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Antibacterial Activity of Honey Bee Extract against Antibiotic Resistant Bacteria Isolated from Human Infections

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Abstract

Bacterial resistance to antibiotics drugs in a continuous development due to the wrong and excessive use of antibiotics, a malty-drug resistant bacteria became a real danger so there is a necessity to search for alternatives strategies to improve the quality of antibiotics or find alternative materials, in recent years, numerous studies confirm various bioactivities of natural products among the antibacterial activity.

The aim of this study was to investigate the ability of homogenous whole bee extract of *Apis mellifera* to overcome microbial resistance to antibiotics as natural antimicrobial agent besides its activity as a complementary to increase the quality, potency and the ability of the antibiotic against resistance bacteria has been investigated. Whole bee extract has been tested on multi drug resistant bacteria isolates from different kind of human infections separately as antimicrobial agent alone beside using by mixing with the tested antibiotics agents that have been used in the study.

The results of this study indicate a significant effect of antimicrobial activity by homogenous whole bee extract against the isolate *Pseudomonas aeruginosa and Klebsiella pneumonia* then which bacterial growth inhibition observed in isolates beside the significant antimicrobial effects of the bee extract on the bacterial isolates some complementary effects with the resistance antibiotics in which homogenous bee extract indicate complementary effect for most types of tested antibiotics been observed in that case bee extract could have antimicrobial effects on resistant bacteria to antibiotics and improving the quality and ability of the resistance antibiotics to be more significant.

Keywords: *Pseudomonas aeruginosa, Klebsiella pneumoniae,* Antibiotics resistance, Bee extract, *Apis mellifera*.

INTRODRODCTION

Bee venom (BV) is a rich source of secondary metabolites from honeybees *Apis mellifera* It contains a variety of bioactive ingredients including peptides, proteins, enzymes, and volatile metabolites [1], that have advantageous potential in treating inflammation and central nervous system diseases, as well as in recent times in treatment of tumors [2-3].The BV constitutes approximately 50% of its dry matter, Is melittin (MEL), MEL is 26 amino acids long peptide{4-6}. Modern pharmacological studies showed that MEL exerts various antitumor effects by inhibiting tumor cell growth [7-8], promoting tumor cell apoptosis [9-12]. It is also reported to have strong hemolytic activity [13]. The component of BV also exhibits antioxidant, anti-inflammatory and antibacterial properties [14].Also possess ant diabetic properties and display beneficial effects against cardiovascular disease, gut symbiosis, and chronic kidney disease been reported, it has an excellent clinical safety profile, with no known toxic effects described so far [15].Natural antimicrobial agents such as *Apis mellifera* venom have different mechanisms to inactivate bacteria [16-17]. As apitherapy is alternative therapy relies on the usage of honey bee product [18]. Therapeutic application of bee-venom has been well investigated against Gram-negative and Gram-positive bacteria [19]. The aim of this study was to investigate the ability of whole bee extract *Apis mellifera* to overcome microbial resistance to antibiotics as natural antimicrobial agent besides its activity as a complementary to increase the quality, potency and the ability of the antibiotic against resistance bacteria has been investigated.

MATERIAL AND METHODES

Samples collection: Samples were collected from Egyptian patients suffering from infections, Bacterial isolates obtained from Kasir Al-Ainy hospital central microbiology laboratory. The swab samples were suspended in normal saline (0.8%) and diluted up to 10-4. 100µl from the final dilution 10-4 was streaked on the Nutrient Agar (NA) medium using a spread plate method on nutrient agar medium and incubated at 37°C for 18-24h, All single colonies that have were subculture on the Macconkey agar medium to obtain stock from the single colony, the same colony smear stained by gram stain to differentiate between the Gram+ve and Gram-ve bacteria by using the same loop that used for sub culturing [20].

Preparation of Whole bee extract: One hundred life bees were collected from bee hive and killed by freezing. These bees were washed with 70% ethyl alcohol then by sterile distilled water. The bees were homogenized in 10ml sterile normal saline and then centrifuged at 1500 rpm for15 minutes. The

supernatant was collected after centrifugation and sterile normal saline was added to reach a total volume of 10ml [21].

Antibiotics Susceptibility: Test on bacterial isolates by Disk diffusion by the Kirby-Bauer method is a standardized technique for testing rapidly growing pathogens, Briefly, a standardized inoculums (i.e., direct suspension of colonies to yield a standardized inoculum is acceptable) is swabbed onto the surface of MH agar (i.e., 150 mm plate diameter), Because reproducibility depends on the log growth phase of organisms, fresh subcultures are used. Filter paper disks impregnated with a standardized concentration of an antimicrobial agent are placed on the surface, and the size of the zone of inhibition around the disk is measured after overnight incubation [22]. This study done on two isolates which had significant character of malty–drug resistant activity, isolates identified by 16sRNA, Multiple antibiotics disk from different kind of antibiotics types been used alone and with 20µL of bee extract solution. Antibiotics: Amikacin (AK), Cefalexin (CN), Azithromycin (AZM), Doxycycline (DO), Tetracycline (TE), Ciprofloxacin (CIP), Ofloxacin (OFX), Levofloxacin (LEV), Cefuroxime (CXM), Cefoxitin (FOX), Ceftriaxone (CTR), Cefaclor (CEC), Amoxiclav (AMC), Tazobactam (TPZ), Cefepime (FEP), Meropenem (MEM), Trimethoprim + Sulfamethoxazole (SXT), Ceftazidime (CAZ), Polymyxin (PB) and Ampicillin (AMP).

Isolates identification

DNA extraction: A full loop of bacterial growth been used for DNA extraction DNA extraction involves lysing the cells and solubilizing for Cell Lysis DNA extraction kit from Jene Bioscience been used: Add 300µl Lysis Buffer and 2µl RNase A to the cell pellet Vortex vigorously for 30-60 sec Add 8µl Proteinase K and mix by pipetting Incubate at 60 °C for 10 min and cool down for 5 min Add 300µl Binding Buffer and vortex briefly Place tube on ice for 5 min Centrifuge for 5 min at 10,000 rpm.

PCR amplification: Was done according to Huangby using Taq PCR Master Mix Kit (Qiagen, Cat. No. 201443) PCR reactions were done in 100µL mixture reaction, according to the instruction of the manufacturer [23]. PCR was performed with the following parameters: preheating at 95°C for 1 min, 35 cycles of 95°C for 1 min, 48.3°C for 1 min and 72°C for 1 min; finally at 72°C for 10 min as a final extension step. The PCR products were separated using 1% agarose gel electrophoresis with ethidium bromide stain (0.5 µg mL) and purified using QIA-quick Gel Extraction Kit (Qiagen, Cat. No.28704). Purified PCR products were sequenced directly using a Prism Big Dye v3.1 kit (Applied Biosystems, Cat. No. 4336917) on an ABI 310 DNA automated sequencer (Applied Biosystems). All samples were analyzed in both forward and reverse directions. For sequence comparisons of gene sequences of bacterial types, sequence alignment was performed using themultiple- alignment algorithm in Meg align (DNASTAR, Window version 3.12e)

Sequencing and phylogenetic analysis: The amplicons of the correct size were extracted and purified from 1% low-melting-temperature agarose gel using a NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Germany), according to the manufacturer's instructions. The gel purified PCR products were got sequenced from outsource on automated Applied Biosystems DNA sequencer 3100 (ABI prism) and compared with published sequences using the BLAST programme. All the sequences were submitted to Genbank (Table-2). For establishing the genetic and evolutionary relatedness, the sequences were subjected for sequence pair distance and phylogenetic analysis along with ten reference published sequences (Table-2). All the sequences were edited and compiled using Ediseq programme from Laser Gene Biocomputing software Package (DNASTAR Inc.). Multiple sequence alignment was performed using the Clustle-W and nucleotide divergence/identity was calculated using Meg Align programme from Laser Gene Biocomputing software Package (DNASTAR Inc.). The phylogenetic trees were reconstructed with the phylip computer program package [24], using neighbour-joining analysis using MEGA software version 6. The genetic distances were computed using Kimura 2-parameter model [25] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The nucleotide sequence of the *Esherichia coli* K- 12 was used as the out-group for phylogenetic tree reconstruction. The bootstrap values were calculated 1000 replicates of the alignment [26].

RESULTS

Isolates identified morphologically (Table 1) and by by 16sRNA as *Pseudomonas aeruginosa* and *Klebsiella pneumoni* (Figurem 1). *Klebsiella pneumoniae* is an encapsulated Gram-negative bacillus belonging to the Enterobacteriaceae family. First described in lung tissues of dead pneumonia patients, *K. pneumoniae* is a bacterium able to infect humans, causing different types of infections, including respiratory and urinary tract infections, soft

tissue infections, surgical wounds and sepsis. After E. coli, *K. pneumoniae* is the second most important opportunistic enterobacterium causing nosocomial and community infections. However, it is a ubiquitous species in nature, although environmental strains are more susceptible to antibiotics than clinical ones [27]. Nosocomial infections caused by *K. pneumoniae* multi-antibiotic resistant have become an increasing public health concern. *K. pneumoniae* produces various pathogenic virulence factors, including capsular polysaccharide, lipopolysaccharide, fimbriae, siderophores and resistance [28-29]. Increased global infections by multidrug- resistant bacteria closely associated with limited drug treatments imply the failure of empirical treatments and strengthen the need for antibacterial therapies based on antimicrobial sensitivity tests [30].

In this study antibiotic resistance for many of used antibiotics occurred Table (2).

Table (1) Sample sources and morphological characters of bacterial isolates

Isolates codes	*Gram stain*	*Sample source*	*Colony character*	*Shape of bacterial cells*
1UR	G-	urine	colonies are greyish, thick, white, moist, smooth, opaque	Rod shape
2SP	G-	sputum	yellow mucoid colonies, opaque	Rod shape
3SK	G+	skin	Circular, Convex, Smooth, yellow, Opaque	Cluster
4UR	G-	urine	White colonies, smooth, opaque	Rod shape
5UR	G-	urine	colonies are off- white, thick, white, moist, smooth, opaque	Rod shape
6SK	G+	skin	White, smooth, opaque, mucoid	Cocci
7SK	G-	skin	Greenish coloring. large, opaque, flat	Rod shape
8UR	G-	urine	colonies are greyish, white, smooth, opaque	Rode shape
9UR	G-	urine	colonies are greyish, thick, white, smooth, opaque	Rod shape
10SK	G+	skin	Circular, Convex, Smooth, yellow	Cocci
11SK	G+	skin	Convex, Smooth, yellow, Opaque	Cluster
12UR	G-	Urine	large, opaque, flat	Rod shape

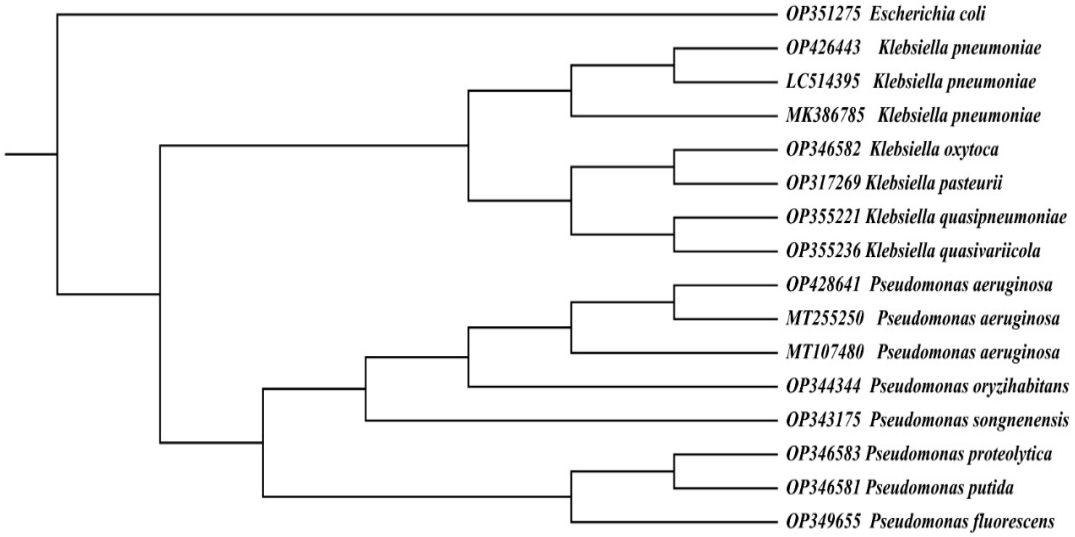


Fig. (1) Phylogenetic analysis

Table (2) Antibiotic effect on *Klebsiella pneumonia* OP426443 ***and*** *Pseudomonas aeruginosa* OP428641

Antibiotices	*Klebsiella pneumoniae OP426443*	*Pseudomonas aeruginosa OP428641*
AK	18mm	12mm
CN	15mm	0
AZM	0	0
DO	15mm	0
TE	10mm	0
CIP	0	9mm
OFX	0	0
LEV	0	0
CXM	0	9mm
FOX	14mm	0
CTR	0	0
CEC	0	9mm
AMC	0	0
TPZ	0	0
FEP	0	0
MEM	17mm	0
CAZ	0	0
PB	0	20mm
AMP	0	9mm
SXT	0	0

The humongous whole bee extract showed antimicrobial effect on the tested *K. pneumonia* OP426443 that isolated from sputum as 9mm inhibition zone in a compare with most antibiotics types that been tested on whole bee extract had a positive result on *K. pneumoniae* as antimicrobial agent but it doesn't enhance the activity of the resistant tested antibiotics. In the other hand the second isolate was *Pseudomonas aeruginosa* is a non-fermentative gram-negative bacterium with an extraordinary ability to colonize a large variety of ecological niches, particularly moist environments. Currently, *P.aeruginosa* is one of the major pathogens causing hospital-acquired infections, in particular affecting patients with impairment of immune defenses or admitted in the Intensive Care Unit (ICU) {31-32}. This organism is not only intrinsically resistant to a wide range of antimicrobials, but also has an extraordinary capacity for developing resistance to commonly used antimicrobials through the selection of mutations in chromosomal genes or by horizontal acquisition of resistant determinants. The increasing prevalence of multidrug-resistant (MDR) strains is a cause of concern as it compromises the selection of appropriate empirical and definitive antimicrobial treatments. This situation is associated with worse outcomes and higher mortality, particularly in patients with severe *P. aeruginosa* infections, including bacteremia and ventilator associated pneumonia {33} Increasing resistance to different anti-pseudomonal drugs particularly among hospital strains has been reported worldwide (34), and this is a serious therapeutic problem in the management of disease due to these organisms. In this study *P. aeruginosa* OP428641 isolated from urine had resistant activity to wild ranges of antibiotics (Table3).

Homogenous whole bee extract showed 12mm of inhibition zone of *Pseudomonas aeruginosa* growth around the filter disk, whole bee extract also showed activity enhancement for some antimicrobial agents either in increasing the effect of the inhibition zone before and after using with antibiotic for the susceptible antibiotics or alternate the antibiotic activity from zero inhibition zone to indicatable inhibition zone in *Pseudomonas aeruginosa* Cefalexin (CN) , Azithromycin (AZM), Doxycycline (DO), Ofloxacin (OFX, Levofloxacin (LEV), Cefoxitin (FOX), Ceftriaxone (CTR), Amoxiclav (AMC), Tazobactam (TPZ), Cefepime (FEP), Meropenem (MEM), Ceftazidime (CAZ), Sulfamethoxazole (SXT) all these antibiotic types indicated positive result from zero growth inhibition zone to palpable result with (Table 4).

Table (3) Effect of Bee extract on *Klebsiella pneumonia* OP426443

Ab Isolates	AK	CN	AZM	DO	TE	CIP	OFX	LEV	CXM	FOX	Whole bee extract
***Klebsiella pneumoniae* OP426443**	18mm	15mm	0	15mm	10mm	0	0	0	0	14mm	9mm
	AK+B	CN+B	AZM+B	DO+B	TE+B	CIP+B	OFX+B	LEV+B	CXM+B	FOX+B	
	18mm	15mm	0	14mm	10mm	0	0	0	0	11mm	
	CTR	CEC	AMC	TPZ	FEP	MEM	CAZ	PB	AMP	SXT	
	0	0	0	0	0	17mm	0	0	0	0	
	CTR+B	CEC+B	AMC+B	TPZ+B	FEP+B	MEM+B	CAZ+B	PB+B	AMP+B	SXT+B	
	0	0	0	0	0	0	0	0	0	0	

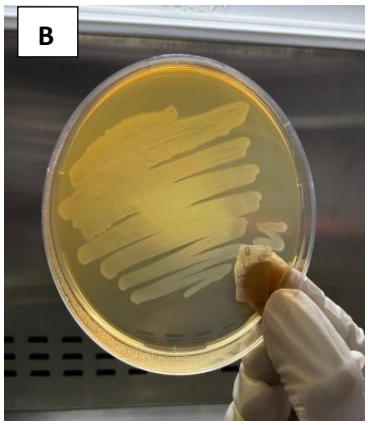
Table (4) Effect of Bee extract on *Pseudomonas aeruginosa* OP428641

Ab Isolates	AK	CN	AZM	DO	TE	CIP	OFX	LEV	CXM	FOX	Whole bee extract
Pseudomonas aeruginosa OP428641	12mm	0	0	0	0	9mm	0	0	9mm	0	12mm
	AK+B	CN+B	AZM+B	DO+B	TE+B	CIP+B	OFX+B	LEV+B	CXM+B	FOX+B	
	12mm	10mm	15mm	15mm	0	17mm	10mm	9mm	11mm	14mm	
	CTR	CEC	AMC	TPZ	FEP	MEM	CAZ	PB	AMP	SXT	
	0	9mm	0	0	0	0	0	20mm	9mm	0	
	CTR+B	CEC+B	AMC+B	TPZ+B	FEP+B	MEM+B	CAZ+B	PB+B	AMP+B	SXT+B	
	10mm	17mm	10mm	13mm	15mm	14mm	12mm	20mm	14mm	6mm	

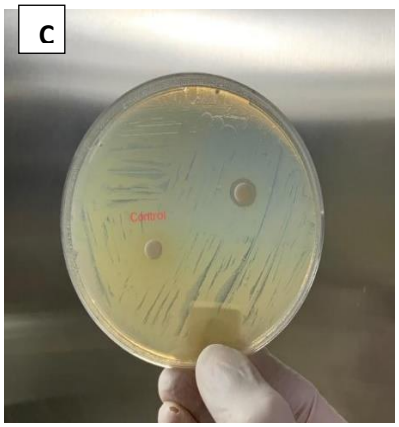
DISCUSION

The venoms of most insects including honey bees consisted of peptides, enzymes, protein, and a verity of smaller molecules. The pharmacological and biochemical activities of the various stinging insect venoms remarkably convergent [35]. Most venoms induce immediate pain, contain phosphlipases, hyaluronidase, and other enzymatic activities, and are capable of destroying red blood cells [36].The antimicrobial activity of homogenous whole bee extract against the tested pathogens may be due to the presence of several peptides like melittin, apamin, adolapin, mast cell degranulation peptide, enzymes, biologically active amines and non-peptide component [37,38]. As a result, to increasing types of infections with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* that are exhibiting resistance to multiple antibiotics, there is an urgent need to develop new treatment and protocols depends on natural products [39].Antimicrobial activity against bacterial species like *Escherichia coli* [40]. Also, another study reported effective antimicrobial against *B.burgdorferi* which is a causative agent of Lyme disease [41]. These antimicrobial activities or anti-inflammatory of bee venom referrer to the presence of some bioactive substances such as adolapin, apamin, melittin, and mast cell degranulation peptides [42]. Bee venom peptides mechanism of action for targeting and killing various types of microbes has been studied for last year's [43-44]. This peptide which is one of the major components of bee venom have the ability to integration into target phospholipidbilayers 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 led to cell death and killing the microbes [46-47]. Furthermore, other reported mentioned that killing mechanisms of bee venom could be due to inhibiting DNA synthesis by ROS productions. The mechanism of much antibacterial and antitumor drug relationship with DNA topoisomerase [48].

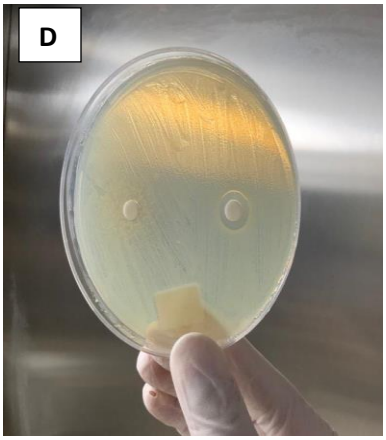
Klebsiella pneumonia OP426443



B- *Pseudomonas aeruginosa* OP428641

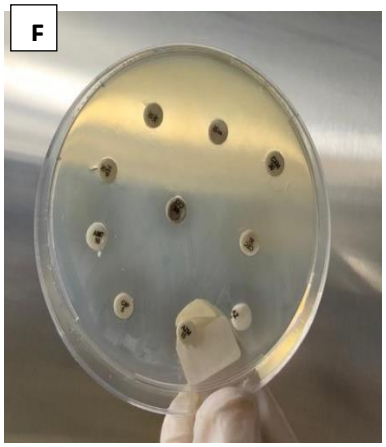


C- Bee effect on *Klebsiella pneumonia* OP426443



D- Bee effect on *Pseudomonas aeruginosa* OP428641

E-Bee effect on *Pseudomonas aeruginosa* with antibiotecs OP428641



F-Bee effect *on Pseudomonas aeruginosa* OP428641 with antibiotecs

Fig. (2) Showing inhibition zone of growth

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