

Full Length Research Paper

Investigation of the antimicrobial and hemolytic activity of venom of some Egyptian scorpion

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Leirus quinquestriatus, *Androctonus amoreuxi* and *Androctonus australis* are venomous scorpion in Egypt. Their venom is complex mixture of salts, neurotoxins, peptides and proteins which has therapeutic applications, and can rapidly kill a broad range of microbes. Estimations of total proteins of the venom indicated that *L. quinquestriatus* had the highest value than the others (10.64 ± 0.04). Hemolytic activity of human erythrocytes was detected and showed that all concentrations (20, 8 and 5 mg/ml) of tested scorpion species crude venom have hemolytic activity against human erythrocytes. The present study was conducted to evaluate the antimicrobial activity of the three Egyptian scorpion's venom against four Gram-positive and negative bacterial strains (*Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii* and *Kliformella pneumonia*) in addition to one fungus species *Candida albicans*. The results show that *L. quinquestriatus* venom has a significant antibacterial effect against *B. subtilis* and *C. freundii*. In contrast, *A. amoreuxi* and *A. australis* venoms do not have a noticeable effect on tested microbes. So, the aim of the present study was to investigate the antimicrobial activity of crude venom against Gram-negative, as well as Gram-positive bacteria, fungi and to evaluate the hemolytic activity of the three investigated species on human erythrocytes.

Key words: Crude venom, *Leirus quinquestriatus*, *Androctonus amoreuxi*, *Androctonus australis*, hemolytic activity, antibacterial activity.

INTRODUCTION

Scorpions are venomous arthropod animals belonging to the class Arachnida (Gouge et al., 2001). All scorpions are venomous but approximately 50 of them have enough poison to kill a person (Osnaya-Romero et al., 2001; Isbister et al., 2003). Scorpions use venoms for immobilization of prey and protection against predators, and these venoms consist of a complex of several toxins that exhibit a wide range of biological properties and actions (Petricevich, 2010).

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 (Shittu et al., 2007). Scorpion venoms contain a great

variety of biologically active low molecular weight proteins responsible for various pathological effects as, neurotoxins, enzyme inhibitors, hemolytic toxins (Possani et al., 1999, 2002). In recent years, venoms and venom components from different venomous animals have shown potential antimicrobial activity, this includes snake (Perumal Samy et al., 2007; Shittu et al., 2007; Al-Ahmadi et al., 2010), scorpion (Conde et al., 2000, Torres-Larios et al., 2002) and spider venom (Kozlov et al., 2006; Kuhn-Nentwig et al., 2002a, b). Many studies detected the toxicity of the three scorpion species used in the present study. *Leirus quinquestriatus* is a venomous species which are incremented in most stings in Saudi Arabia (Antoplosky et al., 2009). While, *Androctonus australis* is one of the

most dangerous scorpions in the world, with very potent venom. This species are medically important, and cause several deaths each year (Gaban, 1997). *Androctonus amoreuxi* venom caused teratogenic effect on rats (Ismail et al., 1983).

Hemolysis due to envenomation by scorpion is so common that when a patient is suspected of been stung by a scorpion, the first clinical test is to check the presence of hemoglobin in the patient's urine (Radmanesh, 1990). First reports on the hemolytic activity of scorpion venom have been available since 1918 (Balozet, 1971). Starting in 1996, possible pore-forming peptides from the venom of *Scorpio maurus palmatus* were assumed to be responsible for the induction of leak currents in *Xenopus laevis* oocytes (Debont et al., 1996) and cardiac cells of the rat (De Plessis et al., 1999). Scorpion sting cause severe hemolysis which fluctuated according to scorpion species and venom concentration. So, the present study was designed to characterize the antimicrobial activity against one fungus species, Gram positive and negative bacteria, as well as hemolytic activity of three Egyptian scorpion crude venom: *L. quinquestriatus*, *A. australis* and *A. amoreuxi*.

MATERIALS AND METHODS

Sites of collection

Scorpions were collected by professional hunters during the period of April to October 2012. Three species of scorpion, *L. quinquestriatus*, *A. australis* and *A. amoreuxi* were collected from Aswan, Borg El Arab and Baltim in Egypt. Then determination of scorpion age and sex as female adult scorpion was according to pectin, body length and color. Their length range between 9-9.5, 9.5-10, and 8.5-10, respectively, and they were kept them in glass containers according to the geographical areas. Then, the scorpions were anesthetized to collect the venom samples immediately at collection to avoid stress.

Venom samples

Twenty telsons from each collected species of scorpions were used. Venom solution was prepared using the maceration method in which telsons were removed from anesthetized scorpions at the point of their articulation with the last caudal segment (Ozkan and Filazi, 2004; Ozkan et al., 2006). All telsons were weighed, ground to a fine powder and dissolved in physiologic saline solution (PSS; 0.9% w/v NaCl) and kept at 4°C for 72 h. The venom solution was centrifuged at 10000 g for 10 min at 4°C. Supernatant was removed and immediately lyophilized and stored at -20°C until use.

Protein assay

Protein concentrations were determined by using a commercial kit (Biomed Diagnostics, 30175 Hannover, Germany) using bovine serum albumin as a standard.

Hemolytic assay

Hemolytic activity of the crude venom was assayed with heparinized human red blood cells rinsed three times in 5 mL

phosphate buffered saline (PBS - 50 mM NaH_2PO_4 and 150 mM NaCl, pH 7.2) and centrifuged for 5 min at 3,000 rpm. Red blood cells were then incubated at room temperature for 1 h in deionized water as positive control (100% hemolysis), in PBS as negative control or blank (0% hemolysis), or with venoms at three concentrations (5, 8 and 20 mg/mL) in PBS. Three replicas of the samples were centrifuged at 12,000 rpm for 5 min. The supernatant was separated from the pellet, and its absorbance was measured at 570 nm and its standard deviation (SD) was calculated. The percentage of hemolysis was calculated using the following equation:

$$\text{Absorbance of sample} / \text{Absorbance of control (100\% hemolysis)} \times 100$$

Gram-positive and negative bacterial strains

Different Gram-positive and negative bacteria in addition to a fungus were used as indicator organisms in this study. *Citrobacter freundii*, *Kliformella pneumoniae*, *Bacillus cereus* and *Bacillus subtilis*, as well as *Candida albicans* were used. These microorganisms were kindly provided by Bacteriology Unit, Botany Department, Faculty of Science, Tanta University. The indicator organisms were grown on nutrient agar plates at 37°C for 24 h.

Preparation of venom dilution for antimicrobial activity

Two concentrations were used from each scorpion's crude venom (20 and 10 mg/ml). The extracted venom from each scorpion species were diluted with PBS.

Evaluation of antimicrobial activity

Overnight culture of microorganisms was prepared and 5×10^5 cfu ml^{-1} in 100 μl Potassium-Sodium-Phosphate buffer, (pH 7) were spread onto nutrient agar plates. 50 μl of each prepared concentration of the crude venom was loaded into well made in the center of inoculated plates. The plates were incubated at suitable temperature (37°C) overnight. The antimicrobial activity was determined by the agar well diffusion method (Galvez et al., 1986). The largest inhibition zone of the indicator bacterium indicates the most antimicrobial-producing test organisms. Nutrient broth or agar (Lapage et al., 1970): Peptone, 5 g; Beef extract, 3 g; sodium chloride, 5 g; distilled water up to 1000 ml and pH was adjusted to 7.

Statistical analysis

Data were statistically processed three times for scorpion species, and standard variations (SD) were estimated, also coefficients were calculated between species using Pearson correlation (r2).

RESULTS

Total protein contents

Total protein contents is shown in Table 1 and Figure 1 show that the highest value of protein concentration was in *L. quinquestriatus* (10.64 ± 0.04 mg/ml), then *A. amoreuxi* which was 9.18 ± 0.13 mg/ml, while the lowest value was in *A. australis* (8.6 ± 0.053).

Table 1. Total protein contents of the three scorpion species.

Specimen	Total protein content (mg/ml)
<i>Leiurus quinquestriatus</i>	10.64 ± 0.04
<i>Androctonus amoreuxi</i>	9.18 ± 0.13
<i>Androctonus australis</i>	8.6 ± 0.053

Hemolytic activity

Different concentrations of scorpion venom disrupted human erythrocytes. Hemolytic activity of scorpion venom is dose dependent for each species as shown in Table 2 and Figure 2. Hemolytic activity of *L. quinquestriatus* (Lq) show high value range between 98.18 and 28.03%. *A. amoreuxi* (Ax) concentrations show high values close to each other ranging between 95.4 and 89.5 %, while the lowest values obtained for the concentrations of *A. australis* venom (As) ranged between 65.91 and 10.03%, which showed the highest coefficient between different concentrations ($r^2 = 0.98$).

Antimicrobial effect of scorpion venom

Antimicrobial effect of venom of three species of scorpion using two concentrations (20 and 10 mg/ml) were tested on Gram-positive bacteria (*B. cereus* and *B. subtilis*), Gram-negative bacteria (*C. freundii* and *K. pneumoniae*) and one species of fungus (*C. albicans*) as shown in Figures 3 to 7. All venom concentrations of both *A. amoreuxi* and *A. australis* have no effect on all species of bacteria and fungus. While, *L. quinquestriatus* venom inhibited two bacterial strains; *B. subtilis* and *C. freundii*.

Inhibitory zone of different venom concentrations of *L. quinquestriatus* is shown in Table 3 and Figures 3 and 4, which illustrated that, the higher the concentration, the higher the inhibitory zone. The most effective results were observed on *B. subtilis*, it showed inhibitory zone of 19.66 ± 0.95 and 17 ± 0.127 with both concentrations of *L. quinquestriatus* venom. While, it showed inhibitory zone of 17.66 ± 0.255 and 15.33 ± 0.337 on *C. freundii*.

DISCUSSION

Scorpions are soil animals exposed to microorganisms such as bacteria and fungi that inhabit the soil. Thus, it is anticipated to produce some antibacterial substance for predation and self -protection. As described in the literature, venom consists of many different substances like proteins and enzymes, which are responsible for its biological activities (Petricevich, 2010). So, the present study measured total protein of scorpion crude venom. First reports on the hemolytic activity of scorpion venom have been available since 1918. Starting from 1996, possible pore forming peptides from the venom of

Scorpio maurus palmatus and *Opisthophthalmus carinatus* (both Scorpionidae) were assumed to be responsible for the induction of leak currents in *Xenopus laevis* oocytes and cardiac cells of the rat (Du Plessis et al., 1999).

Hemolysis can be induced by several protein toxins from animals, plants and microbes, particularly marine animals (Parker and Feil, 2005). Some of these venoms affect biological membranes by inducing the formation of pores or channels in natural and model bilayer lipid membranes (García-Sáez et al., 2011; Savva et al., 2013). Thus, hemolytic activity induced by protein toxins has been used as a sensitive toxicological tool to investigate the targeting and attachment of proteins to cell membranes (Sabirov et al., 1993).

In the present study, three concentrations of different venom of investigated samples were used to estimate percentage of hemolysis of human erythrocytes. The study found that the percentage of erythrocyte hemolysis was concentration dependent. The highest percentage of hemolysis was recorded with high venom concentration (20 mg/ml) which reached 98.18% in *L. quinquestriatus*. While, hemolysis percentage of 8 mg/ml of *A. amoreuxi* venom (94.45%) is more than that represented by the same concentration of *L. quinquestriatus* venom (78.7%). Thus, we assumed that the hydrophilic pores in erythrocyte cell membrane induced by scorpion venom caused colloid osmotic burst that resulted in erythrocyte lysis. In addition to pore-forming mechanisms, lipid peroxidation of erythrocyte membranes plays an important role in the hemolysis induced by hemolytic protein toxins, resulting in cell membrane disorder (Parker and Feil, 2005; García-Sáez et al., 2011). Valavi and Alemzadeh-Ansari (2008), reported that *Hemiscorpius lepturus* scorpion has hemolytic activities. Also, crude venom of wolf spider *Lycosa singoriensis* has hemolytic effect on human erythrocytes in a dose-dependent manner (Liu et al., 2009). On the other hand, some toxins in both scorpion venom as hadrurin and in centipedes as scolopendrin I showed hemolysis activity at different concentrations, and it reached 75% hemolysis under the effect of hadrurin from scorpion (Wenhua et al., 2006).

The present study shows also the antimicrobial effect of scorpion species venom, because scorpion species often use to spray venom on their own bodies to disinfect them from possible saprophytic organisms including bacteria and fungi, showing that venom of these scorpions could contain some sort of antibiotic potential (Torres-Larios et al., 2002). Scorpion venom contains peptides which exhibit anti-microbial properties (Gordon et al., 2005; Brown and Hancock, 2006). In this study, the sensitivity of five microbial strains was tested against the crude venom of three scorpion species. Four Gram-negative and positive bacteria and fungal strains were studied.

From the three scorpion species, only *Leiurus quinquestriatus* crude venom with its two concentrations were effective only against *Bacillus subtilis* and *Citrobacter freundii* with inhibition zone ranging between

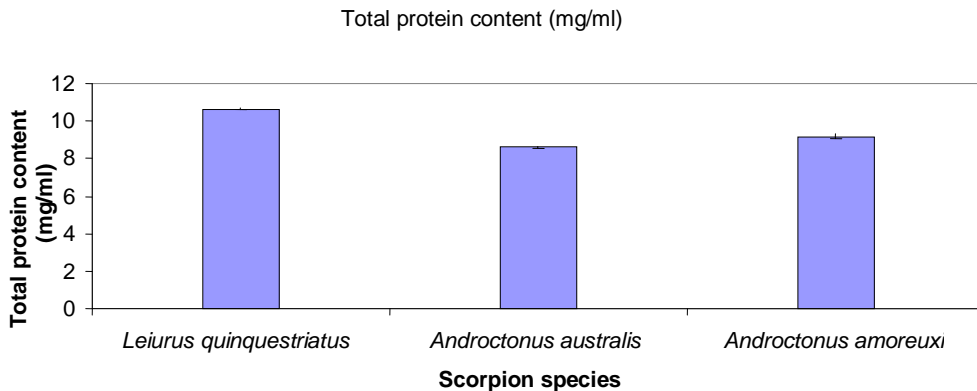


Figure 1. Total protein contents (mg/ml) of scorpion species.

Table 2. Absorbance (560 nm) and percentage of hemolysis (%) due to different concentrations of scorpion venom.

Specimen/concentration	Absorbance at 560 nm	Hemolysis (%)
<i>Leiurus quinquestriatus</i> (Lq)		
20 mg/ml (Lq 1)	1.996 ± 0.05	98.18
8 mg/ml (Lq 2)	1.6 ± 0.028	78.7
5 mg/ml (Lq 3)	0.57 ± 0.022	28.03
r2	0.695	
<i>Androctonus amoreuxi</i> (Ax)		
20 mg/ml (Ax 1)	1.94 ± 0.050	95.4
8 mg/ml (Ax 2)	1.9 ± 0.066	93.45
5 mg/ml (Ax 3)	1.82 ± 0.041	89.5
r2	0.747	
<i>Androctonus australis</i> (As)		
20 mg/ml (As 1)	1.34 ± 0.0317	65.91
8 mg/ml (As 2)	0.314 ± 0.094	15.44
5 mg/ml (As 3)	0.204 ± 0.148	10.03
r2	0.989	

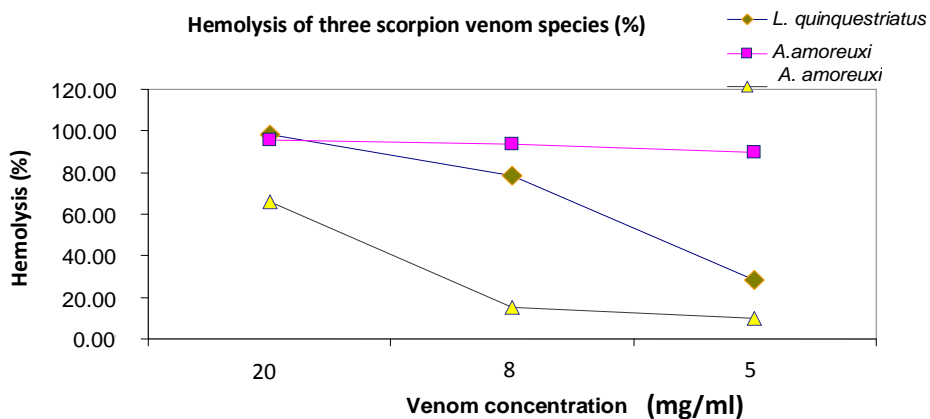


Figure 2. Percentage (%) of hemolysis of three scorpion venom.

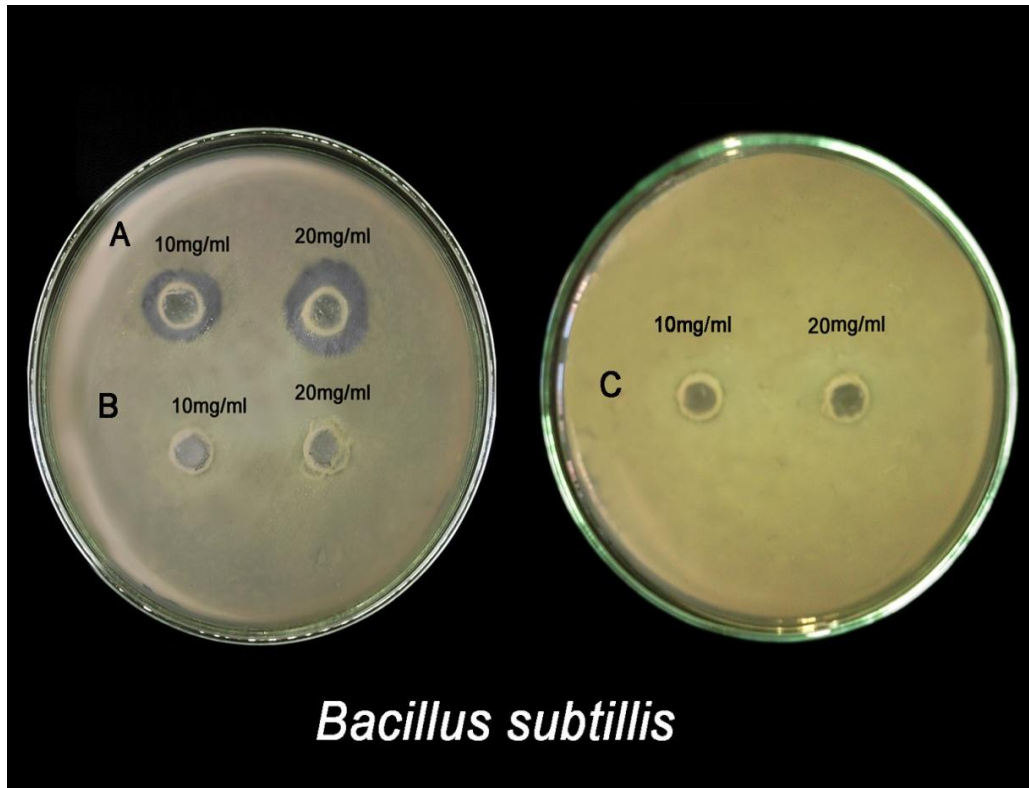


Figure 3. Antibacterial activity of different concentration of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *B. subtilis*.

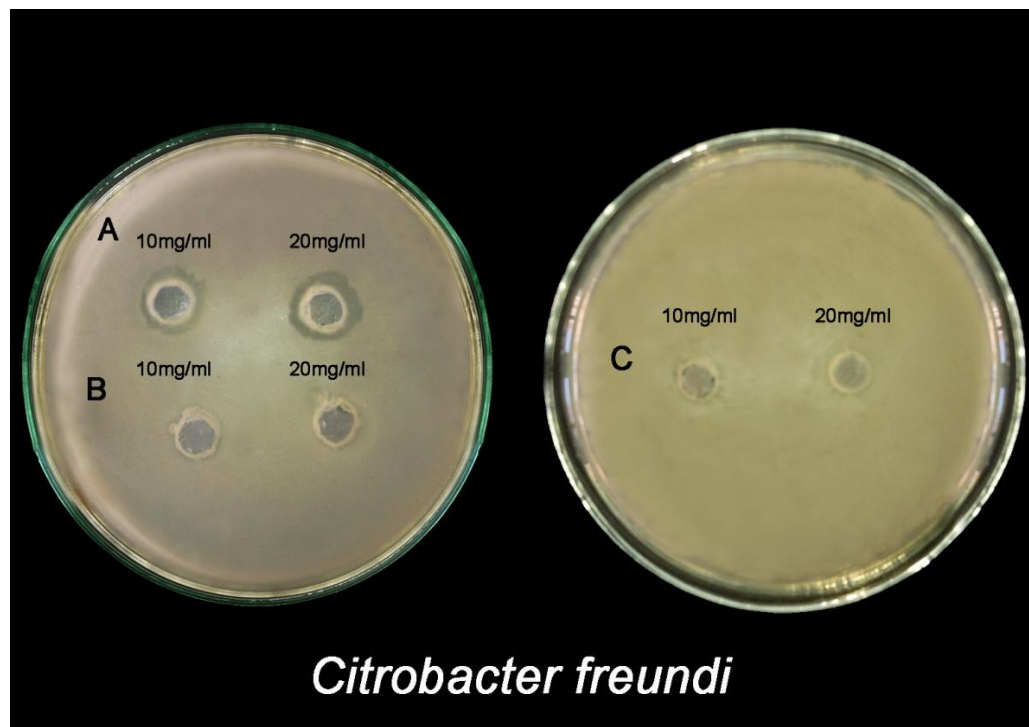


Figure 4. Antibacterial activity of different concentration of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *C. freundii*.

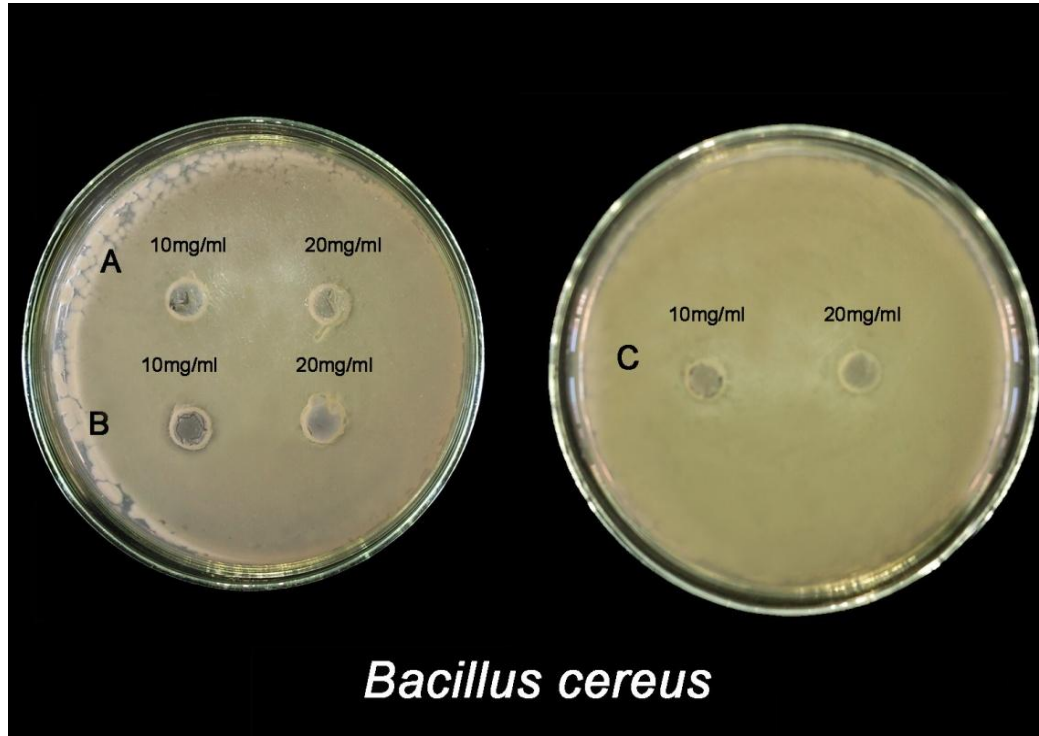


Figure 5. Antibacterial activity of different concentrations of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *B. cereus*.



Figure 6. Antibacterial activity of different concentrations of crude venom of *Leiurus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *K. pneumoniae*.

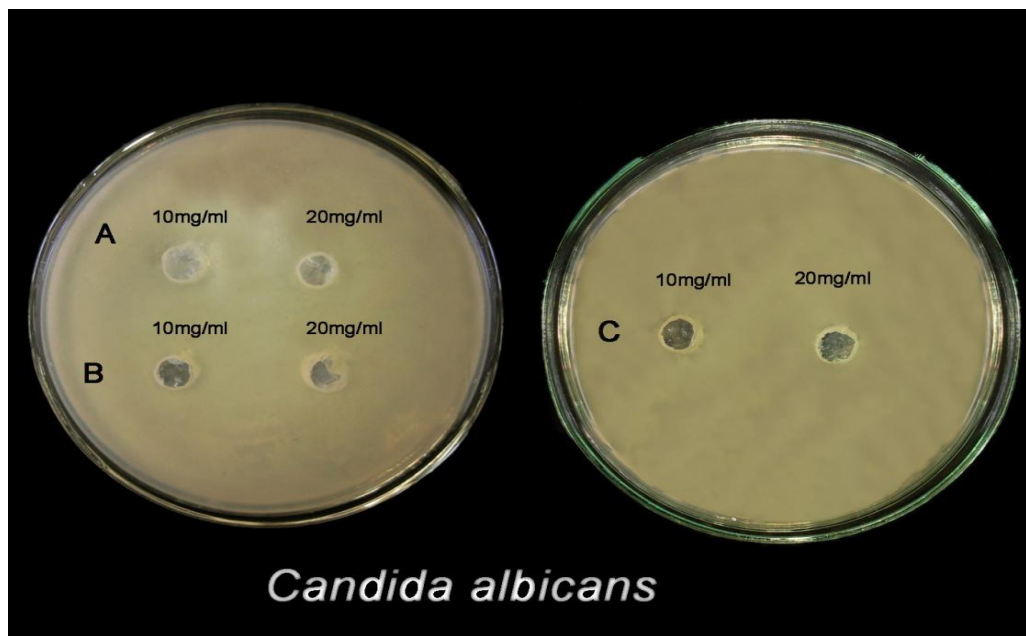


Figure 7. Antimicrobial activity of different concentrations of crude venom of *Leiorus quinquestriatus* (A), *Androctonus amoreuxi* (B), *Androctonus australis* (C) on *C. albicans*.

Table 3. Inhibitory zone (mm) of *L. quinquestriatus* against two bacterial strains.

Bacteria strain	Concentration	
	<i>Leiorus quinquestriatus</i>	
	20 mg/ml	10 mg/ml
<i>Bacillus subtilis</i>	19.66 ± 0.95	17 ± 0.127
<i>Citrobacter freundii</i>	17.66 ± 0.255	15.33 ± 0.337

19.66 and 15.33 mm. While, all the bacterial strain (*B. cereus*, *K. pneumoniae*), and fungus (*C. albicans*) were resistant to this crude venom. These results consistent with that of Ahmed et al. (2012) which found that scorpion *Heterometrus xanopus* has antibacterial effect on *B. subtilis* with inhibition zone of 30 mm, while, *Escherichia coli* showed resistance against the same venom. This study was compatible with Liu et al. (2009) study on wolf spider *Lycosa singoriensis* which was effective only on *B. subtilis* under very low concentration (3 mg/ml) and has weak effect on *C. albicans* fungus. Interestingly, *Buthus martensii* venom was detected only against Gram-positive bacteria but not against Gram-negative one (Gao et al., 2007). Because venom consists of many different substances like proteins and enzymes which are responsible for its biological activities, therefore, these compounds may interact with specific molecules of some bacteria while not affecting other strains. Herein, we believed that the venom of *A. amoreuxi* and *A. australis* may lack effective proteins responsible for its antimicrobial activity for all strains; while *L. quinquestriatus* venom

may be have effective proteins which affect some microbial strain (*B. subtilis* and *C. freundii*). Moreover, *L. quinquestriatus*, possesses venom that is the most toxic of all scorpions. This venom contains "histamines, enzymes, enzyme inhibitors, and the potent neurotoxins, chlorotoxin and charbydotoxin (Neurophysiologywordpress.com).

Interestingly, *L. quinquestriatus* showed the highest protein content, and this elucidated the increase in its hemolytic and antimicrobial activity. Further studies are needed using a wider spectrum of Gram-positive and negative bacteria to determine the *L. quinquestriatus*, *A. amoreuxi*, and *A. australis* venom for detection of their active components.

Finally, in the present study, all venom concentrations of three scorpion species showed hemolytic activity and the hemolysis percentage depending on the protein contents of their crude venom. *A. australis* venom showed the lowest hemolytic activity as compared to the other species. However, not all of them showed antimicrobial activity on the above mentioned strains. As

a result, we recommended separating the antibacterial polypeptides from *L. quinquestriatus* venom to avoid hemolytic activity of its crude venom, using other microbial strains. Furthermore, there should be application of lower concentration of *A. australis* venom on wide range of microbes, due to its lower hemolytic activity.

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