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Antibacterial Activity of Bee Venom against Multidrug-resistant *Acinetobacter baumannii* locally isolates

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ABSTRACT

Bee venom is a complex mixture of proteins, peptides and low molecular components. Nowadays its components have been characterized, the therapeutic application of bee-venom has been well investigated against Gram-negative and Gram-positive bacteria. To determine antibacterial activity of bee venom against Multidrug-resistant *Acinetobacter baumannii* locally isolates. Bee venom was assessed for their antibacterial activity against four Multidrug-resistant *Acinetobacter baumannii* bacterial strains by using MIC depends on resazurin stain, well diffusion methods and Reactive Oxygen Species (ROS) generation test. The minimum inhibitory concentration (MIC) of BV were determined with values (31.25 mg/ml) and bee venom can inhibit the growth of MDR *Acinetobacter baumannii* and may be one of the killing mechanisms by Reactive Oxygen Species (ROS) production which lead to DNA destruction. Bee venom had antibacterial activities against MDR *Acinetobacter baumannii* which confirms the previous work that suggested that bee venom can inhibit growth and survival of some bacterial strains.

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INTRODUCTION

Acinetobacter baumannii is a gram-negative, non-lactose fermenting organism that is increasingly recognized as a major pathogen causing nosocomial infections including bacteremia, ventilator-associated pneumonia, meningitis, urinary tract infection, and wound infection particularly in patients admitted to intensive care units (Townsend *et al.,* 2015). Community-acquired Acinetobacter infections have also increased over the past decade. The organism is characterized by its tendency to acquire resistance to multiple classes of antimicrobials including carbapenems, aminoglycosides, and fluoroquinolones (Zowawi *et al.,* 2015).

Bee venom is a complex mixture of proteins, peptides and low molecular components. Nowadays its components have been characterized. The main components are proteins and peptides. The composition of apitoxin (bee toxin) was complex it contains many biochemical and pharmacologically active substances including histamine, dopamine and melittin (Hegazi *et al.,* 2014)

An important enzyme in bee venom is the phospholipase A and Hyaluronidase, many insects and snakes' venom were involved in the presence of Hyaluronidase which analyzes hyaluronic acid to simple unite, antimicrobial activity of BV was reported against both Gram-negative and Gram-positive bacteria such as *Staphylococcus aureus, Streptococcus mutans* and *Streptococcus pyogenes*, The Gram-negative strains *were Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa*. (Jenkins *et al.,* 2011).

The current objective of the study, to investigate the antimicrobial activity of BV against Multidrug-resistant *Acinetobacter baumannii*

MATERIAL AND METHODS

Bee Venom

Archive of Iraqi honey bees were selected for this study, this was located in farm in the technical institute /AL-Kut, the bee venom was collected by using electro stimulation method inside of hive, impulses were generated in voltage 18-27 V, duration 2 seconds, after dryness of the venom by air scraped with sharp scalpel and collected by dry sterilize tube and stored until used. To obtain a stock solution, a 250 mg of lyophilized BV were dissolved in 1 ml of distilled water then serial dilution have been done with a range of (125 – 3.91) mg/ml

Bacterial Strains

Four strains of *Acinetobacter baumannii* (ATCC 29532, ATCC 29736, ATCC29853 and ATCC27734) which were previously isolated from clinical samples were used in this study, these bacteria were diagnosed and classified in medical microbiology laboratories at the Faculty of Medicine, Nahrain University, all bacterial strains were estimated to be multidrug resistance to the second and third generation cephalosporine, Amikacin, furthermore all isolated were resistant to Carbapenems (Imipenem and Meropenem) and quinolones (Ciprofloxacin and Levofloxacin) by conventional methods such as disc diffusion and MIC as well as by molecular methods by determined their ability to produce bla $_{NDM}$-1gene and qnrA-gene.

This study approved by the Institutional Review Board (IRB) in the College of Medicine /AL-Nahrain University. The study was conducted in the Microbiology Department at the College of Medicine Al-Nahrain University.

Evaluation of Anti-Bacterial Activity

Minimum Inhibitory Concentrations (MIC)

Broth microdilution methods were used to conduct this test, where 100 microliters of bacterial suspension turbidity equivalent to the McFarland 0.5 standard (1.5 x10^8 organisms/ml) were added to each desired well. Then 100 microliters of bee venom with different concentrations were dispensed in each well; plates were incubated at 37 ° C for 24 hours. The MIC of the bacterium was investigated using resazurin (Himedia) after adding 20 µl of dye for all wells, the reduction of resazurin was checked for the change of colour (from blue to lilac, mauve, pink mauve and pink) for 2–4 h. Resazurin is a blue non-fluorescent dye that is converted to pink and fluorescent resorufin (pink) in the presence of a respiring organism.

Well diffusion assay test

The efficacy of bee venom was tested as an antibacterial agent against *Acinetobacter baumannii*. The bacteria were cultured in test tubes containing 2 ml of Brain heart infusion broth. The tubes were incubated at 37°C for 24 hours. The inoculum density of *Acinetobacter baumannii* was adjusted by using 0.5 McFarland standard tubes then plated onto Muller-Hinton agar in three directions by dipped sterile swabs in suspension. Wells (6 mm diameter) were punched in the plates using a sterile stainless-steel borer. 50 microliters of bee venom were placed with concentrations ranging from 125 mg/ml to 31.25 mg/ml. Distilled water was used as a negative control while Ceftriaxone was used as antibiotic control culture plates were incubated at 35°C for 72 hr when the incubation was complete, the diameter of the inhibition zone around the disks was measured and compared with the breakpoints of a clinical laboratory institute (CLSI) (CLSI Document, 2009).

Reactive Oxygen Species (ROS) generation test

Reactive oxygen species contains molecules that damage DNA and RNA and oxidize proteins and lipids (lipid peroxidation). After ascertaining the ability of bee venom to kill *Acinetobacter baumannii* by the MIC and well diffusion methods. The susceptibility of bee venom to Reactive Oxygen Species (ROS) production, has been investigated, the test was performed according to Mohamed *et al.,* 2017.

In brief, mixing the same volume (2 ml) from bacterial suspension with Acrydine Orange / Ethidium bromide (1 mg/mL, 0.3 mg/mL, respectively) then incubated at room temperature for 5 min. and then prepare slide smear from mixture left to dry then examined using a fluorescent microscope. Survival bacteria were appeared orange while dead bacteria take red colour. Compared with bacterial suspension before treatment with Acrydine Orange / Ethidium bromide.

RESULTS

In this study, the antimicrobial activity of bee venom to inhibition the bacterial growth of MDR *Acinetobacter baumannii* were investigated, by using resazurin as a colour indicator for metabolic activity. The MIC was defined as the end points of the lower concentration of colour changes from the colour blue to pink, which is in current study 31.25 mg/ml figure (1). Meanwhile, well diffusion methods showed the high efficacy of bee venom in inhibiting bacterial growth with significant differences compared to negative control figure (2). Furthermore, killing mechanisms of bee venom were determined by Acrydine Orange / Ethidium bromide, and results showed significant differences

between the untreated sample with bee venom as control and sample treated with bee venom. Suggesting that bee venom penetrates the cell wall of the bacteria which stimulate ROS productions then causes bacterial killing by DNA damage Figure (3):

Overall results in the current study, bee venom can inhibit the growth of MDR *Acinetobacter baumannii* and may be one of the killing mechanisms by Reactive Oxygen Species (ROS) production which lead to DNA destruction

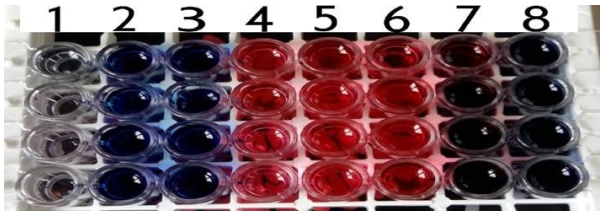

Figure 1: Determination of MIC for bee venom against four strain MDR Acinetobacter baumannii using resazurin dye

Column 8 no contamination occurred while preparing the plate (only dye and bee venom) Column 7 the highest concentration 250 mg/ml, Column 6, 5, 4 shows a change of resazurin natural colour (blue/purple) to the reduced form (red-colourless). Column 4 shows the final colour changes, therefore, the concentration of that column (31.25 mg/ml) was taken as the MIC value, Column 1 negative control (only bee venom and bacterial suspension).

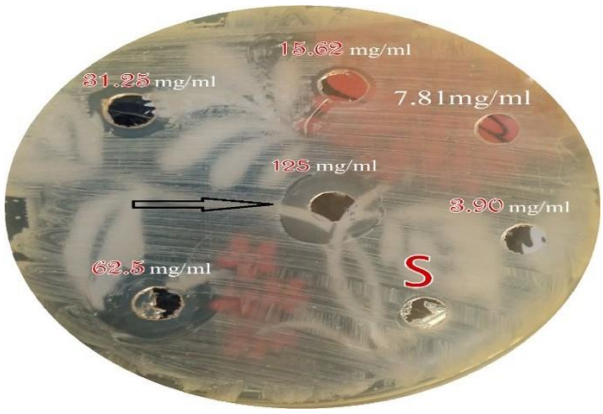

Figure 2: Well diffusion methods the highest concentration of 125 mg/ml, while S refer to a standard which is only D.W.

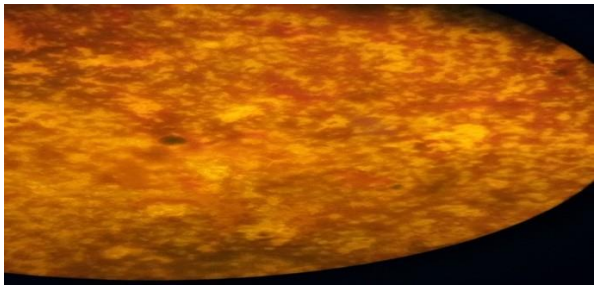

Figure 3: Reactive Oxygen Species (ROS) generation test. In this test, dead bacteria are shown to be red due to Ethidium bromide using a fluorescent microscope

DISCUSSION

As a result, to increasing types of infections with *Acinetobacter baumannii* strains that are exhibiting resistance to multiple antibiotics, there is an urgent need to develop new treatment protocol depends on natural products (Han *et al.,* 2011). Natural components such as BV are potential candidates to address this need due to the anti-inflammatory properties of bee venom as well as antimicrobial activity have (Han *et al.,* 2016). Park *et al*., clearly demonstrated that 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. Although Hegazi *et al*. have been reported that bee venom has antibacterial activity against *E. coli* (Hegazi and EL-Feel 2015) While another study was done by Socarras *et al*. reported effective antimicrobial against *B. burgdorferi* which is a causative agent of Lyme disease (Socarras *et al.,* 2017).

These anti-inflammatory and antimicrobial activities of bee venom referrer to the presence of some bioactive substances such as adolapin, apamin, melittin, and mast cell degranulating peptides (Son *et al.,* 2017). Furthermore, bee venom comprised biological active material included (histamine, epinephrine) which belong under amines groups, in addition to few non-peptide components including free amino acids, carbohydrates and lipids. (Hegazi *et al.,* 2017) In last years, bee venom added as an antiphotoaging product in cosmetic as well as in the treatment of acne-inducing bacteria (Han *et al.,* 2010)

In the current study, the minimum inhibitory concentration was measured to evaluating the efficacy of bee venom as an antibacterial agent by using Resazurin assay which is in an easily identified colour change occurring at cell densities meaningful for MIC testing. The obtained MIC in the current study of BV was 31.25 mg/ml indicate that BV is effective against MDR *Acinetobacter baumannii* strains.

In last years, there has been a focus on bee venom peptides and its mechanism of action for targeting and killing various types of microbes (Raghuraman, 2007) (Carter *et al.,* 2013) (Lee *et al.,* 2018)

This peptide which is one of the major components of bee venom have ability to integration into target phospholipid bilayers found on cell membrane in low concentrations, while in high concentrations it homodimerizes to form pores, releasing Ca2+ ions or disrupting phospholipid head groups, this lead

to cell death and killing the microbes (Andersson *et al.,* 2013) (Bandyopadhyay *et al.,* 2013).

Furthermore, other reported mentioned that killing mechanisms of bee venom could be due to inhibiting DNA synthesis by ROS productions. The mechanism of many antibacterial and antitumor drugs has a relationship with DNA topoisomerase (Jung *et al.,* 2013).

In conclusion, bee venom had antibacterial activities against MDR *Acinetobacter baumannii* which confirms the previous work that suggested that bee venom can inhibit the growth and survival of some bacterial strains.

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