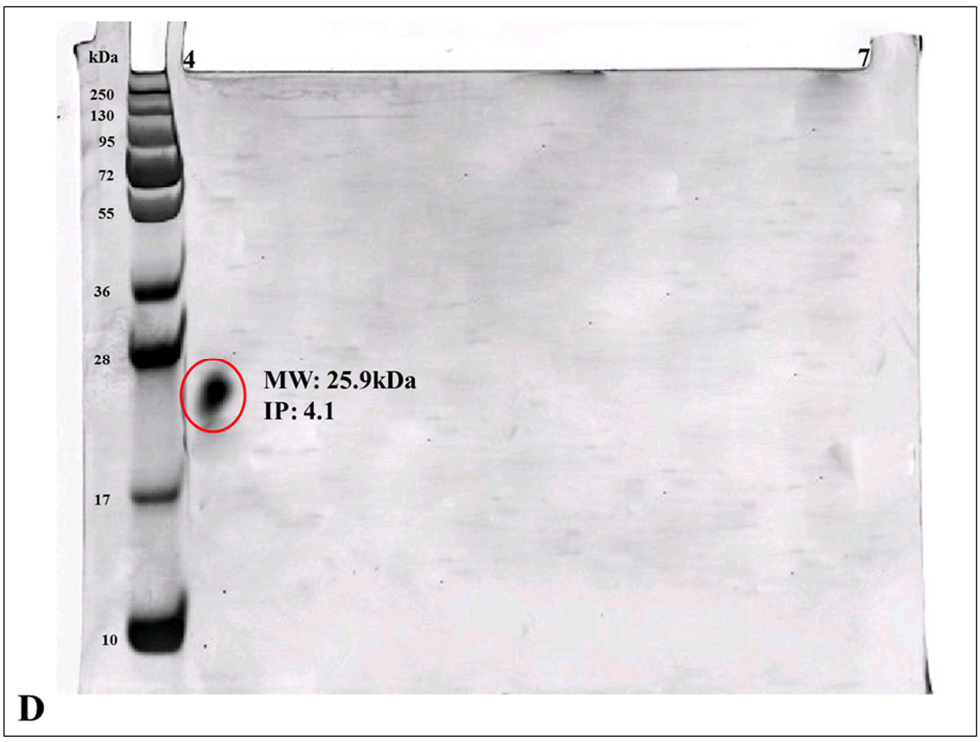


Figure 4

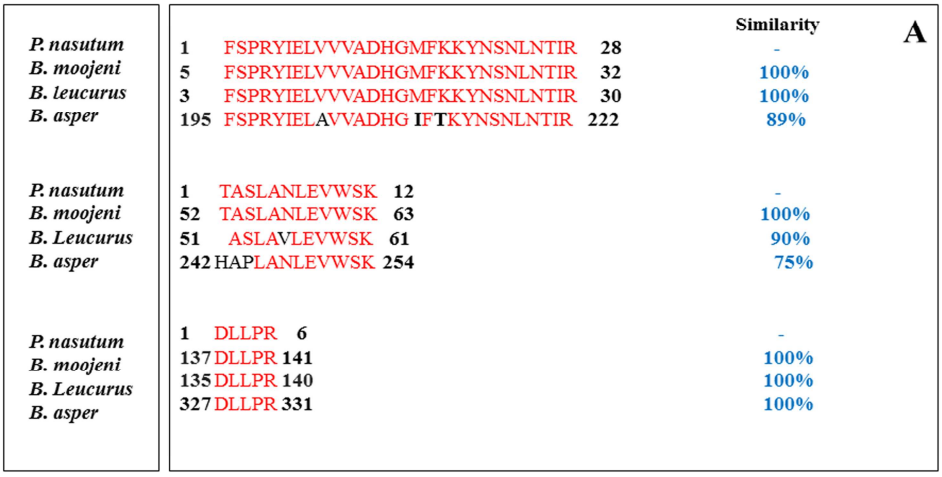

A
Similarity
P. nasutum
B. moojeni
B. leucurus
B. asper
1 FSPRYIELVVVADHGMFKKYNSNLNTIR 28
5 FSPRYIELVVVADHGMFKKYNSNLNTIR 32
3 FSPRYIELVVVADHGMFKKYNSNLNTIR 30
195 FSPRYIELAVVADHG IFTKYNSNLNTIR 222
-
100%
100%
89%
P. nasutum
B. moojeni
B. Leucurus
B. asper
1 TASLANLEVWSK 12
52 TASLANLEVWSK 63
51 ASLAVLEVWSK 61
242 HAPLANLEVWSK 254
-
100%
90%
75%
P. nasutum
B. moojeni
B. Leucurus
B. asper
1 DLLPR 6
137 DLLPR 141
135 DLLPR 140
327 DLLPR 331
-
100%
100%
100%

Figure 4.

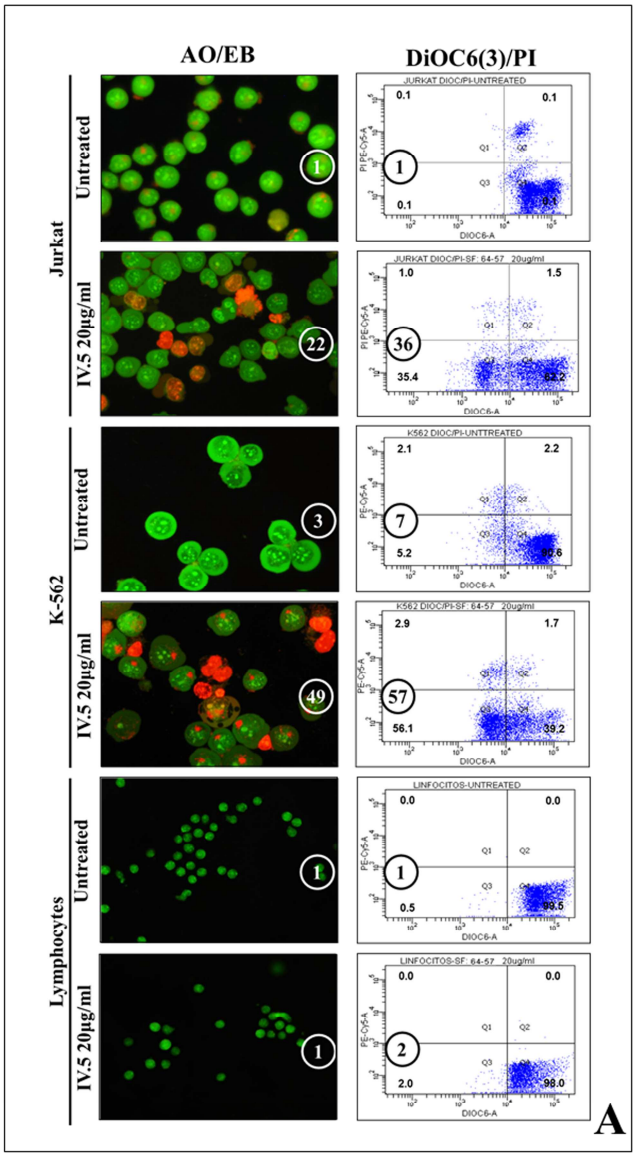

AO/EB
DiOC6(3)/PI
Jurkat
Untreated
IV.5 20µg/ml
K-562
Untreated
IV.5 20µg/ml
Lymphocytes
Untreated
IV.5 20µg/ml
1
22
3
49
1
1
JURKAT DIOC/PI-UNTREATED
JURKAT DIOC/PI-SF: 64-57 20ug/ml
K562 DIOC/PI-UNTTREATED
K562 DIOC/PI-SF: 64-57 20ug/ml
LINFOCITOS-UNTREATED
LINFOCITOS-SF: 64-57 20ug/ml
A

Figure 5

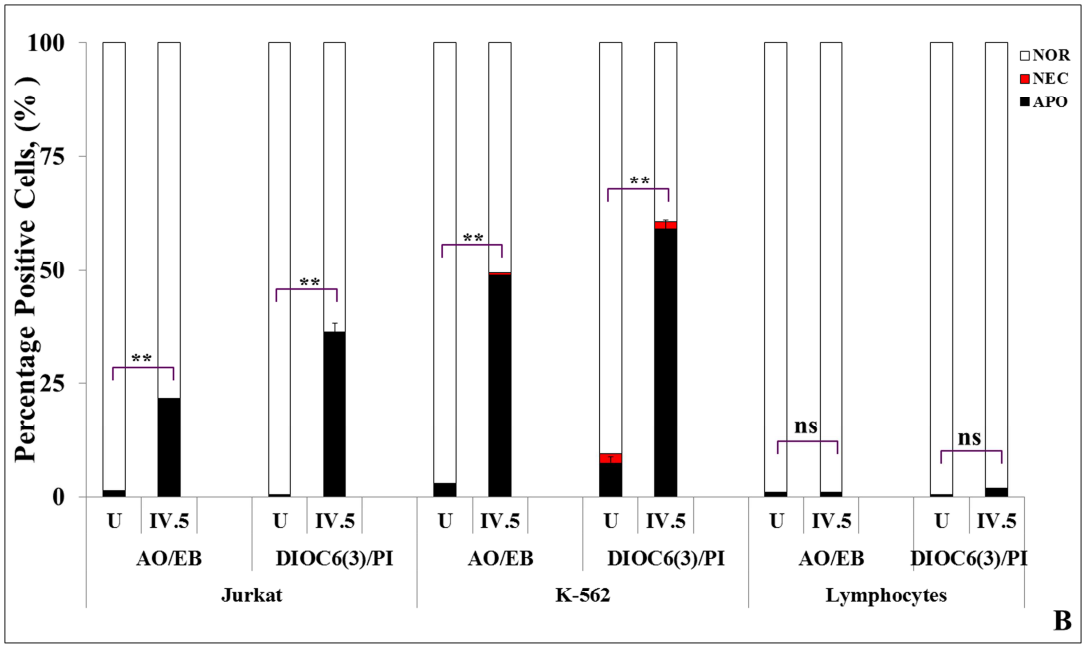
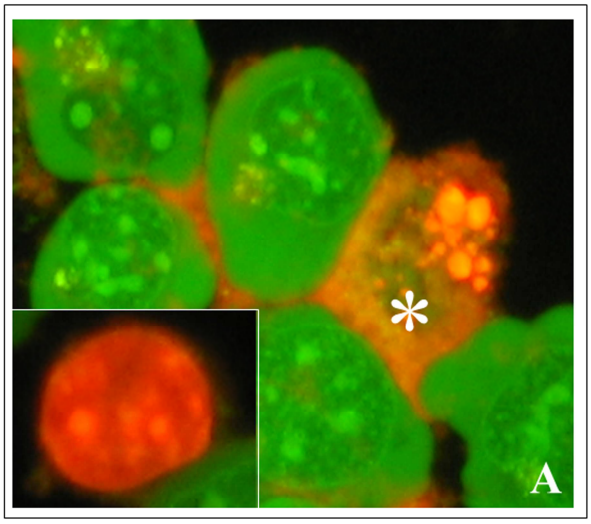


Figure 6



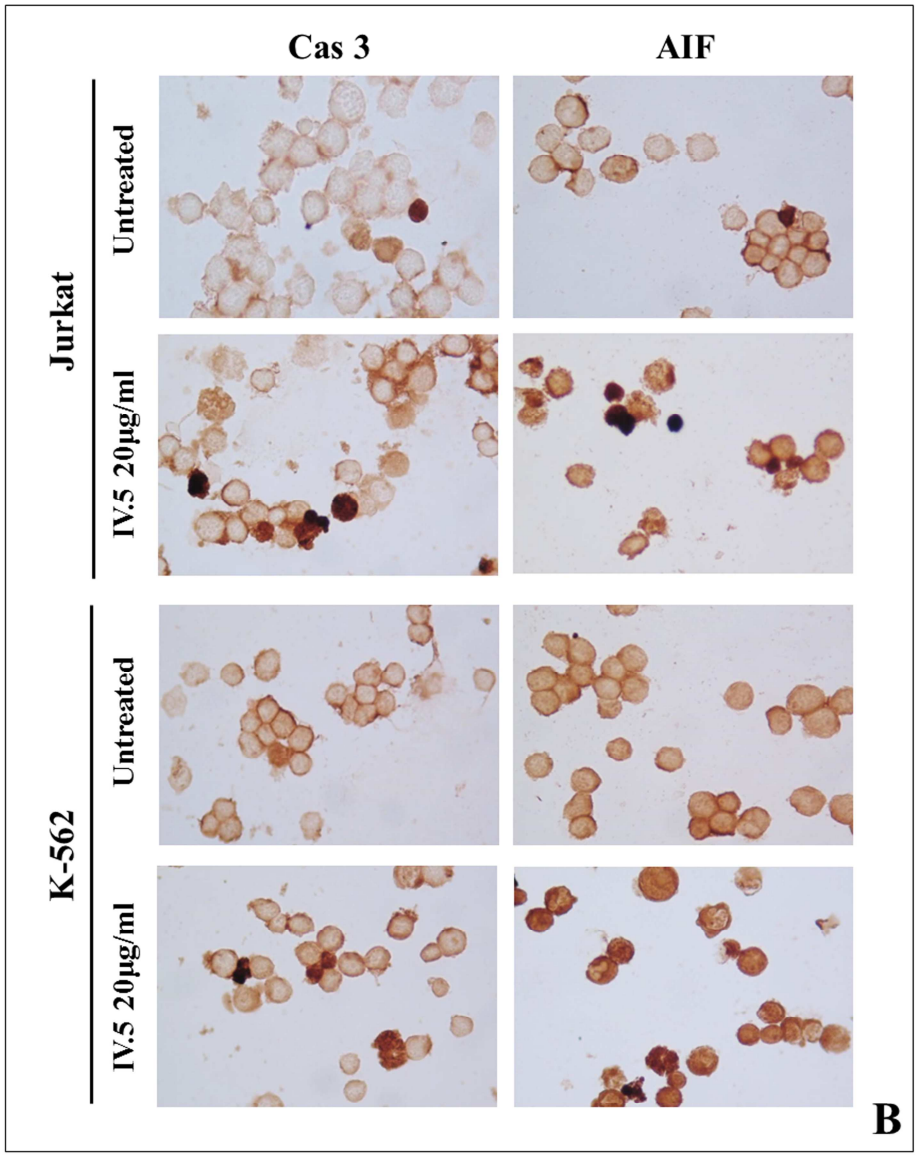
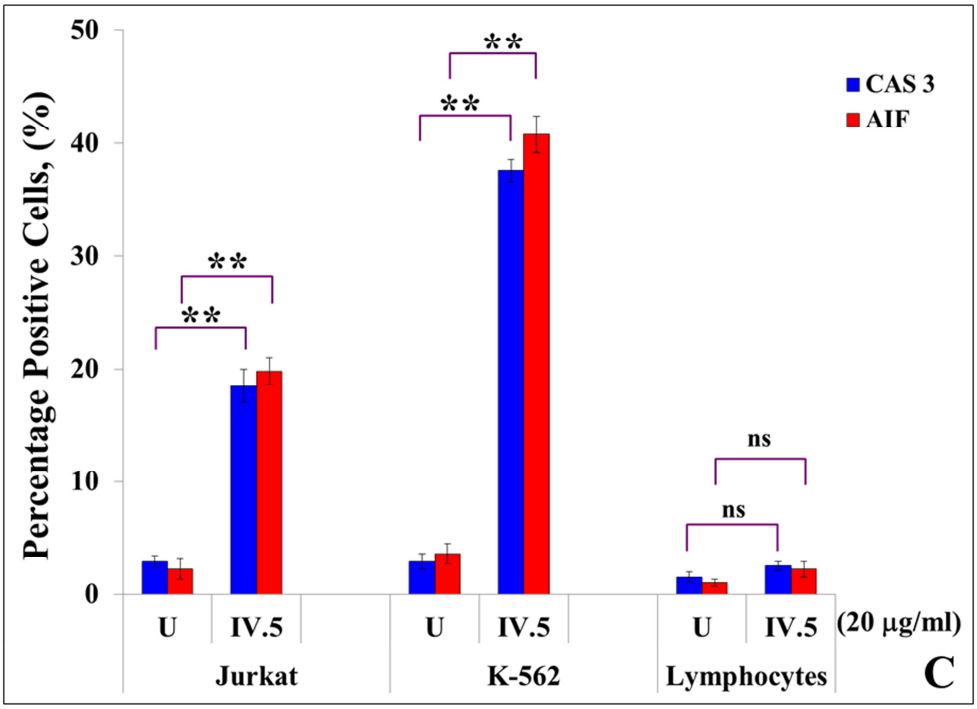


Figure 6

Figure 6



Purification of Nasulysin-1: a new toxin from *Porthidium nasutum* snake venom that specifically induces apoptosis in leukemia cell model through caspase-3 and apoptosis-inducing factor activation

Highlight points

- Nasulysin-1 is a new snake venom metalloproteinase
- Nasulysin-1 is a SVMP with a 26 kDa protein and pI 4.1
- Nasulysin-1 specifically induces apoptosis in leukemia (Jurkat and K562) cells
- Nasulysin-1 induced apoptosis is via caspase-3- and AIF- dependent mechanisms

Ethical Statement.

The authors declare that this manuscript contains original work and that it has not been published or submitted for publication elsewhere. If accepted it will not be published elsewhere in the same form, in English or in other language, without the written consent or the publisher. Additionally, authors declare no conflicts of interest and all authors have approved the final article.