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# Biological assays on the effects of Acra3 peptide from Turkish scorpion *Androctonus crassicauda* venom on a mouse brain tumor cell line (BC3H1) and production of specific monoclonal antibodies

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ABSTRACT

Constitutes of the venom scorpion are a rich source of low molecular mass peptides which are toxic to various organisms, including man. *Androctonus crassicauda* is one of the scorpions from the Southeastern Anatolia of Turkey with public health importance. This work is focused on the investigation of biological effects of Acra3 peptide from *Androctonus crassicauda*. For this purpose, Acra3 isolated from crude venoms was tested for its cytotoxicity on BC3H1 mouse brain tumor cells using tetrazolium salt cleavage and lactate dehydrogenase activity assays. To determine whether the cytotoxic effects of Acra3 was related to the induction of apoptosis, the morphology of the cells and the nuclear fragmentation was examined by using Acridin Orange staining and DNA fragmentation assay, respectively. Caspase 3 and caspase 9 activities were measured spectrophotometrically and flow cytometric assay was performed using Annexin-V FITC and Propidium Iodide staining. Furthermore toxic peptide Acra3 was used as an antigen for immunological studies.

Results showed that Acra3 exerted very strong cytotoxic effect on BC3H1 cells with an $IC_{50}$ value of 5 μg/ml. Exposure of the cells to 0.1 and 0.5 μg/ml was resulted in very strong appearance of the apoptotic morphology in a dose dependent manner. On the other side, not any DNA fragmentation was observed after treatment of the cells. Caspase 3 and 9 activities were slightly decreased with Acra3. Results from flow cytometry and lactate dehydrogenase activity assays indicate that Acra3 exerts its effects by inducing a stronger necrosis than apoptosis in BC3H1 cells. To evaluate its immunogenicity, monoclonal antibody (MAb) specific for Acra3 antigen (5B9) was developed by hybridoma technology using spleen and lymph nodes of mice and immunoglobulin type of antibody was found to be IgM.

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We suggest that Acra3 may exert its effects by inducing both necrotic and apoptotic pathway in some way on mouse brain tumor cells. These findings will be useful for understanding the mechanism of cell death caused by venom *in vitro*. Anti-Acra3 monoclonal antibody can be further used as a bioactive tools for exploring the structure/function relationship and the pharmacological mechanism of scorpion peptide neurotoxins.


## 1. Introduction

Scorpion venoms are known highly complex mixtures of components such as enzymes, peptides, nucleotides, lipids, mucoproteins, biogenic amines and other unknown substances. Among them peptides plays a fundamental role (Bosmans and Tytgat, 2007; Possani and Rodriguez de la Vega, 2006). Four different groups of toxic peptides have been described, which specifically interact with ion channels for sodium ($Na^+$), potassium ($K^+$), calcium ($Ca^{2+}$) and chloride ($Cl^-$). Based on the length of the sequences, generally two groups of peptides were identified: The first group is formed by long chain peptides containing 60–76 amino acids residues which mainly affect sodium channels. The second group is represented by short chain peptides containing 21–40 amino acids residues which are active on potassium, chloride and calcium channels (Possani et al., 2000). While most long chain scorpion toxins are known to be specific for $Na^+$ channels, there are small number of peptides are known specific to $K^+$ channels and also the peptides without disulphide bridges have been identified and characterized from scorpion venom (Bergeron and Bingham, 2012; Zeng et al., 2005). The venom alters the activity of the enzymes, receptors, or ion channels, thus disarranging the autonomic central and peripheral nervous system, the cardiovascular and neuromuscular systems, blood coagulation and homeostasis. In addition, peptides from scorpion venoms have a great potential as pharmacological tools (Calvete et al., 2009).

In Turkey, twenty-two different species of scorpions distributed into four families are reported (Koç and Yağmur, 2007). Among these species *Androctonus crassicauda* belongs to the Buthidae family play an important role in deadly accidents in humans (Altinkurt and Altan, 1980). The accidents mostly can cause severe pain, hyperemia, autonomic central nervous system and muscle function disturbances through a mechanism not yet fully understood (Dehghani and Khamehchian, 2008; Ozkan et al., 2006; Radmanesh, 1990). Application of specific anti-venom immunotherapy is one of the specific medical treatments for scorpion sting (De Roodt et al., 2010). In Turkey, horse anti-venom against to *A. crassicauda* crude venom has been prepared by Ministry of Health since 1942 (Adiguzel et al., 2007).

The biochemical composition of *A. crassicauda* venom is poorly known, despite the fact that it is most dangerous scorpion and many serious envenomation cases of humans have been reported. Although the crude venom consist of at least 80 different peptide components of molecular masses vary from 267 Da to 44,541 Da, up to date a few number of peptide toxins were described by our group (Caliskan et al., 2006). Recently, another new lethal peptide named as Acra3 composed of 66 amino acids residues with a molecular mass 7620 Da has been discovered from the *A. crassicauda* crude venom (Caliskan et al., 2012a). On the other hand, recently apoptotic and the anti-proliferative effects of *A. crassicauda* crude venom on SH-SY5Y and MCF-7 cells were reported (Zargan et al., 2011a). Cell death through apoptosis or necrosis is known to play an important role on normal tissue homeostasis (Jang et al., 2003). Apoptosis is a programmed cell death characterized by biochemical (mitochondrial fragmentation, activation of protease caspases) and morphological alterations (nuclear condensation and fragmentation, cytoplasmic shrinkage and membrane blebbing). Beside, necrosis is characterized by mitochondrial changes, uncontrolled osmotic pressure and finally swells and ruptures of cells. Up to date, several groups have declared principal toxicity mechanism of peptide toxins from venomous animals using *in vitro* tests with various cell lines, indicating their apoptotic or necrotic effects (D'Suze et al., 2010; Heinen and da Veiga, 2011).

Our previous results showed that Acra3 is highly toxic to mice when injected at low concentration whereas it was non-toxic to insects and crustaceans. Amino acid sequence of Acra3 was similar to $Na^+$-channel specific scorpion toxins with between 60 and 76 amino acid residues cross-linked by 4 disulfide bridges. Although the presence of these structural similarities, Acra3 was not found effective on 6 different sub-types of $Na^+$-channels *in vitro* (Nav1.1–Nav1.6) (Caliskan et al., 2012a). In the present study, cytotoxic effects and antigenic properties of Acra3 and crude venom were investigated both *in vitro* and *in vivo* methods, respectively. First of all, the crude venom was obtained by the electrostimulation of animals and then several fractions were fractioned by means of high performance liquid chromatography (HPLC) separations. Cytotoxic effects of the crude venom were determined on six different cell types *in vitro* by MTT assay. The cell lines were F2408 (embryonic rat fibroblasts), CO25 (mouse myoblasts) 5RP7 (H-ras active embryonic rat fibroblasts), A549 (human lung adenocarcinoma cells), WM115 (human malignant melanoma cells), NIH3T3 (mouse embryo fibroblasts), and BC3H1 (mouse brain tumor cells). BC3H1 cells were used for further investigations by several assays as described in material methods section. Furthermore, purified Acra3 peptide was used as an antigen for the development of polyclonal and monoclonal antibody (MAb 5B9). Cross-reactivity of monoclonal antibody was tested with enzyme-linked immunosorbent assay (ELISA). 5B9 MAb was produced in a large scale and purified with S300 size exclusion chromatography. As a result of the study, biological and immunological effects were described for Acra3 peptide from Turkish species of *A. crassicauda*.

## 2. Materials and methods

### 2.1. Chemicals, cell line and culture condition

All chemicals were molecular grade reagents obtained from providers. Cell lines F2408, CO25, 5RP7, A549, WM115 and NIH3T3 were used from the cell line collections of Anadolu University (Eskisehir, Turkey). The mouse brain tumor cell line BC3H1 (ATCC 171) was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) as a growth media supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% $CO_2$. Confluent adherent cultures were split using trypsin-EDTA.

### 2.2. Source of animals and chemicals

*A. crassicauda* and *Buthacus macrocentrus* species scorpions were collected from Harran and Akcakale town of Sanliurfa province, *Mesobuthus gibbosus* species were captured Sarıcakaya town of Eskişehir province of Turkey. They were kept alive in individual boxes and brought to the Venom Research Laboratory of Eskisehir Osmangazi University (ESOGU VAL). The crude venoms of scorpions were collected by electrical stimulation of the last segment of the tail the animals. The crude venoms was dissolved in distilled water and spun at 14,000 g for 15 min, the supernatant obtained and vacuum dried using Labconco vacuum concentrator and kept at −20 °C until use as described previously (Batista et al., 2006). The fractions and then Acra3 peptide from the effective fraction were separated and purified by several runs of high performance liquid chromatography from the crude venom. The peptide was freeze dried and stored at −20 °C (Caliskan et al., 2012a). The peptides were freshly prepared for each specific assay by dissolving in water or culture media. The crude venoms of *B. macrocentrus* and *M. gibbosus* were kept −20 °C and finally used for only cross-reactivity assays with anti-Acra3 as closely related antigens.

BALB/c (8 weeks old) mice were housed in our laboratory. Animal housing performed according to the guidelines of our university. The protocols of animal experimentations were approved by the Animal Ethical Committee (number 2006676) of Eskisehir Osmangazi University. All chemical products and reagents were used analytical or molecular grade depending on the protocols.

### 2.3. Viability assay

The viability of cells treated with scorpion venom was determined by the 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by the mitochondrial enzyme of metabolic active cells (Sylvester, 2011). The cells were cultured on to 96-well culture plates at the concentration of $3.5 \times 10^3$ cells per well. After 24 h incubation, medium was discarded and cells were treated with Acra3 at different concentrations (0.1–50 μg/ml). Following 24 and 48 h incubation, the medium was discarded and cells were treated with fresh medium containing MTT (1 mg/ml). Then cells were incubated further 4 h, medium was discarded and the formazan particules were solubilized by the addition of 200 μl per well of dimethylsulfoxide (DMSO). After 20 min of incubation on a shaker in dark at room temperature, the viability of the cell was determined by measuring the absorbance at 540 nm using a microplate reader (ELX 808 IU, Biotek Ins. Inc., Winooski, VT, USA). $IC_{50}$ value was calculated (Fu et al., 2004).

### 2.4. LDH (lactate dehydrogenase) activity assay

The LDH release from necrotic cells into the extracellular fluid was determined after 24 and 48 h of treatment with Acra3 by using the commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Mannheim, Germany). The assay is based on the cleavage of a tetrazolium salt INT (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) in the presence of LDH in the culture supernatant. For this purpose, only BC3H1 cells were cultured on to 96-well culture plates at a concentration of $3.5 \times 10^3$ cells per well. After 24 h incubation, the medium was removed and cells were incubated with a series dose of Acra3 ranging between 0.01 and 10 μg/ml for 24 and 48 h. Triton X-100 (10 μl/ml) was used as a positive control. After incubation, 100 μl of supernatant from the each well was removed and transferred into a new microplate and 100 μl of reaction mixtures was added to per well and incubated further 30 min. The absorbance was measured at 500 nm using the microplate reader.

### 2.5. Acridine orange (AO) staining

In order to investigate whether the cell death is a cause of apoptosis, AO staining was performed to provide the morphological evidence. For this purpose, $1 \times 10^5$ BC3H1 cells were cultured onto 6-well culture plate containing coverslips pre-coated with poly-L-lysine and incubated for 24 h. The medium was discarded and cells were treated with 0.5 and 1 μg/ml of Acra3 for 24 and 48 h. At the end of the incubations, cells were fixed in 70% ethanol for 15 min and washed with phosphate buffer saline (PBS), and then stained with AO (100 μg/ml in PBS). Morphology of the cells was visualized concerning on nuclear deformations under a flouresance photomicroscope (IX7158F3, Olympus, Japan). Abnormal cells were scored from approximately 2500 cells counted in at least 20 different fields of view (Cotter and Martin, 1996).

### 2.6. DNA fragmentation assay

DNA fragmentation is biochemical method which used to assess apoptosis and describes digestion of genomic DNA by endonucleases, leading a ladder of small fragments (Peitsch et al., 1993). Therefore, to investigate the apoptotic cleavage of DNA, $2.5 \times 10^5$ BC3H1 cells were cultured in 25 $cm^2$ culture flasks for 24 h, and then treated with 0.5 and 1 μg/ml of Acra3 for 24 and 48 h. Genomic DNA extraction

was performed using Apoptotic DNA Ladder Kit (Roche) as described by the manufacturer. Extracted DNA was analyzed by loading 10–20 μg of DNA into 1.8% agarose gel containing ethidium bromide (1 μg/ml) at 5 V/cm$^2$. The gel was then photographed on a transilluminator (UVitec Limited, Cambridge, UK).

### 2.7. Caspase 3 and 9 activation assay

Caspases as intracellular cysteine proteases are key enzymes required for the process of apoptosis (Wimmer et al., 2004). In this sense, caspase 3 and caspase 9 activities were investigated in Acra3 treated cells using commercial colorimetric kit according to the manufacturer's instructions (R&D systems, Inc. 1-800-343-7475). 2.5 × 10$^5$ BC3H1 cells were cultured in 25 cm$^2$ culture flasks for 24, and then treated with Acra3 at 5 μg/ml for 6, 12 and 24 h. Also cells were treated with 20 μM of cisplatin as a positive control. The cells were harvested and centrifuged at 250 × g for 10 min. Lysis buffer was added on the cell pellet (25 μl/10$^6$ cells) and incubated on ice for 10 min and then centrifuged at 10,000 × g for 1 min. The supernatant was transferred to a new tube and kept on ice. An equal volume of supernatant and 2X Reaction buffer containing 10 mM DDT (50 μl:50 μl per well) were mixed in a 96-well plate. As colorimetric substrates for caspase 3 and caspase 9, 5 μl of DEVD-pNA and LEHD-pNA were added to each well, respectively, and incubated for 2 h at 37 °C. Caspase activities were determined by measuring the absorbance at 405 nm using the microplate reader.

### 2.8. Flow cytometry assay

To investigate the apoptotic activity induced by the Acra3, BC3H1 cells were analyzed using commercial Annexin-V FITC flow cytometric kit according to the manufacturer's instructions (BD Bioscience). Briefly, 2.5 × 10$^5$ BC3H1 cells were cultured onto 25 cm$^2$ culture flasks and incubated with Acra3 at a different concentration (1 and 5 μg/ml). As a positive control, cells were treated with 20 μM of cisplatin. After 12 h incubation, the cells were washed with cold PBS two times, and then resuspended in 1X Binding Buffer. In a new centrifuge tube, 100 μl of the sample and the mixture of Annexin-V FITC (A) and Propidium Iodide (PI) dyes (5 μl + 5 μl) were added and incubated for 15 min at room temperature in dark. After incubation, 400 μl of 1X Binding Buffer was added to each sample. Flow cytometric analysis was performed on a FACS Calibur (BD Biosciences) equipped with CELLQuest® software.

### 2.9. Immunization

Eight week old BALB/c mice were immunized with 2.5 μg, 5 μg and 10 μg Acra3 antigen emulsified with equal volume of Freund's complete and incomplete adjuvant, respectively, and immunizations were performed intraperitonally. Booster immunizations were done in phosphate buffered saline (PBS). Antigen specific polyclonal antibody activity was detected by ELISA after fourth immunization and optimal immunization dose was determined as 5 μg protein/mouse.

### 2.10. ELISA

The indirect ELISA was used to detect antibody activity for Acra3 antigen (Douillard and Hoffman, 1983). Mice were bled from the tail vein and sera were collected. Microtiter ELISA plates were coated by overnight incubation with 0.5 μg/ml of Acra3 antigen in 10 mM phosphate buffer (PBS) (pH:7.2) at 4 °C. After washing with wash buffer (PBS+ 0.5% Tween-20), the wells were saturated with 200 μl of 1% BSA in PBS for one hour at 37 °C and washed three more times. Serial dilutions of anti-sera (1/25, 1/50, 1/100) or hybridoma supernatant were added to the wells and incubated for one hour at 37 °C. After washing, 100 μl of 1:1000 diluted alkaline phosphatase conjugated polyvalent anti-mouse antibody (Sigma) was added to the wells. After one hour at 37 °C, the plates were washed once again and 1 mg/ml para nitrophenyl phosphate (PNPP) in substrate buffer (0.1 M glycine, 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, pH:10.4) was added. Absorbance at 405 nm was measured by using Bio Tech EIA reader (Bio-Rad 3550, USA).

### 2.11. Fusion

The mouse with the highest antibody titer for Acra3 antigen was boosted intraperitonally. Fusion was carried out by some modifications of classical fusion protocol (Galfre and Milstein, 1981). Briefly, lymphocytes from spleen and lymph nodes (bronchial, axillar, inguinal, popliteal, and intraperitonal) were fused with F0 (ATCC CRL 1646) mouse myeloma cells and polyethylene glycol (PEG 4000, Merck, Germany) was used as the fusing agent. Hybrid cells were selected in HAT medium (5 μM hypoxanthine, 20 μM aminopiterine, 0.8 μM thymidine) and distributed in 96-well culture plates. Positive clones and cross reactivity of monoclonal antibodies with closely related antigens were tested with indirect ELISA. Monoclonal antibody producing hybrid cells were detected in plate 5, line B9 and then sub-cloned by limiting dilution.

### 2.12. Determination of antibody isotype

The class and subclasses of monoclonal antibody was determined by using commercial IsoStrip Mouse MAb subisotyping kit (Roche Applied Science, Indianapolis, IN).

### 2.13. Purification of monoclonal antibodies

Monoclonal antibody 5B9 was produced in large scale in tissue culture *in vitro*. After 45% ammonium sulfate precipitation and dialysis, the monoclonal antibody was purified with size exclusion colon chromatography using the Sephacryl S-300 (Sigma Chemical, USA) (2.5 × 25 cm) as the matrix.

### 2.14. Statistical analysis

All the data demonstrated are mean ± SD. Analysis between the groups was accomplished with one way ANOVA

(analysis of variance) with post hoc analysis by Tukey-HSD and Scheffe multiple comparison method. Any variation with $P < 0.05$ was considered to be significant.

## 3. Results

### 3.1. Collecting of scorpions and purification of Acra3

Scorpions were collected from the different areas as in addressed by Section 2.1. Totally 148 alive *A. crassicauda* scorpions were captured from the inside of the houses, barns, coops and ruined houses. On open land searching, not any *A. crassicauda* was observed. *B. macrocentrus* were collected also from the same locality with *A crassicauda*, but they were found only in open land. *Mesobuthus gibbosus* species also were captured in open land of the area. The soluble venom of *A. crassicauda* was fractioned by HPLC and Acra3 has been purified several steps of chromatographic separations as described earlier (Caliskan et al., 2012a).

### 3.2. In-vitro cytotoxicity of the crude venom, fractions and Acra3 on BC3H1 cells

The soluble venom of *A. crassicauda* was tested for its cytotoxic effects on several types of cell lines such as F2408, CO25, 5RP7, A549, WM115, NIH3T3 and BC3H1 by MTT assay. The exposure with 1, 10, 50, 100 and 250 μg/ml of the crude venom significantly decreased the viability of BC3H1 cells in a dose dependent manner after 48 h exposure; whereas 1 and 10 μg/ml of venom increased the cell number and then following doses decreased the cell viability after 24 h (Fig. 1A). The cell viability after exposure with 250 μg/ml of the venom for 48 h was declined up to approximately 50%. However, the crude venom slightly reduced the cell viability even at 250 μg/ml concentration after 24 h treatment. On the other hand, not any significant effect of the crude venom was observed on F2408, CO25, 5RP7, A549, WM115 and NIH3T3 cell lines (data's not shown, but available under request to the authors). According to these results, we have performed further investigation with only BC3H1 cells by treating with effective HPLC sub-fraction of crude venom containing Acra3 or Acra3 alone. The cells were treated with 20–60 μg/ml of the sub-fractions or 0.01–50 μg/ml of Acra3 alone for 24 and 48 h and analyzed for cell viability by MTT assay. As shown in Fig. 1B and C, the fraction and pure Acra3 significantly inhibited the cell growth in a dose and time dependent manner. IC50 value of the fraction was around 40 and 30 μg/ml (Fig. 1B), and Acra3 was around 5.5 and 3 μg/ml (Fig. 1C) for 24 and 48 h, respectively.

Additionally, the cytotoxicity of Acra3 was detected by measuring the activity of a cytosolic enzyme LDH released into the growth medium following the membrane distribution. As shown in Fig. 2, Acra3 caused a significant elevated LDH activity at all concentrations compared to Triton X-100 as a positive control, with a plateau between concentrations of 0.05–10 μg/ml. However, highest concentration of Acra3 (50 μg/ml) caused a significant increase in LDH levels as 69% and 81% after 24 and 48 h, respectively.

### 3.3. Morphological changes induced by Acra3

As a first evidence of morphological changes after incubation of BC3H1 cells with 0.5 and 1.0 μg/ml of Acra3 were detected after 24 h and 48 h using an inverted microscopy (Fig. 3). Untreated cells growing as a monolayer normally showed flattened fibroblastic morphology after 24 and 48 h as seen in Fig. 3A and D, respectively. Morphology of the cells was significantly changed after treatment with Acra3 compared to control. It was resulted in noticeable growth arrest, rounding and shrinking in shape, and loss of adhesion to the growth surface (Fig. 3B, C, E and F). Most of the cells appeared to be seriously damaged, deformed and small in shape. Some of them with flattened in shape and many granular structures such as vacuoles were also observed after 24 h exposure of Acra3 (Fig. 3B and C). These effects of Acra3 were seen less in the cells after 48 h exposure (Fig. 3E and F).

To investigate the apoptotic morphology for a possible mechanism of cell death, BC3H1 cells treated with 0.5 and 1.0 μg/ml of Acra3 for 24 and 48 h were examined after

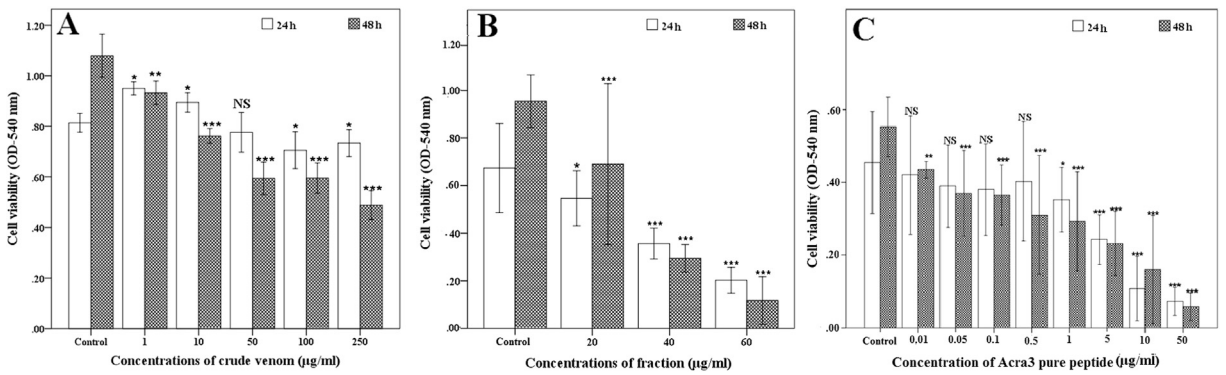


**Fig. 1.** Effects of (A) the crude venom (1–250 μg/ml), (B) the fraction 33.3 RT (20–60 μg/ml) and (C) Acra3 peptide (0.01–50 μg/ml) from *A. crassicauda* scorpion venom on the proliferation of BC3H1 cells measured by MTT assay. Results are shown as optic density ($OD_{540nm}$) ($n = 4$, mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group, NS: non-significant) using one-way ANOVA, Post Hoc, Scheffe test. Test of homogenecity of variances > 0.05 for B and C. Because of the test of homogenecity of variances < 0.05, Dunnett T3 test was performed for A.

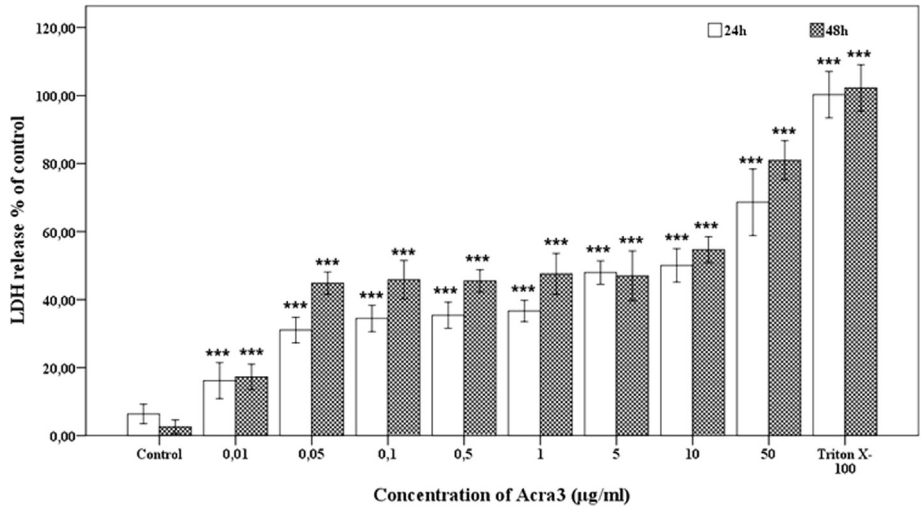


**Fig. 2.** Effect of Acra3 peptide (0.01–50 μg/ml) on cell cytotoxicity on BC3H1 cells determined by LDH release assay. Triton X-100 (10 μl/ml) was used as a positive control. Results are shown as % of control cells ($n = 4$, mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group, NS: non-significant) using one-way ANOVA, Post Hoc, Scheffe test. Test of homogenecity of variances > 0.05.

staining with acridine orange. As presented in Fig. 4A and D, normal cells had rounded nucleus in the center of cytoplasm. However, the percentage of the cells with abnormal nuclei was increased significantly in a dose and time-dependent manner after Acra3 exposure (Fig. 4B, C, E, and F). In these cells, morphology of nuclei was a large fragmented and deformed structure in general and cells were appeared to be neither necrotic nor apoptotic, showing multinucleated-like or cauliflower shape. The cells with these abnormalities were scored and expressed as the percentages of total abnormal cell number (Table 1).

### 3.4. DNA fragmentation assay

DNA fragmentation assay was also performed to investigate the mechanism of cell death (Korsnes et al., 2006). DNA extracted from BC3H1 cells treated and untreated with Acra3 for 24 and 48 h were resolved on 1.8% agarose gel as seen in Fig. 5. Treated cells, with 0.5 and 1.0 μg/ml of Acra3 for 24 (Fig. 5B and C) and 48 h (Fig. 5E and F) did not induce DNA fragmentation in both of concentration compared to the untreated cells (Fig. 5A and D) and the ladder marker control (Fig. 5M).

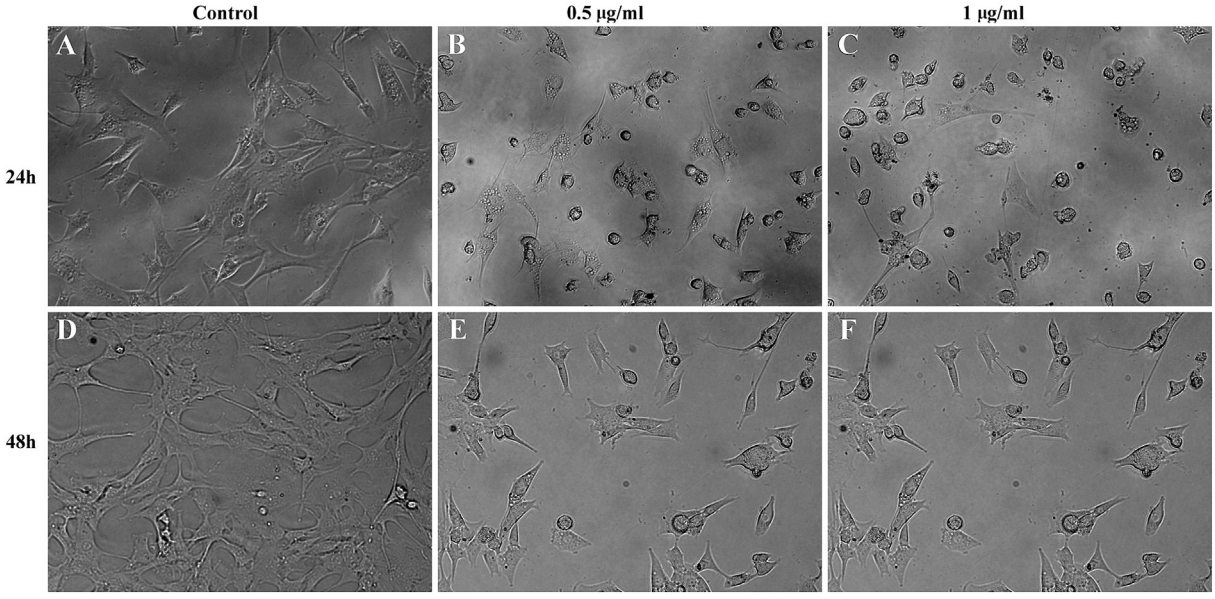


**Fig. 3.** Morphological observation of BC3H1 cells by inverted microscopy. (A) Control cells, (B) treated cells with 0.5 μg/ml and (C) 1.0 μg/ml of Acra3 after 24 h (D) control cells, (E) treated cells with 0.5 μg/ml and (F) 1 μg/ml of Acra3 after 48 h. Magnification: ×200.

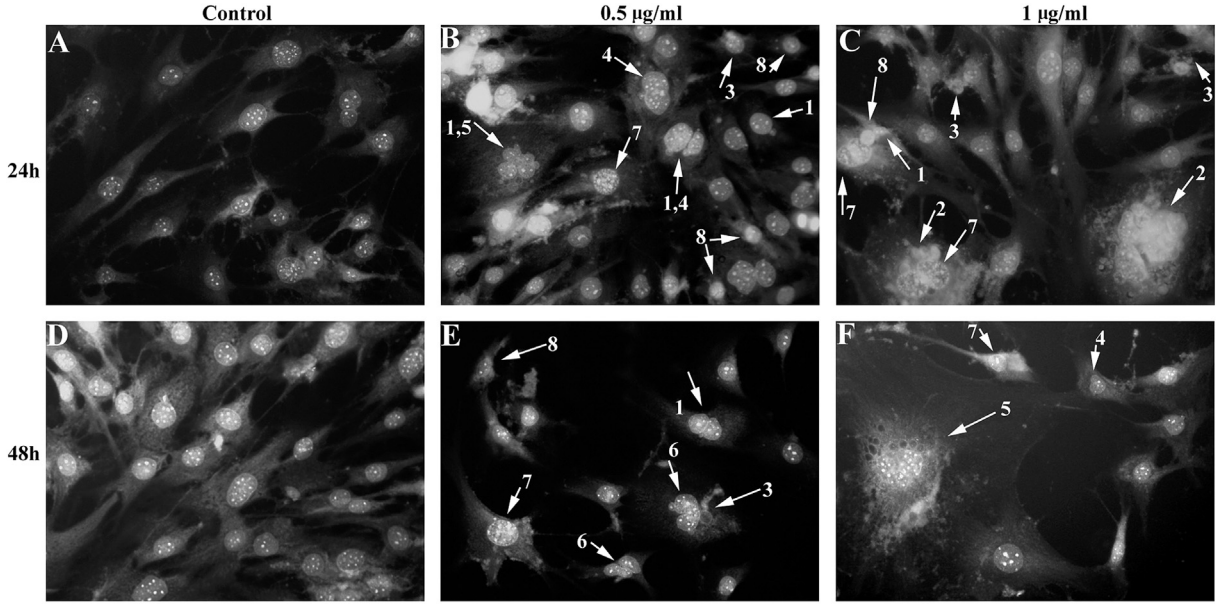


**Fig. 4.** Fluorescence microscopic images of BC3H1 cells treated with Acra3 using acridine orange staining. Control cells (A), treated cells with 0.5 μg/ml (B) and with 1 μg/ml (C) of Acra3 for 24 h. Control cells (D), treated cells with 0.5 μg/ml (E) and 1 μg/ml (F) of Acra3 for 48 h. The numbers show that multinuclear cell (1); fragmented nucleus (2); apoptotic bodies (3); deformed nucleus (4); cauliflower shape nucleus (5); kidney shape nucleus (6); condensed chromatin (7) and shrinked nucleus (8). Magnification: ×400.

### 3.5. Caspase 3 and caspase 9 activities

The caspases are aspartate-specific cysteine proteases which execute apoptosis and the evaluation of their activity is widely used for the detection of apoptosis in various cell types (Benjamin et al., 1998). Here, the induction of caspase 3 and 9 activation were analyzed in BC3H1 cells treated with 5 mg/ml of Acra3 for 6, 12 and 24 h Acra3 did not cause an increase in the level of both caspase 3 and caspase 9 activity, even slightly caused a decrease (See Fig. 6A and B).

### 3.6. Flow-cytometric analysis of apoptosis

Here, a possible induction of apoptosis by Acra3 in BC1H cells was investigated by measuring phosphatidylserine (PS) residues using a flow cytometer (See Fig. 7). In the results, A−/PI− cell population was regarded as normal, while A+/PI− cells were the early apoptotic status. A+/PI+ cells was regarded as late apoptotic and A−/PI+ cells as necrotic or death status (Das Gupta et al., 2007). After incubation of BC1H3 cells with Acra3 for 12 h, the percentage both of A+/PI+ and A−/PI+ was increased in a dose dependent manner as compared to the untreated cells. In untreated cells, a small percentage of late apoptotic and necrotic cells or death cells were also observed spontaneously. The percentage of A−/PI+ as necrotic or death cells was found to be higher than A+/PI+ in both concentration of Acra3. Late apoptotic cells were around 6.3% and 9.4%, necrotic or death cells was 10% and 19.1% in 1 μg/ml and 5 μg/ml of Acra3 treated cells, respectively. On the other hand, the percentage of A+/PI+ late apoptotic cells was very high (93.3%) after treatment of cells with 20 μg/ml of cisplatin used as positive control.

### 3.7. Production and isolation of monoclonal antibodies to Acra3 antigen

In order to produce monoclonal antibody specific for Acra3, mice were immunized with 2.5 μg, 5 μg and 10 μg of Acra3 antigen. The optimum concentration for polyclonal

**Table 1**
Effects of Acra3 on the ratio of morphological abnormalities in BC3H1 cells after 24 and 48 h using fluorescence staining assay. Values were quantified by random counting of AO stained cells from three independent experiments.

| Cell morphology | 24 h (cell number) | | | 48 h (cell number) | | |
|---|---|---|---|---|---|---|
| | Control | 0.5 μg/ml | 1 μg/ml | Control | 0.5 μg/ml | 1 μg/ml |
| Fragmented nucleus | 13 | 162 | 198 | 17 | 113 | 276 |
| Deformed nucleus | 19 | 164 | 266 | 58 | 522 | 802 |
| Chromatin condensation | 7 | 13 | 10 | 5 | 9 | 33 |
| Multinucleated cells | 7 | 73 | 46 | 4 | 46 | 84 |
| Micronucleated cells | 2 | 5 | 27 | 7 | 35 | 20 |
| Total cell number | 2878 | 2217 | 2502 | 2793 | 2223 | 2482 |
| Total abnormal cell (%) | 1.65 | 18.82 | 21.83 | 3.51 | 32.22 | 48.14 |

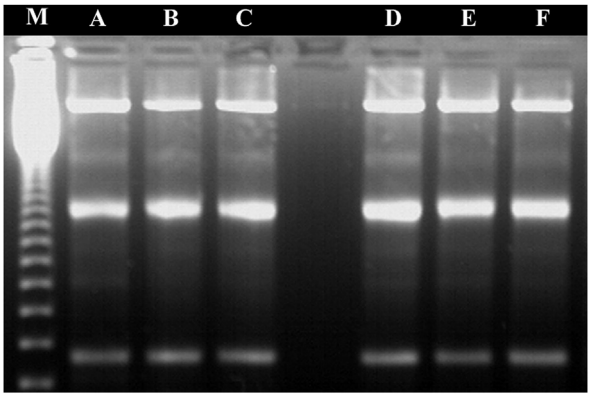


**Fig. 5.** DNA fragmentation analysis of BC3H1 cells by gel electrophoresis. (A and D) untreated cells as a control, treated cells with 0.5 μg/ml (B and E) and 1 μg/ml (C and F) of Acra3, for 24 and 48 h, respectively. M: Molecular weight marker.

antibody response without any toxic effect was observed in 5 μg antigen immunized mice. Mouse with the highest polyclonal antibody titer for Acra3 antigen was selected for fusion. Lymphocytes from spleen and lymph nodes were fused with mouse myeloma cells and after successful fusion, a monoclonal antibody specific for only Acra3 antigen was developed and named as 5B9, referring to plate 5 and line B9.

Cross reactivity of polyclonal antibodies and 5B9 monoclonal antibody was tested with indirect ELISA by coating the plate with other crude venoms and human serum. As it is seen in Fig. 8, 5B9 monoclonal antibody was specific only for Acra3 antigen and immunglobulin type of antibody was determined as IgM by using commercial IsoStrip sub-isotyping kit. Anti-Acra3 monoclonal antibody 5B9 was produced in large scale *in vitro*, and then purified with ammonium sulfate precipitation and S-300 size exclusion chromatography (Fig. 9).

## 4. Discussion

Buthidae family especially its *A. crassicauda*, *Leiurus abdullahbayrami* (earlier wrongly identified *as Leiurus quinquestriatus*), *M. gibbosus* and *Buthacus macrocentrus* species are known as medically important species in the country (Caliskan et al., 2012b). Although the toxicity of the venom from *Leiurus* species has been known to be more effective than the venom from *Androctonus* species (Tulga, 1964), *A. crassicauda* is responsible for all fatal cases of scorpion accident in the country. It was not surprise, because of this species is well known with their extreme anthropotolerance feature (Vignoli et al., 2003) as they were captured from the inside of the houses, barns, coops and ruined houses in the present study. The location of *A. crassicauda* indicates the importance of the species in the public health.

Here, crude venom, fractions and Acra3 from *A. crassicauda* were investigated for their some biological characteristics and compared in BC3H1 cells. Variations in the effects of the crude venom on growth after 24 h exposure might be a result of complexity of the venom. As expected, pure Acra3 was more effective on the cell growth than the crude venom and the fraction. Recently, Zargan et al. (2011a) reported a quite similar result suggesting a variation in the effect of crude venom from *A. crassicauda* with an IC50 value as 200 μg/ml for neuroblastoma and breast cancer cells after 24 h exposure.

The cell death mechanism were investigated in term of necrosis or apoptosis. Here, an increase in LDH release proved the decreased cell viability. However, the plateau in LDH release seen at 0.05–10 μg/ml indicates the cytotoxic effect of Acra3 could not be attributed to only necrosis. Zargan et al. (2011a) also showed that 50–100 μg/ml of crude venom of *A. crassicauda* significantly increased LDH release in neuroblastoma and breast cancer cells after 24 h. This effect can be dependent on Acra3 or other peptides present in the venom. According to cell morphology

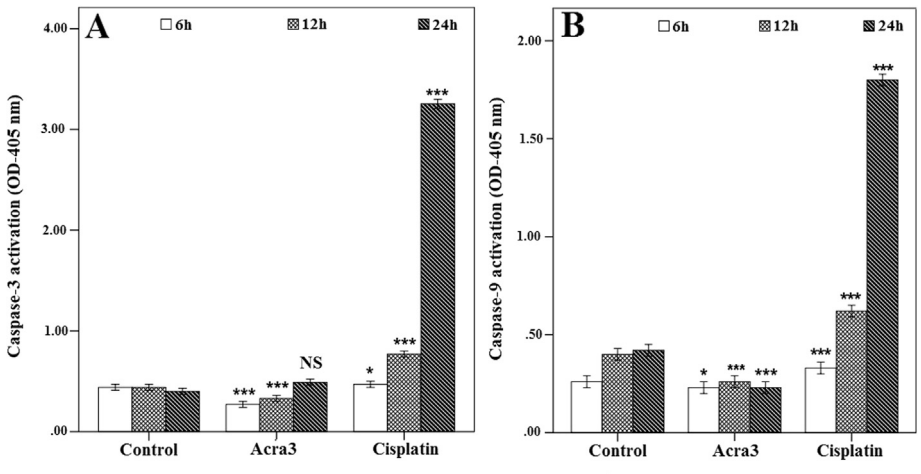


**Fig. 6.** The activities of caspase 3 (A) and 9 (B) were measured in Acra3 treated BC3H1 cells. Results are shown as optic density ($OD_{405nm}$) ($n = 3$, mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group) using one-way ANOVA, Post Hoc (Tukey-HSD and Scheffe) tests. Test of homogenecity of variances > 0.05.

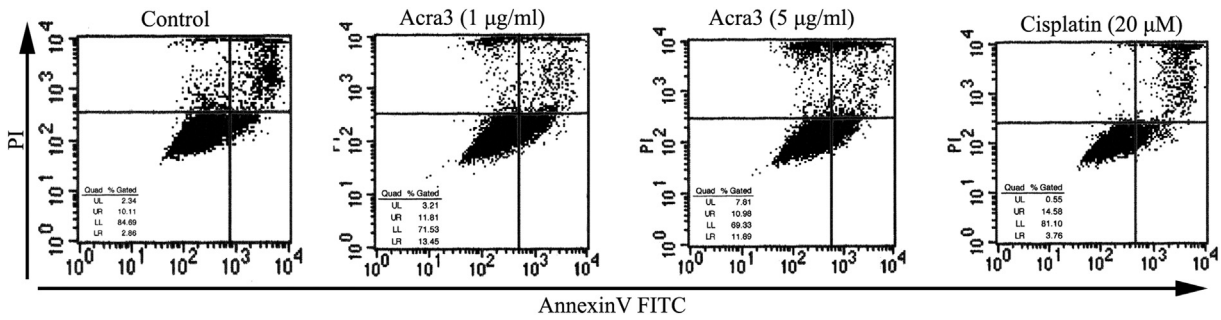


**Fig. 7.** Flow cytometric analysis of BC3H1 cells incubated with or without Acra3. Cells were stained with FITC annexin V and PI then analyzed on an FACS Calibur instrument (Becton Dickinson).

visualized by acridine orange staining, the effects of Acra3 was neither necrotic nor apoptotic, since observation of multinucleated-like or cauliflower shape nuclei. We suggest that the abnormality of nuclei may begin with kidney-like shape, then multinucleation and finally fragmentation while the cell swell. On the other hand, some of the cells showed some characteristics of apoptotic morphology with apoptotic bodies and condensed chromatin (Robertson et al., 2000). A similar cauliflower morphology of nuclei was also reported for A549 cells treated with Taxol and termed as indeterminate morphology (Ramage et al., 2006). These cells could not be easily categorized as normal or apoptotic cells. We suggest that Acra3 may affect microtubule function. Then, fragmented nuclei and multinucleation may be resulted from the cells arrested in the early stages of mitosis. Generally effects of Acra3 on cell morphology were seen much more after 24 h than 48 h. It might be due to insensitivity of remained cells to Acra3 or degradation of Acra3 at end of the 48 h incubation.

As a characteristics of apoptosis, Acra3 did not cause DNA fragmentation in the cells, supporting the results related with necrosis. Also Zargan et al. (2011a) reported that extracted DNA of cells treated with 50 μg/ml of *A. crassicauda* crude venom showed two fragments whereas crude venom did not cause any fragmentation at 100 μg/ml concentrations in SH-SY5Y and MCF-7 cell lines. We can suggest that Acra3 may trigger apoptosis in small number of cells, but cells withdraw the early stages of apoptosis and undergo some other mechanism such as swelling, rupture and/or second stage of necrosis. This postulation is in part in agreement was reported previously; the apoptotic effects of *L. quinquestriatus* scorpion venom on primary human embryonic kidney cell were suggested more prominent in the early stages of toxicity, while other forms of cell damage occurred in the later stages (Omran, 2003). Acra3 did not increase the level of both caspase 3 and caspase 9 activity, even slightly caused a decrease (See Fig. 6A and B). However, enhanced activity of caspase 3 was reported after 24 h treatment of SH-SY5Y and MCF-7 cells with crude venom of *A. crassicauda* (Zargan et al., 2011a). Some other studies also reported increased caspase 3 activity following treatment with venom from different scorpion species on different cell types (Gao et al., 2005; Zargan et al., 2011b). Phosphatidylserine (PS) residues

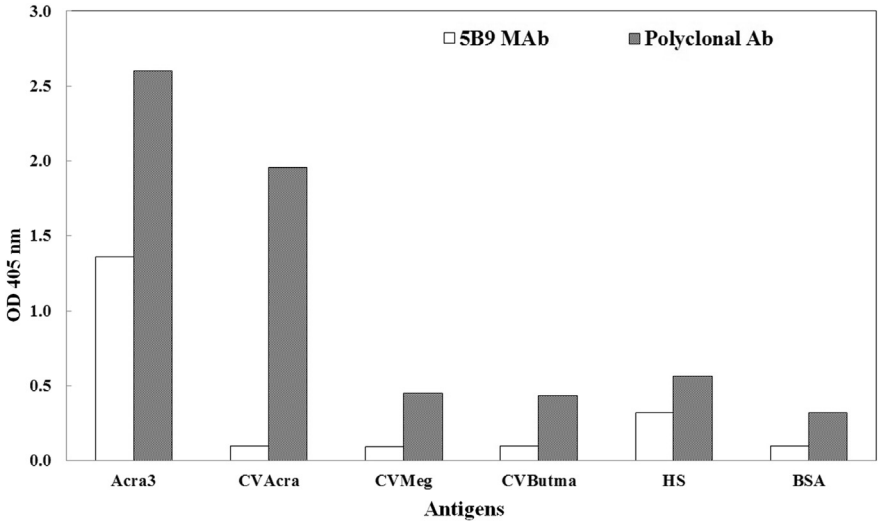


**Fig. 8.** Cross reactivity of 5B9 monoclonal antibody and polyclonal antibody as tested with indirect ELISA. Acra3, CVAcra: Crude venom of *A. crassicauda*, CVMeg: Crude venom of *M. gibbosus*, CVButma: Crude venom of *B. macrocentrus*, HS: Human serum. BSA: Bovine serum albumin.

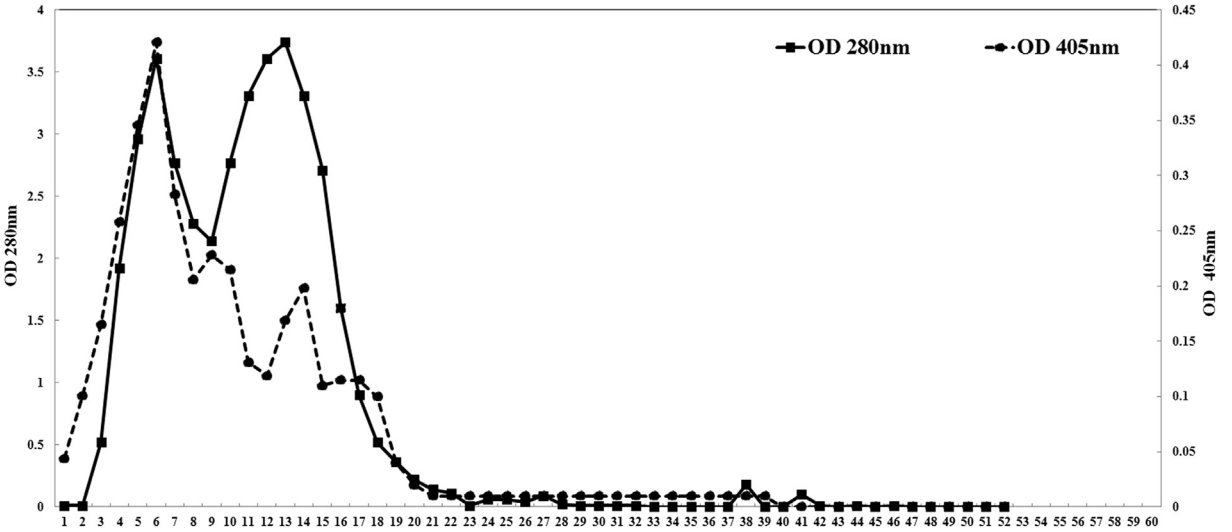


**Fig. 9.** Purification of 5B9 monoclonal antibody using size exclusion column chromatography. Values measured at $OD_{280nm}$ show the protein content of fraction and at $OD_{405nm}$ show ELISA results of the reaction.

normally found in plasma inner surface of the cell membrane can appear on the outer surface of the membrane during apoptotic process. Therefore, PS residues are widely used to detect and measure of apoptosis by binding of Annexin-V (Vermes et al., 1995). In untreated cells, a small percentage of late apoptotic and necrotic cells or death cells were also observed spontaneously. The percentage of necrotic or death cells (A−/PI+) was higher than apoptotic cells (A+/PI+) in both concentration of Acra3. On the other hand, the percentage of late apoptotic cells was quite high (93.3%) after treatment of cells with 20 μg/ml of cisplatin which is used as positive control.

Scorpion peptides are known to inhibit the growth of various cancer cell lines by several mechanisms. Some of these peptides affect specific ion channels such as the inhibition of human pancreatic cancer cell via blockage of calcium activated $K^+$ channels (Jäger et al., 2004). Some of them bind to a specific site on the cell membrane, such as inhibition of glioma cell invasion via matrix metalloproteinase-2 after chlorotoxin exposure (Deshane et al., 2003). Some venom peptides activates the apoptotic pathway such as neopladines and bengalin induction of apoptosis in breast cancer cells and human leukemic cells, respectively (D'Suze et al., 2010; Gupta et al., 2010) and some of them act as agiogenesis inhibitors (Arbiser et al., 2007). Results obtained here indicates that Acra3 exerts its effects by inducing a stronger necrosis than apoptosis specifically in mouse brain tumor cancer cells BC3H1 at least after 12 h treatment. The effect on the induction of apoptosis might be in an earlier stage. On the other hand, primary structure of Acra3, except its 8 cysteines which form 4 disulphide bridges, does not share any conserved amino acid residues with other known antitumoral peptides. For example, chlorotoxin isolated from the venom of *Leiurus quinquestriatus* is specific for chloride channel (Soroceanu et al., 1998) and insect toxin PBITx1 isolated from the venom of *Parabuthus schlecheteri* is specific for $Na^+$ ion channel (Tytgat et al., 1998). In a previous study, although structural similarities of Acra3 to $Na^+$ ion channel specific toxins, it was not found effective on 6 different sub-types of $Na^+$-channels (Nav1.1–Nav1.6). It was suggested that the main target of Acra3 might be another receptor molecules rather than $Na^+$ channels (Caliskan et al., 2012a). Since, BC3H1 is a mouse brain tumor cell lines with properties characteristic of muscle with high-conductance voltage sensitive $Cl^-$ channels (Hurnak and Zachar, 1993), the mechanism of cytotoxicity can be via binding to $Cl^-$ channels or some other molecules. Identification of the toxicity mechanism of Acra3 in term of its direct target is waiting further investigations.

Acra3 is present in relatively quite small amount in *A. crassicauda* venom and is responsible for the lethal effect of the scorpion sting. Since purified toxin peptides can induce polyclonal antibodies in mammals, it is useful to determine the mechanisms of neutralization of the toxins (Clot-Faybesse et al., 1999). Therefore, in the present study, the production of specific mAbs was undertaken. Using low concentration of Acra3 without any toxic effect, it has been possible to obtain polyclonal antibody in mice. Fusion of lymphocytes from mouse and myeloma cells was successfully performed. A monoclonal antibody specific for only Acra3 antigen was developed in few wells, but fifth plate among eight was most effective against to Acra3, then antibody was named as 5B9. Antivenom produced with venom of *A. crassicauda* is known its polyvalent feature in neutralizing venoms of several other scorpion species (Whittemore et al., 1961). Thus, neutralizing effects of 5B9 will be undertaken in our future research.

## 5. Conclusion

In conclusion, these studies describe the biological roles of Acra3 peptide from *A. crassicauda* crude venom which present toxicity on mammals. Scorpion peptides are known to inhibit the growth of various types of cancer cells. As a result, we suggest that different concentrations of Acra3

peptide inhibit the growth of BC3H1 cells due to more than one mechanisms. Moreover, with this report, an antigenic property of Acra3 was first time determined. A monoclonal antibody which recognize and protect against the neurotoxic effect of the Acra3 was produced. Monoclonal antibodies for new active polypeptides are worthwhile expecting the additional available probes for approaching a better understanding of distinction, molecular basis of immunopharmacology and the structure/activity relationship of scorpion neurotoxic polypeptides. The properties determined here indicate that *A. crassicauda* has interesting potential peptides which wait further analysis.

## Ethical statement

The authors declare that this work was performed under the best known ethical respects. All authors have contributed to this manuscript, additionally have read the manuscript and accepted to participate as co-authors. Authors declare that the described work has not been published previously (except in as part of an academic thesis or a congress abstract).

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We would like to thank Dr. Hakan Caliskan and Dr. Umit Sirin from Biology Department of Eskisehir Osmangazi University, who collected scorpion used in this protocol. This work was financed by a grant from the Scientific and Technological Research Council of Turkey (TBAG-106T527) to the authors.

## Conflict of interest

A conflicting interest exists when professional judgment concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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