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ANTIBACTERIAL ACTIVITY OF VENOM FROM FUNNEL WEB SPIDER *Agelena labyrinthica* (ARANEAE: AGELENIDAE)

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ABSTRACT: Since the number of microorganisms that are resistant to antibiotics has been increasing steadily, the need for combating these pathogens requires new pharmaceutical agents. To produce these substances, new models have been developed in recent decades. In our study, the venom of *Agelena labyrinthica* (Clerck, 1757) (Araneae: Agelenidae) was tested against ten bacterial strains, specifically, testing 1/100, 1/10 and 1/1 fractions of diluted venom against these bacteria. While the 1/100 dilution was successful in only one of ten bacterial strains, the 1/10 and the 1/1 were effective on six of ten bacterial strains. The most effective results, among these three different concentrations, were observed on *Bacillus subtilis*. The other five strains that were also sensitive to the dilutions showed similar inhibition zones. Morphological alterations on bacterial cells and comparison with normal cells were accomplished by scanning electron microscopy (SEM). The venom-treated cells, due to their loss of cytoplasm, shrank and presented cell wall depression.

KEY WORDS: *Agelena labyrinthica*, venom, antimicrobial activity.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

There have been many studies on venomous animals and their poisons published in the literature due to a direct connection between these toxins and several biological fields. Similarly to other poisonous animals, such as snakes and scorpions, some spiders can produce venoms that are composed of complex mixtures of biologically active and inactive substances. The major constituents of spider venoms are proteins; polypeptide and polyamine neurotoxins; enzymes; nucleic and free amino acids; monoamines; and inorganic salts (9, 12). Over millions of years, spiders have evolved toxins, with a variety of targets, primarily affecting the nervous systems of their prey. But the main purpose of their venoms remains killing or paralyzing prey (5).

The toxins isolated from spider venom have been invaluable in determining the role and diversity of neuronal ion channels and the process of exocytosis (16).

Due to the development of antibiotic-resistant bacteria, antibacterial peptides have attracted much attention in recent years, in order to aid the search for new therapeutic agents. In 1981, Steiner *et al.* (17) identified the first antibacterial peptides, cecropins, from the *Hyalophora cecropia* moth. In the following years, peptides presenting antibacterial activity were found throughout the entire animal kingdom, ranging from bacteria and different insect orders to amphibia, humans and other mammals (1, 7). Gomesin was the first peptide isolated from a spider exhibiting antimicrobial activities. This highly cationic peptide is composed of 18 amino acid residues including four cysteines that form two disulfide linkages (15). Additionally, spider toxins have an enormous pest control potential that may be employed in agriculture (10, 18).

Kuhn-Nentwig *et al*. (13, 14) investigated antimicrobial activity of the venom from the *Cupiennius salei* spider. They isolated several antimicrobial peptides from it, synthesized their analogs and found that these peptides had lytic activity on human red blood cells and insecticidal effect on *Drosophila melanogaster.* In another study, by Corzo *et al*. (3), five amphipathic peptides – called oxyopinins and exhibiting antimicrobial, hemolytic and insecticidal activity – were isolated from the crude venom of the wolf spider *Oxyopes kitabensis*.

As previously mentioned, prolonged use of broad-spectrum antibiotics has led to the emergence of drug-resistant pathogens, both in medicine and in agriculture. In addition, new threats such as biological warfare have increased the need for novel

and efficacious antimicrobial agents. Thus, the present study aimed to test the venom's antibacterial activity of the palearctic species *Agelena labyrinthica* (Clerck, 1757) (Araneae: Agelenidae).

MATERIALS AND METHODS

Spiders

Five hundred adult *A. labyrinthica* of both sexes were collected in Ankara, Kirikkale and Mersin, Turkey, from June 2003 to September 2006. They were reared in special cages and fed *Oncopeltus fasciatus* (Hemiptera) in the Biology Department of Ankara University.

Venom Milking

In order to milk the spiders, they were first narcotized with ether and subsequently submitted to direct current electric shock (15 V). Electrodes were placed on the prosoma of adult spiders. The venom from fang tips was withdrawn with a micropipette and immediately used. To avoid contamination with saliva, the venom was collected only from the fang tips (19). The amount of venom per spider was approximately 0.1 µL. The animals were fed after milking and then not milked again for at least two weeks.

Test Microorganisms

Antibacterial studies were carried out with a series of ten bacterial strains (*Enterococcus gallinarium* CDC-NJ-4, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* RSHE*, Escherichia coli* ATCC 25922, *Shigella* sp. RSHE, *Escherichia coli* RSHE, *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 7644, and *Pseudomonas aeruginosa* ATCC 27853). These microorganisms (six gram-positive and four gram-negative) were obtained from the Culture Collection of the Microbiology Laboratory, Refik Saydam Hifzissihha Institute (RSHI). The strains were inoculated into Nutrient Broth® (Merck, Germany) and incubated for 24 hours at 37 ± 0.1°C

Determination of Antibacterial Activity

Microorganism cultures that had grown for 24 hours were diluted to 10^{-1} with sterile physiological saline solution (0.85% NaCl) (x10^7 CFU/mL). One hundred microliters

of test microorganisms was inoculated on solid medium surface on plates (Muller-Hinton Agar®, Merck, Germany) and three drops of venom (10 µL) were placed onto each plate. The agar plates containing bacterial strains were incubated at 37 ± 0.1°C for 24 hours. After incubation, all plates were observed for inhibition zones and the diameters were measured in millimeters. All experiments were carried out three times. The following antibiotics were used as positive controls: amikacin, 30 µg/mL (Eczacibasi); vancomycin, 30 µg/mL, (Mayne); penicillin, 10 U/mL (I. E.Ulagay); gentamicin, 10 µg/disc (I.E.Ulagay); rifamicin, 5 µg/mL (Aventis); tetracycline, 30 µg/mL (Sigma); ampicillin, 10 µg/mL (Selva); chloramphenicol, 30 µg/mL (Sigma); and erythromycin, 15 µg/mL (Sigma). Positive control discs were tested on the same microorganisms under the same conditions. The antibiotic supply mentioned above was prepared in appropriate amounts (µg/mL), 20 µL were absorbed onto discs (6 mm), and for negative control, insect saline solution was used (insect saline solution: 1.80 g NaCl, 1.88 g KCl, 0.16 g $CaCl_2$, 0.004 g $NaHCO_3$ and 100 mL of distilled water).

Determination of Bacterial Cell Damage by Scanning Electron Microscopy

The bacterial samples that were susceptible to the venoms (diluted with 1/1 insect saline solution) were prepared for scanning electron microscopy (SEM). The observations were carried out on agar plates. Small portions of agar containing inhibition zones and bacteria were cut out. Small agar pieces were fixed in 3% (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.2) for an hour at room temperature and then washed four times in sodium phosphate buffer, and post-fixed in 1% (w/v) osmium tetroxide in the same buffer for an hour then washed four times in the same buffer. They were then dehydrated in a graded ethanol series. The last stages of dehydration were performed with propylene oxide ($CH_3CH.CH_2.O$). The specimens were then dried in a drying oven at 30^oC overnight (8). The dried specimens were mounted onto stubs by double sided carbon tape. The specimens were coated with a thin layer of gold by a Polaron SC 502 sputter coater, and were examined in a Jeol JSM 6060 LV Scanning Electron Microscope.

RESULTS

Adult individuals of both sexes of spiders were collected and identified as *A. labyrinthica* (Figure 1). The venom was milked from spiders and used in this study to determine antibacterial activity against the test bacterial strains.

In our studies venom diluted 1/100, 1/10 and 1/1 was tested against ten bacterial strains. While venom diluted 1/100 was effective against only one of ten bacterial strains (*B. subtilis*) (Table 1), the 1/10 dilution venom was effective on six of ten strains (*Bacillus subtilis* RSHE, *Escherichia coli* ATCC 25922, *Shigella* RSHE, *Escherichia coli* RSHE, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853) (Table 1). To increase the venom concentration, venom diluted 1/1 was tested against the same strains, and results similar to those of the 1/10 treatment were obtained. Furthermore, the 1/1 dilution presented clearer and larger inhibition zones than observed 1/10 treatments (Table 1). The most effective results were observed against *B. subtilis* at these three different venom concentrations. The other five strains that were found to be sensitive had also shown similar inhibition zones.

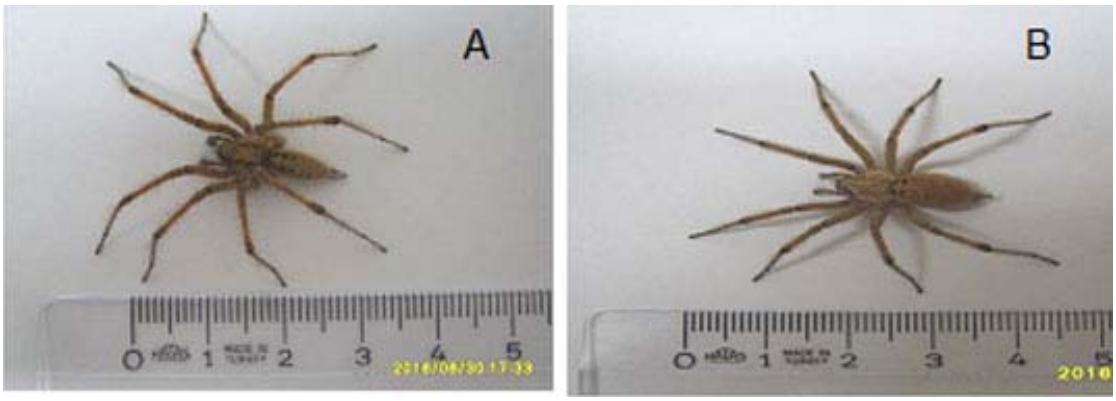

Figure 1. Dorsal view of male (**A**) and female (**B**) of *A. labyrinthica.*

Table 1. The inhibition zones were formed by three different concentrations of venom, negative control and some standard antibiotics. *RSHI: Microbiology Laboratory Culture Collection of Refik Saydam Hifzissihha Institute, (-): No inhibition zone (resistant)

Microorganisms	The diameter of inhibition zone (mm)												
	1/1 venom	1/10 venom	1 /100 venom	Negative control	Amikacin	Vancomycin	Penicillin	Gentamicin	Rifamicin	Tetracycline	Ampicilin	Chloramphenicol	Erythromycin
Enterococcus gallinarium CDC-NJ-4	–	–	–	–	–	12	–	15	13	–	–	–	11
Enterococcus faecalis ATCC 29212	–	–	–	–	16	12	–	16	14	–	–	–	11
Bacillus subtilis RSHI*	23	12	8	–	24	19	22	25	23	12	–	13	24
Escherichia coli RSHI	10	7	–	–	18	–	–	18	–	–	–	–	–
Shigella sp. RSHI	10	9	–	–	20	–	–	19	–	–	–	–	–
Escherichia coli ATCC *25922*	10	8	–	–	16	–	–	17	–	–	–	–	–
*Streptococcus pyogene*s ATCC 19615	–	–	–	–	13	12	–	16	15	–	–	–	12
Staphylococcus aureus ATCC 29213	10	8	–	–	17	15	19	17	27	12	–	–	18
Listeria monocytogenes ATCC 7644	–	–	–	–	25	16	–	27	39	–	–	–	19
Pseudomonas aeruginosa ATCC27853	10	7	–	–	17	–	–	15	–	–	–	–	–

When positive controls were compared with the venom treatments, *B. subtilis* and *S. aureus* were found to be the strains most sensitive to antibiotics and venom. The spider venoms were effective against these bacteria as were standard antibiotics. On the other hand, *Shigella*, *P. aeruginosa* and two different strains of *E. coli* were found to be sensitive to spider venom as demonstrated by effective inhibition zones, although these strains were resistant to antibiotics used for treatment against diseases (Table 1).

The action mechanism of these antibacterial peptides in venom was not determined. To observe the morphological alterations on bacterial cells caused by the venom (diluted with 1/1 insect saline solution) and to compare with normal bacterial cells, we used Scanning Electron Microscope (SEM). The venom-treated bacterial cells, due to the loss of cytoplasm, presented depression of cell wall and shrinkage (Figure. 2). Similar results were found in the other six bacterial strains (*B. subtilis, Shigella*, *S.*

aureus, *P. aeruginosa* and two different strains of *E. coli*.) which were found to be sensitive against spider venom by SEM studies.

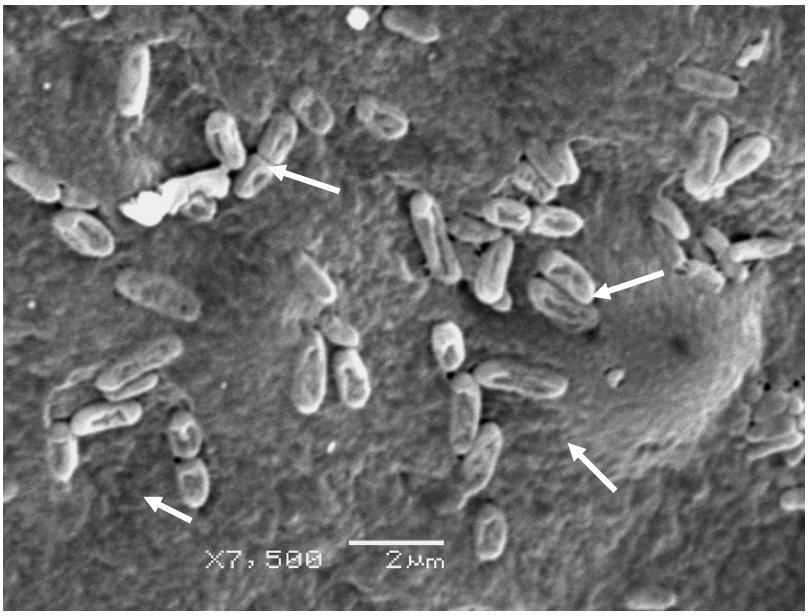

Figure 2. The treated cells (*Shigella* sp.) are shrinking. Note the cytoplasm loss and depressed cell walls (arrows).

DISCUSSION

In our study, three different concentrations of *A. labyrinthica* venom, considered an alternative resource for antibiotics, were tested against ten bacterial strains. While 1/100 diluted venom was effective only against one of ten strains (*B. subtilis*), 1/10 and 1/1 dilutions were efficient against six (*B. subtilis*, *Shigella* sp., *S. aureus*, *P. aeruginosa* and two *E. coli* different strains). In these trials, the most sensitive bacterium was *B. subtilis*. Although the results of venom diluted 1/1 and 1/10 were similar, when the venom concentration was increased, the 1/1 dilution presented a clearer and larger inhibition zone.

Several studies have already identified antimicrobial activity in spider venoms. Yan and Adams (21) identified two peptide toxins that present antimicrobial activity, lycotoxins I and II, in the venom of the wolf spider *Lycosa carolinensis* (Araneae: Lycosidae). Antimicrobial assays showed that both peptides potently inhibit the growth of *E. coli* and *Candida glabrata* yeast. Similarly, in the present study, we found an antibacterial effect in *A. labyrinthica* venom that had inhibited the growth of two *E. coli* strains.

Antimicrobial peptides (AMP) were detected in the venom glands of *Lycosa singoriensis* – another wolf spider species – by Budnik *et al.* (2) and named lycocitin 1, 2 and 3. Both lycocitin 1 and 2 restrain the growth of gram-positive (*S. aureus*, *B. subtilis*) and gram-negative (*E. coli, P. aeruginosa*) bacteria as well as that of fungi (*Candida albicans*) (2). We proved that *A. labyrinthica* crude venom hindered the development of gram-positive (*S. aureus, B. subtilis,*) and gram-negative (two strains of *E. coli, Shigella* and *P. aeruginosa*) bacteria. In another study, seven novel short linear antimicrobial and cytolytic peptides – called latarcins – were purified from the venom of *Lachesana tarabaevi* spider and found to produce lytic effects on cells of diverse origin (gram-positive and gram-negative bacteria, erythrocytes and yeast) (11).

Haeberlia *et al*. (7) isolated five antibacterial peptides from the venom of *Cupiennius salei*, a neotropical wandering spider.

Several antimicrobial peptides were isolated from the venoms of different spider species. These peptides are supposed to increase membrane permeability by formation of either distinct channels or pores and to lyse both prokaryotic and eukaryotic cells (4, 7, 21). To observe morphological alterations after cell incubation in crude venom, scanning electron microscopy (SEM) was employed. When the cells treated with crude venom were compared with the untreated ones, the first appeared to be shrinking and presented degradation of the cell walls. This finding indicates that *A. labyrinthica* venom possesses antibacterial activity against some strains.

It is clear that this spider venom contains components presenting lytic activity, which have not yet been purified or identified. However, the analysis of *A. labyrinthica* venom by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that there were at least seven components ranging from 10 to 40 kDa (22).

Antimicrobial peptide studies are providing new insights into the dynamic interactions between microorganisms and their hosts, and are generating new paradigms for the pathogenesis and treatment of diseases. Given that antimicrobial peptides of higher eukaryotes differ structurally from conventional antibiotics produced by bacteria and fungi, they offer novel templates for pharmaceutical compounds that could be effective against increasingly resistant microorganisms (6).

Many infections acquired in hospitals are caused by potentially fatal bacteria, such as *S. aureus*, that are resistant to many antibiotics like methicillin (20). Our study demonstrated that the venom of *A. labyrinthica* was effective against *S. aureus*.

Consequently, effective new drugs against antibiotic-resistant microorganisms can be developed from spider venoms.

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