ANTIBACTERIAL AND ANTIBIOFILM EFFECTS OF BEE VENOM FROM (APIS MELLIFERA) ON MULTIDRUG-RESISTANT BACTERIA (MDRB)

BY
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Abstract
The prevalence of (MDRB) is increasing worldwide; therefore, this study aimed to identify the most common MDRB in clinical specimens and meet the urgent need to develop new antibacterial drugs to control their intractable infection. Additionally, due to the confrontation of the infection associated with bacterial biofilms, which are difficult to treat, and cause problems to public health, which require real solutions. Bee Venom produced by the glands of (Apis mellifera) is a complex mixture of active peptides, enzymes, and amines. So, it is considered a fertile environment for research to achieve the goal of this study. The results of the specimen’s examination showed that, from a total of 500 clinical specimens, there are 224 specimens exhibited no growth, while 276 were positive. From 276 positive cultures, 317 isolates were obtained. Out of the 317 bacterial isolates, 169 (53.3%) were Gram-negative bacteria (GNB), and 148 (46.7%) were Gram-positive (GP). It was of this number 124 (39.1%) were multidrug-resistant (MDR) isolates of which 89 (71.77%) were Gram-negative type, including Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, and Pseudomonas aeruginosa and 35 (28.23%) were Gram-positive including Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, Vancomycin-resistant Staphylococcus aureus, Staphylococcus haemolyticus and Enterococcus faecalis. Antibacterial assays showed that Bee Venom possesses strong potential effect against MDR isolates including both GNB and GPB. with a wide range of MICs and MLCs concentration-spacing between 3.125 – 50 μg/mL and 6.25 – 100 μg/mL, respectively against all MDR-GNB and GPB. It was found that GPB was more sensitive at lower concentrations of Bee Venom than GNB. In addition, Bee Venom sub-MICs values against the most biofilm bacterial produces namely; E. faecalis, P. aeruginosa, S. aureus, VRSA, S. haemolyticus and E. faecalis exhibited sharp reduction in their biofilms ranged between (63.8- 92%) especially at ½ MICs according to each bacterium, exclude E. faecalis biofilm was moderately affected (39%). While, at another tested sub-MICs showed moderate, weak, and no antibiofilm effects.

Keywords: Multi-drug resistant, MDR, Bacteria, GNB, GPB, MRSA, VRSA, Antibacterial, MIC, MLC, Biofilm, Bee Venom.
Introduction

Antibiotic resistance was considered an essential threat to human health worldwide in clinical practice (Blair et al., 2015). Most bacteria that triggered severe diseases and were once effectively treated with several distinct antibiotic groups have now become resistant often to many antibiotics (CDC, 2013; Laxminarayan et al., 2013). A limited pipeline of new antibiotics production has complicated the problem of antimicrobial resistance, resulting in higher morbidity and mortality rates and higher health care costs (Walker and Fowler, 2011). For instance, 25,000 people in Europe each year dies as a result of MDRB bacterial infections that cost the economy of the European Union € 1.5 billion annually (Walker and Fowler, 2011). Also, over two million people in the U.S. are infected annually with multi-drug resistance bacteria, resulting in 23,000 deaths directly (Hampton, 2013; WHO, 2014). The situation is much worse in developing countries, including Egypt, where no precise estimates are available as most developing countries face a critical shortage of disease detectives and the necessary infrastructure for Health Information System and Surveillance (WHO, 2014). The most crucial virulence factor plays a considerable role in the antibiotic-resistance is the biofilm formation, that described as a sessile microbial community in which cells are connected and integrated into a protective, extracellular polymeric matrix with a surface or other cell (Archer et al., 2014; Kiedrowski and Horswill, 2011; Lister and Horswill, 2014). This nature of multiplication has changed the physiology of gene expression and protein manufacturing (Archer et al., 2014; Kiedrowski and Horswill, 2011). Biofilm growth represents an essential role throughout infection by offering a defense against multiple clearance mechanisms (Lister and Horswill, 2014). The biofilm matrix can hinder certain immunological defenses, like macrophages that show unfinished entry into the biofilm matrix and "frustrated phagocytosis" (Scherr et al., 2014). Also, biofilm cells demonstrate enhanced antibiotic tolerance (de la Fuente-Núñez et al., 2013). Also, biofilms perform a key position in chronic disease progression (Lister and Horswill, 2014). After a biofilm has been established, separate cells can spread from the initial biofilm and either seed fresh sight of infection or arbitrate an acute infection like sepsis (Costerton et al., 1999). MDRB have been put forward several approaches to solve, but the development of new natural antimicrobial agents is the most significant. Therefore, intensive research has focused on developing new approaches to prevent and treat MDR-infection (Blair et al., 2015). Bee venom (apitoxin), is a colorless liquid secreted by the glands of bees (Hegazi et al., 2015). The bee venom has a complicated combination of enzymes, active peptides, and amines (Hider, 1988). Bee venom has, since ancient times been used in primitive therapy for healing diseases because of its biological activity (Son et al., 2007). Therefore, the goal of the current research was to identify the most common MDR-isolates in some clinical specimens and evaluate the antibacterial and antibiofilm activity of Bee Venom from Apis mellifera' on isolated MDR-GNB and GPB.
Specimens and Methods

Venom collection

Bee venom collector device: Bee venom collecting electric shock device CJ 401 (Chung-Jin Biotech Ltd., Ansan, Korea) consists of digital control board, five bee venom collection frames, wire electrodes, and battery. Input / Output Voltage: 12 VDC Collector Frames: 46 cm x 28 cm. Honey bee was subjected to bee venom collecting electric shock device, at the Plant Protection, Department, Faculty, of Agriculture, Al-Azhar University. The device consists of a five bee venom collection frames with wire electrodes installed in parallel to each other. Each frame was placed on the top of the combs in every hive and then was connected to an electro-stimulator. Electrical impulses stimulate the bee workers to sting through latex sheet placed on a glass plate of the device frame. Bees that come into contact with the wires received a mild electrical shock and stung onto the glass sheet. The processing of dry bee venom scraping was implemented by sharp scraper under laboratory conditions, after that dry bee venom was weight, recorded and packed up in the dark glass jars and stored in a cool and dry place.

Specimens collection

In this study, starting from April 2017 to November 2017, a total of 500 clinical specimens were obtained. Specimens types were an abscess, pus, sputum, and urine, from medical analysis laboratories of; Desuoq general hospital Kafr Al sheikh governorate. The specimens were immediately transported in sterilized box, swabs, or tubes to the laboratory for bacteriological analysis (Miller, 2005).

Bacterial isolation and maintenance media

Bacteria were isolated from clinical specimens by agar streaking method onto surface plates of nutrient agar and blood agar media, and then the Petri dishes were placed in the incubator for 24h, at 37°C aerobically. After growth, bacterial colonies were subjected to purification processes and maintained on slants for identification and further use (Barrow and Feltham, 1993).

Antibiotic Susceptibility Testing and Isolates Identification:

The antibiotic susceptibility patterns of Ampicillin (10 mcg), Flucloxacillin (5 mcg), vancomycin (30 μg), (5 mcg), Clindamycin (2 mcg), Levofloxacin (5 mcg), Erythromycin (15 mcg), Kanamycin (30 mcg), Tobramycin (10 mcg), Ofloxacin (5 mcg), Rifamycin (30 mcg), Aztreonam (1 mcg), Gentamicin (10 mcg), Norfloxacin (10 mcg), Ceftriaxone (5 mcg), Cephradine (30 mcg), Tetracycline (30 mcg), Ciprofloxacin (5 mcg) and Oxacillin (1 mcg) were performed using the antibiotic disk diffusion method (CLSI, 2009). Incubation at 37°C for 24 h. The zones of bacterial growth inhibition according to the antibiotic pattern were classified according to bacteria was sensitive, intermediate sensitive, or resistant per antibiotic. All MDR-clinical isolates were primary identified using Morphological characteristics of bacterial colonies according to Bergey's manual, (2009); Collins and Lyne (2004); Cheesbrough (2006). Identification of all isolates was confirmed by secondary identification (Automated) using the Biomerieux Vitek 2 System according to Funke and Funke-Kissling (2004); Funke and Funke-Kissling (2005).
Screening of antibacterial activity of Bee Venom

Bacterial isolates

The most encountered MDRB were, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* (GNB), *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, Vancomycin-resistant *Staphylococcus aureus*, *Staphylococcus haemolyticus* and *Enterococcus faecalis* (GPB).

Inoculum preparation

The bacterial isolates were cultured in Mueller-Hinton broth (MHB) media to a mid-logarithmic phase. The bacterial isolates were then suspended and adjusted by comparison against 0.5 Mc-Farland turbidity standard (1.5 × 10⁸ cfu/ml) tubes. The resulting suspension was further diluted to a final of 5X10⁶ cfu/ml (CLSI, 2009).

Disc diffusion method

This method has been done according to NARMS (2002); Surendra et al. (2011). A sterile cotton swab was used for spreading diluted culture samples at a concentration (5X10⁶ cfu/ml) on (MH) agar plates. The impregnated discs (7-mm) by Bee Venom at a concentration (200 μg/ml); were then placed on the MH agar’s surface. The plates were investigated after the incubation period between 24 to 28hrs, at 37°C and the inhibition zones were determined. The means ±SE of results each experiment was calculated using Microsoft Excel.

Broth microdilution method

Diluted culture inoculum at a concentration (5X10⁶ cfu/ml) prepared from a fresh subculture of tested bacteria in Broth media. Bacterial suspension in MHB then loaded in the wells of polystyrene plate exclude three wells contains only MHB media as (background control). Bee Venom sample at a concentration (200 μg/ml) was used in each well except three wells containing bacterial suspension without Bee Venom as (Growth control). After an incubation period of 24h at 37 °C, the O.D.620nm was measured using absorbance microtiter plate reader at the Bot. and Micro., Dep., Fac., of sci., Al-Azhar University (Sunrise™-TECAN, Switzerland). The results were recorded as means ±SE of the triplicate experiment (NCCLS/CLSI, 2007).

Minimum inhibitory concentrations (MICs) and minimal lethal concentration (MLCs)

The MIC was determined by the broth micro dilution method using 96-well microplates (Saini et al., 2005; NCCLS/CLSI, 2007). The bacterial inoculum concentration of (5x10⁶ CFU/ml) was obtained in each well. Bee Venom sample (1.0 mg) was dissolved in DMSO (1 mL) to obtain 1000 μg/mL stock solution to obtain ten dilutions, were 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781 and 0.390 μg/mL and applied against the microscopic MDR-organisms in MHB media. Three wells containing bacterial suspension without Bee Venom used as (Growth control) and the (background control) are three wells containing media without bacterial inoculum. The O.D.620nm was measured using absorbance microtiter plate reader at the Bot. and Micro., Dep., Fac., of Sci., Al-Azhar University (Sunrise™-TECAN, Switzerland). The lowest concentration showing no growth
was taken as the (MIC). After 24 h incubation, 0.1 ml from each well was sub-cultured in (MHA) plates and overnight incubated at 37 °C. The lowest concentration of VB, which gave a viable count of less than 0.1% of the original inoculums (5x10^6 CFU/ml) was assumed as the minimal lethal concentration (MLC).

**Biofilm formation assay**

All MDR-strains were tested to determine its ability to form the biofilm (quantitively) using tissue culture plate method (TCP) as described by Bekir et al. (2011). MDR-isolates were cultivated overnight in 96-well polystyrene tissue culture microtiter plates at 37°C, with trypticase soy broth supplemented with 0.25% glucose as the growth medium. After incubation, the culture medium was removed and attached bacteria fixed by 95% ethanol, and stained with 1% crystal violet. Optical density (570 nm) was measured. Isolates exhibit O.D.570 nm > 0.1 considered as positive for biofilm production. Biofilm production was interpreted as strong, moderate, or low, according to Stepanović et al. (2007). The experiment was performed in triplicate.

**Antibiofilm activity of Bee Venom**

Antibiofilm activities of Bee Venom was determined at five concentrations sub-MICs against most biofilm producing strains were, *P. aeruginosa* (50 μg/mL), *E. cloacae* (25 μg/mL), *S. aureus* (6.25 μg/mL), Methicillin-resistant *S. aureus* (12.25 μg/mL), Vancomycin-resistant *S. aureus* (12.25 μg/mL), *S. haemolyticus* (6.25 μg/mL), and *E. faecalis* (3.125 μg/mL). Isolates were incubated with Bee Venom in microtiter plates, and the procedure was performed as previously described according to Bekir et al. (2011). For each isolate, three replicates values were considered.

**Results**

**Bacterial isolates and antibiotic resistance patterns**

Results of examined all clinical specimens, showed that, there are 224 specimens exhibited no growth, while 276 were positive. A total of 317 isolates were obtained from 276 positive specimens. Two hundred and ninety-one of these isolates (91.8%) obtained from single infection specimens, while 26 bacterial isolates (8.2%) obtained from mixed infection specimens. Out of the 317 bacterial isolates, 169 (53.3%) were Gram-negative, and 148 (46.7%) were Gram-positive. It was found that, out of 317 bacterial isolates; 124 (39.1%) were MDR-isolates of which 89 (71.77%) were Gram-negative type, including *Escherichia coli* (39/89, 43.82%), *Klebsiella pneumoniae* (27/89, 30.33%), *Enterobacter cloacae* (14/89, 15.73%) and *Pseudomonas aeruginosa* (9/89, 10.11%), and 35 (28.23%) were Gram-positive including *Staphylococcus aureus* (12/35, 34.3%), Methicillin-resistant *Staphylococcus aureus* (8/35, 22.86%), Vancomycin-resistant *Staphylococcus aureus* (6/35, 17.14%), *Staphylococcus haemolyticus* (6/35, 17.14%) and *Enterococcus faecalis* (3/35, 8.57%), figure (1).
Figure 1. Percentages of MDR-GNB and GPB isolates from clinical specimens.

Antibacterial activity of Bee Venom

The antibacterial activity of Bee Venom was evaluated by disk diffusion and broth microdilution methods in order to confirm the results obtained. It was found that the results of both methods show the antibacterial strength of bee venom at 200μg/mL. The results included in the table (1) and illustrated in figure (2) demonstrated 100% growth inhibition percentage against all tested MDR-isolates. In particularly, S. aureus (figure 3), E. faecalis and S. haemolyticus showed the highest inhibition zones diameter, were 37±0.75, 36.5±0.75 mm and 35±0.69 mm, respectively (table 1 & figure 1). While, Methicillin-resistant and vancomycin-resistant types of S. aureus exhibited the lowest diameter of inhibition zones than other (GNB) were 25.4±0.98 and 28±0.95 mm, respectively (table 1 & figure 1). Bee Venom had a marked increased inhibition zones diameter against MDR-isolates of E. coli and E. cloacae with 34±1.45 and 31±1.2 mm, respectively. While, P. aeruginosa and K. pneumoniae inhibition zones diameter were less in size than the previous two organisms with 21.6±0.75 and 25±0.85 mm, respectively (table 1 & figure 2).

Table 1: Antibacterial activity (disc-diffusion and broth microdilution methods), MICs and MLCs of Bee Venom for tested MDR-isolates

<table>
<thead>
<tr>
<th>MDR-strains</th>
<th>Inhibition zone (mm)</th>
<th>Mean growth inhibition (%)</th>
<th>MICs (μg/mL)</th>
<th>MLCs (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>34±1.45</td>
<td>100±0.20</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>25±0.85</td>
<td>100±0.11</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>31±1.2</td>
<td>100±0.24</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>21.6±0.75</td>
<td>100±0.12</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>S. aureus</td>
<td>37±0.75</td>
<td>100±0.32</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>MRSA</td>
<td>25.4±0.98</td>
<td>100±0.34</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>VRSA</td>
<td>28±0.95</td>
<td>100±0.33</td>
<td>12.5</td>
<td>50</td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>35±0.69</td>
<td>100±0.14</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>36.5±0.75</td>
<td>100±0.40</td>
<td>3.125</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Abbreviations: MIC, minimum inhibitory concentration; MLC, minimum lethal concentration; MDR, multidrug resistant. Data expressed as MEAN ± SE of 200 (μg/mL) of Bee Venom. The determination was performed in triplicates.
Figure 2: Histogram represents the antibacterial activity of Bee Venom on MDR-isolates by inhibition zone diameter (mm), (gray column) and mean growth inhibition (%), (black column).

Figure 3. Inhibition zone (mm) produced by Bee Venom at 200 μg/mL against MDR-
S. aureus.

Minimum inhibitory concentrations (MICs) and minimal lethal concentration (MLCs)

From the obtained results, Bee Venom showed strong antibacterial activity with a wide range of MICs and MLCs concentration-spacing between 3.125 – 50 μg/mL and 6.25 – 100 μg/mL, respectively against all MDR-GNB and GPB one. It was found that GPB was
more sensitive at lower concentrations of Bee Venom than GNB since *E. faecalis* was the highest sensitive organism with 3.125 μg/mL, and the MLC value was at 6.25 μg/mL. Moreover, then in the sensitivity comes both *S. aureus* and *S. haemolyticus* seemed to be the most sensitive after the previous bacterium 6.25 and 12.5 μg/mL for MICs and MLCs, respectively for both MDR-isolates. However, compared with the previous GPB MRSA and VRSA isolates needed higher concentrations of Bee Venom 12.5 μg/mL (MICs) and 50 μg/mL (MLCs) to inhibit their growth or even death altogether, table (1) and figure (4). In contrast, GNB were less influential than the previous organisms except for *E. coli* a stronger effect had been reported, where it required less concentration (12.5 μg/mL) and was similar to prevent growth (MIC) as well as, murder (MLC) compared to other GNB. It was found that the highest MICs and MLCs values were against both *P. aeruginosa* and *K. pneumoniae*, where MICs were at 50 μg/mL and MLCs at 100 μg/mL for each bacterium. The MIC and MLC values for the last remaining MDR-isolate, namely, *E. cloacae* were recorded at 25 and 50 μg/mL, respectively table (1) and figure (4).

![Figure (4): MICs and MLCs in μg/mL of Bee Venom against tested MDR-bacterial isolates.](image)

**Biofilm formation by MDR-bacterial isolates**

Biofilm production of MDR strains makes the treatment using conventional antibiotics more difficult. The ability of tested MDR-GNB and GPB isolates in the current study was investigated by crystal violet staining of culture in 96 polystyrene well plates. Among the 9 tested isolates biofilm formation was strong in 6 (66.7%), namely; *E. cloacae, P. aeruginosa, S. aureus, VRSA, S. haemolyticus* and *E. faecalis* with optical densities (O.D.570nm) were, 1.3, 1.1, 1.43, 1.12, 1.34 and 1.5, respectively. Only, *E. coli* demonstrated weak biofilm pattern 0.211 O.D.570nm, while *K. pneumoniae*, and MRSA showed a moderate biofilm production were 0.41 and 0.64 (O.D.570nm) respectively, figures (5&6).
Figure 5. Microtiter ELISA plate showing biofilm formation types.

Figure 6. Biofilm formation of tested MDR-isolates O.D. = Optical density.

Anti-biofilm formation effect of Bee Venom against MDR-GNB and GPB isolates

The anti-biofilm activity of VB sub-MICs was assessed against the most MDR-biofilm producers. From the obtained results (figure 7) it was found that planktonic growth of *E. cloacae* was not affected by sub-MIC concentrations of Bee Venom, while biofilm formation was strongly inhibited by 84%, 68% at ½ MIC and ¼ MIC respectively. Moderate attenuation 39% was showed at 1/8MIC, figure (7).
**Figure 7. Effect of sub-MIC of Bee Venom on biofilm formation of MDR-E. cloacae**

Biofilm formation of *P. aeruginosa* considered a key factor for organism survival and resistance. Bee Venom sub-MICs was nearly totally inhibited *P. aeruginosa* biofilm by 92% at ½ MIC. Other sub-MICs showed moderate to strong antibiofilm inhibition effect ranged between 28% to 77% with increasing sub-MIC concentration, figure (8).

**Figure 8. Effect of sub-MIC of Bee Venom on biofilm formation of MDR-P. aeruginosa.**

*S. aureus* considered as the gold standard Gram-positive biofilm producing bacteria, sub-MIC concentration Bee Venom had a potential relatively high effect of eradicating the biofilm by 63.8% at ½ MIC. While 37.8% and 18% biofilm eradication were achieved at ¼ and ⅛ of MIC. However, the *S. aureus* biofilm was not affected under low concentrations, figure (9).
Figure 9. Effect of sub-MIC of Bee Venom on biofilm formation of MDR-S. aureus.

The biofilm producing MDR-VRSA isolate with different Bee Venom sub-MICs were screened for its ability to eradicate the formed biofilm to the wells of microtitration plate. Sub-inhibition doses of Bee Venom were relatively steep (78%) in biofilm depletion of this bacterium while the cell growth was not affected. Also, a 55% reduction in biofilm was obtained at ¼ of MIC, figure (10).

Figure 10. Effect of sub-MIC of Bee Venom on biofilm formation of MDR-VRSA.

As well as, the obtained results showed that, Bee Venom sub-inhibitory concentrations was able to decrease the biofilm production of *S. haemolyticus* by 68% and 35% at ½ and ¼ MICs respectively. While, low concentration showed low to no effect, figure (11).
It was found that *E. faecalis* biofilm was less affected at ½ MIC only 39% reduction, compared to all MDR-GNB and GPB biofilms examined in this study at half sub-inhibitory concentration. As well as, at ¼ MIC showed low attenuation effect 19% while, other concentrations were completely ineffective, and this may be attributed to the low MIC value of this bacterium figure (12).

**Discussion**

MDRB will continue to persist and spread around the world. They cause clinical failure in the treatment of infectious diseases by decreasing the efficacy of antibiotic therapy and tend to boost infection and government health problems’ seriousness, incidence and expenses (*Alekshun and Levy*, 2007; *O’Neil*, 2016). Today the continuous implementation of new or enhanced antibiotics into clinical and agricultural environments has produced resistance to almost all recognized antibiotics (*Barton*, 2014; *Clatworthy et al.*, 2007). It is, therefore, highly crucial for human health to proceed to produce new or improved antibiotics (*Thomsen et al.*, 2016). So, in this study, we aim to identify the MDRB in some clinical specimens against major antibiotics used in the treatment of
bacterial infection. Moreover, then evaluation of the antibacterial activity of bee venom as a natural antimicrobial to control these MDRB, also to evaluate its ability to eliminate the MDR-bacterial biofilm, which is a key factor in drug-resistance.

In the current study, A total of 317 isolates were obtained from 276 positive clinical specimens. Out of the 317 bacterial isolates, 169 (53.3%) were GN, and 148 (46.7%) were Gram-positive. It was found that, out of 317 bacterial isolates; 124 (39.1%) were MDR isolates of which 89 (71.77%) were GP type, including *E. coli*, *K. pneumoniae*, *E. cloacae*, and *P. aeruginosa* and 35 (28.23%) were Gram-positive including *S. aureus*, Methicillin-resistant *S. aureus*, Vancomycin-resistant *S. aureus*, *S. haemolyticus* and *E. faecalis*. In this study, 39.1% of isolates were resistant to antibiotics and therefore considered MDR isolates. This finding was in the same direction with the study conducted in Egypt by El-Mahallawy et al. (2015), who reported that MDR was identified in 69% of bacteria isolated from positive blood cultures. Increased resistance in bacteria may be due to the uncontrolled use of antibiotics and their overuse lead to the rapid and extensive spread of antimicrobial resistance (Llor and Bjerrum, 2014). Antimicrobial overuse is occurring in multiple sectors (human, animal, agriculture) (Aarestrup et al., 2008; O’Neil, 2016). Microorganisms under the pressures of antimicrobial choice improve survival through acquisition and expression of resistance genes and then share them with other bacteria and processes such as gene overexpression and silencing, variety in the phase (Collignon et al., 2018). Some important resistance genes, such as beta-lactamases, are millions of years old (Gaze et al., 2013; Perry and Wright, 2014). Soil and other environmental matrices are wealthy causes of very varied bacterial and genetic communities (Ruuksen et al., 2016). Thousands of tons of antimicrobials are manufactured every year and are brought into the environment (Singer et al., 2016; Van Boeckel et al., 2015). Treatment plant and pharmaceutical waste, especially if not handled properly, can discharge elevated levels of antimicrobial substances into the surface water (Aubertheau et al., 2017; Singer et al., 2016; So et al., 2015). Antimicrobial residues are components of animal manure, human sewage, and aquaculture alongside fecal bacteria and resistance genes (Ruuksen et al., 2016; Wang et al., 2018). Waste treatment and manure composting decrease levels of certain but not all antimicrobials and microorganisms that are brought to the soil after land use of person and animal biosolids (Rahube et al., 2016). Antibiotic residues can contribute to the development of antibiotic resistance in bacteria through selective pressure (Laxminarayan et al., 2013). Specifically, maybe even the exposure of a bacterium to a single molecule of an antibiotic can favor natural selection for resistance, or a mutation developing resistance (Lundborg and Tamhankar, 2017). Genes coding ESBLs are often plasmid-mediated and thus can be transferred between different bacterial strains within or between species (horizontal gene transfer), and this helps antibiotic resistance spread (Chandran et al., 2014). Besides, ESBL-producing bacteria have shown co-resistance to quinolones, sulphonamides, and aminoglycosides (Maina et al., 2013). In our study the most predominate bacterial isolates were GNB, in contrary, with our findings by Zahran et al. (2017) exhibited that, the predominant bacteria isolated from wound infection were GP including *S. aureus* (27.4%), followed by coagulase-negative staphylococci (CoNS) (19.4%), Gram-negative *K. pneumoniae* (12.2%), *E. coli* and Enterobacter spp. (each 9.7%), *P. mirabilis* and *P. aeruginosa* (each 5.6%). Also, their study reported that *S. aureus*
(27.4%) was the predominant organism, and 88.3% of *S. aureus* isolates were MRSA. As well as, another study was in harmony with our results in respect to the MDR-VRSA, their study found that a lot of *S. aureus* are resistant to several antimicrobial agents such as vancomycin which is regarded as the last staphylococci therapy choice (Jensen and Lyon, 2009). Vancomycin resistance can be caused by the overproduction and retention of the cell wall content (including decreased autolytic exercise), by the activating cell wall structure that leads to cell wall thickening and reduced vancomycin access to its active site (Howden et al., 2010). In consistence with our findings, *S. haemolyticus* was frequently isolated from human specimens and presented the highest level of antimicrobial resistance among the coagulase-negative staphylococci (CoNS), (Becker et al., 2014). Also, our findings were in agreement with another study in Egypt by Tohamy et al. (2018) according to their results, the recovered clinically relevant GNB such as *E. coli*, *K. pneumoniae*, *A. baumannii*, and other GNB.

From the obtained results, Bee Venom exhibited strong antibacterial activity with a wide range of MICs and MLCs against all MDR-GNB and GPB one. Also, our results demonstrated that GPB was more sensitive at lower concentrations of Bee Venom than GNB. Our results are in harmony with another study in Egypt by Hegazi et al. (2015) who investigate the antibacterial activity of Bee Venom against five pathogenic bacterial strains, including *S. aureus*, *S. pyogenes*, *K. pneumoniae*, *E. coli*, and *P. aeruginosa*, their study showed that Bee Venom exhibited antibacterial activity against all five bacterial strains and differed according to the type. Also, in another study by Hegazi et al. (2014) reported that the antimicrobial activity of Bee Venom has been documented for both GPB and GNB, including *E. coli* (*E. coli*) and *Salmonella* spp, *E. cloacae*, *E. coli*, and *C. freundii*, *S. aureus*, and coagulase-negative *Staphylococcus* and *E. coli*. From the obtained results, it found that Bee Venom exhibited strong antibacterial effect against MDR-*E. coli* and this result was supported by Perumal Samy et al. (2007) and Hegazi et al. (2015) by their studies the strong antibacterial activity against *E. coli* has been reported. The antimicrobial activity of bee venom was documented in earlier studies. Park et al. (2013) demonstrated that honey bee venom inhibited 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 several peptides presences, such as adolapin, apamin, melittin, mast-cell-degranulating peptides, biologically-active amines, enzymes, and non-peptide components (Leandro et al., 2015). Čujová et al. (2014) reported that honey Bee Venom contained melittin, which is more active against GPB than GNB.

As well as, it was found that, antimicrobial peptides (AMPs), which are typically less than 100 amino acids in length that exhibit antimicrobial activity can be obtained from the poisons of various animals, such as bees (Perumal Samy et al., 2017). AMPs have a broad antimicrobial spectrum and are not affected by classical mechanisms of resistance to conventional antibiotics. AMPs interact primarily with the lipids of cytoplasmic membranes or cell walls, leading to membrane permeabilization, cell lysis, and death (Brogden, 2005). AMP interaction with the lipid monolayer as described by Brogden (2005) can cause peptide aggregation forming pores, lipid and peptide combination forming a toroidal pore, or direct membrane disruption (O’Brien-Simpson et al., 2018).
Bacteria within biofilms are more resistant than those in the planktonic or sessile state. Studies have shown that biofilm cells can withstand up to 1000 times as many antibiotic concentrations as their planktonic peers, and are even prepared to endure in biocidal and UV-exposed settings (Otter et al., 2015). This makes it very hard to eradicate them once they have reached their biofilm form (de la Fuente-Núñez et al., 2013).

Our results demonstrated that among the tested isolates biofilm formation was strong in 66.7% of MDR-isolates, namely; *E. cloacae*, *P. aeruginosa*, *S. aureus*, VRSA, *S. haemolyticus* and *E. faecalis*. Bacteria generate biofilm because the virulence factor performs a major part in infection by protecting against many clearance mechanisms (Scherr et al., 2014). The biofilm matrix can hinder certain immunological defenses, like macrophages that show unfinished entry into the biofilm matrix and "frustrated phagocytosis" (Scherr et al., 2014).

From the obtained results, Bee Venom exhibited strong antibiofilm effect against tested MDR-GNB and GPB. As mentioned above, AMP in Bee Venom interact with the lipid as described by Brogden (2005) and therefore, cause peptide aggregation forming pores, lipid and peptide combination forming a toroidal pore, or direct membrane disruption (O’Brien-Simpson et al., 2018). This distinctive intervention system enables AMPs to work on bacteria at various stages of biofilm, like structure, attachment, and dispersion (Batoni et al., 2016). In a previous report, the AMP Macropin, from bee venom, was recognized and declared to be made of 13 amino acids thus, Macropin is less than Melittin, which makes it more economical to synthesize (Monincová et al., 2014). Macropin had strong antibiofilm activity against MDRB including *S. aureus* and *P. aeruginosa* through a decrease in MBIC concentration of the peptide combined with antibiotics indicated that it could inhibit biofilm formation successfully (Dosler and Karaaslan, 2014).

**Conclusion**

In the current study (39.1%) from bacterial isolates were MDR. Of which (71.77%) were Gram-negative type, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa* and (28.23%) were Gram-positive including *Staphylococcus aureus*, MRSA, VRSA, *Staphylococcus haemolyticus* and *Enterococcus faecalis*. Also, this study revealed the potential effect of Bee Venom as antibacterial to control MDR-isolates as well as, its remarkable ability to eliminate biofilm, making bee venom a promising antibacterial that can be used in many different fields.

**References**


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التأثير الضد بكتيري ضد البئوفيلم لسم النحل من (عسل النحل) على البكتيريا المقاومة للعديد من العقاقير

للمادة الدكترية

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يتزايد انتشار البكتيريا المقاومة العديد من المضادات الحيوية في جميع أنحاء العالم، وذلك كان الهدف من هذه الدراسة هو تحديد بعض أنواع هذا البكتيريا الأكثر شيوعا في الانتباب السريارية، ومن ثم تلبية الحاجة الملحة لإيجاد أدوية جديدة ضد بكتيريا وذلك للسيطرة على العدوى المستعصية بواسطة هذا النوع من البكتيريا، بالإضافة إلى ذلك، تهدف هذه الدراسة إلى مواجهة العدوى المزدوجة بسبب علاجها (Apis (Bee Venom).

كما أن هناك تفشي مشاكل للصحة العامة التي تتطلب حلولاً حيّية. سم النحل (Bee Venom) الذي تنتجه عادة عن مزيج معد من البكتيريا النشطة والإلزامات والأمنات، وذلك، يعتبر بيئة خصبة للبحث لتحقيق هذه الدراسة.

أظهرت نتائج فحص العينات، أنه تم مجموعه 500 عينة سريارية، هناك 224 عينة لم تظهر أي نمو ميكروبي، في حين أن 276 عينة كانت إيجابية. من 276 المزارع الإيجابية تم الحصول على 317 عزلة بكتيرية.

من بين 317 عزلة بكتيرية، 169 (53.3 %) كانت حاملة للجرام (GN) و 148 (46.7 %) كانت حاملة للجرام (GP). وكان من هذا العدد 124 (39.1 %) كانت مقاومة للعديد من المضادات الحيوية (MDR).

أما في ذلك الإحصائيات كولاي، كليليسلا نمون، إمبيرياكتر كلاكا، سيديمونس إريجيرو، و 35 (23 %) عينة كانت مصحوبة الروحية عن، ستافلوكوكس أوريوس، ستافلوكوكس أوريوس، ستافلوكوكس هيمولينكوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيدتوكوكس.

أظهرت فحوصات النشاط الضد بكتيري أن سم النحل يمكن ذكرها جيدة من قبل عزلات البكتيريا المقاومة للعديد من المضادات الحيوية، والتي تم إيجادها بما في ذلك كل من البكتيريا السالسا والموجبة الجرام. مع وكان بين 3-156 MLCs مدى تركيز واسع من أقل تركيز مثبط للنحل البكتيري MICs وكذلك أقل تركيز مثبط MICs. غير أن هذا الدراسة لم تظهر تركيزات مثبط للنحل البكتيري MICs إلى C. البكتيريا السالسا الموجبة الجرام.

كما أظهرت النتائج أيضا أن البكتيريا موجبة الجرام أكثر حساسية للتركيزات المنخفضة لنحل النحل من البكتيريا السالسا الجرام.

ضد أكثر Bee Venom-MICs بالإضافة إلى ذلك، تعتبر التركيزات تحت المثبط للنحل البكتيري العزلات البكتيرية المنتجة للعسل النحلية، والتي كانت، إمبيرياكتر كلاكا، سيدمونس إريجيرو، ستافلوكوكس أوريوس، ستافلوكوكس أوريوس مقاومة للفلاكوس، ستافلوكوكس هيمولينكوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيدتوكوكس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس.

في كل البكتيريا، أظهرت هذه التركيزات انخفاضًا حادًا في تكون البئوفيلم الخاصية بهذه العزلات تراوح بين 2-80% (98.73 %) وفقًا لكل دفعة، باستخدام و Sub-MICs يمكن تحديد التركيز المثبط للنحل البكتيري هذه العزلات البكتيرية تحت التركيز المثبط للنمو MICs:

بالإضافة إلى ذلك، تُعتبر التركيزات تحت المثبط للنحل البكتيري العزلات البكتيرية المنتجة للعسل النحلية، والتي كانت، إمبيرياكتر كلاكا، سيدمونس إريجيرو، ستافلوكوكس أوريوس، ستافلوكوكس أوريوس مقاومة للفلاكوس، ستافلوكوكس هيمولينكوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيدتوكوكس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس.

هذه العزلات البكتيرية مثيرة للشوكة الحيوية، والتي كانت، إمبيرياكتر كلاكا، سيدمونس إريجيرو، ستافلوكوكس أوريوس، ستافلوكوكس أوريوس مقاومة للفلاكوس، ستافلوكوكس هيمولينكوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيدتوكوكس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس.

هذ العزلات البكتيرية مثيرة للشوكة الحيوية، والتي كانت، إمبيرياكتر كلاكا، سيدمونس إريجيرو، ستافلوكوكس أوريوس، ستافلوكوكس أوريوس مقاومة للفلاكوس، ستافلوكوكس هيمولينكوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيدتوكوكس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس، ستافلوكوكس فيكاليوس.