Original Article
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Investigating Antibacterial Effects of *Latrodectus Dahli* Crude Venom on *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*

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ABSTRACT

Background and Objectives: Nowadays, infections with antibiotic-resistant bacteria are among the most important causes of mortality worldwide. This has attracted the attention of researchers to seek suitable alternatives for antibiotics. The venom of many toxic species such as arthropods has antibacterial properties. In this study, we investigated antibacterial effects of crude venom of *Latrodectus dahli* on *Escherichia coli, Staphylococcus aureus*, and *Bacillus subtilis*.

Methods: Lyophilized crude venom of *L. dahli* was dissolved in 50 mM Tris-HCl buffer. Protein concentration was determined by the Bradford assay. Then, the bacteria were exposed to different concentrations (31.25-250 ng/mL) of the crude venom. Inhibitory activity of the venom against the bacteria was determined by MTT assay and determining minimum inhibitory concentration (MIC).

Results: Results of the MTT assay showed that the crude venom significantly inhibited the growth of *E. coli* (31.25 and 62.5 ng/mL), *S. aureus* (at 250 ng/mL) and *B. subtilis* (at 125 and 250 ng/mL). In the MIC experiment, the crude venom significantly inhibited the growth of *E. coli* (at concentrations of 31.25 and 62.5ng/mL), *S. aureus* (at concentrations of 31.25-250 ng/mL) and *B. subtilis* (at concentrations of 31.25-250ng/mL).

Conclusion: The crude venom of *L. dahli* and its components showed relatively strong antibacterial effects.

Keywords: Spider venoms, Black Widow Spider, Antibacterial agent, Drug-resistance.

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INTRODUCTION

Recently, researchers have focused on antimicrobial peptides (AMPs) to combat antibiotic resistance (1, 2). These peptides contain deformed amino acids that are not found in polypeptides made by ribosomes. Research has shown that these compounds have great medicinal potential. Selective toxicity is an essential characteristic of an antimicrobial agent. Ideally, such compounds have affinity for one or more microbial determinants that are easily accessible, common to a broad spectrum of microbes and relatively immutable. Nature seems to develop a class of molecules that meet these constraints in the evolution of AMPs, which initially target microbial cells, and thus fulfill the criteria mentioned above for identifying molecular determinants of pathogens. AMPs have amphipathic features that mirror phospholipids, thus allowing them to interact with and exploit vulnerabilities inherent in essential microbial structures such as cell membranes (3). Until now, the antibacterial activity of more than 1000 peptides from different eukaryotic and prokaryotic sources have been investigated to find a suitable antimicrobial alternative for antibiotics (4, 5).

It has been demonstrated that venom of snakes, scorpions and spiders has strong antibacterial effects (6). Some studies have also shown that AMPs have anticancer effects (7, 8). Peptides from arthropods are cationic and amphiphilic and do not contain cysteine residues (9). Spiders are members of arthropods and have more than 60 families and 35000 species. They live in nearly every habitat on earth (10). *Latrodectus* spider, also known as the black widow, is a member of the *Theridiidae* family (11). The venom of this spider contains α-latrotoxin, a 130 kDa mammalian neurotoxin that has been used for studying exocytosis in cells (12). In this study, we investigate antibacterial effects of the venom of black widow spider on *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*.

MATERIALS AND METHODS

3-(4,5-Dimethyl-2-thiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) powder, flat bottom 96-well plates and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. Tris-HCL and crude venom of the black widow spider were purchased from Merck (Germany) and Razi Institute (Iran), respectively. Lyophilized venom was dissolved in 250 µL of Tris-HCl buffer (50mM), incubated at 4°C and then kept in a freezer at -20 °C.

The Bradford assay was used for determining protein concentrations. Bovine serum albumin (BSA) was used for plotting the standard curve and microplate spectrophotometer (Epoch, Biotek) was used for measuring the absorbance of the samples at 595 nm.

E. coli (ATCC 25922), *S. aureus* (ATCC 25923) and *B. subtilis* (ATCC 6633) were purchased from the Persian Type Culture Collection (PTCC).

In brief, the bacteria were cultured in Mueller Hinton broth media inside of a 96-well plate until reaching turbidity of 0.5 McFarland (Q-lab). Minimum inhibitory concentration (MIC) was calculated as the lowest concentration of venom that inhibited bacterial growth (13).

The bacteria were exposed to different concentrations of the crude venom (31.25-250 ng/mL). Tetracycline (50 µg/mL) and bacterial suspension were used as positive and negative controls, respectively. In addition, Mueller Hinton broth was used as a blank. The final volume for each well of 96 plate was set as 100 µL. The assay was repeated three times for each concentration.

After 15 to 18 hours of incubation at the defined condition, cell density was measured at 605 nm (14). MIC was calculated using the following equation: $\mathrm{MIC} = \left(1 - \frac{OD\ sample - OD\ blank}{\mathrm{OD\ negative\ control} - OD\ blank}\right) \times 100.$

After 15 to 18 hours, 5 µL of MTT dye (5 mg/mL) were added to all wells and the plate was incubated for one hour in dark at 37 °C. Then, 100 µL of DMSO was added to each well and the plate was incubated for two more hours in dark. Absorbance was read at 595 nm (15). All experiments were carried out in triplicate. Cell viability was calculated using the following equation: $viability = \frac{OD\ sample - OD\ blank}{\mathrm{OD\ negative\ control} - OD\ blank} \times 100.$

Results were reported as mean ± standard deviation (SD) and data were analyzed in the GraphPad Prism 6.1 software (GraphPad Software Inc., USA) using ANOVA and the Tukey test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The results of MIC assay indicated that 31.25 and 62.5 ng/mL of the crude venom had significant inhibitory effects on the growth of *E. coli* cells. However, the inhibitory effect of the control antibiotic was much stronger than that of the crude venom. The MIC values of the crude venom were 9.3±4.61, 9±3.60, 5.7±4.04 and 3.7±3.05 percent against *E. coli* at the concentrations of 31.25, 62.5, 125 and 250 ng/mL, respectively. The MIC analysis of the antibiotic for these cells showed that 99.16 ± 0.40 percent of the cells were inhibited when using 50 µg/mL of the venom (Figure 1A).

Results of the MTT assay showed that 31.25 and 62.5 ng/mL of crude venom have significant inhibitory effects on the growth of *E. coli* compared with the control. However, the antibiotic showed stronger antibacterial activity compared to the crude venom. The viability of *E. coli* was 85.7±5.09, 90.3±1.15, 93.7±0.87 and 98±3.46 percent when using 31.25, 62.5, 125, and 250 ng/mL of crude venom, respectively. The viability of *E. coli* was 4.64 ± 3.67 percent when using 50 µg/mL of the antibiotic (Figure 1B).

Concentrations of 31.25-250 ng/mL of crude venom had significant inhibitory effects against *S. aureus*. MIC of the crude venom was 5±1, 3.7±2.08, 3±1, and 2 percent at the concentrations of 31.25, 62.5, 125, and 250 ng/mL, respectively. However, the inhibitory effect of the antibiotic was much stronger than the crude venom against *S. aureus* (Figure 2A). Results from MTT showed that 250 ng/mL of the crude venom had significant inhibitory effect on the growth of *S. aureus*. However, the viability of *S. aureus* treated with tetracycline (50 µg/mL) was much lower (16.7±5.03 percent) than those treated with venom (Figure 2B).

The crude venom at concentrations of 31.25-250 ng/mL had significant inhibitory effect on the growth of *B. subtilis*. MIC of the crude venom was 7.7±0.87, 7.7±1.32, 7.9±2.08 and 7.8±4 percent at the concentrations of 31.25, 62.5, 125 and 250 ng/mL, respectively. Based on the results, 50 µg/mL of antibiotic showed stronger inhibitory activity than the mentioned concentration of crude venom (Figure 3A).

In the MTT assay, concentrations of 125 and 250 ng/mL of crude venom had significant inhibitory effects on the growth of *B. subtilis*. *B. subtilis* viability was 97±2, 98±1.73, 90.3±3.02 and 91±2 percent at concentrations of 31.25, 62.5, 125 and 250 ng/mL, respectively. However, tetracycline showed higher inhibitory effect against this bacterium compared to the venom (Figure 3B).

Figure 1-(A) Inhibitory effects of different concentrations of crude venom against *E. coli* in the MIC assay. (B) Viability of *E. coli* after exposure to 31.25-250 ng/mL of crude venom in the MTT assay (1.5×10^8 cells/well). Tetracycline (50µg/mL) and medium without crude venom were used as the positive and negative control, respectively (ns: non-significant,*: $p<0.05$,: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).**

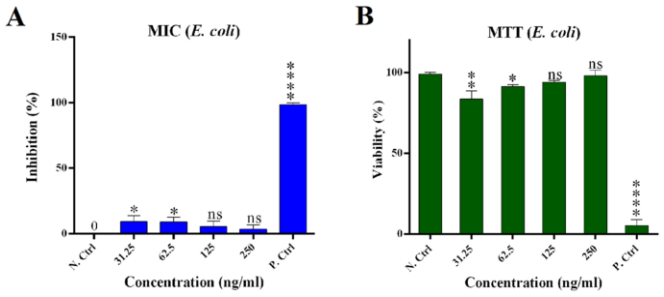


Figure 2- (A) Inhibitory effects of different concentrations of crude venom against *S. aureus* in the MIC assay. (B) Viability of *S. aureus* after exposure to 31.25-250 ng/mL of crude venom in the MTT assay (1.5×10^8 cells/well). Tetracycline (50µg/mL) and medium without crude venom were used as the positive and negative control, respectively (ns: non-significant,*: $p<0.05$,: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).**

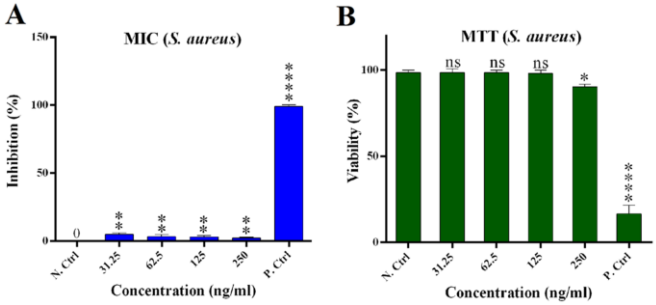
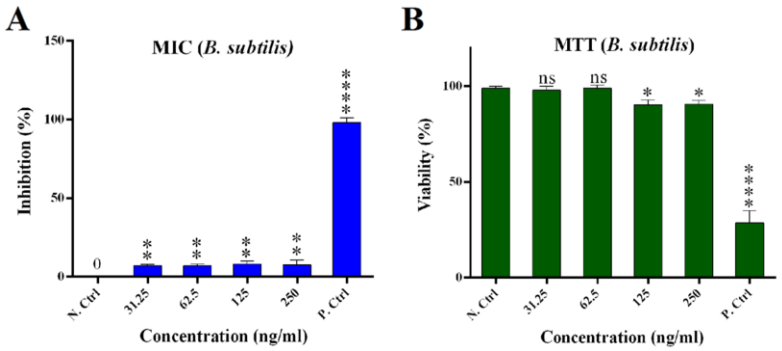


Figure 3- (A) Inhibitory effects of different concentrations of crude venom against *B. subtilis* in the MIC assay. (B) Viability of *B. subtilis* after exposure to 31.25-250 ng/mL of crude venom in the MTT assay (1.5×10^8 cells/well). Tetracycline (50µg/mL) and medium without crude venom were used as the positive and negative control, respectively (ns: non-significant,*: $p<0.05$,: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$).**



DISCUSSION

Since the discovery of penicillin in 1940, various antibiotics have been proposed for the treatment of bacterial diseases and control of bacterial epidemics (16). The emergence of antibiotic-resistant bacteria along with the side effects of antibiotics, namely allergic hypersensitivity and immunosuppression (17, 18) encouraged researchers to seek for new generations of antibiotics or alternative antimicrobials (19-21). Recently, isolation of antibacterial molecules from animals, such as AMPs has been considered for the treatment of diseases. Venoms of different species such as snakes, spiders, insects, centipede and amphibians are rich sources of biologically active and therapeutic compounds including AMPs (4, 6, 22-24).

The innate immune system of arthropods has evolved a complex arrangement of constitutive and inducible AMPs that immediately destroy a large variety of pathogens. In this complex system, several enzymes, low-molecular-mass compounds, neurotoxins, antimicrobial and cytolytic peptides interact together, resulting in extremely rapid immobilization and/or killing of prey or aggressors (25). Many studies have shown that the venom of snakes, scorpions and spiders have antibacterial effects (6). As the largest venomous animals with more than 50000 species (26), spiders and their venom have been long used in traditional medicine for treatment of various diseases (27). In this study, we investigated the inhibitory effects of crude venom from black widow spider (*L. dahli*) against a number of bacteria using the MTT assay and MIC determination. Disc diffusion and well diffusion methods had low reproducibility and were not suitable for this study. Since the venom was water insoluble, paper discs (as a filter and block) were used in the disc diffusion assay for evaluating the antibacterial activity of the crude venom and its components during culture (28-31).

Some studies have suggested that the antimicrobial effect of venom is promoted by the increased permeability of the bacterial cell membrane induced by antimicrobial proteins present in spider venom (32). The results indicated that 31.25, 62.5, 125 and 250 ng/mL of the crude venom had significant antibacterial effects against all tested bacteria. A study performed by Amirmozafari et al. in 2015 revealed that concentrations of 100, 250 and 500 µg/mL of *Tarantula Cubensis* venom (Theranekron) have no antibacterial effect on *E. coli* strain K-12, *S. aureus* and *Pseudomonas aeruginosa* (33).

A study carried out by Lei et al. in 2015 showed that protein components purified from the *Latrodectus tredecimguttatus* egg as Latroeggtoxin-IV (1.8 µg/disc) has higher antibacterial activity against *S. aureus* compared to *B. subtilis* and *E. coli* (34). In 2008, Benli and Yigit reported that the venom of *Agelena labyrinthica* has more inhibitory effects on *B. subtilis* compared to the *S. aureus* and *E. coli* (19). In our study, the results of MTT assay showed that the crude venom could significantly reduce the viability of *E. coli* (at 31.25 and62.5 ng/mL), *S. aureus*

(at 250 ng/mL) and *B. subtilis* (at 125 and 250 ng/mL). In the MIC experiment, the crude venom significantly inhibited the growth of *E. coli* (at 31.25 and 62.5 ng/mL), *S. aureus* (at 31.25-250 ng/mL) and *B. subtilis* (at 31.25-250 ng/mL). Altogether, these results indicated that the crude venom significantly inhibited the growth of *E. coli* at low concentrations and *S. aureus* and *B. subtilis* at high concentrations.

CONCLUSION

The crude venom of black widow spider, *L. dahli*, has relatively strong antibacterial effects. This suggests that the crude venom of this spider may be a suitable source of antibacterial.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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