Investigation of the Antibacterial Effect of Venom of the Iranian Snake *Echis carinatus*

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Abstract

Although some venoms and their isolated compounds have been shown to have antibacterial properties, most have not been investigated for such activity. *Echis carinatus* is one of the most venomous snakes in the world and has an effective haematotoxic venom that destroys endothelial cells and causes haemorrhagia.

In this study, the antibacterial activity of Iranian snake *Echis carinatus* venom against six different bacteria (*Staphylococcus aureus*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes*, *Bacillus subtilis*, *Salmonella typhimurium* and *Escherichia coli* O157:H7), were investigated. Crude venom (100µg/ml) and different standard antibiotic disks as positive controls were tested by the gel diffusion method. Since the results showed that Echis carinatus venom has a significant antibacterial effect against S. aureus and MRSA, the minimum inhibitory concentrations (MIC) were also determined for these two susceptible bacteria: this was 80µg ml⁻¹ against both strains. Also, the results determined that *Echis carinatus* venom dose not have a noticeable effect on other tested bacteria.

Keywords: antibacterial, venom, *Echis carinatus*.

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Introduction
The discovery of penicillin by Alexander Fleming in 1928 changed the world of medicine. From that time, the challenge between bacteria and antibiotics started and still continues (Raghunath, 2008). The majority of bacteria such as Pseudomonas, Klebsiella, Entrobacter, Acinobacter, Salmonella, Staphylococcus, Methicillin Resistant Staphylococcus aureus (MRSA), Enterococcus and penicillin-resistant Streptococcus pneumoniae (PRSP) vancomycin-resistant enterococci have developed several ways to resist antibiotics. Such bacteria are becoming a serious clinical problem throughout the world (e.g., Ang et al., 2004). Therefore, the discovery of new effective antibacterial agents or developing antibacterials with a new mechanism is continuously necessary.

Natural products are an important source of medicinal compounds. A wide variety of organisms produce such bioactive compounds and some of these natural substances have been shown to be able to kill bacteria (Wenhua et al., 2006; Jensen et al., 2006; Perumal Samy et al., 2006 and Shittu et al., 2007). Snake venoms contain a great variety of biologically active proteins responsible for various pathological effects. Venoms include toxins which are high potency compounds with selective and specific activities. They can be useful and valuable as pharmacological tools in drug research, as potential drug design templates and as therapeutic agents (Harvey et al., 2004 and Koh et al., 2006). In recent years, venoms and venom components from different venomous animals have shown potential antibacterial activity. This includes snake (Stiles et al., 1991 and Perumal Samy et al., 2007) and scorpion venoms (Haeberli et al., 2000; Conde et al., 2000 and Tores-Larios et al., 2002). In addition, venom of the common honey bee (Apis mellifera) displays antimicrobial properties (Fennell et al., 1967, 1968). Its major component, mellitin, is more active against gram-positive than gram-negative bacteria. Also, venom of wasps has been reported to have antibacterial properties (Dani et al., 2003 and Perumal Samy et al., 2007), and spider peptide toxins have also been recognized for their antimicrobial properties (Yan and Adams, 1998; Corzo et al., 2002; Budnik et al., 2004; Benli and Yigit, 2008). Recently described potent antimicrobial peptide Latarcin 2a (Ltc2a) from Lachesana tarabaevi spider venom showed a broad-spectrum antibacterial activity (Kozlov et al., 2006).

To date, only a few studies have been made on the antimicrobial activities of snake venoms (Perumal Samy et al., 2006). In 1948, Glaser investigated antibacterial activity of Crotalus venom (Glaser, 1948) and then in 1968 Aloof Hirsch and his colleagues reported an antibacterial lytic factor from the venom of the cobra Hemachatus haemachatus (Aloof-Hirsch, 1968).

Echis carinatus is one of the most venomous viper snakes in the world, found specifically in India, Pakistan, Afghanistan and Iran (Backshall, 2007). It has also shown a potential therapeutic ability. Echistatinas and Ecarin are two medicinal drugs, isolated from Echis carinatus venom. Echistatin is one of the most potent disintegrin polypeptide which has platelet aggregation inhibitor activity and used as an anticoagulant while Ecarin is an enzyme used in the ecarin clotting time (ECT) test to monitor anticoagulation during treatment with hirudin. (Garsky et al., 1989; Tans and Rosing, 2001; Guerranti et al., 2002; Nowak G., 2004 and Gawade, 2007).

The aim of the present study was to investigate the antibacterial activity of Echis carinatus crude venom against Gram-negative bacteria (E.coli O157:H7, Salmonella typhimurium), as well as Gram-positive (Staphylococcus aureus, Methicillin-Resistant Staphylococcus aureus (MRSA), Bacillus subtillis, Listeria monocytogenes) bacteria.

Materials and Methods

Venom:
Lyophilized crude venom of Echis carinatus
was a kind gift of the Razi Vaccine and Serum research Institute, Tehran-Iran.

**Micro-organisms**
The Gram-positive bacteria including *Staphylococcus aurues* (ATCC: 25923), *Listeria monocytogenes* (ATCC: 7644), *Bacillus subtilis* (ATCC: 6633) and Gram-negative bacteria including *Salmonella typhimurium* (ATCC: 14028) and *E.coli O157.H7* (ATCC: 35150) were purchased from Mast Laboratories LTD-UK and Methicillin-Resistant *Staphylococcus aureus* (MRSA) bacteria obtained from RCC (Razi Culture Collection).

**Standard antibiotics**
The standard antibiotics including tetracycline, neomycin and streptomycin were purchased from Liofilchem S.r.l Diagnostic Company (Italy) and used for comparison with the venom.

**Methods**

**Disc-diffusion**
Lyophilized crude venom (100 µg) was dissolved in 1ml of 50mM Tris-HCl buffer (pH 7.4) and stored at 4°C for the assay. Antibacterial susceptibility tests were performed by the disc-diffusion method (Bauer et al., 1966). Pure cultures were prepared by subculturing the test strain into 10 ml of Brain Heart Infusion broth (BHI broth) (Merck), following incubation at 37°C for 24 h. The concentration of the resulting culture was determined by preparing serial dilutions and surface plating on SPC agar (Merck). The culture media was diluted and adjusted to 0.5 McFarland standards, containing $1.5\times10^6$ cfu ml$^{-1}$, in order to inoculate the same dose of bacteria in repeating the experiment. The absorbance of the cultured media was also determined at 600 nm, using a spectrophotometer apparatus (Jenway 6105, Essex, England).

A sterile cotton swab was used for spreading diluted cultures on Mueller-Hinton (MH) agar plates. Sterile blank paper discs (7mm diameter) were then placed on MH agar surface and 20µl of venom sample were added per disc in five replicates. Antibiogram disks including, Streptomycin (10 µg/disk), tetracycline (30 µg/disk) and neomycin (30 µg/disk) were used as positive controls. The plates were incubated at 37°C for 24h and the zones of inhibition were measured. The experiments were performed at least in seven replicates.

**MIC**
Minimum inhibitory concentration (MIC) of *Echis carinatus* venom was determined by broth tube dilution technique (Wu and Hancock, 1999). Cultures of bacteria were prepared by inoculating of stock bacteria on MH agar at 37°C for 24h. In the Muller Hinton broth (MHB) 3-4 colonies were grown to a mid logarithmic phase with absorbance of 0.1 at 600 nm (equal to $1.5\times10^6$ CFU ml$^{-1}$).

The venom was prepared in the range of 1.25-160 µg ml$^{-1}$ concentrations using Tris-HCl buffer (pH 7.4) (1mol l$^{-1}$). Twenty micro liter of reconstituted venom was added to 200µl of mid-logarithmic phase of bacterial culture in a 96 well plate.

The bacterial control was made by adding 200 µl inoculated bacterial culture in one well, while negative control was prepared by adding 20 µl of 1mol l$^{-1}$ of Tris-HCl buffer (pH 7.4) to 200 µl uninoculated MH broth.

In order to compare the antibacterial effect of the venom with a standard antibiotic, 20µl of tetracycline solution in Tris-HCl buffer with the same concentrations of the venom (from 160 to1.25 µg ml$^{-1}$), were added to 200µl of cultured media. The microplates were incubated at 37°C for 24h. The inhibition of bacterial growth was determined by ELISA reader apparatus (ELx 800-Bio-TEK Instrument, INC) measuring the absorbance at 560 nm.

**Results**
In order to evaluate the antibacterial effects of *Echis carinatus* venom, we used the disc-diffusion method with 20 µl of venom (100
µg/ml) and also different standard antibiotics. In five independent experiments, the venom has shown no antibacterial effects against *E. coli* O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes* and *Bacillus subtilis*. However, standard antibiotics were shown to be effective (Table 1).

In contrast, the venom was effective against *Staphylococcus aureus* and MRSA: maximum inhibitory zones were 11 and 8mm on average, respectively, in at least seven experimental repeats, while the average of inhibition zones of standard antibiotics on both of these two bacteria were 25, 20 and 15 mm for tetracycline, neomycin and streptomycin respectively (Table 1, Figures 1 and 2).

The minimum inhibitory concentration (MIC) determined on the susceptible bacteria, *Staphylococcus aureus* and MRSA, was 80µg ml⁻¹ of crude venom for both strains. The assays were performed in five replicates.

### Table 1: *In vitro* antibacterial activity of *Echis carinatus* crude venom tested by disc-diffusion and compared to some standard antibiotics. Each number is presented as mean ± SD of inhibition zone in mm, (n =7).

<table>
<thead>
<tr>
<th>Microorganism Antibiotics / Venom</th>
<th><em>L. monocytogenes</em></th>
<th><em>S. typhimurium</em></th>
<th><em>B. subtilis</em></th>
<th><em>E. coli O157:H7</em></th>
<th>MRSA</th>
<th><em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. carinatus</em> Venom</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>3.0 ± 0.13</td>
<td>8.2 ± 0.5</td>
<td>11.09 ± 2.3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
</tr>
<tr>
<td>Neomycin</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
<td>25 ± 0.0</td>
<td>20 ± 0.0</td>
<td>20 ± 0.0</td>
<td>20 ± 0.0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>15 ± 0.0</td>
<td>15 ± 0.0</td>
<td>15 ± 0.0</td>
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<td>15 ± 0.0</td>
<td>15 ± 0.0</td>
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</tbody>
</table>

Figure 1: Antibacterial effect of *Echis carinatus* venom against *Staphylococcus aureus* (A) and Methicillin Resistant *Staphylococcus aureus* (MRSA) (B), in compare with three different standard antibiotics, tetracycline, neomycin and streptomycin.

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Discussion

Researchers have reported antibacterial effects of some venoms including Echis carinatus venom against some bacteria (Perumal Samy et al., 2006; 2007). Herein it was clear that Echis carinatus venom has not a wide spectrum antibacterial effect against the mentioned bacteria, although a significant activity against S. aureus and MRSA in comparison with the standard antibiotics, tetracycline, streptomycin and neomycin has been observed. This is in agreement with the results of Gopalakrishnakone (Perumal Samy et al., 2006).

As described in the literature, venom consists of many different substances like proteins and enzymes. Which responsible for its biological activities. Therefore, these compounds may interact with specific molecules of some bacteria while not affecting other strains. Herein we conclude that the venom of Echis carinatus lacks effective proteins responsible for its antibacterial activity for some specific strains while it was effective against S. aureus and MRSA.

It has been reported that phospholipase A₂ (PLA₂) can have antibacterial effects (Nunez et al., 2004; Barbosa et al., 2005; Perumal Samy et al., 2006 and Xu et al., 2007), while Echis carinatus venom contains PLA₂ activity (Kemparaju et al., 1994), and this may be responsible for its antibacterial properties.

However, Ferreira and his colleagues reported that, Lachesis muta venom had PLA₂ activity, with no antibacterial effect. They concluded that presence of PLA₂ in the venom does not guarantee the antibacterial activity. Therefore, it is possible that Echis carinatus venom may have a specific mechanism or unknown molecule that exhibit antibacterial effect on the susceptible bacteria.

To determine whether Echis carinatus venom can influence other pathogens, further studies are needed using a wider spectrum of Gram-positive and Gram-negative bacteria and also other concentrations of this venom. Fractionated venom for detection of active components can improve investigation of its antibacterial activity.

References


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بررسی اثر ضد باکتریایی زهر مار جعفری ایران

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چکیده

اگر چه برخی از زهرها و ترکیبات مشتق شده از آنها، نشان داده اند که دارای خواص ضد باکتریایی هستند اما بیشتر آنها برای بیان چنین قابلیتهایی مورد بررسی قرار نگرفته اند. در دنبال فعالیت‌هایی در زمینه با کشف و مشخص کردن باکتری‌های وارد، باکتری‌ها نیز وارد و در فاصله‌ای مسیر می‌شوند که آن‌ها بتواند به جایی با کثرت‌های کافی و با در هم مرکبات متفاوت باکتری‌ای مطرح می‌گردد. این امر باعث شد تا باکتری‌هایی در این گروه در پژوهشگاه‌های جدید از این نوع پیدا شود و نشان داده شود که آن‌ها کاملاً با باکتری‌های مطرح می‌باشند.

واژگان کلیدی: ضد باکتری، زهر، مار جعفری