Journal of Plant Protection and Pathology

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Antibacterial Activities of Honeybee Venom Produced under Different Storage Conditions

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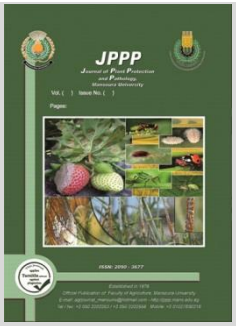

ABSTRACT

The work was evaluated, the activity of bees venom as antibacterial, which was collected from local honey bee hybrid (Carniolian hybrid *Apis mellifera carnica*) under different storage conditions against six pathogenic bacterial strains, including four Gram-positive bacteria, *Staphylococcus aureus; Staphylococcus epidermidis; Pseudomonas aeruginosa* and *Bacillus subtilis* and two Gram-negative; *Salmonella enterica* and *Escherichia coli*. Different samples of bee venom had an inhibitory effect against all types of investigated bacteria. The minimum inhibitory concentration of bee venom was determined. From these data, we can report that, when increasing the concentration of bee venom give more effectiveness against both bacterial strains "Gram-positive and negative bacteria".

Keywords: Honey bee,bee venom andantibacterial activity.

INTRODUCTION

The bee's venom is a very clearly liquid and bitter taste with acidic reaction and aromatically odour, and completely dissolves in water. Bee venom contains various pharmacologically active polypeptides. Boss among them are apamin, melittin and mast cell degranulating peptide that is known as peptide 401.The last component have anti-inflammatory effect in rat models of chronic and acute inflammation (Shipolini, 1984). The Apamine peptide substance is a very important in bee's venom and it gives him good qualities (Dotimas and Hider, 1987). A small peptide represented less than 2% of bee's venom dry weight; it has about of 2.0 Killo Dalton-KDa, 18 amino-acid residues and neurotoxic properties, and has no side effect on a great variety of mammal cells (Hider, 1988). Bee's venom was dried at surrounding-temperature for about (20 minutes), the original weight loses about (65% to 70%). Pure dried bee's venom (0.1 mg) can be collected per bee-sting after liquid evaporation (Simics, 1994). In many previous years, several studies were conducted on honey bee products, e.g. wax; venom; honey; pollen; propolis and royal jelly, which are very important due to their nutritive value or pharmacological activity, they impact different biological and medical aspects for human health, and successful therapy of nervous system, such as, limb pain; neuritis; back pain; neuralgia; ear inflammation and articulates polyneuritis (Munstedt and Bogdanov, 2009). The bee's venom therapy is a part which used within all bees' products. It has been utilized in the over a significant time as an "alternative therapy" to treat the Lyme disease; chronic fatigue syndrome and various sclerosis. The bee's venom rich source of biogenic amines; peptides and enzymes contains (18 active components) at least (El-Bassiony and Khalil, 2007).

This study was conducted to evaluate the bee's venom as antibacterial activity, which was collected from local honey bee "Carniolian honey bee hybrid" against six strains of pathogenic bacteria "Gram-positive and negative bacteria".

MATERIALS AND METHODS

Honeybee venom bioassay

Bee venom was collected from local honeybee hybrid (Carniolian hybrid every 30 days. samples were divided according to preservation method: the first sample saved in -4^{0}C directly, the second sample: lyophilized then saved in -4^{0}C, the third sample: action solution (30 gm venom: 2 ml distilled water) and then centrifugal (12000 roll/4 degree Celsius for 15 minutes) then lyophylized and saved in -4^{0}C. This experiment was carried out at "Zewail City of Sci. & Technology"-6th October City-Giza-Egypt. The bacteria were placed on the medium and the bee venom concentrations added on the plate and the colonies were counted to monitor the bacterial growth. Six different concentrations of honeybee venom were used in this study as follow; 10, 20, 40, 80, 160, and 360 µg/ml.

Tested bacteria

Six species of bacteria including Gram positive and negative were used as follow:

- **Gram–positive:**

-Staphylococcus aureus.
-Staphylococcus epidermidis.
-Pseudomonas aeruginosa.
-Bacillus subtilis.

- **Gram–negative:**

-Salmonella enterica.
-Escherichia coli.

Determination minimum of inhibitory concentration "MIC"

Determined values of minimum inhibitory concentration (MIC) were conducted by spotting technique. Each of bacteria-strain grown overnight in "tryptic soy broth" (TSB;Oxoid-Basingstoke-UK) at

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DOI: 10.21608/jppp.2020.158439

degree medium temperature 37°C. Overnight culture was used to start a culture day. Each culture used to determination of "MIC" at about (10^6) colony-forming-units CFU/ml. Then, microbial cultures (100µl) were transmitted into sterilized of Petri-dishes with "tryptic soy agar" (TSB;Oxoid-Basingstoke-UK) with degree medium temperature to cover surface plate area. Prepared aqueous materials were diluted to six different concentrations (10, 20, 40, 80, 160 and 360 µg/ml), and (10µl) of each dilution was spotted on overlay for each culture. Controls were conducted by these bacterial cultures. "MICs" defined as lowest concentration of each sample at which visible inhibition of bacteria growthing was induced and measure of diameter for each inhibition area by a regular ruler/cm.

Microbial growth curve and growth reduction

Rate values of reduction were determined for samples and displaying antimicrobial properties by using a modified method "micro-dilution broth" Sokmen *et al.*, 2004, in 96well micro-plates (Greiner bio-one, CELLSTAR®). Each bacteria-strain grown overnight in "tryptic soy broth" (TSB;Oxoid-Basingstoke-UK) at degree medium temperature 37°C. Overnight culture was used to start a culture day and the reduction rates were determined for each culture at about (10^6 CFU/ml). Later, 200µl of microbial-cultures were transmitted into a 96well microtiter plate by using a micropipette "multichannel". Briefly, the samples were diluted in sterile water and tested against day cultures of *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Salmonella enterica* ATCC 25566, *Pseudomonas aeruginosa* ATCC 10145, *Bacillus subtilis* ATCC 35854, and *Staphylococcus epidermidis*. Micro-plates were putted in an incubator at medium temp., (37°C) for each bacteria-strains and growthing was observed by absorbance measure at (630nm) over (90min) by using a micro-plate reader, (FLU0star-Omega; BMG-Labtech®). Reduction rate was monitored for each concentration and recorded in comparison with the control sample over the experiment time.

RESULTS AND DISCUSSION

Data presented in Table (1), summarized the MIC values of tested samples of venom against selected bacteria-strains. Antibacterial activity of the 3 materials was investigated in comparison with the control against *E coli; S. aureus, S. epidermidis, S. enterica, P. aeruginosa, and B. subtilis.* Viable counts of different bacteria "Gram positive and negative" in (TSB broth) illustrated in Figs 1-6. Data obtained revealed the major of tested materials were more active of antibacterial relatively, against of all the tested bacteria-strains, when compared with controls. On the other hand, more than (70%) reduction in bacteria growths were spotted in supplemented cultures in highly concentrations (> 40µg/ml.). Throughout these data given, we can conclude that, when the concentrations are increasing the efficacy is very highly against the tested "Gram negative and positive" bacteria, Figs 1-6.

The results are agreement with some researches, e.g. Hegazi, *et al.*, (2014), mentioned that, the "BV" appeared inhibition against bacteria-strains growthing and its vitality and highlighted that, the (BV) can be utilized as antimicrobial complementary material against pathogenic-bacteria.

Table 1. Minimum Inhibitory Concentration (MIC) of tested honey bee venom on different microorganisms (µg /ml).

	Sample 1*		Sample 2**		Sample 3***	
	Concentration (mg/ml)	Inhibition zone (cm)	Concentration (mg/ml)	Inhibition zone (cm)	Concentration (mg/ml)	Inhibition zone (cm)
Salmonella enterica	10	0.6	10	0.5	20	0.5
Escherichia coli	20	0.4	20	0.5	20	0.5
Staphylococcus aureus	40	0.5	40	0.4	80	0.4
Staphylococcus epidermidis	40	0.4	40	0.6	40	0.4
Pseudomonas aeruginosa	40	0.4	40	0.4	40	0.6
Bacillussubtilis	40	0.5	80	0.6	80	0.3

Where: Sample 1*: Honey bee venom saved in -4^{0}C directly., Sample 2: Honey bee venom was lyophilized then saved in -4^{0}C., Sample 3***: action solution (30 gm venom : 2 ml distilled water) and then centrifugal (12000 roll/4 degree Celsius for 15 minutes) then lyophylized and saved in -4^{0}C.**

Microbial growth curve

The higher concentration of venom appeared significant "P≤0.001" as antimicrobial activity when compared with their identical lower concentration. Differences of reactions were recorded in the tested bacteria "Gram positive and negative". Generally, the tested bacteria (Gram-positive) were soft susceptible to high concentrations of the tested samples than Gram-negative bacteria.

The results in Fig. (1), indicated that the10 µg/ml concentration was found to have a less significant effect on *Salmonella* in comparison with high concentrations when honey bee venom was saved in -4^{0}C directly. The highest reduction rate was occurred with highly concentrations of venom (>10µg/ml.). However, sample 2 and 3 showed that the highest reduction rate that was observed at high concentrations of venom (>20 µg/ml.), while the lower concentrations of venom (10&20µg/ml.), appeared an incompletely reductions in bacteria-growth rates.

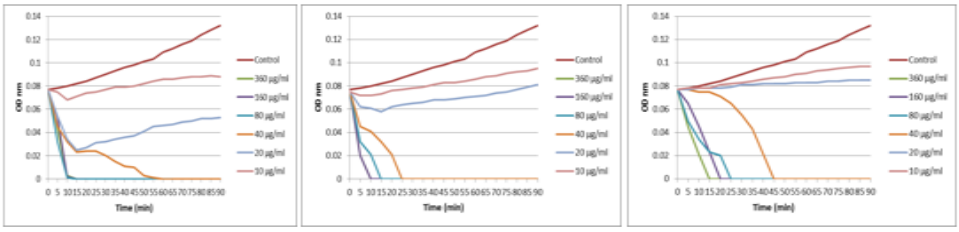

Figure 1. Effect of bee venom (µg /ml) on the growth rate of *Salmonella enterica*.

Data arranged in Fig. (2) indicated that the concentrations of 10 and 20µg/ml of sample 1 were found to have a less significant effect on E. coli in comparison with high concentrations. The highest reduction rate was observed at the high concentrations of venom (>20 µg/ml). Highconcentration treatments (>20 µg/ml) of samples 1and 3 showed the highest reduction rate in comparison with sample 1 where most of bacterial cells numbers were declined to undetectable limit after 30 min of treatment.

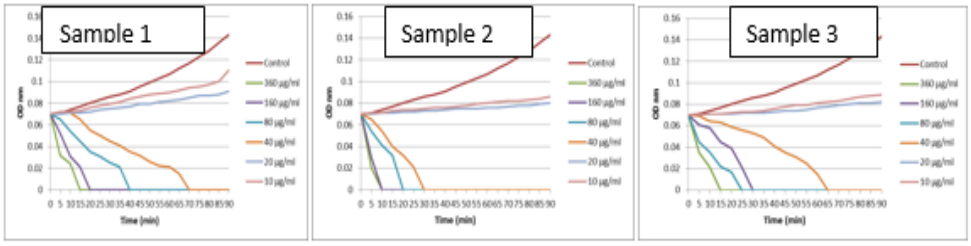

Figure 2. Effect of bee venom (µg /ml) on the growth rate of *E.coli.*

The obtained results in Fig (3) cleared that both concentrations 10 and 20µg/ml showed a less significant effect on *S.aureus* in comparison with high concentrations. The highest reduction rate showed at highly concentration of venom (>20µg/ml.). Highly concentrations (>20µg/ml.) of all samples showed a significant (p>0.05) decrease in all bacterial cell numbers. Most of bacterial cells numbers decreased to undetectable limit after 30 min of treatment except with concentration 20 µg/ml where most cell numbers were declined after 60 min of treatment in most venom samples.

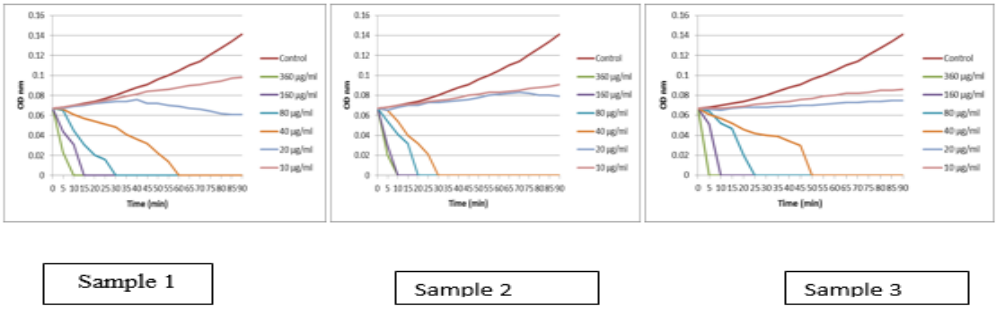

Figure 3. Effect of bee venom (µg /ml) on the growth rate of *S.aureus.*

The samples (1 and 3) show that similar patterns of reduction with*S.epidermidis* Fig(4).The concentration of20µg/ml showed a significant killing effect on *S. epidermidis* in comparison with the (low concentration10 µg/ml) which showed a limited effect in reducing the bacterial count in comparison with other samples. Most of bacterial cells numbers were declined to undetectable limit after 50 min of treatment with high concentrations of venom (>20 µg/ml).

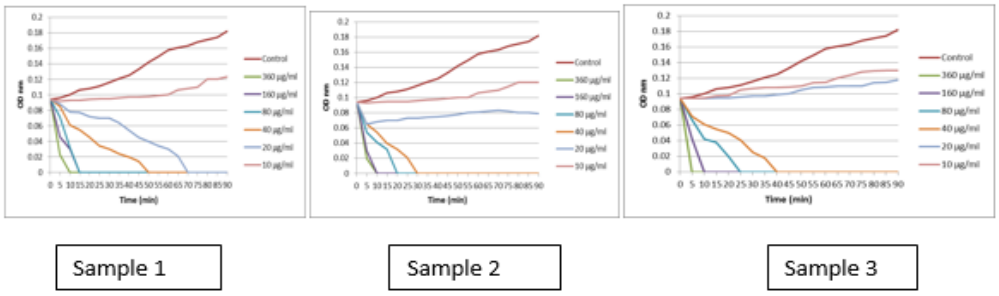

Figure 4. Effect of bee venom (µg /ml) on the growth rate of *S.epidermidis.*

The effect of bee venom on *P. aeruginosa*shown in Fig. (5) It is clear that venom sample number 2 showed the highest reduction rate (P> 0.05) with most concentrations, except concentration 10µg/ml, on *P. aeruginosa* numbers. Samples 2 and 4 showed a less significant effect in comparison with other samples.

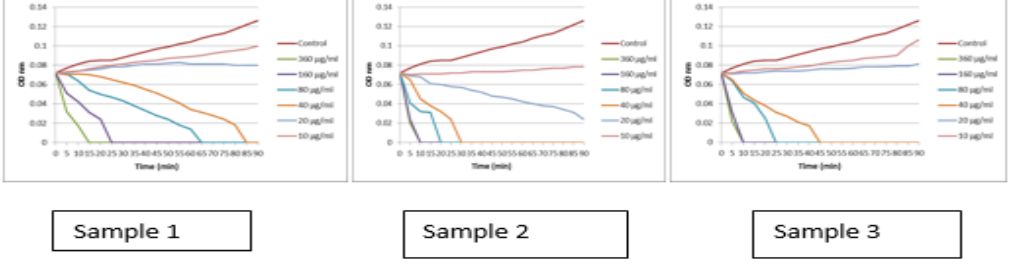

Figure 5. Effect of bee venom (µg /ml) on the growth rate of *Pseudomonas aeruginosa.*

With *Bacillus*,Fig. (6) sample 1 revealed that the lowest reduction effect on bacterial counts where most bacterial cells tolerate concentrations up to 40 µg/ml. On the other hand, sample 3 showed the best reduction effect where all concentrations, except 10 µg/ml, managed to reduce the numbers of bacterial cells significantly (P> 0.05) to undetectable limit after 20 min of treatment except with concentration 20 µg/ml which reduced the numbers of bacterial cells to undetectable limit after 55 min of treatment.

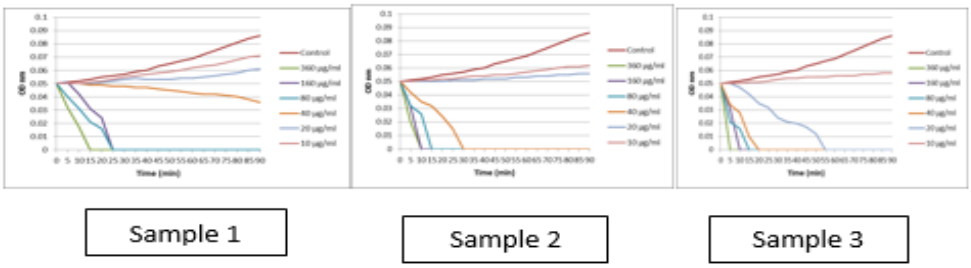

Fig. 6. Bee venom Effect (µg /ml) on the growth rate of *Bacillus subtilis.*

The obtained results indicated that the higher concentrations of venom showed a better killing effect on bacterial cells. Different factors effect of bee venom on bacterial cells and its mechanism. For example, the different concentration of bee venom, the type of bacteria, the bacteria Gram-positive like *Staphylococcus aureus* and *Bacillus subtilis*, has a large thick cell-wall than, bacteria Gram-negative because layer of "peptidoglycan". However, results showed that the bacteria "Gram positive and negative" were more inhibition at highly level of tested venom samples. Slightly sometimes, the strains of "Gram positive" showed high sensitivity than, strains of "Gram negative" maybe occur due to differences of structure in outer membranes between these bacteria, although the outer membranes of Gram-negative rich in lipopolysaccharide compounds, as it reduces diffusion rates of any macromolecules.

Antimicrobial activity of bee venom has primary been referred to the action of peptides mainly melittin-peptide and this compound is responsible for pore formation in the cytoplasmic membrane of both gram positive and gram negative organisms, this compound is a non-cell selective cytolysin(Beven and Wroblewski, 1997; Matsuzaki, 1997;Oren and Shai, 1997).Subbalakshmi*et al.*, (1990) mentioned that these peptides (melittin) from bee venom exhibited the potential of antibacterial activities towards both the bacteria "Gram-positive and negative". It had been suggested that variations in quantity and composition of bee venom may be referred to bee race (pure race or hybrid) and age, location of bee, season of the year and to the quality of nutrition in bee colonies (Marz*et al.*, 1981; Inoue *et al.*, 1987; Omar, 1994;Robinson and Otis, 1996). It is most likely that potency of bee venom against microorganisms is largely dependent on bee venom protein bands and its molecular weights (Noure*t al.*, 2004).

The antimicrobial activity of honeybee venom may bedue to the presence of several peptides like melittin, apamin, adolapin, mast cell degranulating peptide, enzymes, biologically active amines and non-peptide, component (Kwon, *et al.*, 2002).Fennell *et al.*, (1968) reported that the venom contains mellitin that is active against Gram- positive more than Gram-negative bacteria. These results were in agreement with Kondo and Kanai (1986) who found mycobacteria and staphylococci were affected by bee venom fraction (melittin), but not *E. coli*. Ortel and Markwrdt(1955) quantitatively determined the zones of inhibition. They found that Gram-positive organisms were sensitive at lower concentrations of bee venom than Gram-negative. In contrast, a stronger activity on *E. coli* had been reported previously for bee venom (Stocker and Traynor, 1986 and Samy *et al.*, 2007). Although, in earlier study Hegazi *et al.*(2002) showed that bee products had a weak effect against *E. coli*.Hegazi *et al.*, (2015) reported that both bee venom of pure and hybrid bees exhibited antibacterial activity against all five bacterial strains and differs according to the type. Bee venom exhibited antibacterial activity against different bacterial strains. The minimum inhibitory concentration of BV was studied. These results indicate that BV inhibits the growth and survival of bacterial strains and that BV may be an efficient antimicrobial agent against pathogens even if bee venom collected by different methods.

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النشاط المضاد للبكتريا في سم النحل المنتج تحت ظروف تخزين مختلفة

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تهدف الدراسة لإختبار النشاط المضاد للبكتريالسم نحل العسل الناتج من الهجين الكرنيولى تحت ظروف تخزين مختلفة ضد ستة أنواع من البكتريا الممرضة ، أربعة منها موجبة لجرام وهى : *Staphylococcus aureus; Staphylococcus epidermidis*; *Pseudomonas aeruginosa* and *Salmonella enteric* and *Escherichia coli* ونوعين سالبين لجرام وهما : *Bacillus subtilis*. وقد أوضحت النتائج المتحصل عليها أن سم النحل فى ظروف التخزين المختلفة أظهر نشاطا مضادا لكل أنواع البكتريا المستخدمة ، وتم تحديد أقل تركيز مثبط (MIC) ، كما أن الزيادة فى تركيز السم المستخدم أظهر فاعلية أكثر ضد كلا من البكتريا الموجبة والسالبة لجرام .