Archives of Razi Institute, Vol. 74, No. 2 (2019) 183-189

Short Communication

Scolicidal activity of *Mesobuthus eupeus* venom against the protoscolices of *Echinococcus granulosus*

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Received 05 February 2017; Accepted 13 January 2018
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ABSTRACT

Hydatidosis is an important zoonosis caused by a parasitic tapeworm, namely *Echinococcus granulosus*. This infection is distributed worldwide and affects the health as well as economic loss in both humans and animals. In most cases, the disease needs chemotherapy with or without surgery. Conventional drugs have some major problems, including drug complications, harmful side effects, and also progressive resistance. According to the importance of biological productions as alternative medicine, a large number of studies confirmed that whole venom and many peptide ingredients of the scorpion venom have various different medical benefits, including antimicrobial properties, due to the mechanism of blocking gated ion channel. In this study, the venom peptides of *Mesobuthus eupeus* scorpion were purified using gel filtration chromatography and subsequently ion exchange chromatography, followed by the determination of the molecular weights of the proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure. After collecting the hydatid cysts fluids from the liver of infected sheep, protoscolices were derived, washed, and encountered to the whole venom as well as eight different fractions of toxin 30, 60, 120, and 240 min after the exposure. In the next step, the viability of protoscolices was determined by eosin staining. The obtained results revealed that a venom fraction under 10 kDa killed all protoscolices after 30 min. Moreover, it was found that the scolicidal activity of fractions increases according to the time of exposure. As a result, it can be concluded that *M. epeus* venom peptides under its LD50 (1/2 LD50) can properly and quickly destroy the protoscolices of hydatid cysts at the level of applied concentrations and such components are good alternatives to treat hydatidosis.

Keywords: Hydatid cyst, Protoscolices, *Mesobuthus eupeus*, Venom, Treatment

Activité Scolicidal du Venin de *Mesobuthus eupeus* contre les Protoscolices d'*Echinococcus granulosus*

Résumé: L'hydatidose est une zoonose importante causée par un ver solitaire parasite, à savoir *Echinococcus granulosus*. Cette infection est répandue dans le monde entier et affecte la santé humaine et animale, induisant des pertes économiques considérables. Dans la plupart des cas, la maladie nécessite une chimiothérapie avec ou sans chirurgie. Les médicaments classiques présentent des problèmes majeurs, notamment des complications médicamenteuses, des effets secondaires nocifs et une résistance progressive. Selon l'importance des productions biologiques en tant que médecine alternative, de nombreuses études ont confirmé que le venin entier ainsi que des nombreux ingrédients peptidiques du venin de scorpion présentaient divers avantages médicaux, y compris des propriétés antimicrobiennes, en raison du mécanisme de blocage des canaux ioniques. Dans cette étude, les

peptides de venin de scorpion *Mesobuthus eupeus* ont été purifiés par une chromatographie sur gel de filtration suivie d'une chromatographie à échange d'ions, puis par détermination du poids moléculaire des protéines par électrophorèse sur gel de dodécyl sulfate de sodium et de polyacrylamide (SDS-PAGE). Après avoir recueilli les fluides de kystes hydatiques du foie de mouton infecté, les protoscolex ont été extraits, lavés et exposés auvenin entier ainsi qu'àhuit fractions différentes de toxine durant 30, 60, 120 et 240 min. Ensuite, la viabilité des protoscolex a été déterminée par coloration à l'éosine. Les résultats obtenus ont révélé qu'une fraction de venin de moins de 10 kDa avait éliminé tous les protoscolices après 30 min. De plus, il a été constaté que l'activité scolicide des fractions augmente en fonction du temps d'exposition. En conséquence, on peut en conclure que les peptides du venin de *M. epeus* avec sa DL50 (1/2 LD50) peuvent détruire correctement et rapidement les protoscolices des kystes hydatiques dans les concentrations appliquées dans cette étude et que de tels composants sont de bonnes alternatives pour traiter l'hydatidose.
Mots-clés: Kyste hydatique, Protoscolex, *Mesobuthus eupeus*, Venin, Traitement

INTRODUCTION

Canid tiny tapeworm, *Echinococcus granulosus*, is responsible for vesicular hydatidosis, which occurs as a result of the formation of hydatid cyst by the larval stage of taeniid cestode in many internal organs of humans as well as domesticated and wild animals, as intermediate hosts (Thompson, 2017). Hydatidosis seriously affects human health and imposes costs to the involved people due to the required medication, surgery, or both. Moreover, it is responsible for many economic losses in the livestock industry (Ito & Budke, 2017). Chemotherapy has been the first line treatment of hydatidosis for a long period of time. In case the cysts are large in size or drugs are not strong enough to suppress them, surgery is an inevitable method. In these situations, surgical removal of cysts along with systematic chemotherapy before and after the surgery is performed to destroy the protoscolices (Thompson, 2017). Prior to the removal of cysts, a surgeon has to inject a scolicidal drug into internal space of the cyst to prevent the leakage risk of the viable protoscolices to the adjacent region (Musaev et al., 2017). The broad use of drugs in spite of progressive resistance against many scolicidals and also side effects of these chemicals, especially their longitudinal use obliged us to investigate effective biological products for the treatment of such diseases. Substances derived from venomous animals, including scorpions, are new candidates for future drugs and can play as new approaches with fewer side effects. The literature revealed that the scorpion and snake venoms have considerable effects on some human life-threatening parasites, including *Plasmodium*, *Leishmania,* and *Tryanosoma*. The scorpion venoms include many pharmacological peptides, which attract the attention of scientists in the field of drugs development (Perumal Samy et al., 2017). The scorpion *Mesobuthus eupeus* is the most widely dispersed species of the family Buthidae. Antimicrobial peptides (AMPs) isolated from *M. eupeus* venom showed inhibitory activity against both Gram-positive bacteria and Gram-negatives, which could inhibit HIV-1 by direct interaction with viral particles (Gao et al., 2009; Cao et al., 2012). Various types of venomous scorpions are distributed in Iran among which *M. eupeus* is one of the most dominant scorpions of this geographical zone. This opportunity leads us to examine the peptides of the venoms of this species as biological products to evaluate their effects on the protoscolices of hydatid cyst. Therefore, the current study aimed to purify the peptides from the venom of *M. eupeus* by ion exchange chromatography and determine the activity of fractions on the protoscolices of hydatid cyst caused by *Echinococcus granulosus*.

MATERIAL AND METHODS

Venom. The *M. eupeus* scorpions were collected from different zones of Khuzestan Province located in the southwest of Iran using nocturnal UV light inspection (31°19′-32°73′N, 48°41′-49°4′ E). After a precise identification of species by morphological key diagnosis (Navidpour et al., 2008), the scorpions were milked by electric stimuli and the venom lyophilized in the freeze-dryer instrument (Oukkache et al., 2013). In the next step, the total protein of exploited venom concentration was measured by the usual Bradford spectrophotometric method using Bovine Serum Albumin (BSA) as standard.

Purification of venom fractions. The purification of venom fractions was applied as it was by Shirmardi et al. (2010) with some modifications. Briefly, the first 180 mg of lyophilized venom was dissolved in ammonium acetate buffer with pH=8.6. Insoluble substances, including mucoproteins, were removed by centrifugation and supernatants. The supernatant was applied to a column of Sephadex G-50 (2×80 cm). After loading the venom on the column, elution buffer was gathered in a flow rate of 45 ml/h. The purification of venom fractions was performed by ion-exchange chromatography using diethylaminoethyle cellulose column (DEAE-C, Sigma) at a flow rate of 1 ml/min. The fraction 3 of *M. eupeus* venom was dissolved in 0.05 M Tris-HCl with the pH of 8.6 as stabilizing buffer and then loaded on the column (1×20 cm). Unbound components were washed away and then the elution of the bound elements was performed utilizing a linear gradient of sodium chloride with 0.1-2 M concentrations in stabilizing buffer. The derived fraction from ion-exchange chromatography was preserved for subsequent examinations.

SDS-PAGE. Derived purified fractions from ion-exchange chromatography as well as whole venom were analyzed by SDS-PAGE using 4% stacking gel and 16% resolving gels to obtain protein profile. Denatured was performed by boiling in SDS and β-mercaptoethanol as loading buffer then loaded in the gel. Proteins were stained with 1% Coomassie blue R 250. Molecular mass standard (Vivantis, Malaysia) was used to estimate the molecular weight of the proteins.

Protoscolices of hydatid cysts. Hydatid cysts of *E. granulosus* were collected from infected liver and lungs of sheep slaughtered from the slaughterhouse of Ahvaz and immediately transferred to parasitology laboratory. Hydatid fluid aspirated aseptically and lay down for 45 min to make the sediment of protoscolices. Gathered protoscolices were washed 3 times with normal saline and then the viability of these metacestodes was assessed with 0.1% eosin staining through observing their motility characteristics and muscular movements under the light microscopy. The protoscolices with more than 90% viability were selected for the current experiments (Smyth & Barrett, 1980).

Effect of fractions on protoscolices. Whole venom (1/2 LD50) and its fractions were obtained by ion-exchange chromatography to evaluate the scolosidal activity of *M. eupeus* venom. To this end, 100µl of each fraction was added to 1mL PBS (pH 7.4) containing 5000 protoscolices in each test tube and incubated at 37° C for the time intervals of 30, 60, 120, and 240 mins followed by incubation, 100µl from each sample was removed by pipetting and then eosin 0.1% was added. Afterward, the smear was prepared and studied under light microscopy. The percentage of stained protoscolices as dead and un-stained as live protoscolices applying each fraction was measured. To ensure the accuracy of the test and quality control, a control group (PBS) and formalin as negative and positive controls respectively were considered.

Statistical analysis. Statistical analyses were performed by one-way analysis of variance (ANOVA) to evaluate the differences between tests results using SPSS software (version 16). P-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Gel Chromatography. The 105 mg of protein loaded on the column of Sephadex G-50 Gel Chromatography, including four fractions of F1, F2, F3, and F4. Figure 1

represents the optical density of four fractions at 280 nm.

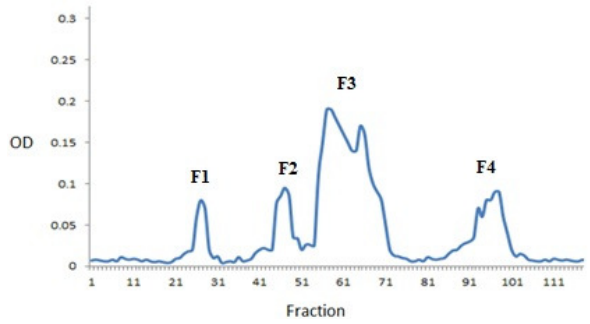

Figure 1. Gel filtration curve of *M. eupeus* venom by Sephadex G-50 column (2×80 cm).

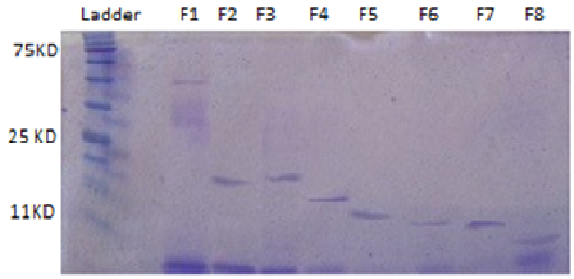

Figure 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of ion-exchange chromatography from scorpion venom. (F1-F8: extracted fractions)

Ion exchange chromatography. The total protein concentration of fraction 3 has been measured from 0.1 up to 2 M of NaCl. Totally, 8 fractions had 1.30, 1.11, 1.92, 2.45, 2.6, 3.2, 1.09, and 0.66 concentrations, respectively. The highest concentration of protein was 3.2 that achieved by elution with 1.25 M NaCl.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. According to SDS-PAGE on products from ion-exchange chromatography, venom fractions had a different protein profile (Figure 2). The proteins of the venom were determined to have 9 detectable bands, which were between ≤8 and ≥60 kDa on 12% polyacrylamide gel electrophoresis.

Protoscolicidal activity of venom fractions of *Mesobuthus eupeus*. The viability of protoscolices under treatment with 8 derived fractions figured out and represented in figure 3. Results revealed that the scolosidal effect of the whole venom on protoscolices significantly increased from 30 to 240 mins of exposure ($P<0.05$); however, it did not kill all of the parasites. Fractions 6, 7, and 8 killed all of the protoscolices after 240 min, fractions 7 and 8 did that effect after 120 min, and only fraction 8 killed all of the protoscolices after 60 and also 30 min with a significant difference in

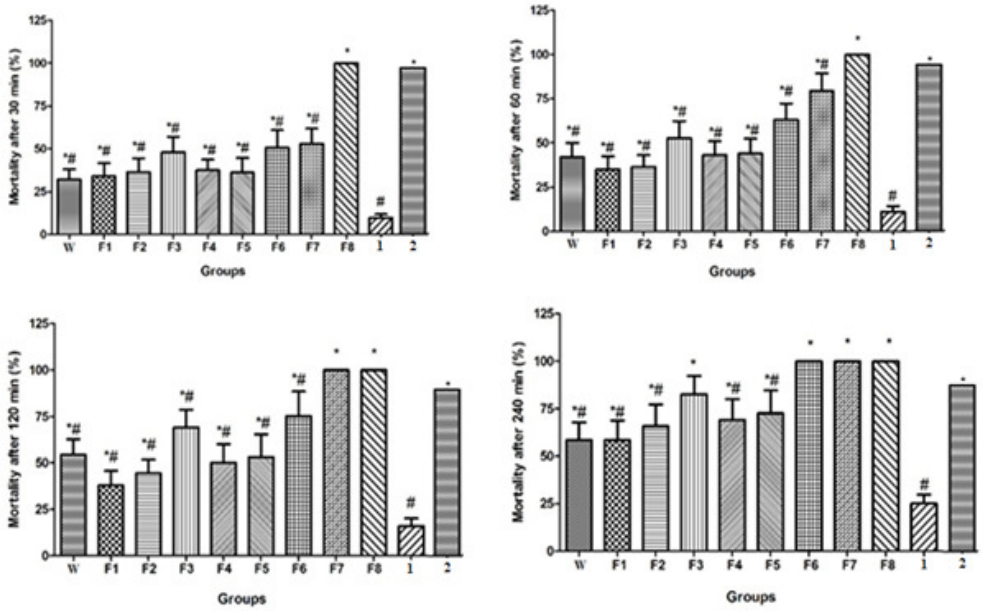

Figure 3. Effect of 8 derived fractions (F1-F8) as well as whole venom (W) on the viability rate of protoscolices, 30, 60, 120, and 240 min after exposure, 1(PBS), 2 (Formalin).

comparison with other fractions ($P<0.05$). Figure 2 demonstrates the molecular weights of extracted fractions by ion-exchange chromatography using SDS-PAGE. As can be seen, the molecular weight of fraction 8 is under 10 kDa. The antibiotic properties of venoms are not confined to scorpions. There are many studies on the antimicrobial effects of whole venoms, fractions, or recombinant peptides derived from snakes, spiders, wasps, honey bees, and also snails (Perumal Samy et al., 2017). It is a fact that the most important compounds in the venom of scorpions are polypeptides, which block sodium, potassium, chloride, or calcium-gated channels, enzymes. Moreover, some of them show enzymatic activities similar to phospholipase A2 (Guillaume et al., 2004; Incamnoi et al., 2013), lysozyme C (Baradaran et al., 2011), and hyaluronidase (Feng et al., 2008). So far, numerous detrimental organisms have been treated by the disturbance or blockage of gated ion channel mechanisms and scorpion venom is also regarded as an appropriate biologic antibiotic compound for such organisms (Possani et al., 2002). This research is considered as a first step in the study of the anti-parasitic properties of this biological process, and it is certainly necessary to study more for use in the treatment of disease. One of the substantial advantages of considering such these targets is the rapid action of a particular drug compound. On the other side, different ion channels, as well as many enzymes, are important targets for anti-parasitic purposes (Wolstenholme, 2011). For instance, praziquantel known as an anti-tapeworm component can destroy the protoscolices of hydatid cysts and interrupt Ca2+ homeostasis (Greenberg, 2005). The treatment of hydatid cyst includes medication or surgery, which is utilized in combination with chemotherapy. It is also common that before most of the surgical cyst removal, protoscolices should be killed by the injection of the suitable scolicidal compound into the cyst cavity to provide an aseptic surgery and resolve the risk of dissemination of infection to the other sites (Bygott & Chiodini, 2009). There are latitude difficulties about chemicals as treatment agents. The side effects of conventional anti-parasitic substances are considerable *per se*. Resistance to many of these drugs is a health-threatening phenomenon. Current used scolicidal agents (e.g., albendazole) take a very long incubation period of about 30 days (Pérez-Serrano et al., 1994); and therefore many authors recommended the combination of this drug with praziquantel or other scolicidals to solve the problem (Urrea-París et al., 2000). This study was a first attempt to examine the scolicidal potential of different fractions derived from *M. eupeus* (a widely distributed scorpion in Iran) venom using ion-exchange chromatography. Regarding parasites, it was reported that the venom of a new world scorpion, *Tityus discrepans*, is effective on the membrane of *Leishmania mexicana* promastigotes (Borges et al., 2006). In addition, the antimalarial and anti-*Trypanosoma* activities of some scorpion venom were also confirmed in some studies (Perumal Samy et al., 2017). The toxicity of scorpion venom for *Ancylostoma caninum* and *Shistosoma mansoni* helminthes was also supported in another study (El-Asmar et al., 1980; Xu et al., 2008). The obtained results of the current study showed that the application of the whole venom regardless of its scolicidal advantages did not significantly affect the rate of mortality of protoscolices. As a result, this compound cannot be suggested for practical implementation. In spite of the whole venom, exploited fractions revealed better results since fraction 8 killed all of the protoscolices after 30 min. It offers that the impurities of venom can inhibit the proper effectiveness of peptides. The administration of the whole venom instead of any other ingredients is easier for such studies; however, the most important advantage of using distinct peptide fraction is to prohibit any unexpected reactions in the host due to non-effective substances that exist in the venom. In the current study, the most effective fraction had a molecular weight of lower than 10 kDa. Peptides disturbing ion-gated channels usually weigh 2-8 kDa

with disulfide-bridges (Possani et al., 1999). Applied fractions elicited from ½ LD50 of the whole venom revealed that the LD50 of fractions is considerably less toxic than the whole venom. Therefore, it is speculated that the suggested quantities of fractions do not harm the tissues around the cysts (Gao et al., 2009; Ortiz et al., 2015). Non-disulfide-bridged peptides are more effective as antimicrobial and antifungal components (Moerman et al., 2002). Ortiz et al. (2015) represented that low mass biologically active peptides derived from the venom of scorpions are more toxic than a pathogenic factor for different organisms. Corzo et al. (2001) and Moerman et al. (2002) mentioned that the anti-Leishmanial and anti-microbial activity of derived fractions from *T. discrepans* venom using Sephadex G-50 gel filtration and ion-exchange chromatography were most effective when molecular weights of ingredients were the lowest. It can be concluded that *Mesobuthus epeus* venom can properly and quickly annihilate the protoscolices of hydatid cyst at the level of utilized concentrations. According to the obtained results of the current study, the scolicidal potential of *M. eupeus* venom peptides turns them into good alternatives to treat these organisms, especially when a surgeon aims to prepare the patient for surgery. It is believed that such scorpion venom ingredients are appropriate biologic alternatives when pumped in more in hand aspirated hydatid cysts as a result of sonography, computed tomography scan, or laparoscopic approaches of diagnosis (Li et al., 2014). Despite all the speculations, the kinetics of this toxicity to protoscolices is not definitely clear and needs many attempts; therefore, it is essential to provide novel insights for the management of cystic hydatidosis.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

This Study was supported by Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Ahvaz, Iran, and Shahid Chamran University of Ahvaz. The authors wish to thank the Vice-chancellor for Research of the Razi Vaccine and Serum Research Institute.

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