

RESEARCH ARTICLE

**ANTIBACTERIAL ACTIVITY AND MECHANISM OF ACTION OF
THE WASP *VESPA ORIENTALIS* VENOM PEPTIDES**

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ABSTRACT

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Recently several insects have been identified as potential carriers of antimicrobial peptides (AMPs), which have potent activity against pathogens. Wasp venom AMPs serve as defense agents against invading microorganisms. AMPs derived from wasp venom have high attention as therapeutic agents against infectious agents with novel mechanisms of action. In the present study, the antibacterial activity of the oriental hornet "*Vespa orientalis* L. (Hymenoptera: Vespidae)" venom was determined against three Gram-positive and three Gram-negative bacteria; and the minimum concentration of the venom that inhibits the bacterial growth was determined. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was used for fractionation of the *V. orientalis* venom peptides. The effect of *V. orientalis* AMPs on bacterial cell membrane and cytoplasm was also investigated; destruction of bacterial cell wall inhibited steps of the synthesis of important molecules and caused cell death. The results proved the highly effective antibacterial properties of the *V. orientalis* venom. Therefore, peptides of the *V. orientalis* venom could be potential alternative agents to the currently-used antibiotics.

INTRODUCTION

Insects provide experimentally tractable and cost-effective model systems to much product development for many purposes in many aspects. Wasps are a valuable source of both chitosan from their chitin^[1,2] and antimicrobial peptides (AMPs) from their venoms^[3]. Vespid venoms consist of a mixture of allergens and pharmacologically compounds made up of proteins^[3]. Stinging hymenopteran species produce venoms that include enzymes (hyaluronidases and phospholipases), neurotoxin, peptides, ionized molecules, and

neurotransmitters. AMPs are part of the wasp venom, which act as defense against microorganisms harbored by its insect victim^[4].

The widespread and inappropriate use of antibiotics against pathogenic microorganisms had led to increase microbial resistance^[5]. This resistance is responsible for increasing costs of hospitalization and treatment, which burdens the health care system. It is suggested that within ten years no antibiotics effective treatment against pathogens would be available^[6]. Current investigations for determining new

antibacterial agents to overcome resistance are a must. We examined in the current study the antibacterial activity of oriental hornet "*Vespa orientalis*" venom peptides using a variety of antimicrobial susceptibility tests and have utilized some antibiotic-resistant pathogenic bacteria to determine the potential of *V. orientalis* venom to serve as an alternative therapeutic agent for bacterial infection.

MATERIAL AND METHODS

Collection of *V. orientalis* samples

Oriental hornet "*V. orientalis*" samples were collected during summer season (July – September, 2019) from hornet traps, which settled between the honey-bee nests at the department of honey bee researches, Institute of plant protection, Ministry of Agriculture (Giza, Egypt). The traps were placed to trap *V. orientalis* during feeding on honey and bee workers.

Venom extraction

The venom was collected from the *V. orientalis* by the electrical shock method^[7]. The collected venom was packed in opaque glass vials and kept at 5.0°C till used.

Bacterial isolates

Gram-positive bacteria such as *Bacillus subtilis* (RCMB015), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Streptococcus mutans* (RCMB017), as well as Gram-negative bacteria such as *Escherichia coli* (RCMB010052), *Klebsiella pneumonia* (RCMB003), and *Salmonella typhimurium* (RCMB006), were used to measure the activity of the *V. orientalis* venom. These genera were obtained from the regional center for mycology and biotechnology, AL-Azhar University (Cairo, Egypt). The bacterial cultures were obtained on nutrient agar (Hi media) by slant streak technique according to the method of Mackie and McCartney^[8]. Stock culture of each bacterial strain was maintained in 20% glycerol and stored at –80°C.

Antibacterial activity of *V. orientalis* venom

The classical agar disk diffusion method^[9] was used during measuring the antibacterial activity of *V. orientalis* venom. Paper disk, about 6.0 mm in diameter were loaded with the venom extract (30µg) and spread on bacterial agar plates, and left for 2 hours in a refrigerator for diffusion, then incubated at 37°C for 24 hours. The resultant inhibition zones were measured according to the method of Holder and Boyce^[10]. The inhibitory action of tetracycline antibiotic against the same bacterial strains was used for comparison.

Determination of the minimum inhibitory concentrations (MIC)

Different concentrations of *V. orientalis* venom were used (from 2×10^{-7} to 20.0 mg/mL). The microtiter broth dilution method was used to estimate the minimum inhibitory concentration of *V. orientalis* venom against all tested bacterial types according to the method of Jalaei *et al.*^[9]. The percentage of inhibition of bacterial growth was calculated as follow^[9]:

Bacterial growth inhibition (%) = $100 - [(O.D \text{ of venom containing wells} - O.D \text{ of background control}) / (O.D \text{ of growth control} - O.D \text{ of background control})] \times 100$

Where O.D is the optical density readings of the bacterial growth, tetracycline antibiotic was used for comparison.

Transmission electron microscopy (TEM)

The changes in the shape and the structure of the treated bacterial strains with *V. orientalis* venom were detected using TEM (JEOL1010, JEOL Inc., Peabody, MA, USA) technique^[11].

Venom protein analysis

One dimensional gel electrophoresis was carried out using sodium dodecyl sulfate (SDS)-polyacrylamide gel slabs (15%) as described by Laemmli gel method^[12], which was used to characterize the protein configuration of *V. orientalis* venom.

RESULTS

Antibacterial activity of *V. orientalis* venom comparing with tetracycline

The crude *V. orientalis* venom caused a significant inhibition rate in bacterial growth as follows: 19, 18, 10, 18, 16 and 7 mm for *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus mutans*, *Escherichia coli*, *Klebsiella pneumonia*, and *Salmonella typhimurium*, respectively, compared with lower values of inhibition of tetracycline (10, 14, 16, 15, 14 and 19 mm, respectively) except against *Streptococcus mutans* and *Salmonella typhimurium*, where its inhibitory activities were high. Most of

tested bacterial strains showed a susceptibility to the tested venom; whereas MRSA was the most sensitive one. Also, the present findings indicate that the crude venom is more effective against gram-positive than gram-negative bacteria. Results of MIC in Table “1” confirmed the activity of *V. orientalis* venom against all tested bacteria. The bacterial inhibition activity after venom application was higher compared with that formed by tetracycline, except for *Salmonella typhimurium* and *Streptococcus mutans* where tetracycline was effective than the *V. orientalis* venom.

Table 1: Minimum inhibitory concentrations (MIC) and percentage of inhibition of *Vespa orientalis* venom against Gram-positive and Gram-negative bacteria compared with tetracycline.

Bacteria	MIC of <i>Vespa orientalis</i> venom (mg/mL)	Inhibition (%)	MIC of tetracycline antibiotic (mg/mL)	Inhibition (%)
Gram-positive bacteria				
MRSA	2.0×10^{-8}	4.74	2.0×10^{-3}	2.5
<i>Bacillus subtilis</i>	2.0×10^{-7}	14.38	2.0×10^{-5}	7.19
<i>Streptococcus mutans</i>	2.0×10^{-3}	8.88	2.0×10^{-7}	11.11
Gram-negative bacteria				
<i>Escherichia coli</i>	2.0×10^{-4}	8.6	2.0×10^{-3}	6.7
<i>Klebsiella pneumonia</i>	2.0×10^{-4}	16.0	2.0×10^{-3}	9.39
<i>Salmonella typhimurium</i>	2.0×10^{-2}	3.1	2.0×10^{-6}	10.85

MRSA: methicillin-resistant *Staphylococcus aureus*

V. orientalis venom protein fractions

The electrophoretically separated protein fractions of *V. orientalis* venom are shown in Table “2”. The results revealed that the total number of peptide bands resolved on 15% SDS polyacrylamide gel were seven polypeptide bands with molecular masses 310.0, 227.8, 186.8, 116.5, 73.1, 68.9, and 60.9 kDa and percentage amount 4.5, 2.6, 3.2, 2.4, 12.8, 6.3, and 2.8%, respectively.

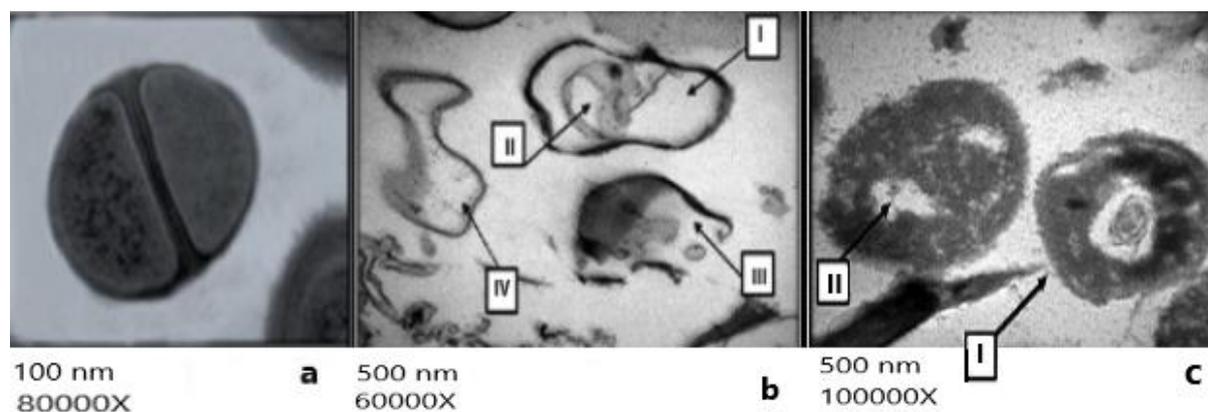
Ultrastructural changes of *V. orientalis* venom-treated bacteria

The ultrastructural sections of untreated MRSA, *Bacillus subtilis*, and *Escherichia*

coli (Figures 1a, 2a and 3a) showed intact cell membrane and cell wall, uniform cell cytoplasm with a distinct membrane, and clear normal cell division and structure. Treatment of MRSA with the *V. orientalis* venom (MIC: 2.0×10^{-8} mg/mL) showed contraction of cytoplasmic contents, loss of cytoplasmic electron density, cell rupture and loss of its contents, and the cell appeared empty (Figure 1b). The examination of the ultrastructure of MRSA cells after treatment with tetracycline (MIC: 2.0×10^{-3}) showed that cells were marginally influenced with moderate film blebbing and cytoplasm misplaced its electron thickness properties (Figure 1c).

Table 2: Relative concentrations of fractionated protein bands detected in venom of *Vespa orientalis*.

Rows	Marker	Venom	
	Molecular weight (kDa)	Molecular weight (kDa)	Amount (%)
1		310.0	4.5
2	245	227.8	2.6
3	180	186.8	3.2
4	135		
5	100	116.5	2.4
6			
7	75	73.1	12.8
8			
9		68.9	6.3
10	63	60.9	2.8
11	48		
12	35		
13	25		
14	20		
15	17		



Figures 1: Transmission electron micrograph of methicillin-resistant *Staphylococcus aureus* (MRSA): (a) untreated, (b) treated with *Vespa orientalis* venom (minimum inhibitory concentrations: 2.0×10^{-8} mg/mL), I and II: loss of cytoplasmic electron density, III: cell rupture and loss of its contents, IV: contraction of cytoplasmic contents, and (c) treated with tetracycline antibiotic (minimum inhibitory concentrations: 2.0×10^{-3} mg/mL) normal cell division, I: membrane blebbing, II: cytoplasm lost its electron density properties.

Treatment of *Bacillus subtilis* with the *V. orientalis* venom (MIC: 2.0×10^{-7} mg/mL) showed detachment of membrane layers (Figure 2b). Tetracycline (MIC: 2.0×10^{-5} mg/mL) also induced membrane detachment in *Bacillus subtilis* (Figure 2c).

The ultrastructure of untreated *Escherichia coli* showed a distinct membrane with clear

normal cilia for movement (Figure 3a). Treatment of *Escherichia coli* with the *V. orientalis* venom (MIC: 2.0×10^{-4} mg/mL) showed membrane blebbing, detachment, and folding (Figure 3b). Tetracycline (MIC: 2.0×10^{-3}) induced shrinkage of cytoplasmic material (Figure 3c).

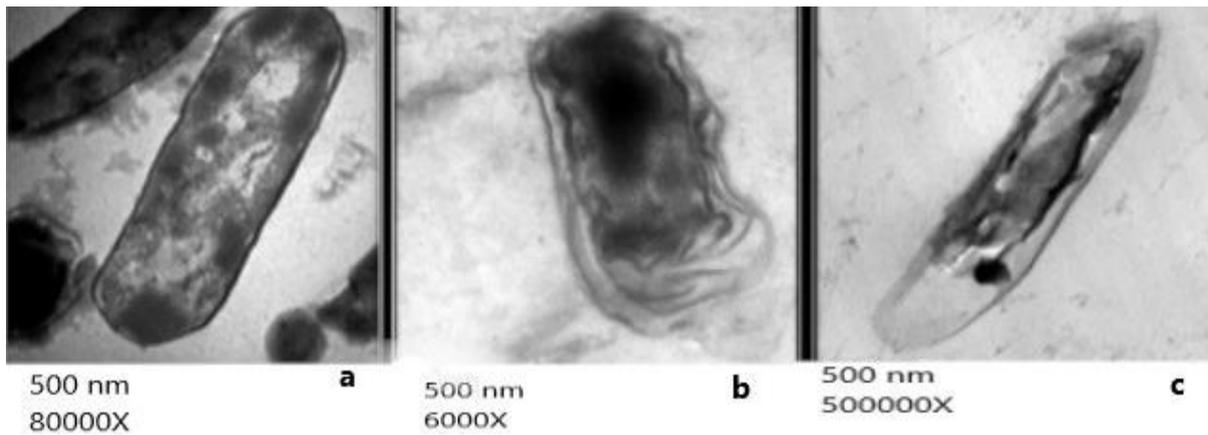


Figure 2: Transmission electron micrograph of *Bacillus subtilis*: (a) untreated, (b and c) treated with *Vespa orientalis* venom (minimum inhibitory concentrations: 2.0×10^{-7} mg/mL) and tetracycline (minimum inhibitory concentrations: 2.0×10^{-3} mg/mL), respectively, showed membrane detachment.

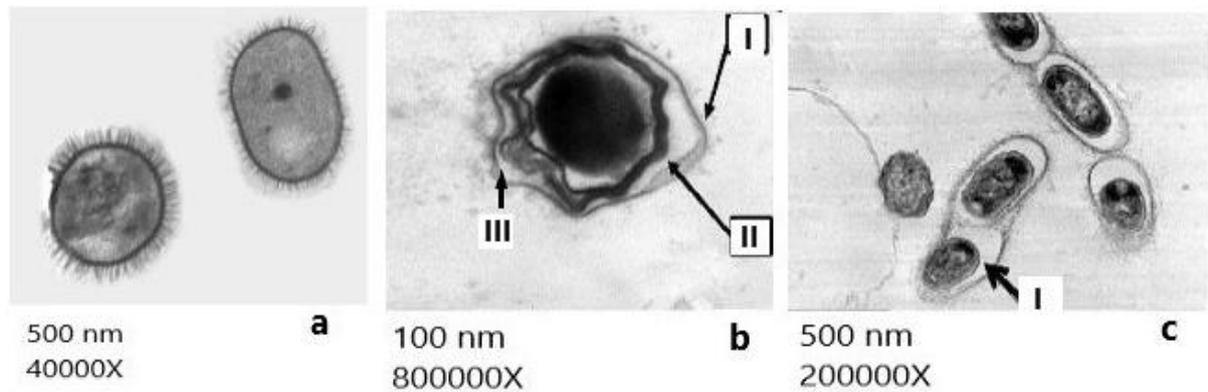


Figure 3: Transmission electron micrograph of *Escherichia coli*: (a) untreated, (b) treated with *Vespa orientalis* venom (minimum inhibitory concentrations: 2.0×10^{-4} mg/mL), I: membrane blebbing, II: membrane detachment, and III: membrane folding (c) treated with tetracycline (minimum inhibitory concentrations: 2.0×10^{-3} mg/mL). I: shrinkage of cytoplasmic material.

DISCUSSION

Bacterial diseases are considered major global health problems, with emerging of antimicrobial resistance. Commonly, simple infections may become life-threatening after increasing resistance to traditional antibiotics^[13]. The lack of effective treatments against resistant bacteria created a global health problem^[14]. Most of antimicrobial agents are effective against either Gram-positive or Gram-negative bacteria lacking broad-spectrum activity^[15,16]. Gram-negative bacteria are characterized by the presence of a double membrane making difficulty to

treat these bacteria due to many antibiotics cannot penetrate the double membrane or are degraded/modified in the periplasmic space^[17]. The most predominant Gram-negative pathogens are *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* sp., *Serratia* sp., *Pseudomonas aeruginosa*, and *Salmonella typhimurium*^[18,19], most of them acquired resistance to known antibiotics^[20,21].

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^[22,23]. Antibacterial

property has been reported for the venoms of a wide variety of animals including venoms of snakes, scorpions, spiders, and wasps; all of which are predatory or parasitic animals^[24]. However, the actual function of AMPs in these venoms is not clear yet. The *V. orientalis* venom proved its antibacterial activity against Gram-positive and Gram-negative bacteria. In Table “1”, MIC of *V. orientalis* venom against *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus subtilis*, and MRSA was 10-1000 folds active than tetracycline. The antimicrobial property of *V. orientalis* venoms is mostly due to their peptides. Many authors detected the antimicrobial properties of different wasp venoms^[22,25]. Isolated AMPs from the *V. orientalis* venom affected the tested bacterial ultrastructure (Figures 1-3). It may also alter cell permeability leading to efflux of essential ions and nutrients, as well as causing the formation of pores and bacterial death^[26-30]. AMPs can selectively target bacterial membranes due to the presence of lipopolysaccharide and absence of negatively charged lipids on the surface of eukaryotic cells^[31,32]. The broad-spectra of antibacterial activity of AMPs suggested them as excellent candidates for the development of bacterial antibiotics. Few AMPs were tested in clinical study for prevention of diabetic foot ulcers and being effective against antimicrobial-resistant bacteria^[33,34].

The detected ultrastructural changes of venom-treated cells proved that interaction with the cell membrane is one of the proposed mechanisms explaining the potency of *V. orientalis* venom. As proteins, peptides comprise the main components of wasp venoms such as phospholipases, hyaluronidase, serotonin, histamine, dopamine, and adrenaline^[35-37]. Phospholipases hydrolyze the ester bonds and destroy the phospholipid layers in the bacterial cell wall, while hyaluronidase enzyme split polymers and destroy the cellular matrix of the bacterial cells^[35]. Our proteomic approach revealed several peptides of the *V. orientalis* venom, most of

them are previously isolated and identified. Upon separation of the *V. orientalis* venom proteins by SDS-PAGE, seven peptides of low- and high-molecular weights ranging from 60.9 to 310.0 kDa had been observed. Our result was consistent with previously report of Vincent *et al.*^[37], who analyzed venom proteins of *Chelonus inanitus* and detected peptide bands with molecular weights ranged from 300 to 60 KDa. Although high molecular weight peptides are rarely obtained from the *V. orientalis* venoms, our isolated fraction similar to that separated by Pessoa *et al.*^[38] after using SDS-PAGE analysis of *Neoponera villosa* venom. Venomous proteins of high molecular weights are responsible for the paralytic activity of solitary spider wasp “*Anoplius samariensis*” that used spider as a food source for its larvae^[39]. The *V. orientalis* peptide bands of 227.89 kDa and 186.84 kDa were previously identified by Bruschini *et al.*^[40] as undecenyl propanoate and 2- nonanyl acetate in the venom of *Polistes gallicus*. They stated that these volatile compounds may be as an alarm pheromone, which trigger defensive responses from nearby insects^[40]. The peptide band of molecular weight 68.94 kDa isolated from *V. orientalis* venom was detected before by Abt and Rivers^[41] from crude venom of the ectoparasitic wasp “*Nasonia vitripennis*” and characterized as phenoloxidase enzyme, which appears to play an important role in cell death. The peptide band of *V. orientalis* venom with 60 kDa is similar to that detected by Moreno and Giral^[35]. This peptide was identified before as phosphatase enzyme, which is not related to venom toxicity, and was also detected in ant venom of *Paraponera cribrinodis* and *Pogonomyrmex badius*^[42], as well as honey bee venom^[43].

The current study suggested that the *V. orientalis* venom and its active AMPs are biologically active peptides and can help in breaking antibiotic resistance between bacterial pathogens, which may encourage the practical application of *V. orientalis* venom in clinical studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

MHR, DMM, and AKA planned the study. MHR and DMM designed all experiments. AEA and EEE carried out the experiments. AEA and DMM performed venom protein analysis and analyzed TEM results. MHR, DMM, and AEA summarized, discussed, and interpreted the results. MHR, DMM, and EEE drafted the manuscript.

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النشاط المضاد للبكتيريا وآلية عمل ببتيدات سُم الدبور "*VESPA ORIENTALIS*"

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تم تحديد العديد من الحشرات مؤخرًا على أنها ناقلات محتملة للبيبتيدات المضادة للميكروبات، والتي لها نشاط قوي ضد مسببات الأمراض. وتعمل البيبتيدات المضادة للميكروبات لسُم الدبور كعوامل دفاع ضد غزو الكائنات الحية الدقيقة. وتحظى البيبتيدات المضادة للميكروبات المشتقة من سُم الدبور باهتمام كبير كعوامل علاجية ضد العوامل المعدية بآليات عمل جديدة. وفي الدراسة الحالية، تم تحديد النشاط المضاد للبكتيريا لسُم الدبور "*Vespa orientalis* L." (Hymenoptera: Vespidae) ضد ثلاثة أنواع من البكتيريا موجبة الجرام وثلاثة أنواع أخرى من البكتيريا سالبة الجرام، كما تم تحديد أقل تركيز لسُم الدبور "*V. orientalis*" يثبط النشاط البكتيري. وتم استخدام "SDS-PAGE" لتجزئة ببتيدات سُم الدبور "*V. orientalis*". وقد تم دراسة تأثير البيبتيدات المضادة للميكروبات لسُم الدبور "*V. orientalis*" على غشاء الخلية البكتيرية والسيتوبلازم، حيث أن تدمير جدار الخلية البكتيرية يمنع خطوات تخليق جزيئات مهمة ويسبب موت الخلية. وقد أثبتت النتائج فاعلية سُم الدبور "*V. orientalis*" المضادة للبكتيريا، لذلك يمكن أن تكون ببتيدات سُم الدبور "*V. orientalis*" عوامل بديلة محتملة للمضادات الحيوية المستخدمة حاليًا.