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Antibacterial effect of the *Montivipera bornmuelleri* crude venom against *Salmonella enteritidis and Staphylococcus aureus*

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ABSTRACT

Snake venoms are composed of different substances with specific chemical and biological activities. In this study, the antibacterial activity of the *Montivipera Bornmuelleri* crude venom was evaluated against human pathogenic strains of bacteria and against yeast. The effects of different concentrations of the venom and different referenced antibiotic disks were tested by the disc diffusion method. Our results have clearly shown an effective antibacterial effect of the crude venom on *Staphylococcus aureus* and on *Salmonella enteritidis*. The MIC was determined at 125µg/ml for *S. aureus* and at 1000µg/ml for *S. enteritidis*.

Keywords: *Montivipera Bornmuelleri*; venom ; antimicrobial ; *Staphylococcus aureus ; Salmonella enteritidis*.

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INTRODUCTION

The increase in bacteria resistance to conventional antibiotics and undesirable side effects are a public health concern worldwide (14, 26) particularly in developing nations. That's why scientists are trying to find new molecules with different action modes.

It is well known that snake venoms are natural physiological products composed of complex mixtures of different substances of proteins, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins with specific chemical and biological activities (18, 19, 21, 22, 30). The components of snake venoms are considered as possible treatment of blood and cardiovascular disorders, thrombosis, multiple sclerosis, pain, cancer, infection and inflammatory diseases (9; 15; 24; 25). Moreover, an antibacterial activity has been described by Bustillo in 2008, Al Ahmadi in 2010 and Dhananjaya in 2016.

An antimicrobial activity of the crude venom of the Lebanese endemic viper Montivipera *bornmuelleri* found at high altitude above 1800 m (32; 26; 24; 22) on some strains of bacteria has been demonstrated (1). In addition, antimicrobial-resistant Escherichia coli from raw vegetables was reported in Lebanon (12) and the surveillance of antimicrobial resistance in Lebanese hospitals showed a prevalence rate of methicillin-resistant *Staphylococcus aureus* at 27.6% and a mean of ampicillin susceptibility of Salmonella at 81.3% (7).

The antimicrobial results on Montivipera *bornmuelleri* venom (22) and the findings concerning the bacteria resistance to classic antibiotics incited us to explore further the effects of *Montivipera bornmuelleri* venom. Thus, the aim of our work was to demonstrate an antimicrobial activity of the crude venom of *M. bornmuelleri* against new human pathogenic strains of bacteria and on a yeast using two antibacterial assays: disc diffusion and micro dilution.

MATERIALS AND METHODS

The venom

The collection of venom was done in a sterile manner during May-June 2015 from *M. bornmuelleri* vipers caught at Ouyoun el Siman region and at Makmel Mountain in Lebanon. Venom extraction took place at the Biology Department in the American University of Beirut (AUB). The vial used for venom extraction was sterilized under ultraviolet light for at least six hours, and then the venom was freeze-dried and stored at -20 °C in a dry and light-free place at the laboratory of the Holy Spirit University of Kaslik (USEK).

Bacterial strains

To test the antibacterial effects of the venom, certified freeze-dried microbial cultures were employed: Gram positive bacteria: *Staphylococcus aureus* ATCC: BAA-1026, *Enterococcus faecalis* ATCC 29212, Gram negative bacteria: *Salmonella enteritidis* ATCC: 13076, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC11303 and yeast strain: *Candida albicans* ATCC 10231, were purchased from Microbiologics.

Chemicals

The antimicrobial agents: Tetracycline 30 µg/disc, Ticarcillin 75 µg/disc, Gentamicin 10 µg/disc, blank sterile discs (6mm; Ward's science); were obtained from Mast Diagnostics and used in the antibacterial test as drug controls and blank.

Mueller Hinton broth (MHB), Mueller Hinton agar (MHA), potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Scharlau Company, as media to test the bacteria and the candida strains cited above.

Antibacterial assays

Two anti-bacterial assays were carried out:

Disc-diffusion method

The initial stocks of lyophilized venom were prepared in MHB at a concentration of 14 mg/ml.

Further dilution were tested as well (8 and 4mg/ml). The susceptibility test and MIC were performed according to the national committee for clinical laboratory standards (9).

A bacterial suspension from different freeze dried microbial cultures were prepared on MHB according to the turbidity of 0.5 Mc Farland approximately 10^8 CFU/ml.

A serial dilution was made, so the final concentration was adjusted to 10^5.

A volume of 0.1 ml of tested bacteria and candida suspension was homogeneously mixed into petri dishes containing 20 ml of the medium.

Sterile blank paper discs (7mm diameter) were then placed on a Mueller Hinton Agar surface Potato.

Dextrose Agar and 10 µl of different concentrations of crude venom solutions were added per disc in 3 replicates.

Plates were incubated at 37^0C or 27^0C (for candida) for 24h and inhibition zones were measured.

Antibiogram disks including: Tetracycline 30 µg/disc, Gentamicin 10 µg/disc were used as positive control for bacteria strains and Nystatin 10 µg/disc for candida.

The blank disk containing 10µL of MHB as a normal control.

Minimum inhibitory concentration (MIC)

Micro-dilution were carried out to determine the minimum inhibitory concentration according to CLSI M27-A2 method (NCCLS, 2002):

Micro-dilution assay

This method involves the use of small volumes of broth dispensed in sterile, plastic microdilution trays that have round or conical bottom wells.

Bacterial strains which showed the highest diameter of inhibition zones were chosen for MIC determination.

Crude venom of initial concentration of 2 mg/ml was serially diluted in the range: 2000; 1000; 500; 250; 125; 62.5; 31.25; 15.625; 7.812 and 3.906 µg/ml.

Bacterial suspensions were standardized to 0.5 Mc Farland unit, dilutions were adjusted in MHB for each bacteria and in PDB for *Candida albicans*, resulting a final concentration approximately 1.10^6 CFU/ml.

This suspension was inoculated in each well of a microdilution plate already filled with the different concentrations of the crude venom.

Plates were incubated 24h for 37 °C and *C. albicans* for 27°C.

The MHB was used as negative control. Value of MIC was determined as the lowest concentration able to inhibit any visible bacterial or candidal growth.

Data Analysis

The statistical analyses were accomplished using SPSS 16.0. The SD and the significance (P) were estimated by using one way-ANOVA from the same program. Results were considered as significant when $P<0.05$.

RESULTS

The results of our experiments revealed that the crude venom showed significant antibacterial activity against the gram positive bacteria, *S. aureus* and a moderate effect against the gram negative bacteria, *S. enteritidis* (Halo 27 and 16 mm respectively) (table 1). Furthermore, our results have clearly shown that the effects of the 3 different concentrations of the crude venom against all bacteria and the yeast were non dose dependent.

Table 1. Inhibition zones (mm) of crude venom against different types of bacteria strains and *Candida albicans* using the disc diffusion method.

MICROORGANISM	INHIBITION ZONE(MM)						
	V 4[a]	V 8[b]	V 14[c]	T30[d]	Ti75[e]	GM 10[f]	Nys10[g]
S. AUREUS	**24.66±0.88[7]**	**25.67±0.88**	**25±1**	**14**	**15**	**0**	**ND**
E. FAECALIS	0	0	0	0	0	0	ND
S. ENTERITIDIS	**13±0.578**	**14±0.58**	**14.67±0.88**	**25**	**28**	**24**	**ND**
E. COLI	12.67 ±0.88	12.33 ±0.33	12.67±0.33	24	29	25	ND
P. AEROGINOSA	12.67±0.67	12±0.577	12.33±0.88	7	23	22	ND
C. ALBICANS	0	0	0	ND	ND	ND	22mm

Values of inhibition zone diameter in mm± Standard error. a. Crude venom 4mg/mL b. Crude venom 8mg/ml c. Crude venom 14mg/ml d. T30: Tetracycline 30 µg/disc e.TI 75: Ticarcilin 75 µg/disc f.GM: Gentamicin 10 µg/disc g.Nys: Nystatin 10 µg/disc.

Results were considered significant when the inhibition zone was greater than 13mm. This was observed with Tetracyclin and Gentamicin which both presented a diameter between 14-17 and 19-22mm respectively.

However, all other tested bacteria *(E. faecalis, E. coli, P. aeroginosa*) and yeast *(C. albicans*) were less susceptible to the venom. In fact, our results showed that the order of susceptibility of the strains to the venom is as follows: S. aureus > S. enteritidis> E. coli>P. aeroginosa> E. faecalis> C. albicans (fig.1).

The antibacterial effect of the crude venom of *M. bornmuelleri* against bacteria and of candida *albicans* was compared to the effect of four standard antibiotics and it has been shown that the crude venom has a greater effect on *S. aureus* than all the antibiotics but lower or no effect against all the other used bacteria and candida (fig.2 and 3).

Since the crude venom from *M. bornmuelleri* has shown most significant zones against *S. aureus* and *S. enteritidis,* these two strains were selected to evaluate the MIC (table2).

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Table 2. MIC of *M. bornmuelleri* crude venom for macro and microdilution

Microorganism	MIC µg/ml
S. aureus	125
S. enteritidis	1000

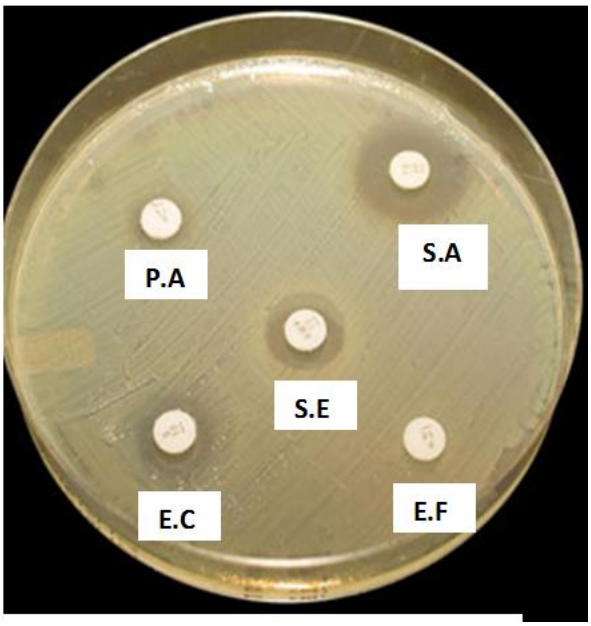

Figure 1: Antibacterial effect of crude venom against different microorganism strains, disc diffusion method

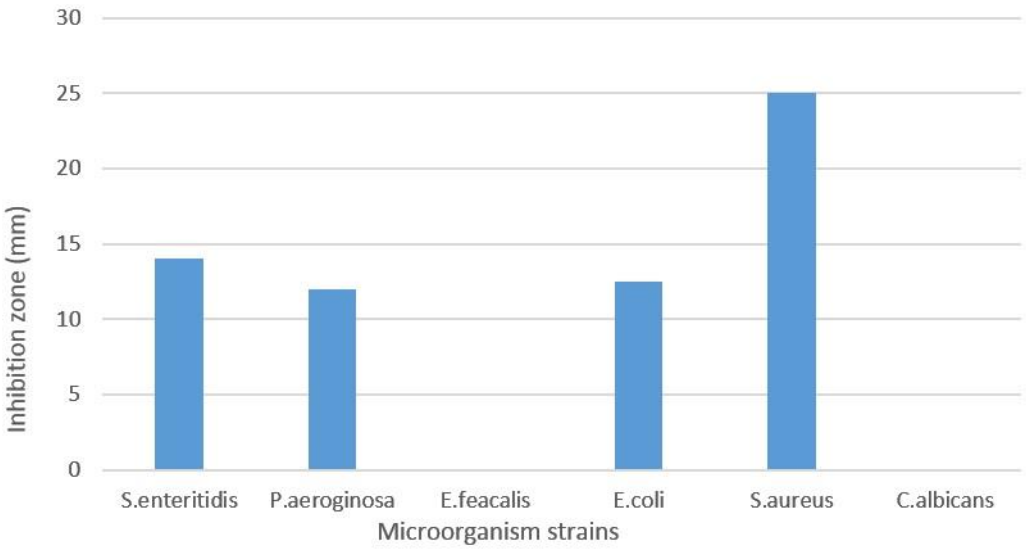

Figure 2: Antibacterial effect of crude venom against different microorganism strains

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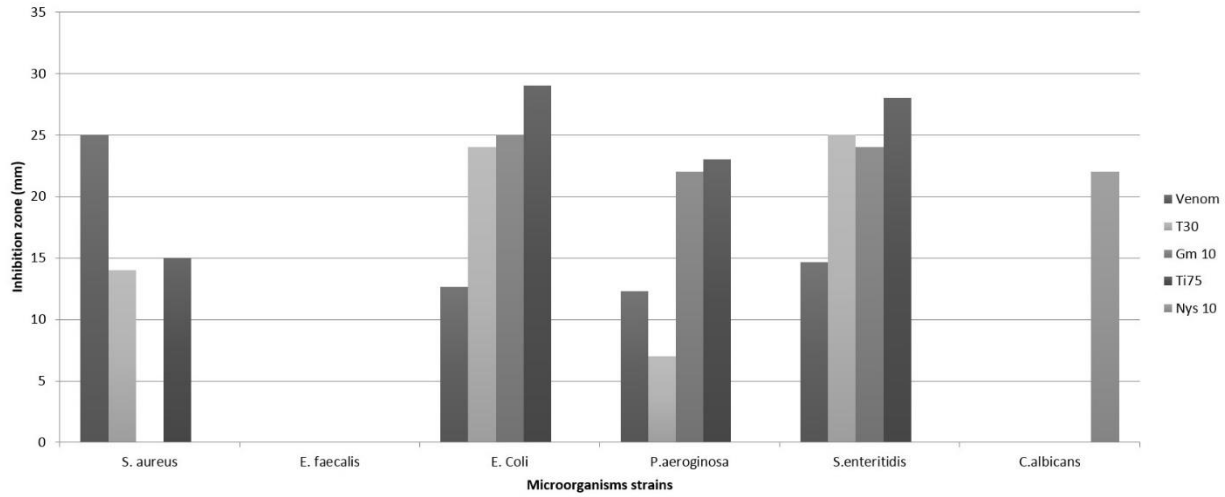

Figure 3: Antibacterial effect of *M. bornmuelleri* venom against different strains in comparison with four standard antibiotics, tetracycline, gentamicin, ticarcillin and nystatin.

As proved by micro dilution method, the MIC values of the venom were within the range of 125-1000 µg/ml (table 2). The MIC value of this venom was found to be much greater than that of the selected antibiotics.

DISCUSSION

The results of the assayed methods used in our study have shown that *S. aureus* and *S. enteritidis* are sensitive to the three concentrations of the *Montivipera bornmuelleri* crude venom in comparison with the standard antibiotics tested: tetracycline, ticarcillin, gentamicin and nystatin. Furthermore, our findings showed that *Montivipera bornmuelleri* crude venom has a non-dose-dependent antibacterial activity since the comparison of the three concentrations of crude venom effects did not show any significant differences ($p>0.05$).

We have noticed the differences in individual susceptibilities where the same venom presented a great antibacterial activity against *S. aureus* while it failed to kill *E. faecalis,* although both being gram positive bacteria. Furthermore, the MIC against *S. aureus* was found to be the lowest among all other tested bacteria (125µg/ml), followed by *S. enteritidis* (1000µg/ml). In fact, these different effects on Gram positive bacteria have been also observed by Bustillo et al., in 2008, by Samy et *al.*, in 2006 and by Al-Ahmadi et al., in 2010.

In addition, it is important to mention that other bacteria have also shown inhibition zones: E.coli and *P. aeroginosa* have presented a halo of 12mm.

This study is a screening test as we are using a crude venom. Regarding the important result obtained, further studies on the *Montivipera bornmuelleri* venom will be conducted to isolate the active components probably responsible of the antimicrobial activity.

Considering the latest research, the antibacterial activity of snake venoms is due to enzymatic components such as L-amino acid oxidase (LAAO) (7, 10, 26, 28, and 30). Therefore, this enzyme may interact with different molecules of some bacteria while keeping other strains intact (5). Effectively, the resistance of the Gram negative bacteria could possibly be attributed to the lipopolysaccharides (LPS) on the outer membrane of the bacteria which affect the uptake of antimicrobial peptide (16). The antimicrobial mechanism of the snake venom is complex and could be affected by many factors as outer membrane composition, net charge of the protein, salinity of the environment (24). These factors could explain the differences of susceptibility among the different bacterial strains.

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