https://dx.doi.org/10.4314/ejppri.v8i3.5

Egypt. J. Plant Prot. Res. Inst. (2025), 8 (3): 249-262

وزارة الزراعة واستصلاح الأراضي
Ministry of Agriculture and Land Reclamation
ARC
مركز البحوث الزراعية
Agricultural Research Center

Egyptian Journal of Plant Protection Research Institute

www.ejppri.eg.net

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The impact of honeybee venom on the bacteria *Klebsiella oxytoca* and *Bacillus pumilus*: An in vitro study

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ARTICLE INFO

Article History

Received:23/7 /2025

Accepted:18/9 /2025

Keywords

Bee venom, *Apis mellifera carnica*, antibacterial activity, *Klebsiella oxytoca* and *Bacillus pumilus*.

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Abstract

Under laboratory conditions, the antibacterial activity of honeybee venom collected from a local Carniolan hybrid honeybee (*Apis mellifera carnica*) against two pathogenic Gram-positive bacterial strains, *Klebsiella oxytoca* and *Bacillus pumilus*, in honeybee colonies, *Apis mellifera* L. (Hymenoptera: Apidae) was compared to tylosin and oxytetracycline antibiotics. Bee venom has a strong antibacterial effect on *Klebsiella oxytoca*, with a minimum inhibitory concentration (MIC) of 1.17±0.098 mg/mL. Tylosin had MIC values of 4.03±0.017 mg/ml. According to mean values, the oxytetracycline antibiotic outperformed all other studied materials in MIC and MBC against *Klebsiella oxytoca and Bacillus pumilus*. There was a significant difference between MIC and MBC when utilizing bee venom on *Klebsiella oxytoca* bacteria ($P \leqslant 0.047$). For *Bacillus pumilus* bacteria, the study showed that oxytetracycline outperformed all used substances in MIC and MBC by an average of 0.59±0.023 mg/mL and 0.79±0.046 mg/mL, followed by bee venom with an average of 0.87±0.040 mg/mL and 0.92±0.011 mg/mL, and tylosin was the least by an average of 3.7±0.145 mg/mL and 3.5±0.115 mg/mL, respectively.

Introduction

Honeybee venom (HBV), specifically from the species *Apis mellifera* L. (Hymenoptera: Apidae) , has several enzymes and peptides that are highly useful in combating a wide range of ailments. The potential therapeutic applications of bee venom extend to the treatment of numerous in vivo or in vitro problems. Multiple research and publications have indicated that bee venom and its components can eliminate bacteria, protozoa, cancer cells, inflammation, and arthritis (Urtubey, 2005; Im *et al*., 2016 and Khalil *et al*., 2021). The bee's abdomen cavity has a gland that produces a substance called honeybee venom, often known as Apitoxin or Apitox (Bhalotia *et al*., 2016; Szabat *et al*., 2019; and Kolayli and Keskin, 2020). According to Ali (2012), dried venom has a light-yellow tint and consists of many volatile chemicals that readily evaporate throughout the collecting process. HBV consists of a mixture of several bioactive compounds, such as melittin (A primary constituent of bee venom), apamin, adolapin, mast cell degranulating peptide, and enzymes (Phospholipase A2 and hyaluronidase). In addition, it contains non-peptide components such as histamine, dopamine, and norepinephrine (Raghuraman and Chattopadhyay, 2007; Moreno and Giralt, 2015 and Wehbe *et al*., 2019). The primary components of the venom

are melittin, which makes up around 50 percent of the venom when it is dry, and phospholipase A2 (PLA2), which makes up roughly 12 percent (Gajski and Garaj-Vrhovac, 2013). Melittin, a primary component of honeybee venom, consists of 26 amino acids and represents about 40-50% of the venom's dry weight (Bogdanov, 2016; Oršolic, 2012 and Badawi, 2021). HBV can inhibit the growth of bacteria, viruses, and fungi. According to El-Seedi *et al.* (2020), the combination of HBV and antibiotics is more effective in treating infections. This means that a lower dosage of each is required, resulting in fewer side effects. Additionally, the bacteria are less likely to develop resistance to the treatments. Microbial infections present a formidable challenge, since medication resistance continues to emerge. Bee venom, which contains bioactive substances such as melittin, apamin, and PLA2, has the potential to function as antimicrobial agents against bacteria, viruses, and fungi (Leandro *et al*., 2015; Han *et al*., 2016; Shin *et al*., 2017; Socarras *et al*., 2017 and Tanuwidjaja *et al*., 2021).

The study aimed to determine the efficacy of honeybee venom in eradicating pathogenic Gram-positive bacteria such as *Klebsiella oxytoca* and *Bacillus pumilus*. This was accomplished by comparing the venom's minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) to antibiotics that are often utilized.

Materials and methods

The present work was conducted to investigate the effectiveness of bee venom against *K. oxytoca* and *B. pumilus*, the bacteria linked to the American foulbrood (AFB) disease. We conducted the study as a laboratory experiment.

1. Bees' venom harvest:

Using an electric shock device, honeybee venom was collected at the Plant Protection Research Institute, Agricultural Research Centre, Dokki, Giza, Egypt from F1 Carniolan hybrid honeybees (*A. mellifera carnica*) (Benton *et al*., 1963). Constantly electric current (18 volts/3 amber/hour) drives the venom collector device (VCD). Collecting bee venom was conducted three times, for thirty minutes, every fifteen days. At the entrance of the hive, we placed the device creating a landing board for returning bees. The technique produces pure, white, and dry venom by use of electrical impulses inducing bee workers to sting through latex sheets on glass plates, therefore eliminating contaminants and avoiding venom oxidation. The wires stung a glass sheet and experienced some electrical shock when they came into touch with the bees. We switch off the VCD and shake the bees off the frames after the collecting is finished. The venom deposited on the glass plate during drying was quickly removed with a sharp scraper; the dried venom was kept at 10 °C in a dark glass jar and kept in a dry, cool condition (Fakhim Zaden, 1998 and Turan and Erkan, 2023).

2. Assay of total protein:

Determination of total protein concentration was conducted using the analytical technique outlined by Warburg and Christian (1942) and Sedmak and Grossberg (1977).

3. Determination of bee venom LD_{50} (British **Pharmacopoeia.,** 2000**):**

Male albino Swiss mice weighing 16–18 grams were used in the study. We prepared the newly made venom solutions in normal saline in increasing concentrations, starting with a dose that kills roughly 0–20% of the animals and progressing to a dose that kills approximately 80–100% of the injected animals. We administered each dosage level to four mice. We administered all injections intravenously and documented the death and survival rates

of the injected animals 24 hrs. later. We calculated the adjusted lethality rate percentage for each dosage level based on the number of survivors and deaths recorded at that dose level. We reevaluated the data to rule out the possibility of accidental survival or deaths due to unusual resistance or susceptibility of the tested animals. We adjusted the number of survivors at each dose level to account for survivors at higher dose levels and adjusted the number of deaths to include deaths at lower dose levels. It is presumed that mice that survived at a certain dose would have survived at a lower dose, and vice versa; mice that died at a certain level would have been killed at any greater dose. We added the number of survivals at higher dosage levels and the number of fatalities at lower dose levels to the uncorrected number of survivals at that dose level. Bradford (1976) determined the accumulated corrected percentage of lethality at each dosage using the accumulated corrected fatalities and corrected survivals at that dose level. The LD50 indicates the deadly action of bee venom, which is affected by its main components, melittin and phospholipase A2. Schmidt (1995) found that mixing the two in the natural 3:1 ratio in bee venom resulted in a lethal activity similar to that of crude bee venom.

Proportionate distance = (% mortality next above - % mortality next below) / (% mortality next above + % mortality next below 50%).

4. Evaluation of antibacterial activity:

We conducted experiments to assess the antibacterial properties of the bee venom we got. Two solutions with concentrations of 100 and 200 mg/mL were created by dissolving bee venom in sterile distilled water. The antibacterial activity of the produced concentrations was assessed using the disc diffusion method on nutritional agar. The pathogenic bacteria were introduced into tryptic soya broth tubes and placed in an incubator at 37ºC for 4 hrs. The turbidity of these cultures was standardized by using a 0.5 McFarland standard. A homogeneous bacterial layer was created on the surface of nutrient agar plates using sterile cotton swabs. The nutrient agar plates were allowed to dry for 30 minutes, after which the discs were saturated with the tested concentration and placed on the surface of the dried plates. The positive control utilized in our experiment was ceftriaxone sodium salt at a concentration of 1.0 mg/mL. The plates were placed in an incubator set at a temperature of 37˚C for 24 hrs. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition (In millimeters).

5. Isolation of the bacteria:

Brood larvae infected with American foulbrood disease were gathered, crushed in 0.5% peptone, and shaken for 24 hrs. to observe any bacterial development. The resulting mixture was then split into two sections, one of which was heated to 100°C for 15 min. and centrifuged at 6,000 g for 45 minutes. To fortify the Brain Heart Infusion Agar medium, 0.1 g of thiamine hydrochloride (Sterilized using Millipore filter paper) was added per liter of sterilized medium , and the pH was adjusted to 6.6 using HCl. The pellet containing the bacterial spores was then streaked on the medium. Autoclaved for 20 minutes at 116°C and 10 pounds per square inch. Media (20 mL/plate) was added to 90-mm-diameter Petri plates, and the dishes were incubated for 72 hrs. at 5°C (Shimanuki and Knox, 1991). Several streaks were used to

purify the emerging colonies, and Gram staining and streaking without heat were also applied to the other portions.

6. Methods of Identification:

6.1. Gram stain:

Bacteria isolated from diseased honeybees were classified by genus and species using morphological, cultural, and biochemical methods, as described by Bradford (1976). Initial bacterial extraction was performed by dissolving the samples in sterile saline, planting them into nutrient plates (USA), and incubating them at 36°C for 24 hrs. at the Microbiology Laboratory, R and sector, VACSERA. Separated bacteria are further purified by taking isolation, growing colonies, and cultivating them on the same seed medium as the original isolation. The isolated bacteria were cultured, and their motility was assessed using a semisolid agar medium. The recovered bacteria were further subcultured on slope agar and utilized as a stock culture for further inoculation in semisolid agar for identification. Identification of the recovered isolate: To examine morphological traits and stain reactivity, dried heat-fixed smears were prepared and stained with Gram's stain, as well as an oil immersion lens microscope. Cultural features include the growing colony's morphological traits (Shape, form, color, texture, pigmentation, and hemolysis). Biochemical tests such as Indole, Oxidase, Urease, and Sugar Fermentation were conducted for the identification of the isolates.

6.2. The Bacterial Identification Biolog® System:

Samples for the Biolog® identification procedure were prepared in accordance with the Biolog Inc. (California, USA) handbook (Gende *et al*., 2009). The data collected on the Biolog® microplates was interpreted using a Microlog TM 4.2 database. Biolog Inc. created the Biolog® system, which is a simpler phenotypic identification method (Gende *et al*., 2008). The basis for the Biolog® microbial identification technique is the way in which bacteria use carbohydrates (Gende *et al*., 2008 and Gende *et al*., 2011). The 96-well Biolog® microplates are available. The Biolog® microplates comprise 96 wells that contain water and 95 different media of explicit carbs and a redox pointer. The redox color, tetrazolium, changes into a purple tone if microbial development happens in a specific well addressing catabolism of the substrate. The variety change of color is considered a positive response. The well-containing water is utilized as a control for the tests. Various microorganisms utilize different carbon sources, relying upon their dietary prerequisites; hence, in view of the positive and negative response, a species-explicit mark can be produced 21. Figure (2) shows a picture of a Biolog® microplate demonstrating the positive outcomes by the variety change (Gende *et al*., 2008 and Gende *et al*., 2011).

7. Determination of minimum inhibitory concentration (MIC):

The MIC was determined using the tube dilution method described by Perrucci *et al.* (2004). A 24 hrs. culture of the bacteria under investigation was diluted in 10 mL of TSB to achieve an inoculum of 108 cfu/mL-1, following the 0.5 McFarland standards. Fifteen concentrations of bee venom (ranging

from 50 to 0.01 mg/mL-1 in sterile distilled water) were prepared in culture tubes. Each tube was then inoculated with 100 µl of the bacterial cell suspension and placed in an incubator at 37°C for 24 hrs. The growth of the bacteria in the broth was assessed by the turbidity of the liquid, and the lowest concentration of bee venom that prevented bacterial growth was recorded as the MIC. The microdilution experiment was performed on flat-bottomed 96-well microtiter plates (Costar, USA) with Brain Heart Infusion Thiamine (BHIT) as the medium, as reported by Brackmann *et al*. (2009). Plates were inoculated with 100 µl of bacteria isolated from infected honeybee larvae in BHIT medium, together with bee venom in successive dilutions. Following 24 hrs. of incubation at 37°C, the absorbance at 600 nm was measured with a kinetic microplate reader. Furthermore, the MIC values for bee venom were established by serial dilution against bacteria obtained from diseased honeybee larvae, following the previously reported approach. The MIC was defined as the lowest dose of antibiotics or pure chemicals that either did not cause bacterial spore germination or inhibited 90% or more of spore germination.

8. Determination of the minimum bactericidal concentration (MBC):

Jobran and Finegold (1994) used serial fold dilutions to measure the minimum bactericidal concentration (MBC) of the bee venom (0.625, 1.250, 2.500, 5.000, and 10.000 µg/mL). Each well was injected with 100 µl (0.5 McFarland) of a standardized suspension of the tested bacterial species, comprising about 1.5x108 cells/mL. The isolated microorganism was then incubated at 37°C for 24, 48, and 72 hrs. Following 24 hrs. of incubation, 0.1 mL of each well was subcultured on Tryptic Soy Broth **(**TSB) agar plates and incubated for an additional 24 hours at 37°C. The lowest dilution of the test resulted in a viable count of less than 0.1% of the initial inoculum (1.5x108 cells/mL) established as the minimal bactericidal concentration (Attalla *et al*., 2007).

9. Statistical analysis:

The data collected for each experiment was calculated for the means and standard deviations using Microsoft Excel, and statistical significance was determined by a t-test and a one-way ANOVA. Differences in survival were considered significant at $P<0.05$ (Steel and Torrie, 1980).

Results and discussion

This study comprised the effect of the bee venom against the microbial causative agents associated with American foulbrood disease (AFB), *K. oxytoca*, and *B. pumilus* under laboratory conditions.

1. Identification and characterization of (A) *Klebsiella oxytoca* and (B) *Bacillus pumilus:*

The microscopic examination and the biological identification of the isolated and stained Gram-positive bacteria are shown in Figure (1).

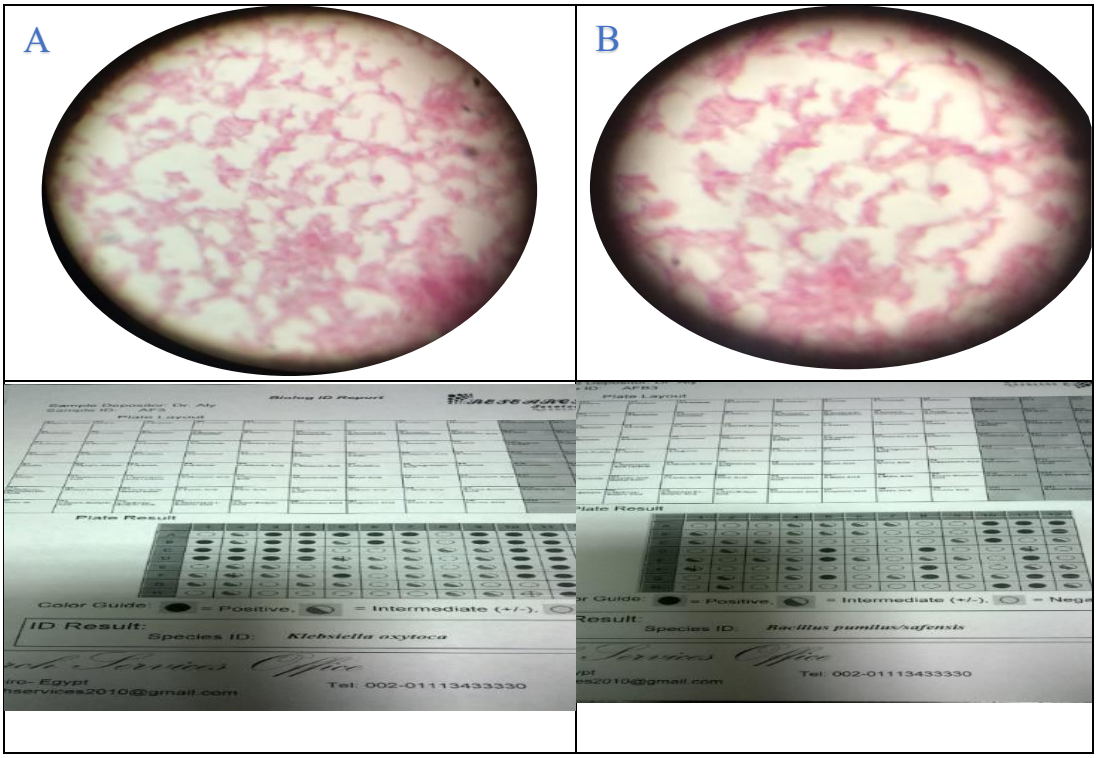
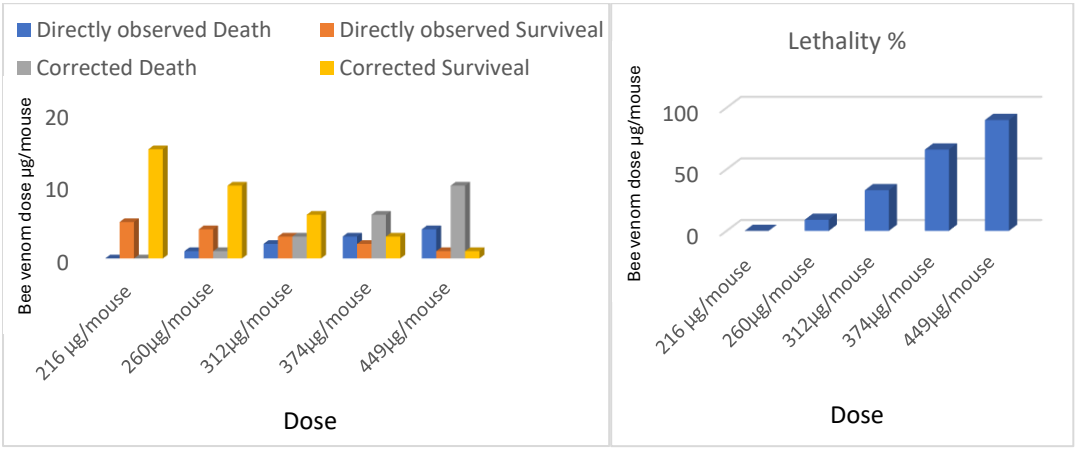


Figure (1): Identification and characterization of (A) *Klebsiella oxytoca* and (B) *Bacillus pumilus*,

2. Determination of bee venom lethality (LD_{50} test):

The lethality percentages (Figure 2) are increasingly dependent on the bee venom dose. As the dose increases, so does the lethality percentage. The result declared that dose 216 µg/mouse was superior to the mice survival and inferior to their death. On the contrary, dose 449 µg/mouse was inferior to the mice's survival and superior to the mice's death.



Ps: Dose increasing factor = 1.2

Figure (2): Carniolan honeybee venom lethality percentages (LD_{50}%) potency.

3. Determination of bee venom total protein content:

The total protein concentration was 1.280 mg/mL on average, as seen in Table (1). In addition, the total protein content of bee venom differs according to the geographical origin of bee venom, honeybee race, and pollen consumption **(**Warburg and Christian, 1942 and Kim, 1997**).** Tanuwidjaja *et al*. (2021) pointed out that melittin and the total protein contents of honeybee venom HBV were 70.10±7.01 and 84.44±3.12 g/100 g, respectively. The total protein content ranged from 47.2% to 77.8% (Costa *et al*., 1999 and

Abrantes *et al*., 2017). Spring and summer are the best seasons for collecting bee venom. In addition, a hybrid race is better than a pure race to collect bee venom. Moreover, good flowering conditions and the availability of food sources (nectar and pollen) are suitable for collecting bee venom **(**Mohany, 2005; El-Shaarawy *et al*., 2007 and Hegazi *et al*., 2014**).**

Table (1): Total bee venom protein content (mg/mL).

Type	Protein content
Hybrid Carniolan race	1.280 mg/mL

4. Determination of bee venom's minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) against the isolated bacteria:

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. It can be determined from broth dilution. Table (2) shows that oxytetracycline had the lowest Minimum Inhibitory Concentration (MIC) on *K. oxytoca* bacteria, at 0.61±0.011 mg/mL. Tylosin came in second, with an average of 4.03±0.017 mg/mL, followed by bee venom at 1.17±0.098 mg/mL. For MBC, tylosin showed the strongest effect, followed by bee venom, and then the lowest was for oxytetracycline, averaging 4.20±0.115, 1.6±0.115, and 0.82±0.017 mg/mL, respectively. There was a significant difference between the MIC and MBC for Oxytetracycline on *K. oxytoca* bacteria (***P***⩽0.0006). A significant difference was observed between MIC and MBC when using bee venom on *K. oxytoca* bacteria (***P***⩽0.047). A non-significant difference was observed between MIC and MBC when using tylosin in accordance with the control on *K. oxytoca* bacteria (***P***⩽0.2191 and 0.2419). Babu *et al*. (1995) declared that the bacteria *K. oxytoca* could also biotransform propionitrile to propionic acid and ammonia. Kao *et al*. (2006) pointed out that the *K. oxytoca* bacterial cells can degrade most nitrile compounds, as *K. oxytoca* can break down acetonitrile and propionitrile. Dias *et al*. (2000) and Kao *et al*. (2006) mentioned that because *K. oxytoca* is able to produce amide and carboxylic acid via the process of nitriles biotransformation, this bacterium can be applied in the industry of amides and organic acid production. Hegazi *et al*. (2014) declared that the lowest MIC (1000 µg/mL) for *Klebsiella pneumoniae* was shown by bee venom from VACSERA. Hegazi *et al.* (2015) noticed that the gram-negative seemed to be the least sensitive bacteria, whereas gram-positive were more affected by tested venoms. Hoenigl *et al*. (2012) stated that *K. oxytoca*, which is produced by *Klebsiella pneumoniae carbapenemase* KPC, was the cause of infection in three patients. One patient developed a urinary tract infection, while the other two patients experienced VAP. We started systemic anti-infective treatment for *K. oxytoca* infections in two of the patients. The third patient with VAP passed away before the commencement of an appropriate anti-infective treatment. *Klebsiella pneumoniae carbapenemase (KPC),* a class A beta-lactamase, presents a significant clinical challenge due to the rapid global dissemination of KPC-producing *Klebsiella pneumoniae* isolates (Bonomo, 2011 and Cuzon*, et al*., 2010).

Table (2): Minimum bactericidal concentration (MBC) and minimum inhibition concentration MIC of bee venom against the isolated bacteria *Klebsiella oxytoca.*

Antimicrobial Activity (*Klebsiella oxytoca*)		Control -	Control +	Oxytetracycline	Tylosin	Bee venom
Mean value	MIC	0.48a±0.017	1.3a±0.057	0.61b±0.011	4.03a±0.017	1.17b±0.098
mg/mL	MBC	0.54a±0.023	1.43a±0.075	0.82a±0.017	4.20a±0.115	1.6a±0.115
LSD 5%		0.080	0.262	0.058	0.324	0.420
F		4.32	1.884	93.025	2.119	8.050
P		0.1062 ns	0.2419 ns	0.0006***	0.2191 ns	0.047*

Means in each column followed by the same letter (s) indicate that there is no significant difference, and different letter(s) in the same column indicate that there is a significant difference at $P \leq 0.05$ according to Duncan's multiple range test.

5. The antimicrobial activity of the tested substances against the isolated bacteria *Bacillus pumilus*:

Table (3) illustrates the effect of tested substances on *B.pumilus* bacteria. Based on the study, tylosin was the best substance in both MIC and MBC, with an average of 3.7±0.145 mg/mL and 3.5±0.115 mg/mL, respectively. It was followed by bee venom, with an average of 0.87±0.040 mg/mL and 0.92±0.011 mg/mL, and oxytetracycline, with an average of 0.59±0.023 mg/mL and 0.79±0.046 mg/mL. There were significant differences between MIC and MBC for oxytetracycline ($P \leqslant 0.0202$). There were no significant differences between the minimum inhibitor concentration MIC and the minimum bactericidal concentration MBC for bee venom and tylosin. *B. pumilus* is a non-spore-forming, anaerobic, Gram-positive bacterium isolated from *Paenibacillus larvae*. It was discovered from the data in Table (3) that bacteria react positively to the catalase test. It could also break down some proteins in milk but not starch or change nitrate into nitrite. It also didn't need any special conditions to grow, as shown by the fact that it could grow in nutrient agar and be negative for casein, positive for Voges-Proskauer, and positive for gelatin liquefaction (Table 3). These results supported the behavioral traits that define the species *B. pumilus* (Alippi, 1999). According to Mash *et al.* (2013), the bacterium *B. pumilus* is sporulating, soil-dwelling, and gram-positive. Agriculture employs it as an antifungal agent in the root microenvironment and as a symbiont to stimulate plant growth (Joo *et al*., 2004 and Thomas, 2004). Researchers have also linked *B. pumilus* to food poisoning, particularly from milk and rice (From *et al*., 2007 and Nieminen *et al*., 2007).

Table (3): The antimicrobial activity of the tested substances against the isolated bacteria *Bacillus pumilus.*

Antimicrobial Activity (*Bacillus pumilus*)		Control -	Control +	Oxytetracycline	Tylosin	Bee venom
Mean value	MIC	0.57a±0.017	1.32a±0.057	0.59b±0.023	3.7a±0.145	0.87a±0.040
mg/mL	MBC	0.43b±0.023	1.31a±0.115	0.79a±0.046	3.5a±0.115	0.92a±0.011
LSD5%		0.0801	0.358	0.143	0.515	0.116
F		23.52	0.006	13.958	1.580	1.415
P		0.0032**	0.9420 ns	0.0202*	0.277 ns	0.3000 ns

Means in each column followed by the same letter (s) indicate that there is no significant difference, and different letter(s) in the same column indicate that there is a significant difference at $P \leq 0.05$ according to Duncan's multiple range test.

3.5. Variance analysis for the investigated substances on both kinds of bacteria:

Table (4) shows the antimicrobial activity of the tested substances against the isolated bacteria *K. oxytoca* and *B. pumilus*. We determined the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for *K. oxytoca* and *B. pumilus* bacteria. The oxytetracycline minimum inhibitory concentration (MIC) for *K. oxytoca* bacteria was found to be 0.61±0.011 mg/mL, and for *B. pumilus*, it was 0.59±0.023 mg/mL. The minimum bactericidal concentration (MBC) for *K. oxytoca* bacteria is 0.82±0.017 mg/mL, and for *B. pumilus* bacteria, it is 0.79±0.046 mg/mL. For tylosin, the minimum inhibitory concentration (MIC) on *K. oxytoca* bacteria was 4.03±0.017 mg/mL and 3.7±0.145 mg/mL on *B. pumilus* bacteria. The minimum bactericidal concentration (MBC) of tylosin was 4.2±0.115 mg/mL and 3.5±0.11 mg/mL on *K. oxytoca* and *B. pumilus* bacteria, respectively. The minimum inhibitory concentration (MIC) of bee venom on *K. oxytoca* bacteria was 1.17±0.098 mg/mL and 0.87±0.040 mg/mL on *B. pumilus* bacteria, respectively. In addition, the minimum bactericidal concentration (MBC) of bee venom on *K. oxytoca* was 1.6±0.115 mg/mL and 0.92±0.011 mg/mL on *B. pumilus*, respectively. Researchers have linked the antimicrobial activity of bee venom to two of its main constituents, melittin and PLA2. These substances, by creating pores in bacteria's membranes, may cause harm to the microorganisms and ultimately cause lysis (Wehbe *et al*., 2019; Carpena *et al*., 2020 and Leandro *et al*., 2015). Pucca *et al.* (2020) suggest that the toxins found in bee venom may have a synergistic effect on toxicity by forming toxin complexes through hetero-oligomerization. The increased bacterial lysis may be caused by melittin-PLA2 hetero-oligomer formation on phospholipid bilayer membranes. Melittin is more likely to attach to the lipid-binding surface of PLA2 molecules through the neutral outer membrane. This can make the lysis process stronger when combined with the molecule. Electrostatic attraction between the phosphate group of phosphatidylcholine, a significant component of cell membranes, and the basic amino acid residues of melittin may facilitate this interaction. The binding of melittin to phospholipids enables the processes of melittin oligomerization, membrane hole formation, and lysis (Pucca *et al*., 2019 and Yang *et al*., 2001).

Table (4): Analysis of variance for the effect of the tested substances on both kinds of bacteria.

Antimicrobial Activity		Control -	Control +	Oxytetracycline	Tylosin	Bee venom
Mean value mg/mL	MIC{K}	0.48bc±0.017	1.3a±0.0577	0.61b±0.011	4.03ab±0.017	1.17b±0.098
	MIC{B}	0.57a±0.0173	1.32a±0.057	0.59b±0.023	3.7bc±0.145	0.87c±0.040
Mean value mg/mL	MBC{K}	0.54ab±0.023	1.43a±0.075	0.82a±0.017	4.2a±0.115	1.6a±0.115
	MBC{B}	0.43b±0.023	1.31a±0.115	0.79a±0.046	3.5c±0.115	0.92bc±0.011
LSD 5%		0.665	0.61	0.0911	0.357	0.56
F		9.36	0.57	17.075	8.047	17.994
P		0.0054**	0.649 ns	0.0008***	0.0085**	0.0006***

Means in each column followed by the same letter (s) indicate that there is no significant difference, and different letter(s) in the same column indicate that there is a significant difference at $P \leq 0.05$ according to Duncan's multiple range test. P.S.: {K} = *Klebsiella oxytoca*; {B} =*Bacillus pumilus*.

Both *K. oxytoca* and *B. pumilus* bacteria can be found in honeybee colonies. They are linked to *Paenibacillus* larvae, which causes American foulbrood disease. Honeybee venom inhibits the growth and survival of gram-positive bacterial strains, *K. oxytoca* and *B. pumilus*. Furthermore, the MIC and MBC of bee venom against various bacterial species lead to variations in its purity, melittin content values, collection and extraction methods, honeybee races, and the pollen feeds that honeybees consume. Standardizing the potential use of bee venom HBV as a complementary antimicrobial agent for pathogenic bacteria requires further studies.

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