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ANTIBACTERIAL ACTIVITIES OF BEE VENOM PRODUCED BY TWO HONEYBEE, *Apis mellifera* L., HYBRIDS

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ABSTRACT

Bee venom (BV) has been reported to have multiple effects, including antibacterial, antivirus, and anti-inflammation effects, in various types of cells (BV) is a complicated combination of active peptides, enzymes, and amines. The aim of this work was to assess the antibacterial action of bee venom obtained from two honeybee hybrids; Carniolian, *Apis mellifera carnica* and Italian, *Apis mellifera Ligustica* against six pathogenic bacteria *q.e.*, four G^+ bacteria; *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, as well as two G^- bacteria; *Salmonella enterica* and *Escherichia coli*. Bee venom collected from two bee hybrids had an inhibitory effect against all types of investigated bacteria compared to control sample. The minimum inhibitory concentration of bee venom was determined. Elevating the levels of bee venom appeared to be very effective against both Gram-negative and Gram-positive. The high concentration (>40µg/ml) of all samples showed a significant ($P \leq 0.05$) decrease in all bacterial cell numbers. The lower concentration (10 µg/ml) showed a limited effect in reducing the bacterial count in comparing with other samples. The use of bee venom, natural and safe bee product as alternative food preservatives and in some pharmaceutical application is promising, but more research should be carried out to standardize its minute composition and quality.

INTRODUCTION

Bee produces many substances, among these the most important substance is apitoxin. This complex chemical is synthesized by the gland located in the abdomen of these insects. Apitoxin of bee venom have 88%water content while 12% comprises of many components like phospholipase A2, hyaluronidase, melittin, histamine. Additionally, it contains peptides such as apamin, secapin *etc*. **(Lima and Brochetto-Braga, 2003)** Bee venom therapy is a form of apitherapy that uses bee venom to treat a variety of ailments. Bee products such as honey, pollen, propolis, royal jelly, wax, and venom are used in apitherapy. It's been used to treat various sclerosis, Lyme disease, and chronic fatigue syndrome since ancient times, and it's still being utilised now. Bee venom is a rich source of enzymes, peptides and biogenic amines and contains at least 18 active components **(El-Bassiony and Khalil, 2007)** for several years ago, many investigations were conducted on honeybee products. Such products, including honey, royal jelly, wax, venom, pollen and propolis are very important due to their nutritive value or pharmacological activity, which influence different biological and medical aspects for human health. Successful treatments of central and peripheral nervous system, such as back pain, limb pain,

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neuralgia, neuritis, articulates polyneuritis and ear inflammation **(Munstedt and Bogdanov, 2009)**. Bee venom is a complicated combination of proteins, peptides and low molecular compounds. Its constituents have now been identified. Proteins and peptides are the most important components. Apitoxin (bee toxin) has a complex content that includes various biochemical and pharmacologically active chemicals like as histamine, dopamine, and melittin **(Hegazi *et al.*, 2014)**. Because of its anti-inflammatory and antibacterial capabilities, natural components such as bee venom are promising candidates to meet this requirement **(Han *et al.*, 2016)**. Bee venom (BV) is a major source of secondary metabolites from honeybees (*Apis mellifera* L.). It comprises peptides, proteins, enzymes, and volatile metabolites, among other bioactive substances. The compounds contribute to the venom's observed biological functions as per its anti-inflammatory and anticancer effects **(El-Seedi *et al.*, 2020)**. The goal of this study was to investigate if bee venom taken from two honeybee hybrids had antibacterial efficacy against six pathogenic bacterial strains, including Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Bee Venom Collection

Bee venom were collected from two local honeybee hybrids (Carniolian hybrid and Italian hybrid) every month (According to the method lined by **Hegazi *et al.* (2015)** and used for the microbial activity experiment. New modern of the electric shock device was used in the present study. The device model used is VC-4FK from Apitronic Canada and depends on using electrical impulses to stimulate the bee workers to sting through polyethylene sheet placed on glass plate which enables the bees to pull out their stings easily. In addition, the polyethylene sheet prevents pollution of bee venom in order to obtain pure dry venom. Bees that contact with the wires received a mild electrical shock and stung onto a glass sheet. The alarm odor, which evaporated from the bees glands and mobilized and irritated the other bees to start to sting. Allowed the venom on the glass plate to dry, in a dark room, in order to prevent the venom oxidation, which may done under light. The dry venom is collected using sharp scraper and quickly packed in dark glass vials. The dry venom stored at -4ºC till use.

Honeybee Venom Bioassay

This experiment was carried out at Zewail City of Science and Technology, 6th of October City, Giza Governorate, 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

The following pathogens, both Gram-positive and Gram-negative, were used:

- Gram–positive: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*.
- Gram–negative: *Salmonella enterica*, *Escherichia coli.*

Determination of the Minimum Inhibitory Concentration (MIC)

MIC values were determined using spotting technique. At 37°C, every strain was grown for 24 hr., in Tryptic Soy Broth (TSB) medium (Oxoid, UK). The 24 hr. -old culture was used to develop a day culture, and the MIC was estimated at approximately 10^6 colony forming units (CFU)/ml for each culture. After that, 100 µl of bacterial cultures were added into a Petri dish with Tryptic Soy Agar (TSA, Oxoid, Basingstoke, UK) medium and

spread to cover the surface area of the plate. The prepared aqueous materials were diluted to six different concentrations (10, 20, 40, 80, 160 and 360 µg/ml) and 10 µl of each dilution was spotted on the overlay of each bacterial culture. Bacterial cultures were used as controls. The MICs were defined as the lowest concentration for each sample that caused observable inhibition of bacterial growth, and the diameter of each inhibition zone was estimated with a regular ruler and expressed in centimetres.

Microbial Growth Curve and Growth Reduction

The reduction rate values were evaluated for all the samples indicating antibacterial activities, by a modified microdilution broth method **(Sokmen *et al.*, 2004)** in 96-well microplates (Greiner bio-one, CELLSTAR®). At 37°C, each bacterial strain was grown for 24 hr., in Tryptic Soy Broth (TSB) medium (Oxoid, UK). The 24 hour -old culture has been used to initiate a day culture, and the decline rate was estimated at approximately 106 CFU/ml for each culture. After that, a multichannel pipette was used to transfer 200 l of microbial cultures into a 96-well microtiter plate. Briefly, the samples were diluted in sterile water and then assessed towards 24 hr.-old cultures of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *S. enterica* ATCC 25566, *P. aeruginosa* ATCC 10145, *B. subtilis* ATCC 35854, and *S. epidermidis*. For all bacterial strains, microplates were incubated at 37°C, and growth was measured at 630 nm over 90 min using a microplate reader (FLU0star Omega, BMG LABTECH®). The reduction rate was monitored for each concentration and recorded in comparison with the control sample over the experiment time.

RESULTS AND DISCUSSION

Table 1 describes perfectly the MIC values of the tested venom samples against the selected bacterial strains. The antibacterial activity of the 2 materials was investigated in comparison with the control against *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella enterica, Pseudomonas aeruginosa,* and *Bacillus subtilis.* The survival curves for various G+ and G^- bacterial strains in TSB broth are illustrated in Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. It could be noticed from the results that the majority of tested materials have relatively higher antibacterial action towards all tested bacteria compared to control sample. In cultures supplemented with the high levels (> 40g/ml), bacterial growth was lowered by more than 70%. Hence, increasing the levels of materials seemed to be especially effective towards G^- and G+ bacteria (Figs. 1-12). These results were in agreement with previous results that reported an antibacterial activity of venom against both G^- and G+ bacteria **(Monk *et al.*, 1996)**. A previous study indicated that the honey bee venom prevented the growth of seventeen G+ strains including two G^-bacteria isolated from bovine mastitis in Korea **(Park *et al.*, 2013)**. The minimum inhibitory concentration of BV was evaluated by **Hegazi *et al.* (2014)** who indicated that BV prevents the growth of pathogens and highlighted that BV seems to be used as complementary antimicrobial substance against pathogens.

Microbial Growth Curve

The high concentrations of venom showed a significant ($P \leq 0.001$) antibacterial action compared to those of lower levels. The responses of Gram-positive and Gram-negative bacteria differed in some ways. G^+ bacteria, on the whole, showed a slight sensitivity to the action of larger levels of the tested venom compared to G^-bacteria.

The results in Figs. 1 and 2 showed that bee venom of Carniolian and Italian hybrid treatments at different concentrations gave the highest reduction rate at high venom concentrations (>40µ g/ml). On the other hand,

Table 1. The minimum inhibitory concentration (MIC) of tested honeybee venom collected from Carniolian hybrid and Italian hybrid on different Gram$^+$ and Gram$^-$ bacteria (µg /ml)

Microorganism	Bee venom1		Bee venom2	
	Concentration (µg/ml)	Inhibition zone (cm)	Concentration (µg/ml)	Inhibition zone (cm)
Salmonella enterica	40	1	80	1
E. coli	40	0.6	40	0.8
S. aureus	40	0.5	80	0.5
S. epidermidis	40	0.5	80	0.7
P. aeruginosa	40	0.5	80	0.8
Bacillus subtilis	40	0.3	80	0.8

Bee venom 1= bee venom collected from carniolian honeybee hybrid.

Bee venom2 = bee venom collected from Italian honeybee hybrid.

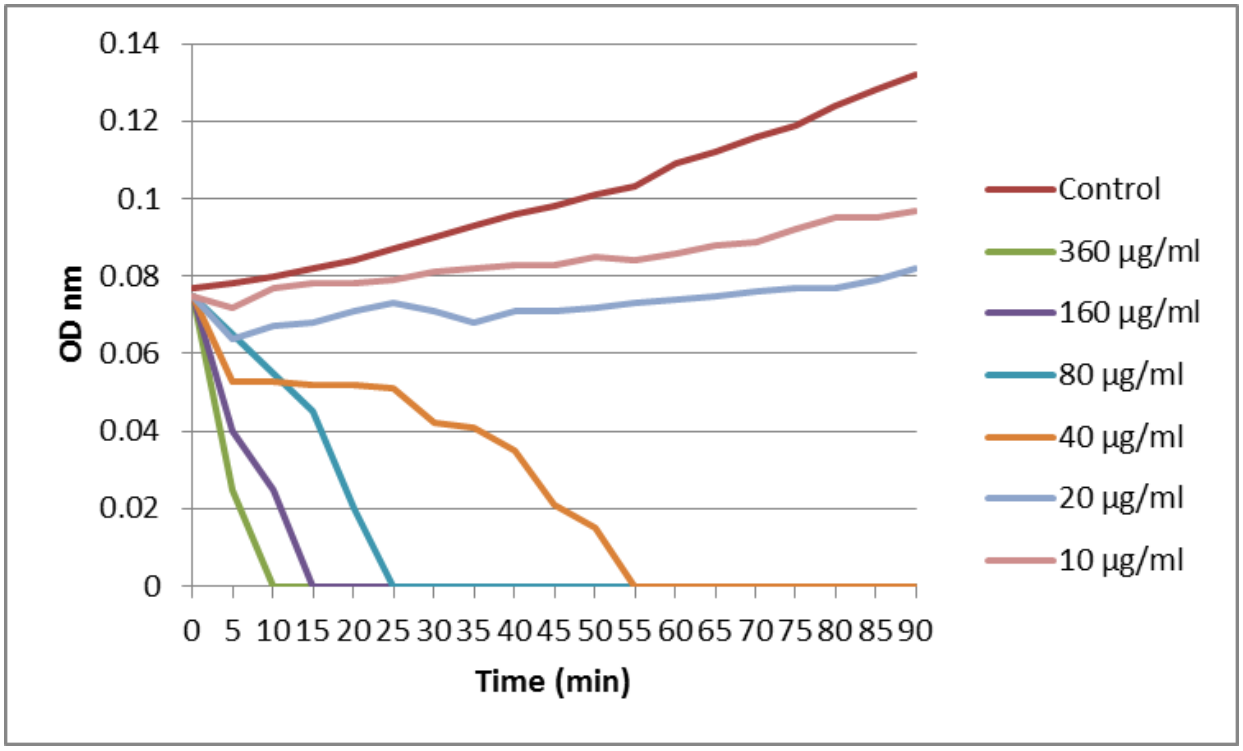

Fig. 1. Effect of Carniolian hybrid bee venom (µg /ml) on the growth rate of *Salmonella enterica*

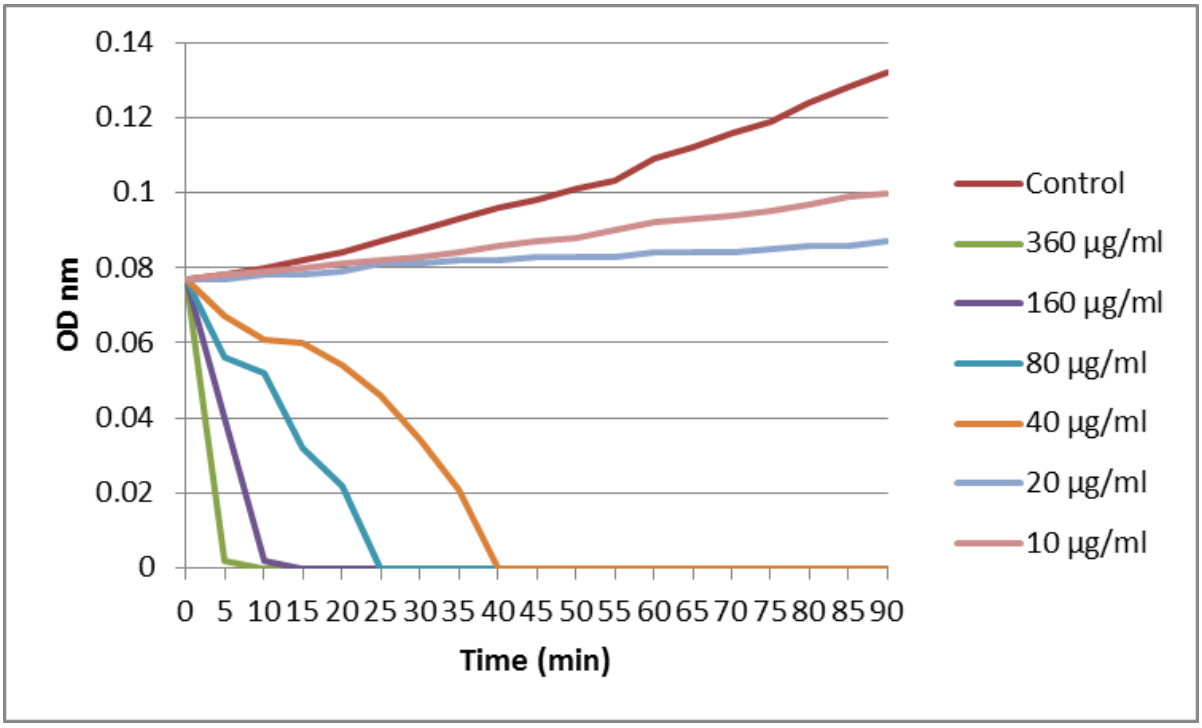

Fig. 2. Effect of Italian hybrid bee venom (µg /ml) on the growth rate of *Salmonella enterica*

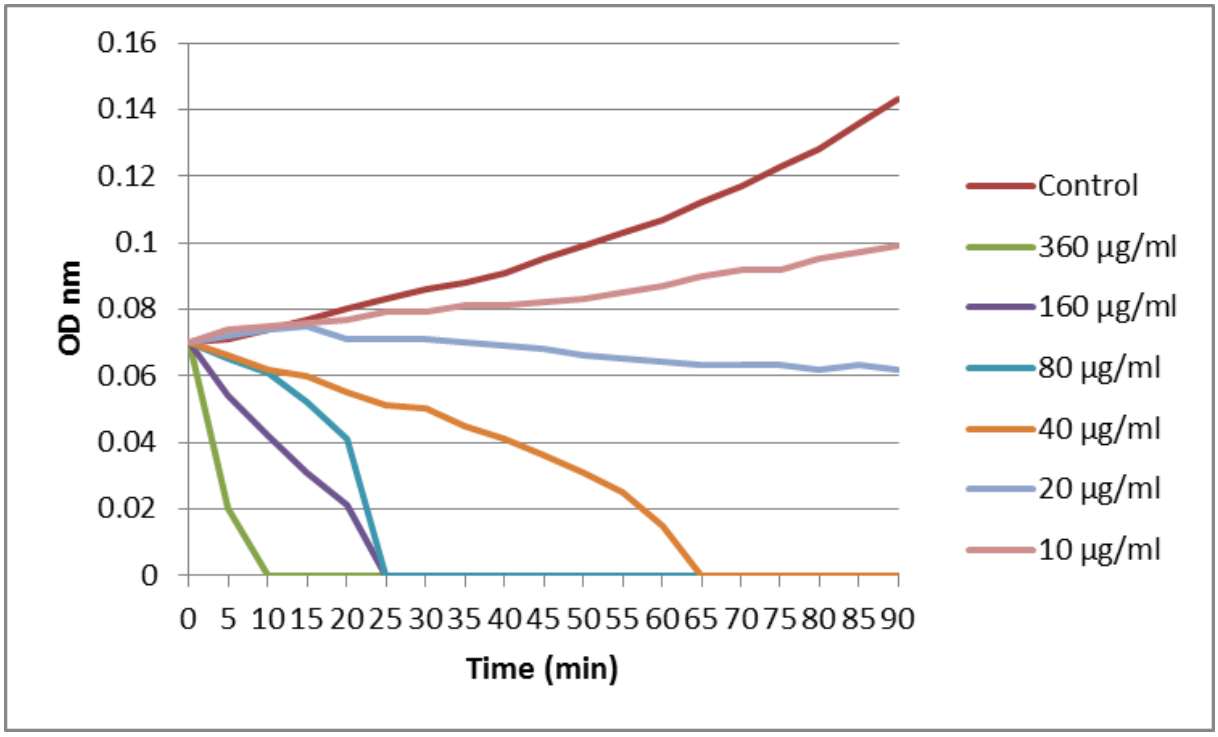

Fig. 3. Effect of Carniolian hybrid bee venom (µg /ml) on the growth rate of *E. coli*

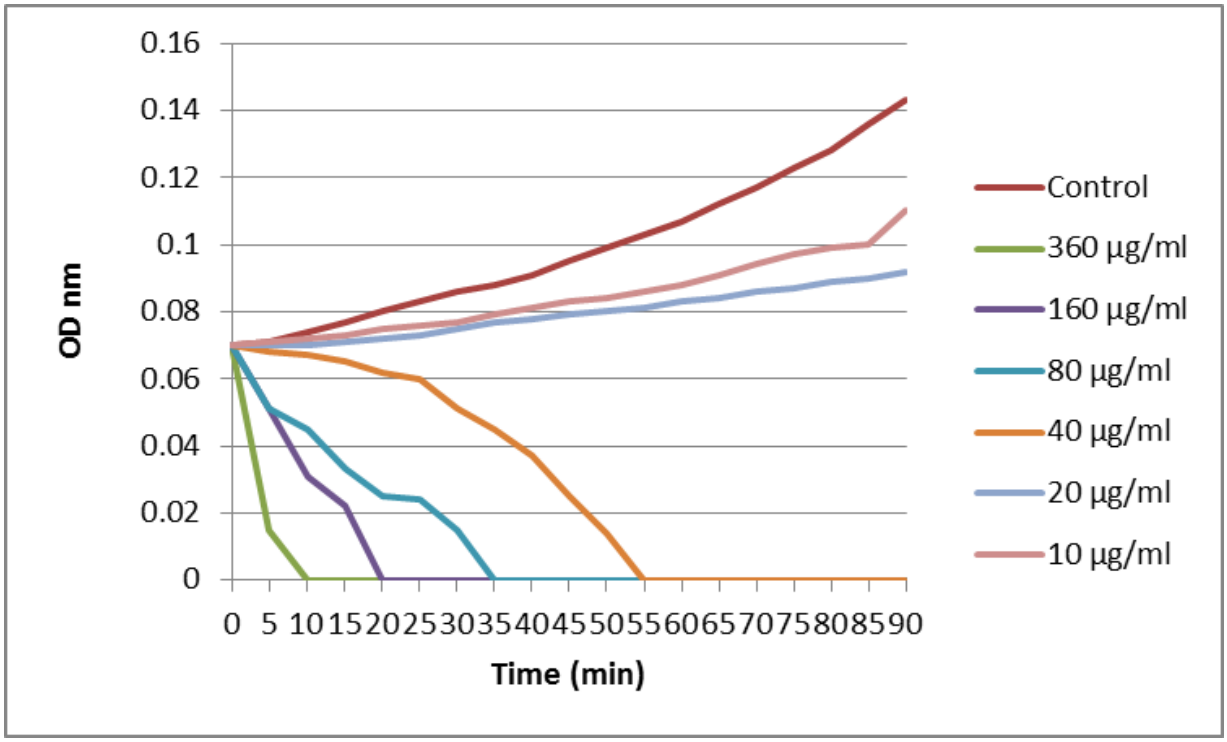

Fig. 4. Effect of Italian hybrid bee venom (µg /ml) on the growth rate of *E. coli*

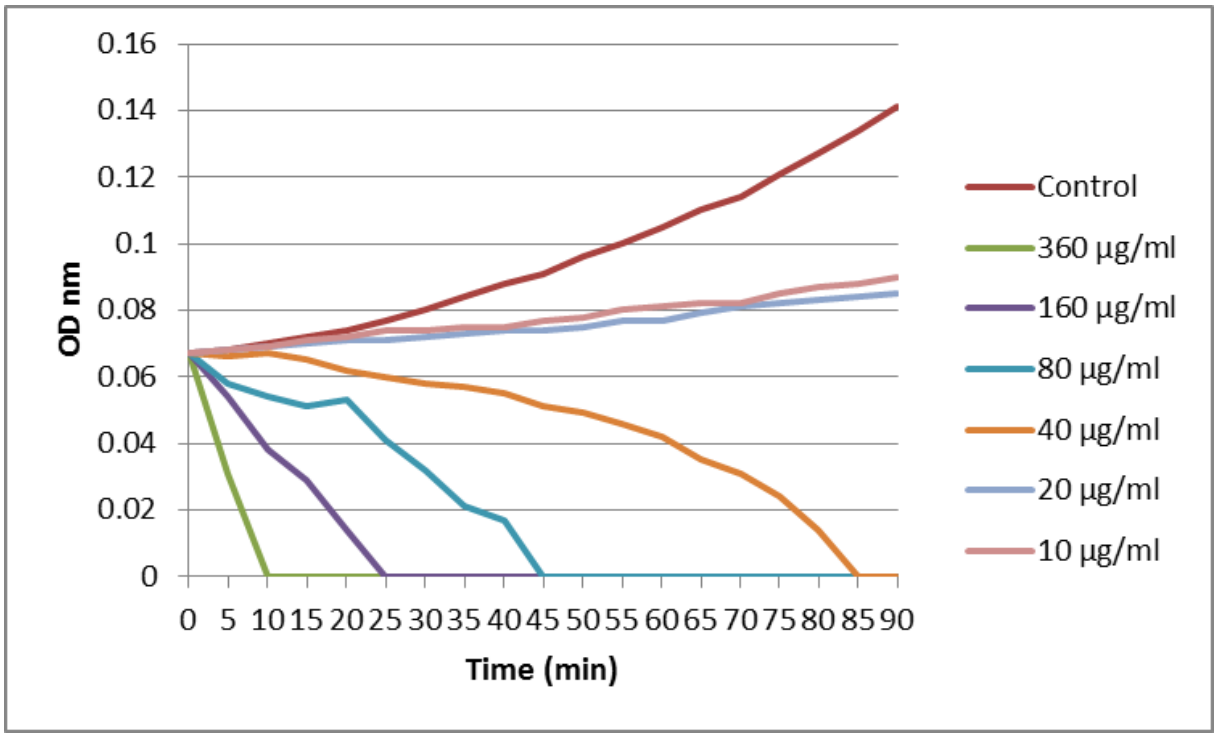

Fig. 5. Effect of Carniolian hybrid bee venom (µg /ml) on the growth rate of *S. aureus*

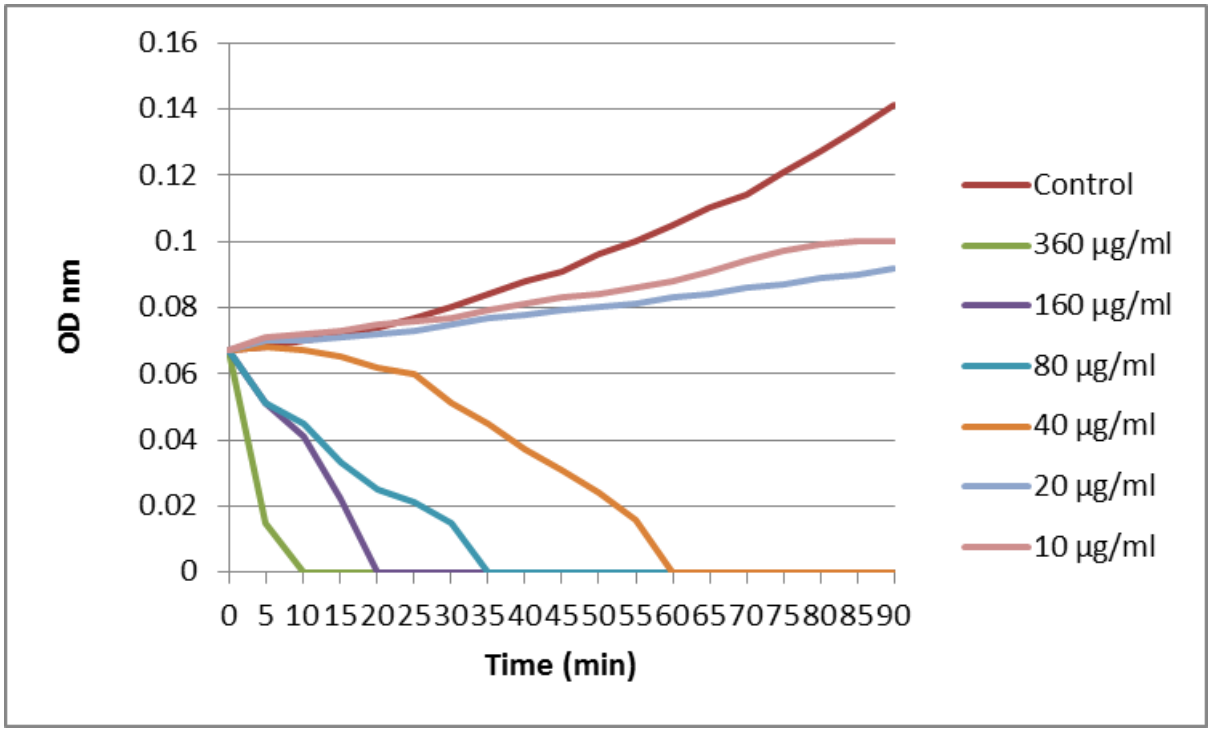

Fig. 6. Effect of Italian hybrid bee venom (µg /ml) on the growth rate of *S. aureus*

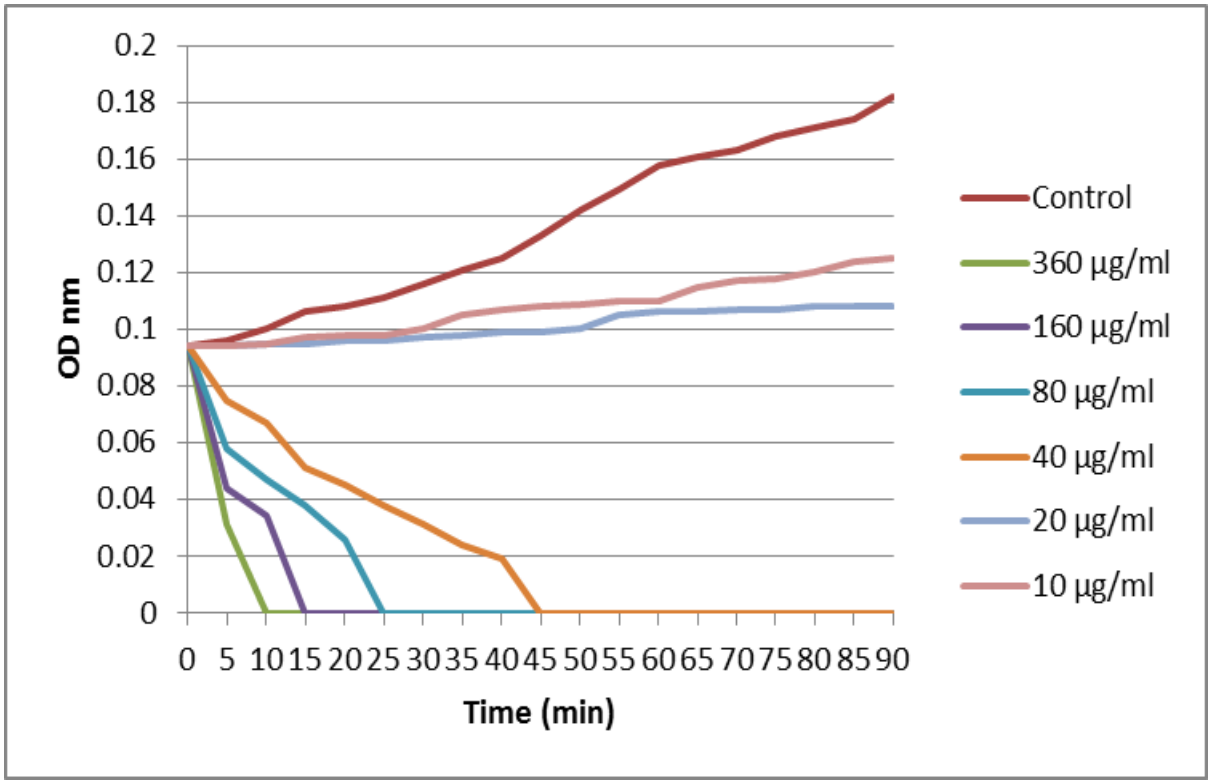

Fig. 7. Effect of Carniolian hybrid bee venom (µg/ml) on the growth rate of *S. epidermidis*

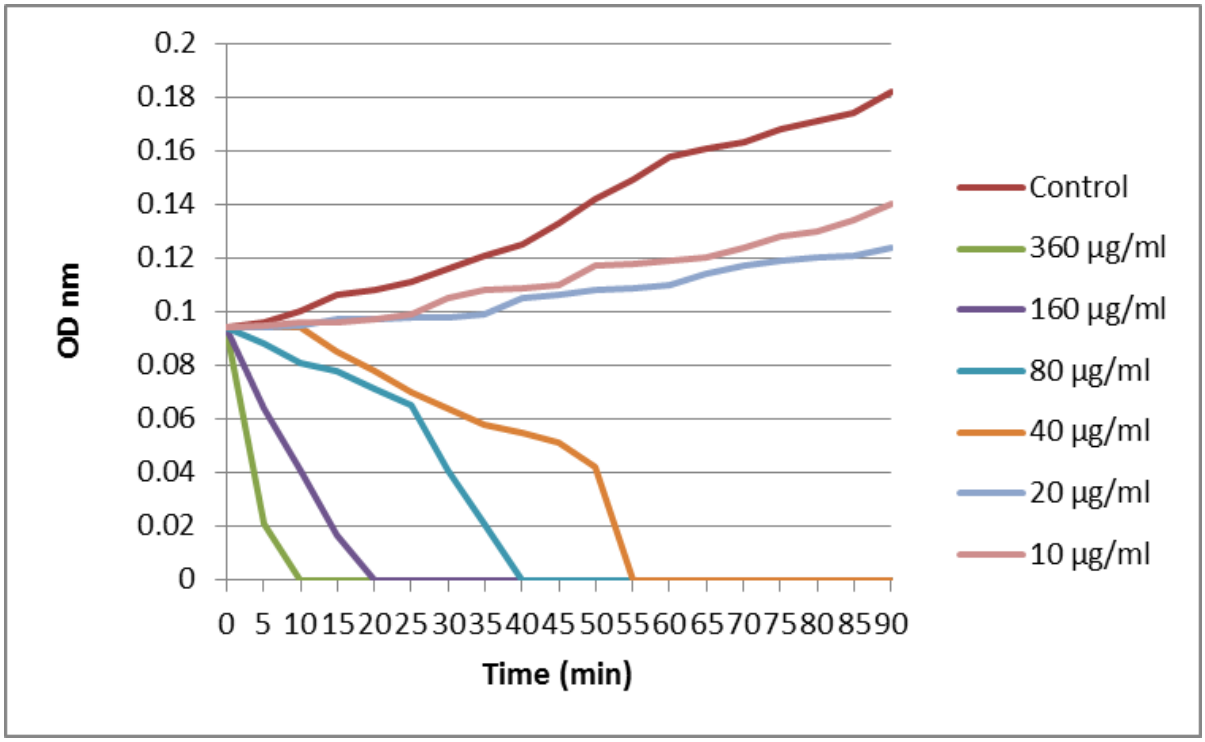

Fig. 8. Effect of Italian hybrid bee venom (µg /ml) on the growth rate of *S. epidermidis*

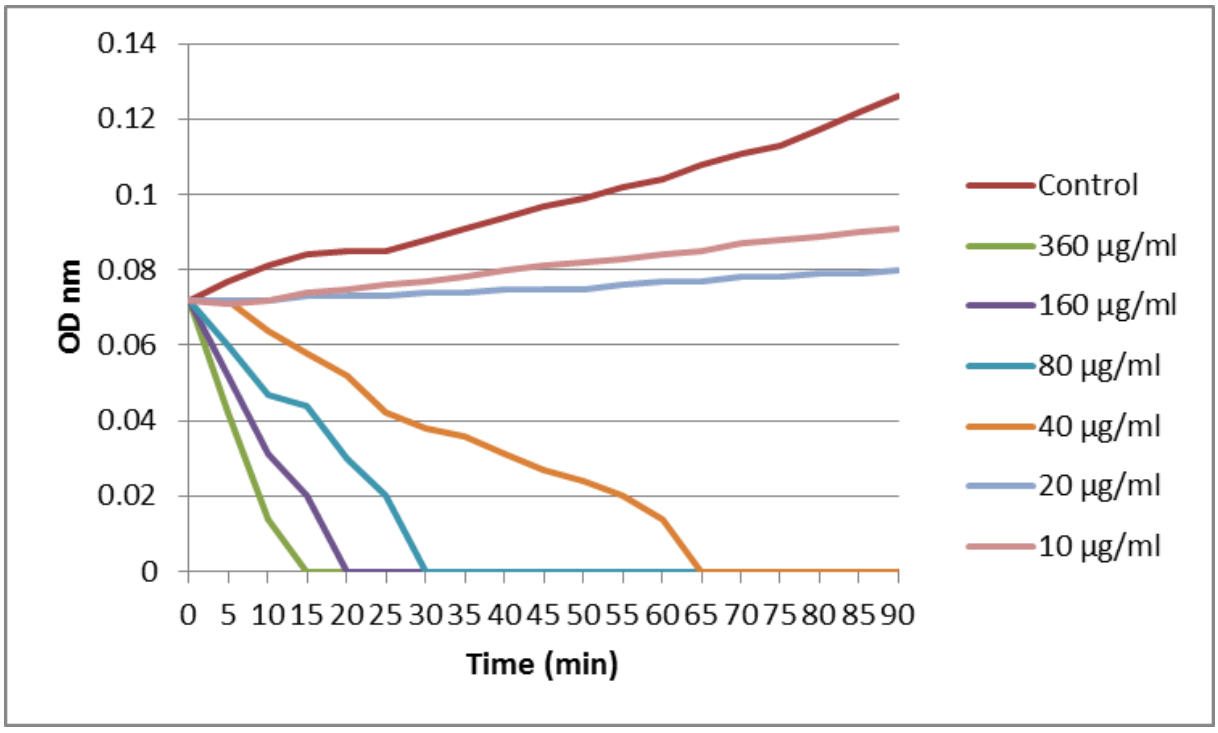

Fig. 9. Effect of Carniolian hybrid bee venom (µg/ml) on the growth rate of *Pseudomonas aeruginosa*

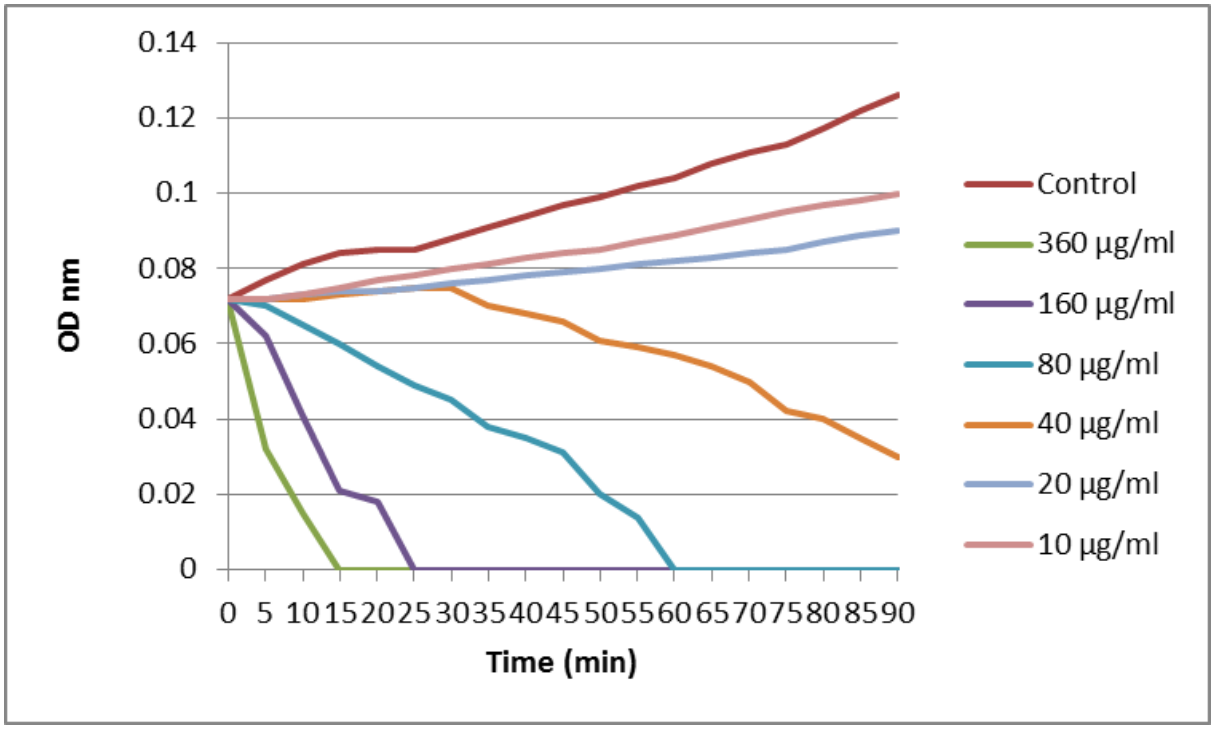

Fig. 10. Effect of Italian hybrid bee venom (µg/ml) on the growth rate of *Pseudomonas aeruginosa*

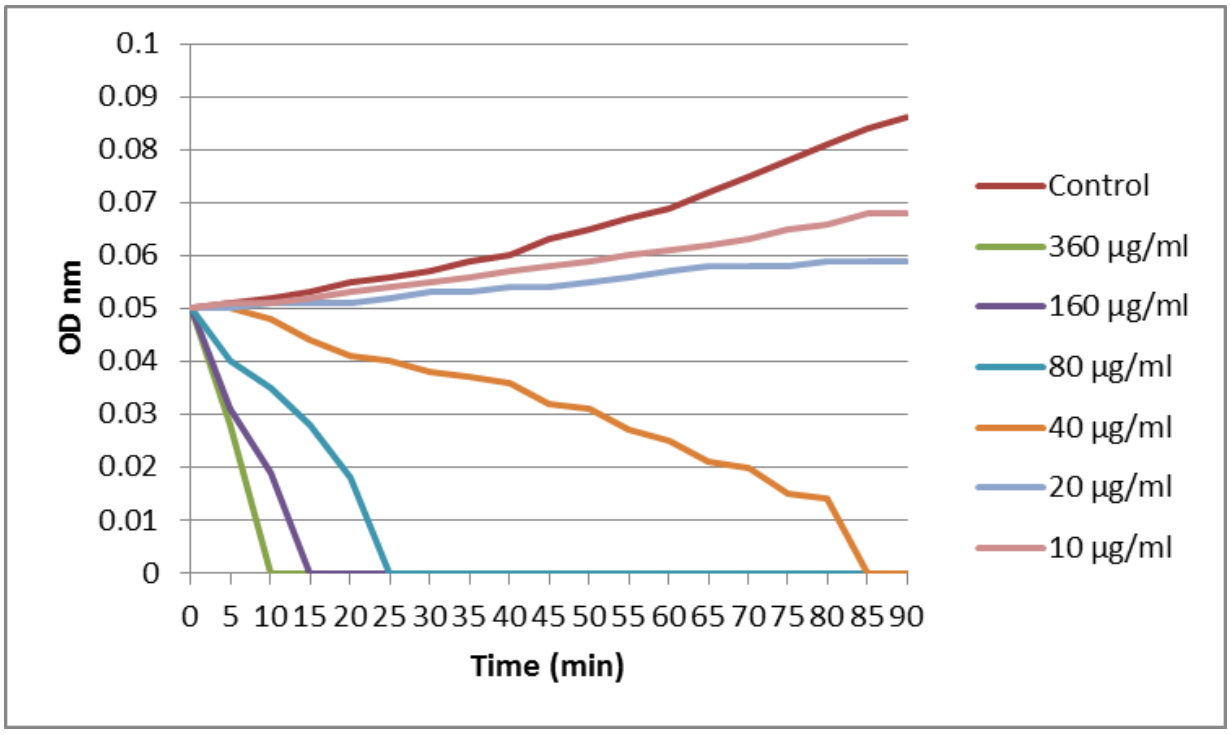

Fig. 11. Effect of Carniolian hybrid bee venom (µg /ml) on the growth rate of *Bacillus subtilis*

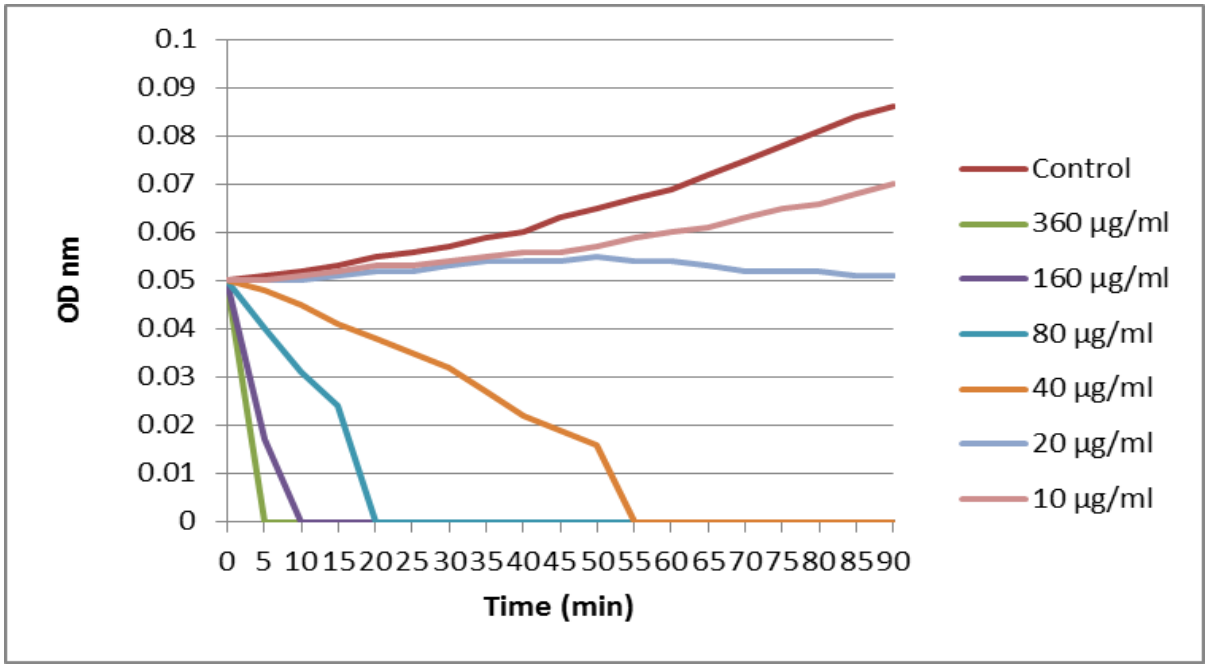

Fig. 12. Effect of Italian hybrid bee venom (µg /ml) on the growth rate of *Bacillus subtilis*

the low venom concentration (10 and 20 µg/ml) indicated an insufficient lowering in the bacterial growth rate. However, the control shows an increase of growth rate by increasing time as shown in Figs. 1 and 2.

Figs. 3 and 4 show that bee venom of Carniolian and Italian hybrid at different concentrations recorded a high reduction rate at high concentrations (>40 µg/ml). Most of bacterial cell's counts were lowered to undetectable limit after 30 min of treatment.

The results showed in Fig. 5 and 6 are quite similar for bee venom collected from the two bee types. Both concentrations 10 and 20µg/ml showed less significant effect on *S. aureusin* comparing with high concentrations while the highest reduction rate was observed with the high venom concentrations (>40 µg/ml). The high concentration (>40 µg/ml) of all samples showed a significant ($P \leq 0.05$) decrease in all bacterial cell numbers. Most of bacterial numbers were 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 with most venom samples.

Results presented in Fig. 7 and 8 show also a similar pattern of reduction with *S. epidermidis*. The concentration of 40 µg/ml showed a significant inhibition effect on *S. epidermidisin* in comparison with the lower concentration (10 µg/ml) which showed a limited effect in reducing the bacterial count in comparing with other samples. Most of bacterial cell's numbers were declined to undetectable limit after 50 min of treatment with high concentrations of venom (>20 µg/ml).

It is clear that venom sample2 showed a less significant effect on *P. aeruginosa* numbers.

The results indicated that the higher concentrations of venom showed a better killing effect on bacterial cells.

In attempt to explain the mechanism of antimicrobial effects of bee venom for specific bacterium, there are several factors that may control such process. For example, because of the peptidoglycan layer, G+

bacteria such as *S. aureus* and *B. subtilis* have a cell wall that is substantially thinner than G^-bacteria. Therefore, the penetration of the venom may be difficult in such case. However, the results showed that both G^+ and G^- strains were suppressed by the high concentrations of the tested venom samples. G^+ strains were somewhat more sensitive than G^- strains in some conditions, and it's probable that this is due to structural differences in the outer membranes of G^+ and G^- strain bacteria, where G^- bacteria had an outside membrane rich in lipopolysaccharide molecules, which slows the diffusion 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)**. It is most likely that potency of bee venom against microorganisms is largely dependent on bee venom protein bands and its molecular weights **(Nour *et al.*, 2004)**. The antimicrobial activity of honeybee venom may be due to the presence of various peptides like melittin, apamin, adolapin, mast cell degranulating peptide, enzymes, biologically active amines and non-peptide, component **(Kwon *et al.*, 2002)**. An efficient action on *E. coli* had been observed previously for bee venom **(Samy *et al.*, 2007)**. Although, in earlier study **Hegazi *et al.* (2002)** indicated that bee products were lower efficient against *E. coli*. **Hegazi *et al.* (2015)** reported that the bee venom from both pure and hybrid bees had an antibacterial action towards all five bacterial strains, but the effect differed depending on the type. Bee venom was found to have antibacterial properties towards a variety of bacterial strains. The minimum inhibitory concentration of BV was evaluated. These findings suggest that BV suppresses pathogen growth and that BV may be a valuable supplemental antibacterial agent against pathogens, despite the fact that bee venom is obtained in a variety of ways. A previous study done by **Cujova *et al.* (2014)** shows that melittin is present in bee venom which has more potential against gram positive bacteria as compared to gram negative bacteria. Mellitin component of the bee venom shows more potent antimicrobial action against gram-positive bacteria compared to gram negative bacteria **Blaylock (2000)**. Different antimicrobial peptides (AMPs) derived from the venom of various bee species have been presented: melittin, mastoparan, melittin apamin, secapin and others **Al-Ani *et al.* (2018)**. These peptides are changeable in length and charge, allowing them to interact electrostatically with negatively charged bacterial membranes. **Ko *et al.* (2020)** Anti-inflammatory action is induced by bee venom therapy employing bee stings. **Lee *et al.* (2004).** The biological, toxicological, and pharmacological effects of bee venom constituents have been intensively researched. The venom peptides mastoparan and melittin display antimicrobial action against a great number of bacteria **Vila-Farres *et al.* (2015) and Choi *et al.* (2015).** Melittin is a representative bee venom peptide with 26 amino acids and is well-known for its antibacterial properties but high cytotoxicity in mammalian cells **Steiner *et al.* (2009). Park *et al.* (2013)** exhibited that honeybee venom suppressed the growth of seventeen Gram-positive and partially two Gram-negative out of 44 bacterial strains isolated from bovine mastitis in Korea. Honey Bee Venom's antimicrobial action can result from many peptides presences, such as adolapin, apamin, melittin, mast-cell-degranulating peptides, biologically active amines, enzymes, and non-peptide components **(Leandro *et al.*, 2015)**. **Cujová *et al.* (2014)** mentioned that honey Bee Venom contained melittin, which is more active towards GPB than GNB.

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الملخص العربي

النشاط المضاد للبكتريا في سم النحل المنتج من هجينين لنحل العسل

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لسم النحل تأثيرات متعددة، كتأثيره المضادة للبكتيريا والفيروسات والالتهابات وذلك في أنواع مختلفة من الخلايا، وسم النحل عبارة عن خليط معقد من الببتيدات النشطة والإنزيمات والأمينات. تهدف الدراسة لاختبار النشاط المضاد للبكتريا لسم نحل العسل الناتج من الهجين الكرنيولى والهجين الإيطالي ضد ستة أنواع من البكتريا الممرضة أربعة منها موجبة لجرام هي: *Staphylococcus aureus* و*Staphylococcus epidermidis* و*Pseudomonas aeruginosa* و*Bacillus subtilis*، ونوعين سالبين لجرام هما: *Salmonella enterica* و*Escherichia coli*. وقد أوضحت النتائج المتحصل عليها أن سم النحل المجموع من كلا من الهجين الإيطالي والهجين الكرنيولي أظهر نشاطا مضادا لكل أنواع البكتريا المستخدمة مقارنة بعينة الكنترول، وتم تحديد أقل تركيز مثبط (MIC)، وأظهرت التركيزات العالية من سم النحل تأثيراً معنوياً ($P\leq0.001$) مضاد للميكروبات مقارنة بالتركيزات المنخفضة ضد كلا من البكتريا الموجبة والسالبة لجرام. أظهر التركيز العالي (> 40 ميكروجرام/مل) لجميع العينات انخفاضًا معنويًا ($P\leq 0.05$) في جميع أعداد الخلايا البكتيرية. أظهر التركيز المنخفض (10 ميكروجرام/مل) تأثيراً محدوداً في تقليل العد البكتيري مقارنة بالعينات الأخرى. وفى ضوء هذه النتائج فإن استخدام سم النحل والذي يتميز بأنه منتج طبيعي وآمن نسبيا فى مجالات حفظ الأغذية والأدوية وهذا يعد أمرا واعدا ولكن يجب إجراء المزيد من الدراسات والابحاث التي تهتم بالتركيب الدقيق لهذا المنتج ومواصفاته القياسية.

الكلمات الاسترشادية: نحل العسل، سم النحل، النشاط المضاد للبكتريا، مضاد للفيروسات، مضاد للالتهابات.

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