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ORIGINAL ARTICLE

Antimicrobial effect of *Dinoponera quadriceps* (Hymenoptera: Formicidae) venom against *Staphylococcus aureus* strains

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Abstract

Aims: *Dinoponera quadriceps* venom (DqV) was examined to evaluate the antibacterial activity and its bactericidal action mechanism against *Staphylococcus aureus*.

Methods and Results: DqV was tested against a standard strain of methicillin-sensitive *Staphylococcus aureus* (MSSA), *Staph. aureus ATCC 6538P* and two standard strains of methicillin-resistant *Staphylococcus aureus* (MRSA), *Staph. aureus* ATCC 33591 and *Staph. aureus* CCBH 5330. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), the rate of kill and pH sensitivity of the DqV were determined by microdilution tests. Bactericidal and inhibitory concentrations of DqV were tested to check its action on *Staph. aureus* membrane permeability and cell morphology. The MIC and MBC of DqV were 6·25 and 12·5 μg ml^{-1} for *Staph. aureus* ATCC 6538P, 12·5 and 50 μg ml^{-1} for *Staph. aureus* CCBH 5330 and 100 and 100 μg ml^{-1} for *Staph. aureus* ATCC 33591, respectively. Complete bacterial growth inhibition was observed after 4 h of incubation with the MBC of DqV. A lowest MIC was observed in alkaline pH. Alteration in membrane permeability was observed through the increase in crystal violet uptake, genetic material release and morphology in atomic force microscopy.

Conclusions: The results suggest antibacterial activity of DqV against *Staph. aureus* and that the venom acts in the cell membrane.

Significance and Impact of the Study: Alteration in membrane permeability may be associated with the antimicrobial activity of hymenopteran venoms.

Introduction

The indiscriminate use of antibiotics has caused a selective pressure of micro-organisms, resulting in frequent emergence of resistant bacteria. Microbial resistance is a global concern in the treatment of infectious diseases, resulting in conventional therapy failure, prolonged illness and higher risk of death (Heinemann *et al.* 2000; Pittet 2005; Bandyopadhyay *et al.* 2013).

Staphylococcus aureus is the most virulent member of its genus and can produce various enzymes such as coagulases, DNAses and beta-lactamase, which confers drug resistance (Plata *et al.* 2009). Methicillin-resistant *Staphylococcus aureus* (MRSA), a beta-lactamase producer, is known for causing serious infections in hospitalized patients. In the last few years, MRSA is estimated to have killed 20 000 people per year and to have increased in the community in the USA (Klein *et al.* 2009). The main

approach of biological researches is the discovery of new therapeutic options for treatment of microbial infections caused by multiresistant bacteria (Kaneti *et al.* 2013).

Venom from the Hymenoptera order (ants, wasps and bees) displays a wide range of functions and biological roles. They are complex mixtures of different substances that include enzymes, peptides, biogenic amines, neurotoxins, amino acids and lipids with a range of pharmacological functions (Palma 2006; Chen and Lariviere 2010), which have great biotechnological potential.

It is generally suspected that the venom of social hymenopterans, such as ants, has properties that allow them to regulate microbial infections in their nests. Venoms produced by ants of the subfamilies Paraponerinae, Ectatomminae, Pseudomyrmecinae, Myrmeciinae, Myrmicinae and Ponerinae are rich in peptides with myotoxic, neurotoxic, cytotoxic or antimicrobial properties (Akre and Reed 2002; Palma 2006; Moreau 2013). *Pachycondyla goeldii*, *Myrmecia pilosula, Crematogaster pygmaea* and *Dinoponera australis* ant venoms have antimicrobial activity against Gram-negative and Gram-positive bacterial strains (Orivel *et al.* 2001; Zelezetsky *et al.* 2005; Johnson *et al.* 2010; Quinet *et al.* 2012).

Dinoponera quadriceps (Ponerinae) is a primitive ant found in north-eastern Brazil (Paiva and Brandão 1995). *Dinoponera quadriceps* venom (DqV) biological action is poorly known. However, a few studies showed antinociceptive (Souza *et al.* 2012) and antiseizure potentials in DqV (Lopes *et al.* 2013). In this context, the aim of this work was to study the antimicrobial activity of DqV against different *Staphylococcus aureus* strains and suggest the antimicrobial action mechanism.

Materials and methods

Dinoponera quadriceps venom

Ant nests were collected (authorization from the SISBIO—licence nr. 28794-1) in 'Serra de Maranguape', a small mountain range in the state of Ceará, north-eastern Brazil. The animals were maintained at the Entomology Laboratory at the State University of Ceará, in standard conditions. To collect venom, ants were seized in the thorax region and its sting was introduced in a tube to induce venom secretion. DqV was then transferred to a tube containing 10 mmol l^{-1} ammonium acetate buffer (pH 6·8), lyophilized and maintained at −20°C until further use (Souza *et al.* 2012). For experimental assays, aliquots were diluted (at final concentrations of 200, 100, 50, 25, 12·5, 6·25, 3·12, 1·56 and 0·78 μg ml^{-1}) with sterile phosphate-buffered saline (PBS;137 mmol l^{-1} NaCl, 2·7 mmol l^{-1} KCl, 10 mmol l^{-1} Na_2HPO_4, 2 mmol l^{-1} KH_2PO_4; VETEC, São Paulo, Brazil; pH 7·4).

Microbial strains

Methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 6538P, *Staphylococcus aureus* CCBH 5330 (MRSA) and *Staphylococcus aureus* ATCC 33591 (MRSA) strains were donated by the Laboratory for Reference Materials of Oswaldo Cruz Foundation, FIOCRUZ. Bacteria cultures were maintained on Nutrient agar (HiMedia Laboratories, Mumbai, India) slope at 4°C and subcultured every 4 weeks. For experimental use, they were subcultured in Brain Heart Infusion (BHI, HiMedia Laboratories, Mumbai, India) broth at 35°C until reaching exponential growth.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration was determined using the broth microdilution method as described in the CLSI (2009). Briefly, microbial strains were added to BHI broth and incubated at 35°C until they reached a visible turbidity equivalent to 0·5 on the McFarland scale (approximately 10^8 CFU ml^{-1}). Cultures were diluted to 10^6 CFU ml^{-1} in Müller–Hinton broth (HiMedia Laboratories, Mumbai, India). An inoculum of 100 μl of microbial culture was added to 20 μl of each concentration of DqV in each wells containing 80 μl of medium and incubated with DqV for 24 h at 37°C in 96-well plates (Techno Plastic Products AG, Transadingen, Switzerland). Sterile PBS and amikacin were used as negative and positive controls, respectively. The MIC was the lowest venom concentration required to inhibit visible turbidity. Experimental groups that showed no observed visible turbidity were subcultured on the surface of a Plate Count Agar (HiMedia Laboratories, Mumbai, India) for colony counting. MBC was considered as the lowest concentration that could inhibit 99% of bacterial growth (Pearson *et al.* 1980).

Rate of kill assessment

The rate of kill of *Staph. aureus* treated with DqV was evaluated as described by Gonçalves *et al.* (2012). Overnight broth cultures were adjusted to a concentration of 10^6 CFU ml^{-1}. Aliquots of experimental and untreated controls incubated at 37°C were removed at intervals of 0, 2, 4, 6, 8 and 24 h after the addition of 12·5 μg ml^{-1} of DqV. Serial dilutions were plated onto plate count agar to count viable cells. Results were expressed as log of colony-forming units (CFU) per ml (log CFU ml^{-1}).

Crystal violet assay

Alteration in membrane permeability induced by DqV was detected by crystal violet assay (Devi *et al.* 2010).

Staphylococcus aureus suspensions were prepared in BHI broth. Cells were harvested at 4500 ***g*** for 5 min at 4°C, washed twice and resuspended in PBS at pH 7·4. DqV (6·25, 12·5 and 25 μg ml^{-1}), antibiotics (amikacin, 3·12 μg ml^{-1}; Sigma-Aldrich, St. Louis, MO), positive control (ethylenediaminetetraacetic acid—EDTA 0·25 mol l^{-1}; Isofar Indústria e Comércio, Rio de Janeiro, Brazil) and negative control (sterile PBS) were added to bacterial suspensions and incubated for 30 min at 37°C. Groups were centrifuged at 9300 ***g*** for 5 min and resuspended in PBS containing crystal violet (10 μg ml^{-1}; Cromato Produtos Químicos, São Paulo, Brazil). After 10 min at 37°C, groups were again centrifuged at 13 400 ***g*** for 15 min, the optical density at 590 nm of the supernatant was measured using a microplate reader (Model synergy HT, Biotek, Winooski, VT), and the percentage of crystal violet uptake in the samples was calculated.

Genetic material-release assay

Release of intracellular DNA and RNA contents in experimental groups was evaluated by increase in optical density at 260 nm, after incubation of DqV with *Staph. aureus*. Cells were harvested by centrifugation at 400 ***g*** for 15 min, the supernatant was discarded, and the pellet was washed twice and then resuspended in PBS (pH 7·4). Samples were adjusted to 2·0 on the McFarland scale and incubated with DqV (6·25, 12·5 and 25 μg ml^{-1}), amikacin (3·12 μg ml^{-1}) and negative control (sterile PBS) for 60 min at 37°C. Groups were centrifuged at 13 400 ***g*** for 15 min, and the absorbance at 260 nm of supernatants was determined (Devi *et al.* 2010).

Atomic force microscopy

Changes in bacterial morphology induced by DqV were examined using atomic force microscopy (AFM; Braga and Ricci 1998). *Staphylococcus aureus* cultures were prepared in BHI broth and treated with DqV (6·25 and 3·12 μg ml^{-1}) for 4 h. Groups were harvested at 4500 ***g*** for 5 min at 4°C and washed twice in PBS (pH 7·4). An aliquot of the suspension was placed on a circular coverslip and air-dried for 15 min. The samples were analysed in a Multimode Atomic Force Microscope Nanoscope III-a (Digital Instruments, Santa Barbara, CA). Scans were performed in air, and amplitude images were acquired by intermittent contact mode using crystalline silicon cantilevers (Veeco-probes) with a spring constant of approximately 40 N m^{-1}, resonance frequency of 242·38 kHz and tip radius of 15 nm. The amplitude images were used to better evidence cell borders and their shape. Cultures of untreated *Staph. aureus* were used as controls.

pH sensitivity assay

The effect of pH on the antibacterial activity of DqV against *Staph. aureus* was assessed by pH sensitivity assay (Gonçalves *et al.* 2012). The MIC was determined on Muller-Hinton broth that was previously prepared and calibrated to different pH ranges (from 5·5 to 9·0).

Statistical analysis

The data were expressed as mean ± SEM and analysed using ANOVA with Tukey's post-test. The statistical significance was considered with a *P*-value of 0·05.

Results

All tested strains were susceptible to *D. quadriceps* venom. The values of MIC and MBC obtained in this study are shown in Table 1. The experiments to investigate DqV action mechanism against this bacterium were performed with *Staph. aureus* ATCC 6538P strain, the most sensitive among all tested strains. Treatment with DqV (12·5 μg ml^{-1}- MBC) was able to kill all the bacteria after 4 h of incubation, as shown in Fig. 1. Additionally, the uptake of crystal violet by *Staph. aureus* was 47% in the absence of DqV, but increased to 60–68% after treatment with MIC, MBC and 2 × MBC of DqV, comparable with the chelating agent (disodium EDTA, 0·25 mol l^{-1}), in which case the uptake increased to 60–65% (Fig. 2). After treatment with MIC, MBC and 2 × MBC of DqV, the optical density at absorbance of 260 nm increased in all concentrations. These results suggest that DqV damages the cytoplasmic membrane and causes release of genetic material (Fig. 3). A pH-dependent inhibitory effect of DqV over *Staph. aureus* was observed. The lowest MIC was found in alkaline pH (7·5–9·0), as shown in Table 2.

Table 1 Minimum inhibitory concentration and minimum bactericidal concentration of *Dinoponera quadriceps* venom against *Staphylococcus aureus* strains

		D. quadriceps venom		Amikacin
Strains	MRSA	MIC (μg ml^{-1})	MBC (μg ml^{-1})	MIC (μg ml^{-1})
Staphylococcus aureus ATCC 6538P	No	6·25	12·5	3·12
Staphylococcus aureus CCBH 5330	Yes	12·5	50	3·12
Staphylococcus aureus ATCC 33591	Yes	100	100	25

MRSA: methicillin-resistant *Staphylococcus aureus*; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ATCC: American type culture collection; CCBH: culture collection of hospital-acquired bacteria.

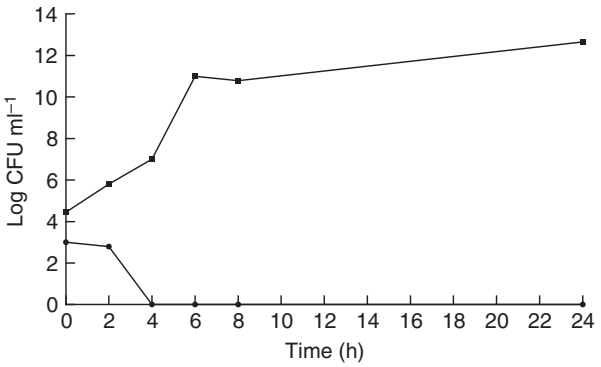

Figure 1 Effect of *Dinoponera quadriceps* venom on *Staphylococcus aureus* ATCC 6538P viability. (12·5 μg ml^{-1}: minimum bactericidal concentration of *Dinoponera quadriceps* venom; control: untreated cells). —■—Control; —●—12·5 μg ml^{-1}.

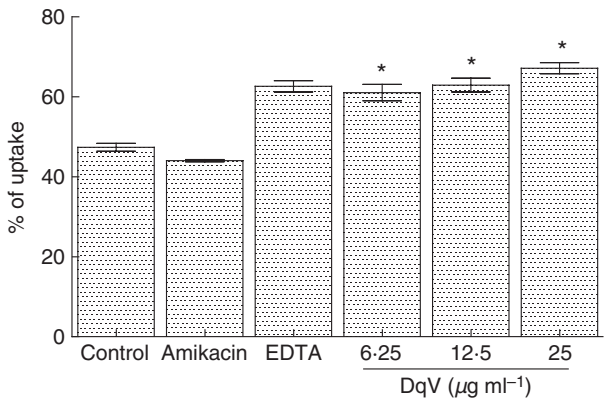

Figure 2 Crystal violet uptake of *Dinoponera quadriceps* venom-treated *Staphylococcus aureus* ATCC 6538P. (DqV: *Dinoponera quadriceps* venom; control: cells treated with PBS; EDTA: cells treated with disodium EDTA 0·25 mol l^{-1}; amikacin: cells treated with amikacin 3·12 μg ml^{-1}). The mean ± SEM for three replicates is shown, *$P < 0.05$.

According to atomic force microscopy, the morphology of untreated *Staph. aureus* was regular and smooth with an intact cell membrane, disclosing its characteristic shape, resembling a 'brunch of grapes'. Fig. 4B,C shows the loss of membrane integrity and cell-to-cell adherence after 4 h of treatment with MIC and MIC/2 of DqV (Fig. 4).

Discussion

Different components found in animal venoms have been studied and identified as important biological active compounds (Pimenta and Lima 2005). These substances have great biotechnological interest, as they are useful to identify new therapeutic targets to treat infections, as well molecular models to development of new drugs, with improvement in efficacy and toxicity.

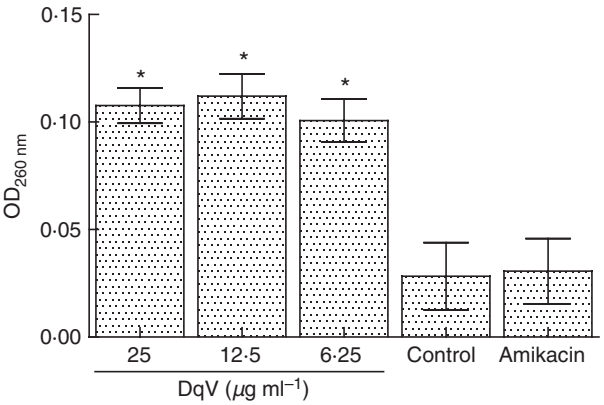

Figure 3 Genetic material release in the supernatants of *Staphylococcus aureus* ATCC 6538P treated with 6·25, 12·5 and 25 μg ml^{-1} of *Dinoponera quadriceps* venom. (Control: cells treated with PBS; amikacin: cells treated with amikacin 3·12 μg ml^{-1}). The mean ± SEM for three replicates is shown, *$P < 0.05$.

Several fractions with antimicrobial properties have been isolated from Hymenoptera venoms. Peptides from the venom of *Agelaia pallipes pallipes (*Protonectin), *Vespa crabro* (Crabolin), *Anoplius samariensis* (Anoplin), *Apis mellifera* (Melittin), *Melecta albifrons* (Melectin), *Polybia paulista* (polybia-MP1 and polybia-CP), *Vespa tropica* (Mastoparan and Vespid chemotactic peptides), *Tetramorium bicarinatum* (Bicarinalin), *Panurgus calcaratus* (Panurgines) have shown antimicrobial activity (Krishnakumari and Nagaraj 1997; Konno *et al.* 2001; Bucki *et al.* 2004; Mendes *et al.* 2004; Cerovský *et al.* 2008; Rifflet *et al.* 2012; Wang *et al.* 2012, 2013b; Čujová *et al.* 2013; Yang *et al.* 2013). Alkaloids from the venom of the *Solenopsis invicta* ant and antimicrobial peptides (pilosulins) found in the venom of the Australian jumper ant *Myrmecia pilosula* exhibit antibacterial activity against Gram-negative and Gram-positive bacteria (Jouvenaz *et al.* 1972; Inagaki *et al.* 2004).

Fifteen different antimicrobial peptides (AMPs), called ponericins, were purified from *Pachycondyla goeldii* venom, and their amino acid sequences were characterized. According to their first most frequent aminoterminal amino acid, these AMPs can be classified into three families: ponericin G (seven peptides), W (six peptides) and L (two peptides). Ponericins G1 and G3 have a broad antibacterial and antifungal spectrum. With exception of W6, ponericins W are active against Gram-positive and Gram-negative bacteria and yeast and have expressive haemolytic and insecticidal actions. Ponericin L2 are active only against bacteria, but not against fungi (Orivel *et al.* 2001). Recently, six peptides (dinoponeratoxins) were isolated from the venom of *Dinoponera australis*. Two of them (Da-3105 and Da-3177) showed 92·9% sequence similarity with ponericin G2 (Johnson *et al.* 2010).

Table 2 Influence of pH on minimum inhibitory concentration of *Dinoponera quadriceps* venom against *Staphylococcus aureus* ATCC 6538P

pH	5·0	5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0
DqV MIC (μg ml^{-1})	12·5	12·5	12·5	12·5	12·5	6·25	6·25	6·25	6·25

DqV: *Dinoponera quadriceps venom*; MIC: minimal inhibitory concentration.

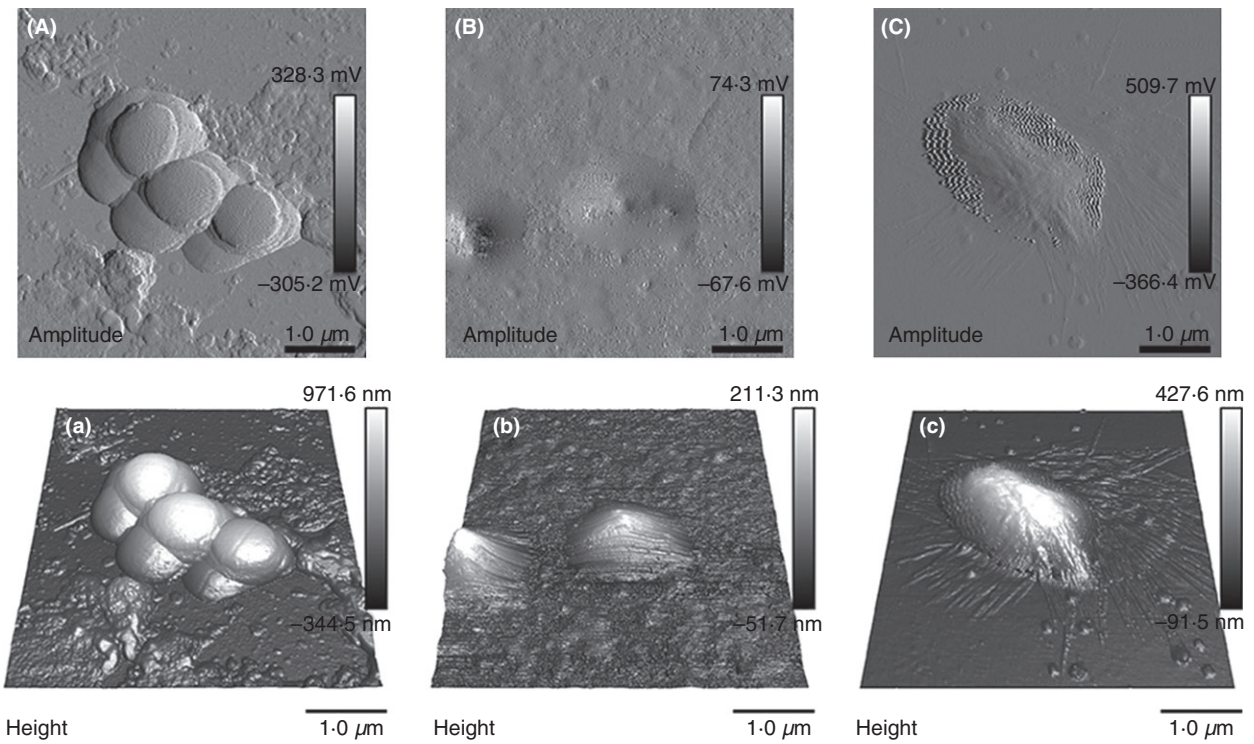

Figure 4 Atomic force microscopy images of *Staphylococcus aureus* ATCC 6538P without exposure to *Dinoponera quadriceps venom* (A and a), after exposure to 6·25 μg ml^{-1} of *D. quadriceps venom* (B and b) and after exposure to 3·12 μg ml^{-1} of *D. quadriceps venom* (C and c). Amplitude images show the morphology of cells in A, B and C. The scale bar in volts corresponds to changes in the oscillation amplitude of the free cantilever. The three-dimensional aspect of the groups is shown in a, b and c.

Cologna *et al.* (2013) demonstrated the presence of compounds similar to antimicrobial peptides found in venoms of other animals, such as temporin-like, dermaseptins-like and ponericins-like in *D. quadriceps* venom. It was also observed a wide variation in the composition of venoms produced by ants from different locations, suggesting that environmental characteristics have great influence on the composition of these venoms. An important fact is that ponericin is one of few common compounds between these ants. It was also observed antimicrobial potential over bacterial and fungi strains in synthetic peptides similar to ponericins obtained after proteomic analysis of *D. quadriceps* venom.

In this study, we demonstrated the antimicrobial effect of DqV against three different *Staph. aureus* strains, suggesting the presence of antimicrobial peptides in this venom.

Recently, our group produced a *D. quadriceps* venom gland cDNA library to investigate the toxin repertoire in the venom used in this work. It was observed that dinoponeratoxins are major components, but also peptides pilosulin-like were found, and these peptides reported primarily for its antimicrobial effect (Torres *et al.* 2014). However, these antimicrobial peptides have been subjected to further analysis and molecular modelling in order to reduce its toxic effects, such as the pilosulins (Zelezetsky *et al.* 2005).

The greater susceptibility of the bacteria at higher pHs is a valuable characteristic, as it represents good evidence of a possible effectiveness in inhibiting bacterial growth in physiologic pH. Furthermore, this venom was able to completely kill the bacteria after a short incubation time (4 h), suggesting a quick action mechanism, such as membrane leakage. To determine the effect of DqV on bacterial membrane, crystal violet uptake and intracellular

content release assays were performed. The crystal violet method verifies the increase in membrane permeability through its uptake after incubation with DqV. Crystal violet has low penetrability in normal cells, but in the presence of membrane lesions, it accumulates in micro-organisms and other cells. Releasing of intracellular content was determined by increase in absorbance at 260 nm in culture supernatant after 30 min. DqV caused an apparent alteration in the structure of *Staph. aureus* membrane, as about 60% of crystal violet uptake was observed, as well significant release of DNA and RNA contents. These effects suggest that DqV cause rupture or formation of pores in *Staph. aureus* membrane. Amikacin, an antimicrobial which does not act on membrane molecules, did not show any effect in these experiments. Usually, over 50% of amino acids found in antimicrobial peptides are hydrophobic, which implicates in their interaction with cell membranes in different micro-organisms (Hancock and Diamond 2000; Teixeira *et al.* 2012).

Images obtained by AFM confirmed that DqV caused important changes in *Staph. aureus* membrane structure, as well loss of cell-to-cell adherence. Other Hymenoptera-isolated antimicrobial peptides such as melittin (Lee *et al.* 2013), polybia-MPI (Wang *et al.* 2013b) polybia-CP (Wang *et al.* 2012) and protonectin (Wang *et al.* 2013a) also show important effects on microbial membranes.

In conclusion, *D. quadriceps* venom showed potential antibacterial activity against *Staphy. aureus* strains. The venom was able to alter membrane permeability at bacteriostatic and bactericidal concentrations. DqV is therefore good candidates for future researches.

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Conflict of interest

No conflict of interest declared.

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