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In vitro antibacterial effect of wasp (Vespa orientalis) venom

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Abstract

Background: The emergence of antibacterial resistance against several classes of antibiotics is an inevitable consequence of drug overuse. As antimicrobial resistance spreads throughout the globe, new substances will always be necessary to fight against multidrug-resistant microorganisms. Venoms of many animals have recently gained attention in the search for new antimicrobials to treat infectious diseases. Thefore, the present study aimed to study the antibacterial effects of wasp (Vespa orientalis) crude venom.

Two gram-positive bacteria (Staphylococcus aureus and Bacillus subtilis) and two gram-negative ones (Escherichia coli and Klesiella pneumonia) were compared for their sensitivity to the venom by determining the inhibition zone (Kirby-Bauer method) and minimum inhibitory concentration (MIC). A microbroth kinetic system based on continuous monitoring of changes in the optical density of bacterial growth was also used for determination of antimicrobial activity.

Results: The venom exhibited a well-recognized antimicrobial property against the tested bacterial strains. The inhibition zones were determined to be 12.6, 22.7, 22.4 and 10.2 mm for S. aureus, B. subtilis, E. coli and K. pneumonia, respectively. The corresponding MIC values were determined to be 64, 8, 64 and 128 μg/mL, respectively. The MIC_{50} and MIC_{90} values of the venom were respectively determined to be 63.6 and 107 μg/mL for S. aureus, 4.3 and 7.0 μg/mL for B. subtilis, 45.3 and 65.7 μg/mL for E. coli and 74.4 and 119.2 μg/mL for K. pneumonia. Gram-positive bacteria were generally more sensitive to the venom than gram-negative ones.

Conclusions: Results revealed that the venom markedly inhibits the growth of both gram-positive and gram-negative bacteria and could be considered a potential source for developing new antibacterial drugs.

Keywords: Wasp venom, Vespa orientalis, Antimicrobial activity, Minimum inhibitory concentration

Background

Since the discovery of penicillin, numerous antibiotics have been developed, primarily to treat bacterial or fungal infections. This fact constituted an important contribution to human and animal health in the fight against infectious diseases [1,2]. The widespread and/or inappropriate use of antibiotics and chemicals against harmful microorganisms has led to microbial resistance [3]. The emergence of antibiotic-resistant organisms comprises a serious worldwide problem. Recent findings on new antibiotic-resistant organisms include multiple-drug resistant (MDR) *Pseudomonas aeruginosa*, MDR *Acinetobacter baumannii* and New Delhi Metallo-β-Lactamase-1 (NDM-1) producing bacteria. Kumarasamy *et al.* [4] reported that the

'superbugs' that produce NDM-1 were resistant to almost all antibiotics, except for polymyxin and tigecycline.

The augmentation in antibiotic resistance is a major challenge in medicine since not many new antibiotics are being produced, and bacteria resistant to currently available drugs are increasing. Seeking novel antibacterial substances and antibiotic combination therapy are the strategic options to overcome the MDR organisms [5,6]. Such initiative has greatly driven the search for new antimicrobial agents with novel mechanisms of action that are broadly effective and less likely to induce antimicrobial resistance. These drugs will be very important, particularly for the treatment of immune compromised patients [7-9].

Despite tremendous advances in biological sciences, the difficulty in identifying new mechanisms to kill bacterial pathogens is discouraging. Thus, finding alternative sources of new drugs or prototypes is of major interest to complementary medicine. In the hope of inventing novel

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antimicrobial agents to control antibiotic-resistant bacteria, natural products are an important source of medicinal compounds. A wide variety of organisms produces such bioactive compounds and some of these natural substances have been shown to be able to kill bacteria [10,11]. Venoms of a vast number of animal species represent complex mixtures of compounds (ions, biogenic amines, polyamines, polypeptide neurotoxins, cytolytic peptides, enzymes etc.) responsible for various effects [12-15]. Venoms can also be useful and valuable as pharmacological tools in drug research, as potential drug design templates and as therapeutic agents [16,17]. In recent years, venoms and venom components from animals have shown potential antibacterial activity. These include venom of wasps, common honeybees, spiders, snakes and scorpions [18-24]. Bearing in mind all these facts, the present study was conducted to evaluate the antibacterial activity of *Vespa orientalis* venom against different strains of gram-positive and gram-negative bacteria.

Methods
Venom extraction
Vespa orientalis specimens were collected from Abarkooh, Yazd Province, Central Iran. Wasps were paralyzed at 4°C, their venom glands were dissected and immersed in liquid nitrogen. The glands were then crushed in a clean mortar using pestle and liquid nitrogen. Twenty milliliters of 0.1 M buffer phosphate (pH = 7.4) was added to the powdered sample immediately after the evaporation of liquid nitrogen. The suspension was then transferred into a clean tube and further homogenized. Each tube containing the sample was centrifuged at $8,000 \times g$ for 15 minutes at 4°C. The supernatant was transferred into another tube, lyophilized and kept at –20°C until further assay.

Antimicrobial assay
Bacterial strains
The microorganisms used in the antibacterial screening assays were: gram-positive bacteria including *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 1010649, and gram-negative bacteria including *Escherichia coli* ATCC 35218 and *Klebsiella pneumonia* ATCC 700603. The bacteria were resuspended in tryptic soy broth (TSB), incubated at 37°C overnight and stored at 4°C.

Agar disc diffusion method
The screening of antimicrobial activity of the crude venom was carried out by agar disc diffusion method using Mueler-Hinton agar (Merck, Germany). Similarly, tetracycline was used for comparison. The bacterial inocula were prepared from the colonies of 24 hour-cultured bacteria on nutrient agar. The inocula were adjusted with McFarland density to obtain a final concentration of approximately 10^5 CFU/mL. The Millipore filter paper discs (Millipore Corporation, USA) were impregnated with either crude venom or tetracycline (30 μg) and applied on the test media previously inoculated with each bacterial strain. Plates were incubated at 37°C and inhibition zones were measured after 24 hours of incubation.

Broth microdilution method
The minimum inhibitory concentration (MIC) of crude venom and tetracycline was also determined using conventional broth microdilution method according to the CLSI guidelines [25]. The adjusted bacterial suspensions were added to each well of sterile microtiter plate containing the test concentrations of antimicrobials (100 μL/well) in Mueler-Hinton broth (MHB, Merck, Germany). Consequently, final inoculum concentration of 1×10^5 CFU/mL was obtained in each well and this plate was incubated for 24 hours at 37°C. The antimicrobial, a non-treated control, and a sterility control were also used. Each assay was carried out in triplicate. The lowest concentration of antibiotic which inhibited the visible bacterial growth was selected as MIC.

Growth curves
Antimicrobial activity of the crude venom was also examined using a 96-well sterile microtiter plate. The crude venom and tetracycline (Sigma, Germany) were serially diluted in MHB at concentrations of 1024, 512, 256, 128, 64, 32, 16, 8, 4 and 2 μg/mL and 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 μg/mL, respectively, in a final volume of 100 μL. Each well was inoculated with 10 μL of the bacterial suspension containing 10^6 CFU/mL. Each test was performed in triplicate. Three wells containing bacterial suspension with no drug (growth control) and three wells containing high concentration of tetracycline (background control) were also included. Optical densities were measured for 24 hours at 37°C using a multi-detection microplate reader (BioTek's PowerWave XS2, USA) at 600 nm and recorded automatically for each well every two hours. Turbidimetric growth curves were obtained depending on the changes in the optical density of bacterial growth for each drug concentration and the drug-free growth control.

For the determination of MIC of crude venom and tetracycline by the microbroth kinetic assay, the percentage of growth at each drug concentration was calculated using the following equation:

$$\% \ Inhibition = 100 - \left[\frac{\text{OD of venom containing wells-OD of background control}}{\text{OD of the growth control-OD of background control}} \right] \times 100$$
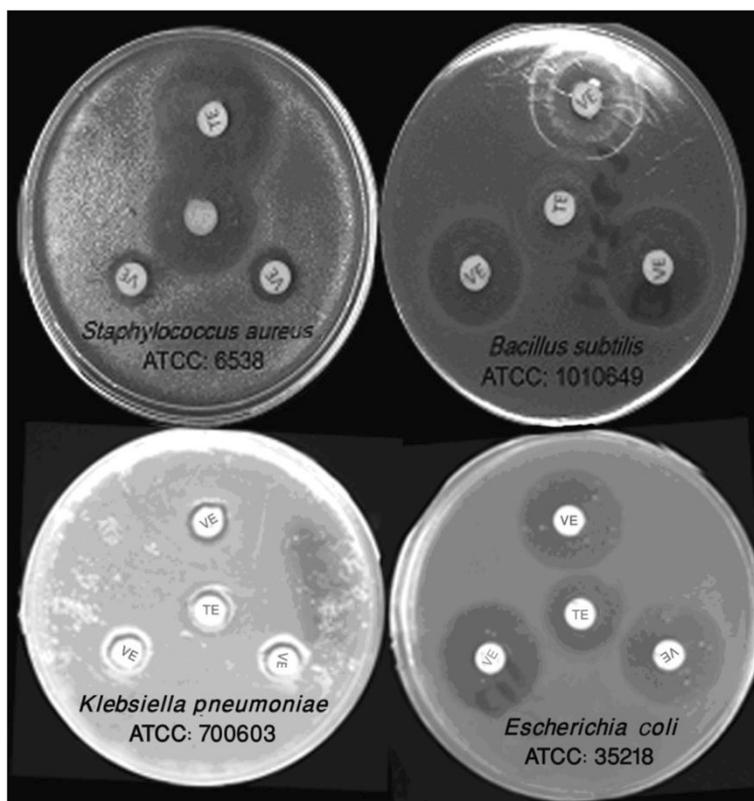
Figure 1 Inhibitory effect of *Vespa orientalis* (VE: venom extract, 30 µg/disc) on the growth of different bacterial strains compared with tetracycline (TE: tetracycline, 30 µg/disc).

Results

Vespa orientalis crude venom displayed a significant effect against different gram-positive and gram-negative bacterial strains emplyed in this study. The corresponding inhibition zones and MICs are listed in Figure 1 and Table 1. The crude venom caused a marked inhibition in bacterial growth with inhibition zones of 12.6, 22.7, 22.4 and 10.2 mm for *S. aureus*, *B. subtilis*, *E. coli* and *K. pneumonia* respectively. The corresponding MICs of the crude venom were respectively found to be 64, 8, 64 and 128 µg/mL using the conventional microdilution method (Figure 1 and Table 1). Growth curves of different bacteria during the incubation period in the presence of various concentrations of crude venom are presented in Figure 1. The MIC_{50} and MIC_{90} of the crude venom against different bacteria determined by microbroth kinetic system were respectively 63.6 and 107 µg/mL for *S. aureus*; 4.3 and 7 µg/mL for *B. subtilis*; 45.3 and 65.7 µg/mL for *E. coli*; 74.4 and 119.2 µg/mL for *K. pneumonia* (Figures 2 and 3; Table 2). All tested bacterial strains were found to be susceptible to the venom and among them, *B. subtilis* was the most sensitive. In addition, the present findings indicate that the crude venom is more effective against gram-positive than gram-negative bacteria.
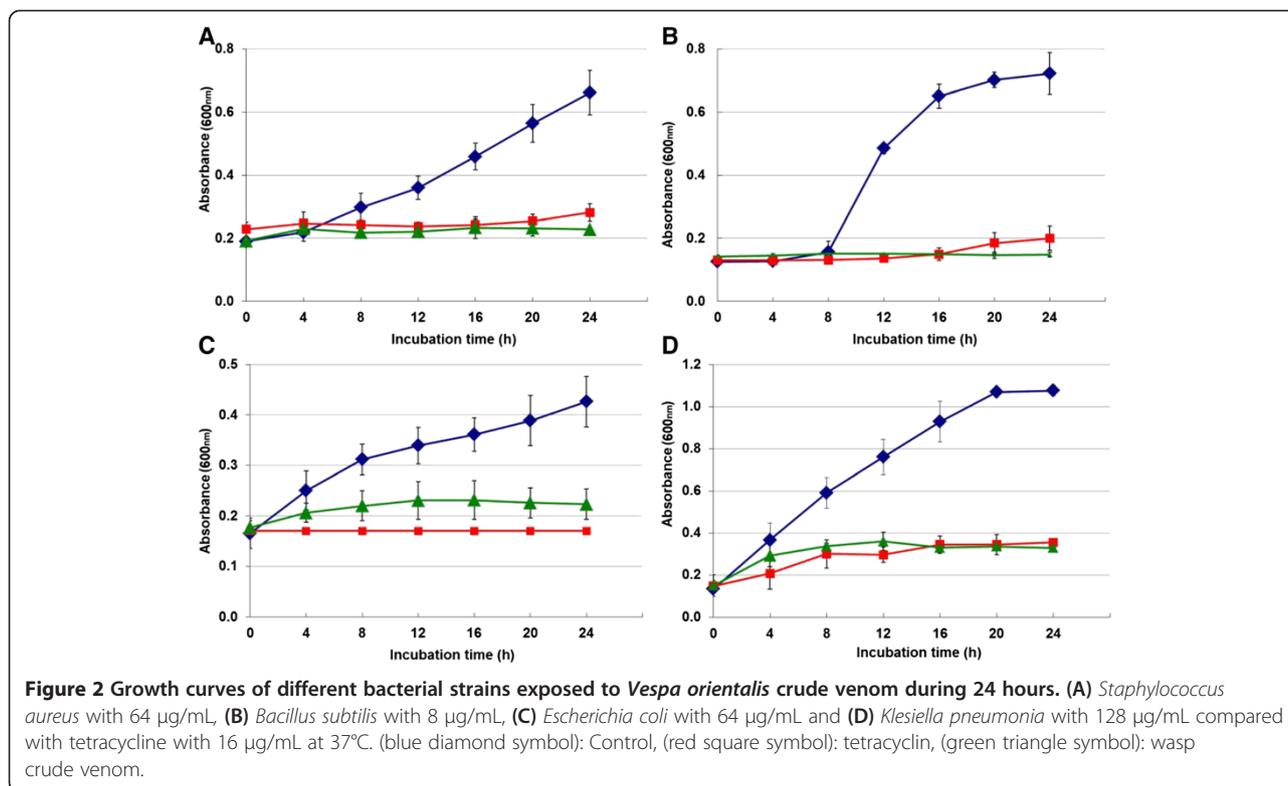
Discussion

The present study describes the assessment of antimicrobial effects of *Vespa orientalis* crude venom using the microbroth kinetic system, which determines the effect of antibacterial agents by continuous monitoring of bacterial culture optical density. The crude venom exhibited activity against both gram-positive and gram-negative bacteria and the MICs obtained in microbroth kinetic system were similar to those found using conventional broth microdilution method. A shorter incubation period was required in the former technique and percentages of growth inhibition were also measurable.

Table 1 Inhibitory effect of *Vespa orientalis* crude venom on different strains of bacteria

Microorganisms	Inhibition zone[1] (mm)		MIC[2] (µL/mL)	
	Venom	**Tetracycline**	**Venom**	**Tetracycline**
S. aureus	12.6 ± 0.31[3]	28.1 ± 0.64	64	0.125
B. subtilis	22.7 ± 0.62	18.9 ± 0.14	8	0.125
E. coli	22.4 ± 0.68	19.3 ± 1.75	64	0.5
K. pneumonia	10.2 ± 0.12	12.1 ± 0.13	128	1

[1]Growth inhibition effects of venom and tetracycline was determined by disc diffusion method (30 µg/disc). [2]Minimum inhibitory concentrations were obtained using serial dilutions. [3]Mean ± SD (n = 3).

Figure 2 Growth curves of different bacterial strains exposed to *Vespa orientalis* crude venom during 24 hours. (A) *Staphylococcus aureus* with 64 μg/mL, **(B)** *Bacillus subtilis* with 8 μg/mL, **(C)** *Escherichia coli* with 64 μg/mL and **(D)** *Klesiella pneumonia* with 128 μg/mL compared with tetracycline with 16 μg/mL at 37℃. (blue diamond symbol): Control, (red square symbol): tetracyclin, (green triangle symbol): wasp crude venom.

To address the rapid emergence of resistance to the classical antibiotics, naturally occurring antibacterial agents are promising candidates in the search for novel therapeutic agents [26]. Antibacterial property has been reported for the venoms of a wide variety of animals including venoms of snakes, scorpions, spiders, conus and wasps all of which are predatory or parasitic animals [27-30]. However, the actual function of antimicrobial agents in these venoms is not clear yet.

Venom components from predator wasps including hornets (genera *Vespa* and *Dolichovespula*), yellow jackets (genus *Vespula*) and paper wasps (genus *Polistes*) have been extensively studied. Their toxins are complex mixtures of amines, small peptides and high molecular weight proteins such as enzymes, allergens and toxins [31-33]. Venoms from these stinging wasps are important weapons both in the defense of the colony and capture of prey. To the best of our knowledge, only a few of its components have been purified and characterized from parasitic Hymenoptera, such as metalloproteinase, serpin, calreticulin-like protein, aspartyl glucosaminidase-like protein and insecticidal toxins [34-38].
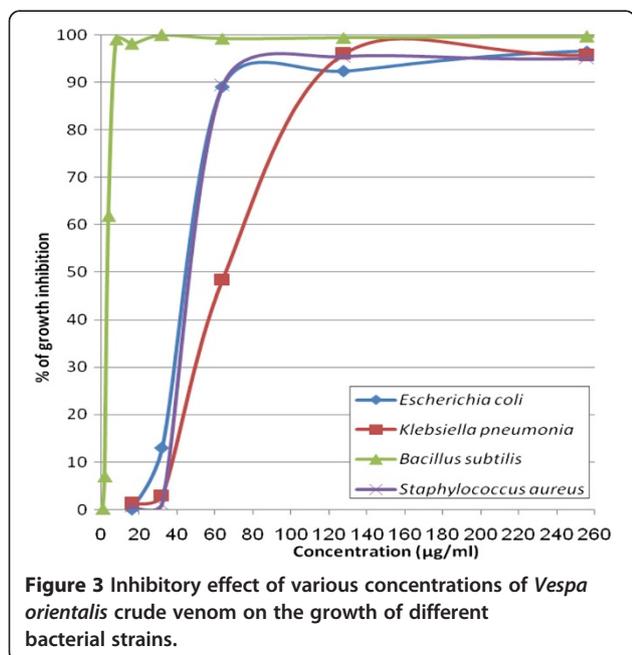

Figure 3 Inhibitory effect of various concentrations of *Vespa orientalis* crude venom on the growth of different bacterial strains.

Table 2 Percent of inhibition action of different concentrations (μg/mL) of *Vespa orientalis* crude venom on the growth of different bacterial strains

Microorganisms	% inhibition						
	10	20	40	50	60	80	90
S. aureus	20.2	31.1	52.8	63.6	74.5	96.2	107.0
B. subtilis	1.6	2.3	3.6	4.3	5.0	6.3	7.0
E. coli	24.9	30.0	40.2	45.3	50.4	60.6	65.7
K. pneumonia	29.6	40.8	63.2	74.4	85.6	108.0	119.2

The antimicrobial property of wasp venoms is mostly due to their peptides. Amphipathic secondary structures with net positive charges are essential to the biological activities of peptides that interact with anionic components of bacterial membranes in different ways, sometimes resulting in irreversible damage to the cell [39].

One of the major targets for antimicrobial agents is the bacterial cell envelope, which is a complex, multiple macromolecular structures that undergoes highly ordered cycles of synthesis and hydrolysis, facilitating cell division while maintaining a protective barrier against environmental stress. There are several different classes of antibiotics that target specific cell envelope structures or enzymatic steps of cell wall synthesis [40]. The biological membrane is a highly dynamic, complicated system, which is composed of weakly interacting protein molecules and lipids [41].

Results of the present study revealed that wasp venom is more effective against gram-positive than gram-negative bacteria, which might be related to the difference in cell envelope structure. Cell wall of bacteria comprises a complex structure that is fundamentally different between gram-positive and gram-negative bacteria. It consists of a polymer of disaccharides cross-linked by short chain peptides, forming a type of peptidoglycan. Cell wall in gram-positive bacteria is thick (15–80 nm), consisting of several layers of peptidoglycans and molecules of teichoic acids. In contrast, cell wall of gram-negative bacteria is relatively thin (10 nm) in and is composed of a single layer of peptidoglycan surrounded by a membranous structure (the outer membrane) which may invariably contain lipopolysaccharides. Thus, the outer membrane is more hydrophobic in gram-negative than in gram-positive bacteria and constitutes a target for being attacked by hydrophobic agents and other antibiotic agents [42,43].

Conclusions

Vespa orientalis crude venom efficiently inhibited the growth of gram-positive and gram-negative bacterial strains, even at a very low concentration when compared to that of tetracycline. The crude venom showed to be more efficient against gram-positive bacteria. As the crude venom is comprised of different proteins and peptides, further investigation is required to determine the potential components that could be used as antimicrobial drugs, especially for treating antibiotic-resistant pathogens.

Ethics committee approval

The present study was approved by the Ethics Committee for Animal Experiments of Shiraz University.

Competing interests

The authors declare that there are no competing interests.

Authors' contributions
JJ, PhD student of pharmacology, Shiraz University, collected the wasps and carried out most pharmacological and biochemical experiments; MF and HR designed the study, supervised the conduction of all experiments, drafted the manuscript and discussed the results. SSS helped conducting some experiments and performed statistical analyses. All authors read and approved the manuscript.

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