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Characterization of Bee Venom and Its Synergistic Effect Combating Antibiotic Resistance of *Pseudomonas aeruginosa*

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Abstract

The bacterial resistance increases day after day augmenting the need for the discovery of new generations of antibiotics, particularly those belonging to the natural origin. During our study, sixty-two bacterial isolates were recovered from 75 clinical specimens, 11 bacterial isolates (18%) were preliminarily identified as *Pseudomonas aeruginosa* through checking their behavior on blood agar medium, MacConkey agar medium, and Gram stain. A susceptibility test was performed using 16 different antibacterial discs, and *P. aeruginosa* exhibited a multidrug resistance phenomenon. Susceptibility of *P. aeruginosa* toward three different antibacterial drugs which are commonly prescribed in the Egyptian medical was estimated, and regretfully the activity of selected drugs was limited hence, bee venom (*Apis mellifera* L.) was investigated as an antibacterial agent and fortunately, it exhibited a promising result. Combinations of bee venom and drugs were made and evaluated, a synergistic effect was easily observed. MIC and MBC for the combinations were determined, in addition to characterization of bee venom was performed. Conclusively, bee venom has a promising antibacterial activity against *P. aeruginosa*, and when it was combined with antibacterial drugs, it gave a superior activity as evidence for synergism. Subsequently, the dose administrated and the side effects would be minimized via combined therapy.

Keywords: *Pseudomonas aeruginosa*; Multidrug resistance, Combined therapy; Bee venom; SDS-PAGE; Antibiotic susceptibility.

1. Introduction

Due to economic variables and caring for lifestyle improvement. Now, people became more aware of their health. Hence, natural drugs will get more interest than other synthetic ones. Plants were extensively studied as sources of several compounds that have a direct relation with human and animal health especially, the treatment of infectious diseases [1]. The reason for bacterial resistance may be due to extreme use of antibiotics, non-adjusted dosage, uncompleted course, or inappropriate antibiotic administration. Before description, physicians should request a report of bacterial culture sensitivity to detect the appropriate antibiotic. Most of the Staphylococcus aureus isolates became methicillin-resistant (MRSA), some of them became vancomycin-resistant (VRSA), which revealed the extent of the resistance problem and how much the world needs to incorporate more advanced antibiotics, especially those belonging to natural origin [2]. Additionally, some microbes could be inhibited by plant extracts such as clove extract which inhibits the fungal growth that infects potatoes and tomatoes in the postharvest stage [3]

Pseudomonas aeruginosa is principal nosocomial Gram-negative bacteria associated with extended hospitalization, and morbidity and mortality increased particularly in immunocompromised persons [4]. It is also considered as one of the most important bacterial pathogens in Europe among Intensive Care Unit patients that threaten human health because of its multidrug resistance [5]. It

invades the wet spaces such as medical ventilators, oxygen respirators, humidifiers, sinks, taps, toilets, and dialysis machines [6]. *P. aeruginosa* has been known as an opportunistic human pathogen that causes high morbidity and mortality in immunocompromised and hospitalized patients [7]. This pathogen can cause both acute and chronic infections. During acute infections, it colonizes different body sites; urinary tract, skin, eye, heart, ear, airway, and lung tissues. promised individuals. Chronic infections are common in the lungs of patients with cystic fibrosis and bronchiectasis, and it accounts for 5% of cases of chronic obstructive pulmonary disease [8, 9]. Urinary tract infections (UTIs) are a dramatic health issue that is considered the second most common infection in the human body and *P. aeruginosa* is capable of infecting the urinary tract [10].

Honeybee venom (Apitoxin, BV) is secreted in the glands of worker honeybees as a defense mechanism, it has long been used to treat an array of medical conditions [11, 12]. It is composed of a mixture of different components; polypeptides and enzymes which exhibit a wide range of biological potentials including antimicrobial, anti-inflammatory cytotoxic, and hemolytic activity especially by melittin, the major compound of BV [13, 14]. Natural products including bee venom, one of many bee products which are rich in bioactive compounds, offer a diversity of activities against a variety of diseases causes [15, 16].

Limited information about the synergistic effect which may be presented through the combination of honeybee venom and antibacterial drugs thus, the current study aimed to investigate the antimicrobial potential of BV individually as well as in combination with some antibacterial drugs in the Egyptian pharmaceutical market involved in the treatment of urinary tract infections (UTI) and respiratory tract infections (RTI) and attempting to overcome the problem of multidrug resistance (MDR) had been shown by *P. aeruginosa* that isolated from Egyptian patient. want.The objective of this template is to enable you in an easy way to style your article attractively in a style similar to that of *EJCHEM*. It should be emphasized, however, that the final appearance of your paper in print and in electronic media will very likely *vary to some extent* from the presentation achieved in this Word[®]

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2. Experimental

1. Collection of the clinical samples

Different clinical specimens had been collected from different parts of the patient's body such as urine, pus, blood, and sputum samples. A total of 75 clinical specimens had been picked from 75 different patients; their ages ranged from 1 to 99 years during a duration period from March 2018 to December 2018. Bacterial isolates were recovered and purified for further investigations.

2. Isolation, and maintenance media

2.1. Tryptic soy agar medium TSA

Clinical specimens were plated for single colony isolation on TSA (22091 NutriSelect™ Plus, Sigma-Aldrich) as an enriched medium, it was prepared by suspending 40 g in 1 L of distilled water, brought to cooking, sterilized, and finally poured into plates for use later.

2.2. Nutrient agar medium

Nutrient agar medium was used for maintenance and purification purposes was prepared by dissolve 28g of nutrient agar powder (Oxoid; CM0003B) in 1L of distilled water. Sterilize by autoclaving at 121°C for 15 minutes. pH should be adjusted to 7.4 ± 0.2 at 25 °C.

3. Purification and preliminary phenotypic identification of bacterial isolates

Growth conditions, macroscopic examination, microscopic examination by Gram stain were checked for preliminary identification of bacterial isolates [17].

3.1. MacConkey agar medium

In parallel, MacConkey agar medium (M7408, NutriSelect™ Basic, Sigma-Aldrich) was used as a normal laboratory protocol, it was prepared by dissolving 50 g of powder in 1 L of distilled water, cooked, and sterilized, and eventually poured in Petri-dishes for further use [18].

3.2. Blood agar medium

Also, a blood agar medium was used for identification of *Pseudomonas aeruginosa* and it was prepared according to [19] by preparation of 5% defibrinated blood in molten sterilized NA medium.

4. Susceptibility of all bacterial isolates to antibacterial discs

During this experiment, table 1 revealed that 16 different antibacterial discs were tested (Bioanalyse® ASD). The susceptibility test was performed by agar

disc diffusion method in which the antibacterial discs were placed onto the surface of Mueller-Hinton agar medium (Code; CM0337, Thermo Scientific, Oxoid Microbiology products) inoculated with the isolated bacterial species, this medium had been prepared by weighing 38 g and up to 1 l with distilled water. The inoculated plate should be chilled for not less than 2 h at 4 °C. The results were expressed as clear inhibition zones for all tested antibacterial discs [2].

Table 1. List of susceptibility test discs which are involved in the evaluation of the resistance level of the clinical bacterial isolates

Code	Name	Conc. (µg)	Category
CTR	Ceftriaxone	30	Cephalosporin
CTX	Cefotaxime	30	Cephalosporin
CAZ	Ceftazidime	30	Cephalosporin
CS	Cefoperazone	75	Cephalosporin
FEB	Cefepime	30	Cephalosporin
AMC	Augmentin	30	Penicillin
CFR	Cefadroxil	30	Cephalosporin
IPM	Imipenem	10	Carbapenems
SXT	S+T*	25	Sulfonamides
TPZ	P+T**	100/10	Penicillin
CIP	Ciprofloxacin	5	Quinolone
OFX	Ofloxacin	5	Quinolone
AM	Ampicillin	10	Penicillin
AX	Amoxycillin	25	Penicillin
TE	Tetracycline	30	Polyketide
LEV	Levofloxacin	15	Quinolone

*S+T means; Sulfamethoxazole/Trimethoprim.
** P+T means; Piperacillin/Tazobactam.

5. Molecular identification of the most resistant bacterial isolate

Molecular identification was performed depending upon 16 rDNA technique in which the DNA was extracted by DNA Extraction Kit (QIAamp DNA Mini Kit (Cat. No. 51304), amplification process involved one set of universal 16S rDNA primers F5`-3` AGA GTT TGA TCC TGG CTC AG and R5`-3` GGT TAC CTT GTT ACG ACT T according to [20]. Taq PCR Master Mix Kit (Qiagen, Cat.201443) was used for PCR Amplification in 50 µL-mixture reaction, according to the manufacturer's instructions. The thermal gradient of PCR parameters was a general denaturation at 95 °C for 1 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C

for 1 min; a final 72 °C for 10 min as a final extension step [21].

PCR products were detected by electrophoresis in 1% agarose gel corresponding to the DNA ladder (100 bp BIORON, Cat. No. 304105). Eventually, the agarose gel was visualized using an ultra-violate (UV) transilluminator to detect amplified 16S rDNA fragments [22]. PCR product of 16S rDNA gene of the bacterial isolate was recovered by using QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28704). The sequencing process was directly performed using the ABI Prism Big Dye Terminator V.3.1 Cycle Sequencing Kit on an ABI 310 DNA automated sequencer (Applied Biosystems). The sequence results generated by the forward and reverse sequencing primers were analyzed with the software program sequencing analysis 5.3.1. Megalign (DNASTAR, Window version 3.12e) was used to align 16S rDNA retrieved from GenBank. Phylogenetic trees were inferred using the neighbor-joining method, which was carried out using MEGA version 2.1 [23]. The resultant neighbor-joining tree and its topology were evaluated by bootstrap analyses [24] based on 1,000 resampling.

6. Susceptibility of *Pseudomonas aeruginosa* to antibiotics from the Egyptian market

Three antibacterial drugs had been selected for this purpose, these drugs are widely described by physicians to treat systemic infections especially urinary tract infections UTI, and respiratory tract infections RTI. The data of the selected drugs were mentioned in detail in table 2 including their names, active ingredients, their forms, their, manufacturers and their concentrations.

Preparation of the tested concentrations of each antibiotic was performed according to the recommended dose as follow; Cefotax was prepared by dissolving 2000 mg in 10 ml of accompanied solution where the final concentration is 200 mg/mL, Cefepime was prepared by dissolving 1000 mg in 5 ml of the attendant solution where the final concentration is 200 mg/mL, and finally, Tavanic vial concentration was 500 mg in 100 ml where the final concentration is 5 mg/ml.

Table 2. Data of the selected antibiotics from the Egyptian pharmaceutical market

Drug	Active ingredient	Form	Company	Conc.
Cefotax	Cefotaxime	Vial	EIPICO Egypt	2000 mg
Cefepime	Cefepime	Vial	PHARCO B	1000 mg
Tavanic	Levofloxacin	Vial	Sanofi	500 mg

7. Bee venom

Lyophilized pure venom of bee venom (*Apis mellifera mellifera*) was provided from ANDI COE, VACSERA, where venom was stored in a brown vial at refrigerator (4 °C).

7.1. Antibacterial activity of bee venom against *Pseudomonas aeruginosa*

The stock solution of bee venom was prepared by dissolving 100 mg in 10 mL saline where the final concentration was 10 mg/ml. A loopful from the overnight growth of fully identified *P. aeruginosa* was transferred to 50 mL Tryptone Soy Broth TSB medium (Oxoid) which was prepared by dissolving 9 g of TSB powder in 300 mL of distilled water, autoclaved at 121 °C for 15 min. the inoculated flask was incubated at 37 °C with vigorous shaking (150 rpm) for 18 h. The inoculum was carefully spread onto the surface of the trypticase soy agar TSA medium. The sterile filter paper discs (Wattman filter paper no. 1 with diameter 6 mm) were impregnated in venom with a concentration of 10 mg venom /1 mL saline. The discs were placed on the surface of the plates suitably spaced apart, and then the plate was incubated at 37 °C for 24 h. The plate was examined for the presence of an inhibition zone for bacterial growth around the disc. The zone of inhibition was indicative of the degree of organism sensitivity [1].

7.2. Minimum inhibitory concentrations MIC for both drugs and bee venom

The bacterial growth was re-suspended and adjusted turbidity to 1.0 McFarland [25] using a spectrophotometer (Perkin Elmer-EZ 301-German) for *Pseudomonas aeruginosa* according to [26]. 100 μL of standard McFarland bacterial growth was pipetted into each well of a microtiter plate (96 Well CELLSTAR®, Greiner Bio-One GmbH). Well, no. 1 was set as positive control while, well no. 2 was set as a negative control (free broth). Wells no. 3-10; serial dilutions of bee venom BV (1 – 1/128) were applied, while, wells no. 11-18; serial dilutions of

AB1(1 – 1/128), also, wells no. 19-26; serial dilutions of AB2 (1 – 1/128), additionally, wells no. 27-34; serial dilutions of AB3 (1 – 1/128), also, wells no. 35-42, 43-50, and 51-58; serial dilutions of a combination of BV and AB1, AB2, and AB3 respectively. This test was designed as triplicate, all the plates were incubated at 37 °C for 24 h, and subsequently, the bacterial growth was turbidimetrically estimated by ELISA reader (Biotek-ELX800- USA) at the wavelength of 630 nm.

7.3. The minimum bactericidal concentrations (MBCs)

According to [1] MBCs were determined by inoculating of loopful which was obtained from the well have no detectable growth and subsequently cultivated on TSA and then incubate for 24 h. The bacterial growth was monitored and recorded.

7.4. Characterization of bee venom
7.4.1. Physical characteristics

According to the standard criteria of ANDI COE, VACSERA from where the bee venom was provided, some physical properties were reported such as color, pH, percentage of dissolved substances, solubility, percentage of active ingredients, and finally the moisture content estimated by Karl Fisher titration method [27].

7.4.2. Total protein

Total protein content was determined at the center of excellence in anti-venom research EGYVAC, VACSERA according to the method described by [28], the reagents kit was purchased from Sigma-Aldrich, St. Louis, MO. And the result was read by spectrophotometer at A260 and A280. Calculations had been according to the following equations:

mg protein/ml = [(1.31 x A280) - (0.57 x A260)] x dilution factor

7.4.3. Determination of LD_{50} of venom

The LD_{50} of the venom was determined according to the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Male albino Swiss mice 16-18 gm. Ascending concentration of 5 dose levels of the freshly prepared venom solution in normal saline were arranged in a geometric progression starting by a dose that kills approximately 0-20 % of the animals and ending by a dose that kills approximately 80-100 % of the injected animals. Each dose level was tested in 4 mice, and all injections were given intravenously, and

deaths and survivals of injected animals were recorded after 24 h. from the time of injection.

The uncorrected % lethality at each dose level was calculated from the numbers of survivals and deaths directly obtained at that dose level. However, to avoid the interference of accidental survivals or deaths resulting from abnormal resistance or susceptibility of tested animals, i.e., the data were recalculated after correction of the numbers of survivals at each dose level by adding consideration of the numbers of survivals at a higher dose level, and correction of the numbers of deaths by adding the number of lower deaths at each dose level. It is assumed that mice surviving at a given dose level would have survived at a lower dose level, and conversely, mice that died at a given dose level would have died at any higher dose level. Therefore, at each dose level, the numbers of survivals at higher dose levels were added to an uncorrected number of survivals obtained at that dose level; and the number of deaths at lower dose levels was added to the uncorrected number of deaths obtained at that dose level. The accumulated corrected % lethality at each dose was determined from the accumulated corrected deaths and accumulated corrected survivals at that dose level [29]

7.4.3.1. Calculation of LD$_{50}$

When the differences in accumulated % lethality at the 5 doses level were statically significant, then the 50%-point dose (LD$_{50}$) was calculated according to [30] from the following equation:

Log. LD$_{50}$ = Log dose next below 50 % + (Log increasing factor x proportionate distance).

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

7.4.4. Determination of the venom protein by SDS-PAGE electrophoresis

The venom protein activity was also assayed by electrophoresis in SDS-PAGE (11% acrylamide) with co-polymerized gelatin (6). The venom samples (10 mg) diluted in 0.05 M TRIS-glycine buffer, pH 6.8, containing 10% glycerol, 0.025 M SDS, and 0.5 mg/mL phenol red were directly applied to the gel. Soon after the run at 150 V for about 3 hours at 4 °C, the gel was then (a) incubated at 37 °C in 0.1 M glycine-NaOH, pH 9.5, for 5 hours, and (b) developed for proteolytic activity with 0.1% Amido Black in methanol: acetic acid: water (3:1:6; v: v: v). The gels were de-stained with methanol: acetic acid:

water (3:1:6) v/ v/v until the clear zones of protein could be seen.

3. Results and Discussion

Seventy-five clinical samples had been collected from different 75 patients during a period from March 2018 to December 2018, some demographic characteristics were reported such as gender, and age of patients, as well as the site from which the samples were collected, cell shape, and aggregation, aeration condition, and Gram reaction. Table 3 revealed the presence of 41 female clinical samples with an observable increase than those belong to male samples which were 21, as well, according to Gram reaction; 46 were Gram-negative while 16 were Gram-positive. It is also reported that 38 belong to pus samples, 13 urine samples, 7 sputum samples, and 4 blood samples. During the investigation of bacterial shapes and aggregations, as shown in figure (1) it is clear to observe that there are 4 different shapes; 11 small rods (17.7%), 35 bacilli (54.8%), 1 pleomorphic bacillus (1.6%), and 16 cocci in clusters (25.9%).
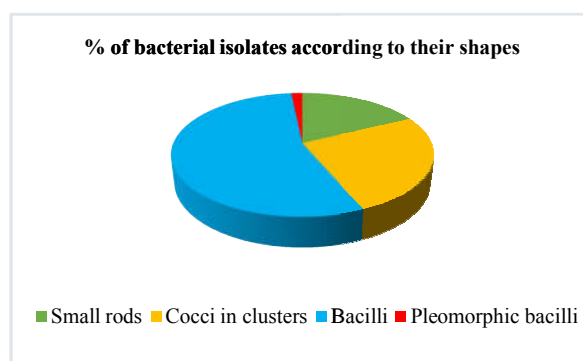

% of bacterial isolates according to their shapes

■ Small rods ■ Cocci in clusters ■ Bacilli ■ Pleomorphic bacilli

Figure 1. 3D-Pie chart represents the diversity of bacterial isolates recovered from the clinical specimens according to their shapes and aggregations

Preliminary identification of bacterial isolates

Sixty-two bacterial isolates were recovered and some criteria had been investigated for preliminary identification such as Gram stain, MacConkey agar medium, and blood agar medium. According to routine preliminary identification, small rods were selected and identified as *Pseudomonas sp.* (11 isolates = 18%). Table (3) and figure (2) revealed the distribution of bacterial isolates among the site of isolation, consequently, pus specimens were the richest due to the presence of 38 (61.3%) bacterial isolates, followed by urine samples which included 13 (20.9%), and blood samples that included 7

(11.3%) bacterial isolates. While sputum samples had the poorest number and diversity of bacterial isolates; 4 (6.4%). Preliminary identification had been achieved in line with [31] who stated that identification outlines for aerobic Gram-negative bacteria that grow on 5% sheep blood agar start with carrying out a Gram stain, observing colony morphology, and evaluating the organism's ability to grow on MacConkey agar by fermenting lactose. As well, molecular analysis became a gradually important technique for the clinical bacteriologist, particularly to identify aerobic Gram-negative bacteria.

Maximum isolates were from pus and sputum samples and least isolates from vaginal swab and cerebrospinal fluid [32]. As well, most of the collected specimens belonged to ear pus 28.6% (111), followed by urine 24.0% (93), wound pus 20.6% (80), stool 14.9% (58), and blood 8.3% (32), this results certainly is highly in harmony with our findings [33].

Table 3. Diversity of the bacterial specimens and their percentages among the clinical specimens from which they were recovered

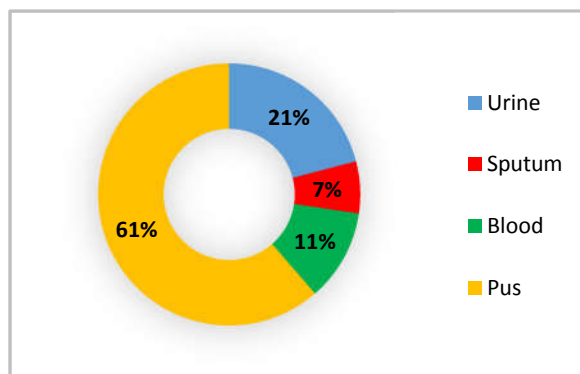| Bacterial isolate	Urine	Sputum	Blood	Pus
Total (62)	13 (20.9%)	4 (6.4%)	7 (11.3%)	38 (61.3%)

Figure 2. The doughnut pie chart represents the bacterial load in each clinical source

Susceptibility of the bacterial isolates to different antibiotic discs

Sixteen antibacterial discs were involved to exhibit their effect on all 11 isolates of *Pseudomonas spp.*, table (4) exposed a noticeable variation whereas the diameters of the inhibition zones ranged from 0 to 24 mm. Obviously, the bacterial isolate 15F99P exhibited multidrug resistance (MDR) to several antibacterial discs with mild response only to ward imipenem (15 mm). This isolate was preliminarily identified as *Pseudomonas sp*. accordingly, it was selected for incoming investigations therefore, it was completely identified depending on a molecular basis, and its 16S rDNA sequence was submitted to the GenBank under accession number *Pseudomonas aeruginosa* MT107480.1.

P. aeruginosa rarely affects healthy individuals but has been recognized as an opportunistic human pathogen that causes high morbidity and mortality in immunocompromised and hospitalized individuals [7]. Multidrug-resistant (MDR) or extensively drug-resistant (XDR) *P. aeruginosa* strains are increasingly prevalent in chronic and nosocomial infections such as wounds and burn patients and are associated with increased morbidity and mortality [34]. Amongst *P. aeruginosa* isolates, maximum resistance (81.8%) was perceived toward piperacillin and meropenem, *P. aeruginosa*, Acinetobacter spp., and Klebsiella spp. are the most pervasive Gram-negative bacteria involved in burn wound infections in hospitalized pediatric patients. A high proportion of these bacterial species exhibited multidrug resistance [35].

Susceptibility of *Pseudomonas aeruginosa* to antibiotics from the Egyptian market

Three different antibiotic drugs were selected from the Egyptian market which were frequently described for either UTI or RTI so, different concentrations of all these drugs in addition to bee venom were prepared and investigated. Moreover, bee venom was combined with each drug individually in different concentrations and all these runs were investigated as antibacterial agents. Table (5) revealed that Cephotax was completely resisted in all tested concentrations, while Cefepime and Tavanic showed antibacterial activity with 12.5 and 0.39 mg/mL respectively for MIC, and 50 and 0.78 mg/mL respectively for MBC values. On the other hand, bee venom exhibited antibacterial activity at values 5 and 10 mg/mL for MIC and MBC respectively. Venom gave a moderate inhibitory effect on E. coli by viability assay, caused high membrane permeability and significant ATP loss where the effect was increased by increased concentration [36].

In combination cases, bee venom and Cephotax could inhibit the growth of *P. aeruginosa* at MIC value (0.625+12.5 mg/mL) clarifying how much the minimizing of bee venom level, while MBC value (2.5+50 mg/mL). In case of bee venom with Cefepime, MIC and MBC values became (1.25+25 mg/mL) and (2.5+50 mg/mL) respectively. Finally, the combination of bee venom with Tavanic exhibited (0.08+0.39 mg/mL) and (0.31+6.25 mg/mL) respectively for MIC and MBC. This result is in agreement with [37] who concluded that the synergism of melittin at its nontoxic dose with doripenem and ceftazidime could be of great therapeutic value as a topical drug against burn infections caused by MDR bacteria.

Melittin is considered a potential antimicrobial agent that inhibits the growth of Acinetobacter baumannii, and it could be used to control bacterial infections and suggest that antimicrobial peptides can serve as the basis for the development of new treatments [38].

Table 4. Diameters of inhibition zones in mm expressing the susceptibility of all bacterial isolates toward different antibacterial discs

Code	CTR	CTX	CAZ	CS	FEP	AMC	CFR	IPM	SXT	TPZ	CIP	LEV	OFX	AM	AX	TE
3F37U	0	0	1	0	15	0	0	15	0	1	17	16	15	6	1	15
15F99P	0	0	0	0	4	0	0	15	0	0	6	4	6	0	0	0
17F37U	0	0	1	1	15	0	1	16	0	0	15	15	16	0	0	0
23F57P	0	2	0	1	R	0	0	15	0	0	1	0	1	0	0	0
27M63P	0	0	1	1	4	0	0	15	1	15	5	15	15	0	1	0
28F35P	1	0	1	0	4	1	0	0	0	2	1	2	5	2	0	10
31M63P	0	0	0	0	4	0	0	15	0	1	7	15	6	0	0	0
40F60P	0	0	0	0	4	0	0	18	2	0	0	15	0	1	0	
44F8P	0	15	15	1	15	0	0	15	3	15	0	0	2	2	0	0
54F99S	0	0	3	0	0	0	0	6	0	0	0	1	4	3	0	0
59M11P	0	1	0	1	2	0	0	15	0	1	0	0	3	2	0	5

Table 5. Detection of MIC and MBC of antibacterial drugs, bee venom, and their combinations against *P. aeruginosa*

Agent	C+	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	C-
Cephotax	0.102	0.126	0.122	0.122	0.122	0.119	0.116	0.116	0.113	0.05
Cefepime	0.171	0.14	0.137	0.144	0.148	0.148	0.158	0.164	0.168	0.053
Tavanic	0.933	0.335	0.35	0.353	0.358	0.362	0.372	0.377	0.387	0.193
Bee venom	1.763	0.111	0.776	1.54	1.596	1.903	1.675	1.623	1.578	0.049
C1	1.369	0.211	0.226	0.306	0.341	0.341	0.767	0.773	1.353	0.056
C2	1.07	0.178	0.206	0.318	0.38	0.56	0.672	0.9	0.995	0.051
C3	1.054	0.286	0.317	0.322	0.341	0.357	0.377	0.41	0.43	0.367

The data in the table represents the OD of *P. aeruginosa* growth measured by spectrophotometer, whereas blue cells represent MIC values, while yellow cells represent MBC values.

Characterization of bee venom and detection of its in vivo toxicity

According to the criteria of ANDI center of excellence, VACSERA; the investigated bee venom was characterized physically and biochemically, and its characteristics were listed in the table (6).

As shown in table 6. The total protein concentration in our bee venom was 1.34 mg/mL, and it also exhibited a similar pattern of protein on SDS-PAGE (figure 3) to the standard. Regarding its toxicity, the LD_{50} value was 190 µg/mouse. It is clearly to observe that the present bee venom has yellowish white color, it is completely soluble with no residues. As well, its pH is 6.2 with moisture content 0.0%. regarding its biochemical characterization, its protein concentration is 1.34 mg/mL that fractionated into 7 clear protein bands on the SDS-PAGE (figure 3). Eventually, the value of

LD$_{50}$ is 190 µg/mouse, and when compared with MIC values (0.08-1.25 mg), it will be noticed that the crude bee venom has a slight toxicity *in vivo*. This result is in a harmony with An, Hyun☐Jin, et al. [39] who reported that the crude be venom is suitable for topical applications for treatment of cutaneous infections.

Table 6. Physical and biochemical characterization of bee venom and its LD$_{50}$

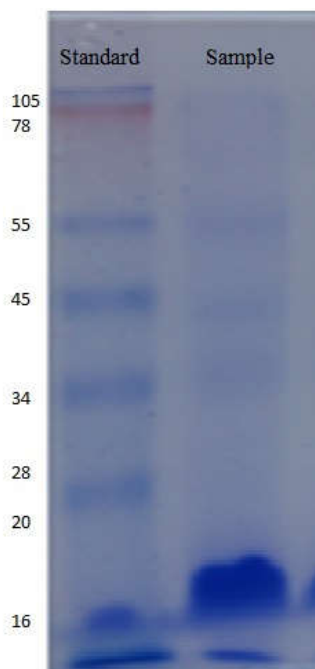| Parameters	Results
1. Physical parameters	
Color	Yellowish white
Solubility	Soluble with no residues
pH	6.2
Percent of solid undissolved substances	0%
Percent of the active ingredients	100%
Moisture content	0.01%
2. Biochemical parameters	
Total protein	1.34 + 0.1mg/ml.
SDS-PAGE	(See figure 3)
LD$_{50}$	190 +1.2 µg/mouse

Figure 3. Protein pattern of our bee venom on SDS-PAGE in corresponding to bee venom standard

4. Conclusion

Our findings emphasize how much important the need to raise awareness of patients about handling antibiotics to limit the emerging of microbial resistance. *P. aeruginosa* represents one of the major pathogens that affect human health causing systemic infections that represents a serious health problem in particular its MDR phenomenon. Bee venom has a promising antibacterial activity with low in vivo toxicity, The synergistic effect of bee venom contributes not only to inactivate the pathogen growth but also to decrease the loading and maintenance dosage, hence, lower side effects of chemotherapy. In further studies, the active protein responsible for the antibacterial activity of bee venom needs to be fully purified with full characterization and may be obtained in the nanoform for maximum benefits.

5. Conflicts of Interest

The authors declare that they have no conflict of interest regarding this article.

8. References

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