ASIA PACIFIC JOURNAL of MEDICAL TOXICOLOGY
APJMT 9;2 http://apjmt.mums.ac.ir June 2020

ORIGINAL ARTICLE

Immunomodulatory effect of a purified peptide fraction isolated from *Mesobuthus eupeus* scorpion venom on astrocytoma cell line (1321N1)

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Abstract

Objective(s): Multiple Sclerosis is a central nervous system disease which belongs to the category of autoimmune diseases. The prevalence of this disease in Iran is approaching the European level. Astrocyte cells are nerve tissues that regulate the immune system activity by secreting various cytokines such as IL- 17. The aim of this study was partial purification of toxin from *M.eupeus* scorpion venom that has immunomodulatory effect on astrocyte cell line (1321N1)
Materials and Methods: In the present study, purified crude venom of *M.eupeus* scorpion. Size exclusion and reverse-phase high-performance liquid chromatography was used for fractionation. The fractional molecular weight was determined by Using SDS and Tricine electrophoresis, Astrocyte cells (1321N1) were selected as functional cells in testing the immunomodulatory effect of venom. The viability of cells were determined by MTT and LDH assays. Astrocyte cells were activated by lipopolysaccharide and the release of interleukin-17 in activated cells was estimated using ELISA kit.
Results: fraction 331 (F331) from RP-HPLC contain the purified peptide with molecular weight of about 4500 Dalton. When activated cells exposed to purified peptide the rate of interleukin-17 release was found to be 85 pg/ml which is almost similar to un-activated cells (78 pg/ml). However in activated cells by LPS without treatment with purified peptide the rate of IL-17 release was found to be 147 pg/ml which was significantly ($P<0.05$) higher than control group.
Conclusion: The purified peptide (F331) from venom of *Mesobouthus eupeus* can inactivate the astrocyte 1321N1 cells activated by LPS as indicated by decreased secretion of IL-17 from the cells.

Key words: Scorpion, Venom, Interleukin-17, Astrocyte, Lipopolysaccharide

How to cite this article: Masihipour B, Zare Mirakabadi A, Haji Hosseini R, Tebianian M. Immunomodulatory effect of a purified peptide fraction isolated from Mesobuthus eupeus scorpion venom on astrocytoma cell line (1321N1). *Asia Pac J Med Toxicol* 2020; 9(2):52-59.

INTRODUCTION

Multiple Sclerosis (MS) is a common disease in many parts of the world (1). Based on the report published by the World Health Organization (WHO), more than 2.5 million people are affected worldwide. The prevalence of this disease in Iran was 8 to 30 people per thousand people. However in recent years the incidence of MS has increased in the country and cities such as Tabriz, Tehran, Isfahan and Mashhad which have the highest incidence of MS. (2)

MS is a central nervous system disease in which the immune system is disrupted. Axons may also be damaged by the lack of nourishment by surrounding cells. (3) . The most prominent features of this disease are myelin destruction, axonal damage and gliosis (scar formation)(4), which are the basic mechanisms which may be involved in pathogenesis of MS is immune cell trafficking and production of cytokines. The current hypothesis points to the role of cells such as T CD4 +, T CD8 +, B, microglia / macrophages (dendritic, astrocytes) in the pathogenesis of MS(5) . Astrocytes are the major antigen-presenting cells in the nervous system, which Have occupied almost 25 to 50 percent of brain volume.(6) Astrocytes are able to maintain the internal environment of the nervous system by removing excess K+ ions ,(7) and produce chemokine such as RANTES, IL-8, MCP-1 and 10 IP- and IL-27, IL-17(8) . Interleukin-17 secretion by astrocytes affects Th17 cells via activating signaling pathways which leads to increased astrocyte activity, producing an inflammatory environment that can induce tissue damage in the CNS (9-11). The drugs available for MS are to correct the disease process. Over the past 20 years, various drugs, including interferon beta was used for treatment(9). Venom therapy is one of the most recent methods of treatment in this field (13). It is suggested that

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animal venom such as snake venom (10), bee venom(11) and scorpion venom are useful in the treatment of autoimmune diseases such as multiple sclerosis (MS)(12). Scorpion venom contains toxins which are effective on cellular channels including potassium (K^+), calcium (Ca^{++}), Chloride (Cl^-) and sodium ($Na^)$ channels. Kaliotoxin peptide, with 37 amino acid residues,can reported to inhibit voltage-dependent potassium channels Kv1.3 that selectively blocks T cell activities and hence decreases T cell sensitivity(13). Another toxin from the scorpion venom that targets the potassium channel (KTx) consists of about 31-39 amino acid residues(14). Venom toxins inhibit the flow of ions through the biological membrane. (17-20) A Kv1.3 channel-based therapeutic approach seems to have an advantage over agents that cause generalized immunomodulation because native and T_{CM} cells would escape inhibition through upregulation of IKCa1 channels, leaving the bulk of the immune response intact. (11)

Hence the present study was undertaken to investigate the immunomodulatory effect of a toxin present in scorpion *Mesobuthus eupeus* venom, based on suppression of IL-17 secretion by activated astrocyte cell line.

METHODS

Preparation of venom

lyophilized *M. eupeus* scorpion venom (150mg) provided by venomous animal department of Razi Vaccine and Serum Research Institute was dissolved in distilled water and centrifuged at 14,000 rpm for 15 minutes. The supernatant was collected and protein content determined by Bradford method (15)

Polyacrylamide Gel electrophoresis

Glycine and tricine SDS-PAGE was performed for determination of protein pattern in the scorpion venom. The advantage of using tricine instead of glycine in electrophoresis is its greater negative charge than glycine and its higher ionic strength, which results in faster mobility of the ion in the protein and better

Separation of small proteins (especially in the range of 1 to 10 KDa).

The concentration of the gel separator was 15%, the density of the condensing gel was 4%, and the molecular marker 6.5 to 200 and 3.4 to 26.6KDa which were used for SDS page and tricine SDS- PAGE respectively. Staining of protein bands was done by Coomassie Brilliant Blue. The molecular weight of the proteins was calculated using the Laemmli method (16).

Size exclusion chromatography

Solubilized crude venom was applied to a 1.6 × 150 cm column containing sephadex G-50 equilibrated with a 0.1 M ammonium acetate buffer (pH 8.6). The flow rate was adjusted at 60 ml/hr. 5 ml of solubilized venom (120 mg) was loaded to the column and the eluted material was collected in 10 ml fractions. The optical absorbance of the eluent was measured at 280 nm. Fractions were separated and active fraction was selected by molecular weight. The fractions were lyophilized and protein content was determined as described above (17).

High performance liquid chromatography (HPLC)

The active fraction from size exclusion chromatography was further purified by applying on a C18 RP-HPLC (Amersham Biosciences,UV-900.P-900) column which was equilibrated with solvent A (H2O, 0.1%trifluoroacetic acid), and eluted with a concentration gradient of solvent B (acetonitrile, 0.1% trifluoroacetic acid) from 0 to 100%, at a flow rate of 0.5 mL/min during 80 min. The peaks were detected through the A280. Each RP-HPLC peak was collected individually and lyophilized and each individual fraction was tested for activity. The active fraction was further purified by repeating the purification until a single peak was obtained (18).

50% lethal toxicity of venom (LD50)

Balb/c male mice weighing (18± 20 g) were used in this research. The ethical code for this study is RVSRI. REC. 9800004. The venom as well as the fractions' lethal toxicity was assayed. Crude venom and fractions were subjected to 4-5 serial dilutions by 1.25 factor using isotonic sodium chloride solution. (From 20 to 100 µg/ml). Aliquot of 0.3 ml of each dilution was intravenously injected into 4 mice. Dilutions ranges covering the entire Mortality range from 0% as non-lethal dose and - 100% as a dose which kills all the animals. The mice dying in each group during 24h following injection were considered as toxicity. The LD50 was calculated using the spearman karber method (19).

Cytotoxicity assay

For cytotoxicity evaluation of crude venom and fractions on 1321N1 cell line growth a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay was used. The assay is based on the cellular conversion of a tetrazolium salt (MTT) into a formazan product that is easily detected.

The prepared astrocyte cells (0.7×10^5 cells well) were added into each well and exposed to various concentrations of crude venom, F_3 fraction and sub fractions at concentrations (40, 20, 10 and 5 µg/mL respectively), for 24 h, then medium in each well was replaced with 100 µL of medium containing 0.5 mg/mL MTT, followed by incubation at 37°C for 3 h. DMSO was added into each well and read at optical density 580 nm using micro plate reader (Bio-Rad 550). In each experiment, 6 wells were used, and experiments were repeated 3 times. As a positive control 50 ug/mL of cisplatin was used. (20)

Determination of IC50

This indicates a level of venom that reduces 50% of the biological activity of the astrocyte cell compared to the control cell.

% viability = 100 - Cell Inhibition Percentage

Cell Inhibition Percentage = (1 – (OD sample wells - OD control wells) × 100

Release of Lactate Dehydrogenase (LDH) enzyme

The necrotic effect of venom, fractions and subtractions on cells were examined by lactate dehydrogenase (LDH) assay. The increase in lactate dehydrogenase activity is proportional to the number of lysed cells in each well(21)

Measurement of cytokine IL 17: Astrocyte cell line grown in wells for 24 hours, was activated by exposure of cells to 50 pg lipopolysaccharide for 8 hours. The activated cells were then exposed to various concentrations (40, 20, 10 and 5 µg/mL) of crude venom, fraction F_3 and sub fractions respectively for 24 hours. The Supernatant of cell culture (control and activated) were used for specific cytokine IL-17 Immunoassay: ELISA kits (ebioscience Company). (22)

Statistical analysis

All the tests were repeated 3 times. Obtained data were analyzed with statistical software Sigma plot 12 using one-way ANOVA. The results expressed as mean ±SD and $P < 0.05$ was considered as significant (23).

RESULTS

Venoms lethal toxicity

The toxicity of venom was tested by determination of LD_{50} of crude venom in mice during 24 hours. The average LD_{50} of crude venom was found to be 78 ± 3.2 µg/mice. The LD50 for fraction MFs3 was almost half of crude venom with 38 ± 4.6 µg/mice. Total protein of the crude venom was found to be 80.4 mg after removal of mucoproteins.

Cytotoxic effect of scorpion venom

The astrocyte 1321N1 cell line cultured on medium containing DMEM high glucose + FBS (10 %) showed spindle-shaped rods when observed under the inverted phase microscope (Figure No 1A). Following the administration of venom on these cells, no significant changes were observed in the shape, size and morphology of the cells (Figure No 1B). However with the increase in venom concentration some changes in appearance like deformed rods sticking together was observed (Figure No. 1C).

Determination of IC_{50}

The IC_{50} (Half maximal inhibitory concentration) was calculated and plotted after drawing the curve and obtaining the line equation using different concentrations of venom and percentage of live cells, the IC_{50} in the 1321N1 astrocyte cell against the *M.eupeus* scorpion crude venom is equal to 82± 3.3 µg/ml

Size exclusion chromatography

After elution from gel filtration column 5 peaks were obtained (Figure No.2) out of which the third peak (F_3) was found to be toxic on mice with low molecular weight when checked with SDS-PAGE. The content of protein in F_3 was found to be 19.26 mg when estimated by Bradford method.

Electrophoretic Profile

The crude venom of *M.eupeus* scorpion contained proteins raging from less than 6 KDa. up to 116 KDa. Fraction F_1 contains molecular weight proteins ranging from 31 to 116.5 KDa and fraction F_2 contains proteins of molecular weight 14.5 to 31 KDa. As it is shown in Electrophoresis (Figure 3.1) Fraction F_3, has small molecular weight proteins ranging from 14.5 to less than 5 KDa. Sub fractions of F3 has molecular weight proteins ranging from3.4 to 6.5 KDa. (Figure3.2A). the molecular weight of F331 is almost 4.5 KDa. (Figure: 3.2B)

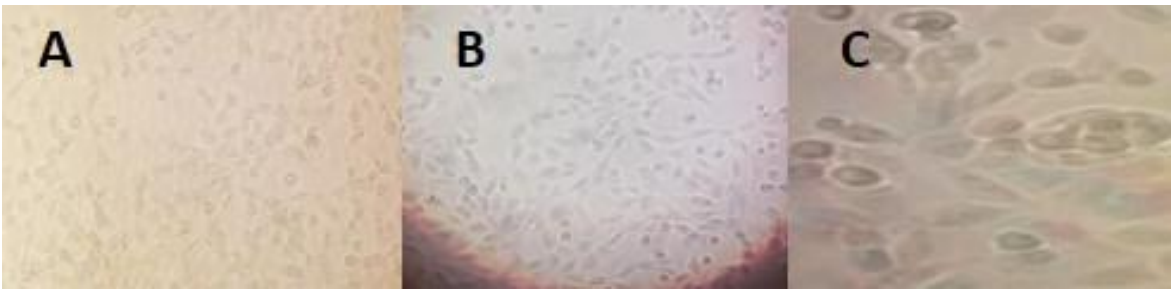


Figure No. 1(A, B, C). A. Astrocyte cell (control), B: Activated Astrocyte cell (with LPS) and C: Exposed Aastrocyte .Cells in Proximity to LD_{50} Levels of Scorpion venom (Zoom × 10X). The morphology of the astrocyte cells (control and activated) is similar and only the number of astrocyte cells in the activated state shows an increase, but due to the increase in scorpion venom the cells are more deformed and stuck together.

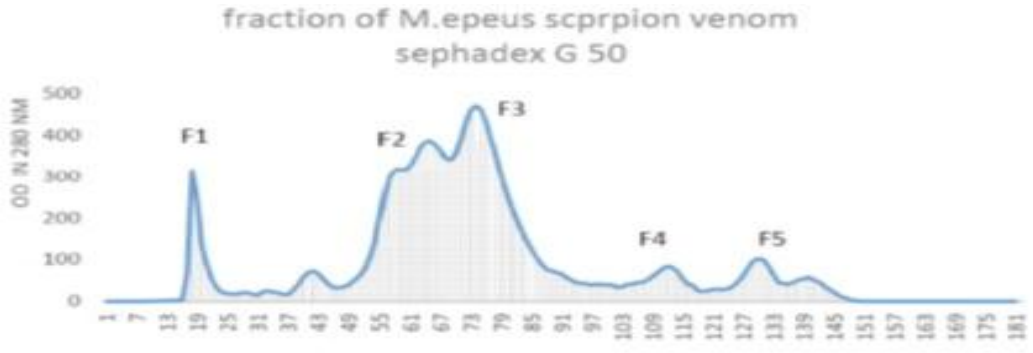


Figure.No2. Fractions of *M. eupeus* crude venom obtained by gel filtration (sephadex G-50) chromatography

Table1. Gel filtration (Sephadex G-50) Efficiency and level of LD_{50}

Purification steps	Fractions	protein content (mg)	LD50 µg/mice
Extraction & dialysis	Crude venom	80.4	78 ± 3.2
Sepadex -G50	F 1	7.98	
	F 2	23.11	
	F 3*	19.46	38 ± 4.6
	F 4	1.12	
	F 5	0.93	
	Total	52.6	

*: toxic Fraction

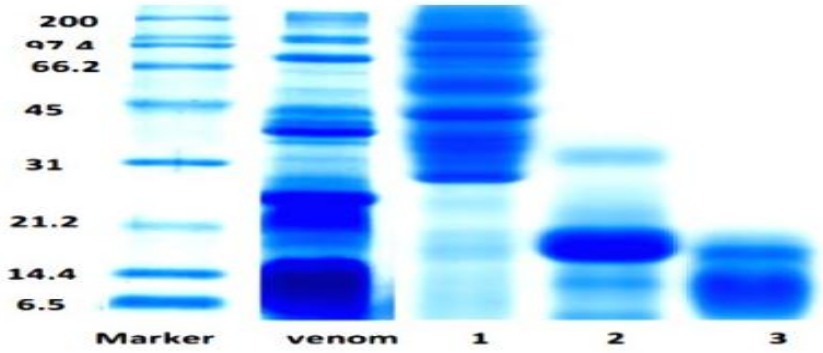
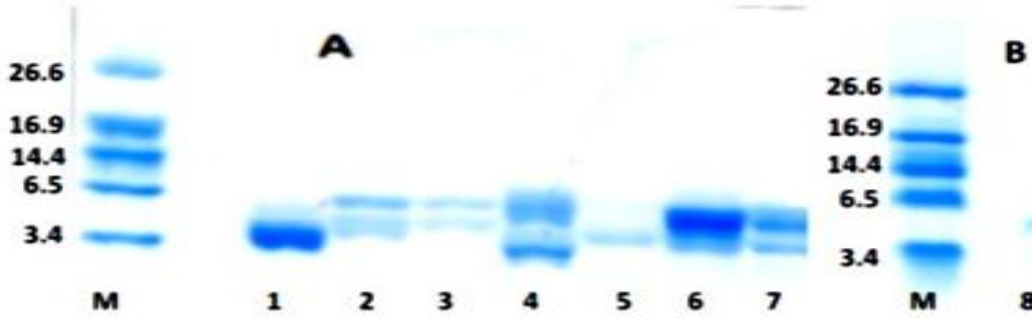


Figure 3. Lane 1: F_1 fraction, Lane 2: F_2 fraction Lane 3: F_3 fraction



M: Marker, lane 1: F_{31} subfraction, lane 2: F_{32} subfraction, , lane 3: $F_{3.3}$ subfraction,, lane 4: F_{34} subfraction,, lane 5: F_{35} subfraction,, lane 6: F_{36} subfraction, , lane 7: F_{37} subfraction, , lane 8: F_{331} subfraction,

Figure 3-2. Tricine SDS electrophoresis of F_3sub fractions of venom against molecular weight marker

Purification of active peptide by RP-HPLC Method:

After loading of F_3 fraction onto RP-HPLC , 7 sub-fractions, including $F_{3.I}$, F_{32} , $F_{33.}$, $F_{34.}$, $F_{35.}$, $F_{36.,}$ F_{37} were obtained.(Figure No.4.A). Fractions were injected into 18- to 20-g mice after being lyophilized to remove toxic acetonitrile content of each fraction. Each fraction was tested on astrocyte (control and activated) cell line. From F33 sub Fraction, 5 sub fractions F_{331}, F_{332}, F_{333}, F_{334} and F_{335} were obtained (Figure No.4.B). F_{331} was a peptide purified from *M.eupeus* scorpion venom (Figure No4.C).

Fraction F_3 from size exclusion loaded on RF-HPLC 7 gained sub-fractions. Sub-fractions were separated in 35% acetonitrile and collected during 22 to 55 minutes retention time. Sub fraction F33 was collected at 33^{Th} minute and final purification carried out by repeating the process.

Determination of cell viability by MTT assay:

The astrocyte cell viability in all groups including control

and activated cells exposed to crude venom, fractions, subtractions and purified peptide were determined. Concentration of 80 µg/ ml of crude venom are shown in (Table:2) 57.7± 2.35 % survival rate of astrocyte cell (control) and lower doses did not show significant toxicity on cells (86.4 ± 1.9 %viability). (Table3) However fraction F3 at concentration of 20 µg / ml showed significant effect ($P<0.05$) on activated astrocyte cells. Although fraction F35 reduced survival rate to 52±1.8 %, but F33 of HPLC did not affect the viability of activated cells by endotoxin (Table3). On the other hand the purified peptide (F331) at 10 µg/ml did not show significant toxicity (viability 89±4.2) on activated astrocyte cells

LDH release rate:

Release of mitochondrial enzyme lactate dehydrogenase (LDH), which indicates the cytotoxic effects of scorpion venom on the cell, was determined. The fraction F_3 showed a significant effect on cells to release lactate dehydrogenase with 95% confidence. However the purified peptide did not show significant necrotic effect of LDH release on activated astrocyte cell line (1321N1) (Table: 4, 5)

Release rate of lactate dehydrogenase enzyme from astrocyte cells (control and active cell). Table 4: Enzyme release following scorpion venom fractions. The effect of F_3 fraction on activated astrocyte cells was significant at $P< 0.05$ and it was not significant at the $P < 0.05$ on astrocyte control cells, Table 5: Sub-fraction F_{35} causes lactate dehydrogenase (LDH) release in the cell and is significant on both activated and control cells.

Interleukin 17 release:

As shown (in Figure 5.A) the level of interleukin-17 released in cultured media by control astrocyte cells was found to be 78±1.9 pg /ml. When the cells exposed to LPS at a concentration of 50ng/ml, the release of IL-17 increased to

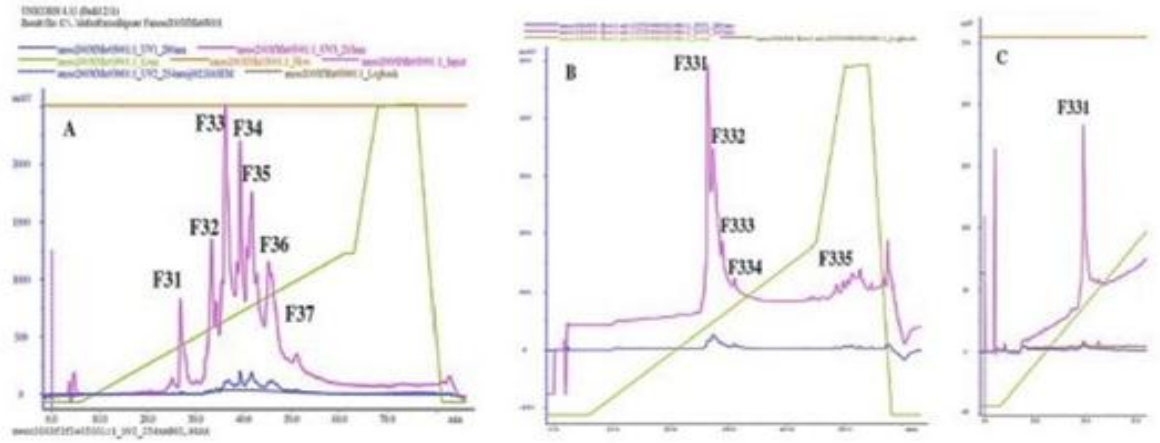


Fig.(4). Purification of active peptide by RP-HPLC(A: F3, B: F33 and C: F331)

Table3. Percentage of cell survival against sub fractions of M.eupeus scorpion venom MTT assay of scorpion venom effect on astrocyte cell , F_3 sub fractions on control and activated astrocyte cell (10 µg/ml)

Sub fractions	F31	F32	F33	F34	F35	F36	F37	F331
Control cell (%Viability)	84±1.3	91±1.7	86±1.9	63±3.1	52±1.8*	87±1.2	89±1.8	87±0.9
Active cell (%Viability)	82±3.4	84±2.7	79±3.3	69±2.1	57±4.5	81±2.5	81±2.5	88±1.3

*: $p < 0.05$ Significant

Table4. Release LDH (U/L) against fractions of M.eupeus scorpion venom.

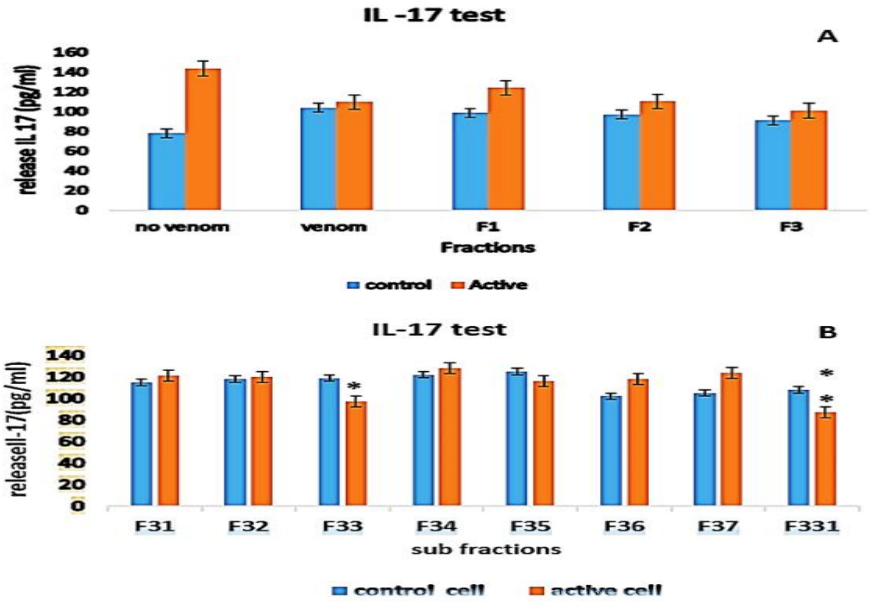
Fractions	F1	F2	F3
Control cell	17±1.4	18.5±2.3	28±2.9
Active cell	19±1.7	19.5±1.9	38±2.4*

*: $p < 0.05$ Significant

Table 5. Release LDH (U/L) against fractions of *M eupeus* scorpion venom

Sub fractions	F31	F32	F33	F34	F35	F36	F37	F331
Control cell	16±0.7	18±0.5	17±0.4	25±0.7	58±0.8**	34±1.1	12±0.7	18±0.6
Active cell	18±0.8	21±0.9	23±0.7	32±1.3	44±0.9*	25±0.9	17±0.9	22±0.8

*: p < 0.05 Significant, **: p < 0.01 Significant



*: significant at P < 0.05, **: significant at P < 0.01

Figure.5. A: Interleukin-17 secretion rate from (control and active) astrocyte cells adjacent to the following fractions of scorpion venom. B: Interleukin-17 secretion is enhanced by the effect of lipopoly saccharide (LPS) on activated astrocyte cells and increases up to about 2.5-fold above normal levels. Scorpion venom fractions have no significant effect on IL-17 secretion in astrocyte control cells, But in activated astrocyte cells scorpion venom has an effect on the secretion of interleukin 17, which, following F_{33I} sub fraction and purified peptide F_{331}, had significantly reduces the secretion of interleukin 17

147± 2.7pg/ml which was statistically significant (p<0.05). Crude venom could reduce the release of IL-17 in cells exposed to LPS (103±3.4 pg/ml). The activated 1321N1 cells when exposed to fraction F3 showed a reduced IL-17 release up 93± 3.3 pg / ml. However partially purified fraction F33 as well as purified peptide reduced the IL-17 of activated cells to 82 ± 2.9 which were statistically significant at P< 0.05 (Figure 5.B)

DISCUSSION

Multiple Sclerosis (MS) is a chronic central nervous system disease (1) and affects about 2.5 million people worldwide (24) . According to available reports, the prevalence of this disease in Iran is about 0.07% (2).The mostly accepted hypotheses in this case is the role of autoimmune mechanisms, including abnormal increase in the number of astrocyte cells (25). Recently the use of venom as a tool for treatments is considered a hope for new effective drugs (15) and this study investigated the potency of scorpion venom as well as its peptides for modulating the hyperactivity of activated astrocytes using the cell line 1321N1. Today, the use of activated astrocyte-cells is MS disease (26). Scorpion venom contain a mixture of peptides, toxins and many other bioactive compounds (17, 18). The cells were activated by LPS. Cell activation was identified by a rise in Interleukin 17 (18). The assay of MTT and LDH release of astrocyte cells exposed to venom was determined and the highest dose of venom that was not cytotoxic to cells was used as treatment dose. (26, 27). In the present study sequential gel filtration and RP-HPLC were used to purify active peptide from *M.eupeus* scorpion venom (27). Results of the present study clearly showed that F331 peptide was able to significantly reduce the secretion of interleukin 17 in the activated astrocytoma cells. Researchers demonstrate that blocking the IL-17 pathways in astrocytes is a promising therapeutic approach for MS disease, which does not interfere with systemic immune responses which is major concern in conventional MS therapy(27).Astrocytes can modulate the excitability of neurons by changing the concentration of potassium ions in the extracellular environment, a process called K^+ clearance (28). It is suggested that astrocytes, in addition to their modulation of neuronal excitability at the synaptic level, are strategically located to act as "synaptic managers" that oversee the overall

synaptic activity (18). Several studies have confirmed that Kv1.3 channel is highly expressed in macrophages, microglia, and TEM cells, suggesting that Kv1.3 plays a crucial role in immune and inflammatory responses to human diseases such as multiple sclerosis (MS). Over 120 different peptides including agitoxin2, charybdotoxin, kaliotoxin, margatoxin, noxiustoxin, and *Pandinus* toxin were isolated and shown to recognize and block with distinct affinities and varieties of different K+ channel(17). Several naturally peptides, especially from scorpion, have been reported to be effective blockers of Kv1.3 channels (29). Most peptides purified from scorpion venom with 33 – 55 residues reported to affect Kv channels have been isolated from species of family Buthidae . The F331, a purified peptide from *M. eupeus* scorpion venom, may modulate the hyperactivity of activated 1321N1 cells, through blocking the K channel. Scorpion venom heat resistant peptide (SVHRP) is a toxin purified from scorpion venom decrease in glial fibrillary acidic protein (GFAP) as an indicator of hyperactivity in astrocyte cell (30) Bin Gao, et al reported the purification ,sequencing and functional characterization of a K+ channel blocker (MeuKTX) with molecular weight of 3.5 KDa from the venom of the scorpion *M.eupeus* (31). However the molecular weight of peptide purified in the present study was found to be 4.5 KDa. Previous reports by some previous research scientists working on toxins blocking Kv1.3 derived from scorpion, sea anemone, snakes, and other animals are found to be 3 to 6.5 KDa (31).Hence the molecular mass of F331 purified from M.eupeus is in the range and confirmed previous reports

CONCLUSION

Based on the results obtained in the present study, the peptide with molecular weight of about 4.5 KDa in venom of scorpion *Mesobouthus eupeus* may act as a modulatory tool for activated astrocyte 1321N1 cells through reduced release of IL17. More studies are require to characterize and confirm the in vivo modulatory action of F331 peptide.

ACKNOWLEDGEMENT

Authors are thankful to the Department of Venomous Animals and Anti venom Production, Razi Vaccine and Serum Research Institute, Karaj, for providing facilities and Supports.

Conflict of interest: None to be declared.
Funding and support: None.

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