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ORIGINAL ARTICLE

Synergistic Effect of the Bee Venom with Traditional Antibiotics on Multi-Drug Resistant Bacteria

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ABSTRACT

Background*: The number of diseases caused by multidrug-resistant (MDR) bacteria is increasing across the world. The potential risk of untreatable MDR pathogenic bacteria has been looming since the beginning of the 21st century. In last years, numerous researches have been conducted and confirmed the antimicrobial activity of natural products. Bee venom (BV) is a potent natural substance that has antibacterial properties. The objective of the current study was to evaluate the antibacterial properties of bee venom alone or in combination with several traditional antibiotics.* ***Methodology****: Fourteen multi-drug resistant bacteria were isolated, molecularly identified using the 16s rRNA gene sequencing technique, and submitted to Genbank database to get accession numbers. The antibacterial efficacy of bee venom was determined using the disc diffusion method.* ***Results:*** *BV showed high effective action with concentration 0.35 µg/mL in combination with six groups of traditional antibiotics if compared to the antibiotics or bee venom alone. The addition of BV increased the effectiveness of the antibiotics and increased their antagonistic potency. Bee venom chemical composition was assessed using GC/MS giving Azoxystrobin and Tebuconazole as major compounds.* ***In a conclusion****, our study addressed the issue of antibiotic resistance by offering a natural substance as a supportive to pharmaceutical therapies.*

INTRODUCTION

The pathogenic bacteria are bacteria which cause diseases, the majority of bacterial species are benign and frequently helpful, but others can spread infectious diseases, less than 100 of these pathogenic species are thought to exist in humans [1]. pathogens can spread disease once they enter the body, the host is all that a pathogen requires to grow and survive [2]. The pathogen enters a host's body, avoids the immune system's defenses, and uses the host's resources to replicate before leaving and infecting a new host, depending on the type, there are various methods that pathogens can be spread, they can be transmitted by skin-to-skin contact, biological fluids, airborne particles, feces, touching a surface, and more [3].

One significant public health concern is the emergence of multidrug resistance to antibiotics in commensal microorganisms, Commensal bacteria are opportunistic pathogens that cause a significant portion of hospital and community-acquired bacterial infections, examples of these bacteria *Streptococcus pneumoniae* and *Escherichia coli* [4].

The number of diseases caused by multidrug-resistant (MDR) bacteria is increasing across the world, and the dangerous of untreatable pathogenic bacteria has been looming since the beginning of the 21st century, while antibiotics have allowed the development of several fields of medical practice [5]. The prevention and control of diseases that are resistant to several drugs is one of the most significant areas where novel antimicrobials are required [6].

Numerous researches conducted in the last several years the antibacterial activity of natural products in a variety of bioactivities, bee venom is a potent natural substance that has antibacterial properties. BV was first used therapeutically in Ancient Egypt (4000 BC) to treat the inflammatory diseases like rheumatoid arthritis, tendinitis, fibrosis, lupus, and multiple sclerosis in Traditional Chinese Medicine and other historical methods[7].

Therapeutic application of bee venom has been well investigated against different type of bacteria, Gram-negative and Gram-positive as *Escherichia coli*, *Staphylococci aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and others [8-9]. A gland in the bees' abdominal cavity secretes Api-toxin, or bee venom (*Apis mellifera*), bees frequently employ this clear, acidic liquid with no smell as a weapon in their defense against potential predators, the venom of honeybees, which is marketed under the names Apitoxin or Apitox, is a mixture of several substances [10]. BV is composed of a very complex mixture that contains at least 18 active components, including peptides, enzymes, and amines [11]. So, the purpose of the current study was to evaluate the antibacterial properties of bee venom alone or in combination with several traditional aantibiotics.

METHODOLOGY

Collection and processing of samples

Thirty bacterial samples were collected randomly from waste water near hospitals, the samples were collected using sterilized test tubes that being transferred to the laboratory for the bacterial isolation.

Microbial isolation and purification

The collected samples were streaked onto nutrient agar plates. All of these dishes were then placed in an incubator and kept at 37°C for 18 to 24 hours. Bacterial colonies that had developed on the dishes were isolated and purified on new Petri dishes. A pure culture of each distinct colony type was obtained.

Antibiotic susceptibility test

A systematic method for evaluating the effectiveness of antibiotics against fast-growing infections is disk diffusion using the Kirby-Bauer method [12]. In short, Muller Hinton (MH) agar was surface-swabbed with a standardized inoculum (direct suspension of colonies to create a standardized inoculum is acceptable). Fresh subcultures were utilized since reproducibility is dependent on the log growth phase of the organisms. After an overnight incubation period, filter paper disks coated with an antimicrobial agent at a standardized concentration were placed on the surface, and the size of the zone of inhibition surrounding the disk was measured. Twenty antibiotics belong to six different groups according to the mode of action were used, with definite concentrations indicated in brackets, Trimethoprim (25mcg), Streptomycin (10mcg), Amikacin (30mcg), cefalexin (10mcg), Azithromycin (15mcg), Doxycycline (30mcg), Tetracycline (30mcg), Ciprofloxacin (5mcg), Ofloxacin (5mcg), Levofloxacin (5mcg), Cefuroxime (30mcg), Cefoxitin (30mcg), Ceftriaxone (30mcg), Cefaclor (30mcg), Amoxicillin (25mcg), Penicillin (110mcg), Meropenem (10mcg), Ceftazidime (30mcg), Ampicillin (10mcg), Cefepime (30mcg). The agar dilution assay involved the inoculation of bacteria into a nutrient agar medium that has doses of antibiotics. Agar disc diffusion method was frequently employed. After the incubation period, the inhibition zones surrounding the discs were measured in millimeters (mm) using a metric ruler. These measurements were then compared with the Clinical Laboratory Standards Institute (CLSI, 2020) to determine which of the three inhibition zones was susceptible (S), intermediate (I), and resistant (R).

Extraction and preparation of bee venom

Venom sacs of bees were extracted in an ethanol-filled container. Using a vortex, the solution was thoroughly mixed and prepared as described by Hegazi *et al.*[8]. At 37ºC, the solution was allowed to dry. In sterilized water, the dried venom was dissolved, finally ethanol was evaporated and 4% dimethyl sulfoxide was added to make definite concentration.

Gas chromatography/mass spectrometry (GC/MS) analysis of the bee venom

By comparing the retention durations and mass spectra of each chemical with those from the National Institute of Standards and Technology (NIST), WILEY library database in the GC/MS instrument, the compounds were tentatively identified [9].

Antimicrobial activity of bee venom

The antimicrobial activity of the bee venom was evaluated using the disc diffusion method, a technique based on the diffusion of antimicrobial compounds in a solid medium[8]. The diameter of this inhibition zone was measured in millimetres, with larger diameters indicating higher susceptibility of the bacterial strain[13].

Antimicrobial interactions

The initial stock concentration of each extract was mixed with antibiotics Trimethoprim, Streptomycin, Amikacin, Cefalexin, Azithromycin, Doxycycline, Tetracycline, Ciprofloxacin, Ofloxacin, Levofloxacin, Cefuroxime, Cefoxitin, Ceftriaxone, Cefaclor, Amoxicillin, Penicillin, Meropenem, Ceftazidime and Ampicillin, Incubation condition were maintained at 37°C for 24 hours. the obtained results were compared with those of antibiotics tested alone on the same strains using the same method, as outlined by Moussaoui and Alaoui [14].

Bacterial molecular identification

Genomic DNA extraction was performed in accordance with conventional bacterial protocols in order to identify the isolated bacteria. Particular primers were used to amplify the 16S rRNA gene: reverse primer R1 (GGT TAC CTT GTT ACG ACT T) and forward primer F1 (AGA GTT TGA TCC TGG CTC AG). A UV transilluminator was used to visualize the DNA bands. The PCR products were sequenced at SIGMA-Biotech in Constance, Germany, and the NCBI Genbank database (www.ncbi.nlm.nih.gov) was used to align the resulting DNA sequences.

RESULTS

Multi-drug resistance study

Six groups of antibiotics were used to treat a total of twenty bacterial isolates. Trimethoprim (25mcg), Streptomycin (10mcg), Amikacin (30mcg), Cefalexin (10mcg), Azithromycin (15mcg), Doxycycline (30mcg), Tetracycline (30mcg), Ciprofloxacin (5mcg), Ofloxacin (5mcg), Levofloxacin (5mcg), Cefuroxime (30mcg), Cefoxitin (30mcg), Ceftriaxone (30mcg), Cefaclor (30mcg), Amoxicillin (25mcg), Penicillin (110mcg), Meropenem (10mcg), Ceftazidime (30mcg), Ampicillin (10mcg), and Cefepime (30mcg). The findings demonstrated that the fourteen bacterial isolates that were chosen to complete our analysis were those that showed resistance to at least three classes of antibiotics.

Molecular Identification for the fourteen MDR bacterial isolates

A total of fourteen bacterial isolates were obtained, the MDR isolates (designated as Bac1to Bac14) were selected. The Blastx program (BLAST), National Centre for Biotechnology Knowledge, was utilized to compare the DNA sequences with unknown sequences. The bacterial isolate Bac1 was identified as ***Pseudomonas mendocina*** using 16S rRNA gene sequencing technique. Sequence of the strain was submitted to Genbank database and had accession number **OR534004.** The bacterial isolates Bac2, Bac12, and Bac14 were identified as ***Klebsiella pneumoniae*** using 16S rRNA gene sequencing technique. Sequences of strains were submitted to Genbank database and had accession numbers, respectively **OR534220, OR533569,** and **OR534221 (Table 1).** The bacterial isolates Bac3 and Bac13 were identified as ***Escherichia coli***. Sequences of strains were submitted to Genbank database and had accession numbers **OR534005** and **OR534006**, respectively**.** Bac4 to Bac11 were identified as ***Pseudomonas aeruginosa*** under accession numbers **OR534222, OR534224, OR534225, OR534226, OR534227, OR534228, OR534229** and **OR534230**, respectively**. Fig. (1)** shows the phylogenetic trees and accession numbers of the fourteen MDR bacterial isolates.

Table 1: Shows the molecular identification of the fourteen MDR bacterial species and their accession numbers on Genbank database

Bacteria no	Bacterial species	Accession number
Bac 1	*Pseudomonas mendocina*	OR534004
Bac 2	*Klebsiella pneumoniae*	OR534220
Bac 3	*Escherichia coli*	OR534005
Bac 4	*Pseudomonas aeruginosa*	OR534222
Bac 5	*Pseudomonas aeruginosa*	OR534224
Bac 6	*Pseudomonas aeruginosa*	OR534225
Bac 7	*Pseudomonas aeruginosa*	OR534226
Bac 8	*Pseudomonas aeruginosa*	OR534227
Bac 9	*Pseudomonas aeruginosa*	OR534228
Bac 10	*Pseudomonas aeruginosa*	OR534229
Bac 11	*Pseudomonas aeruginosa*	OR534230
Bac 12	*Klebsiella pneumonia*	OR533569
Bac 13	*Escherichia coli*	OR534006
Bac 14	*Klebsiella pneumonia*	OR534221

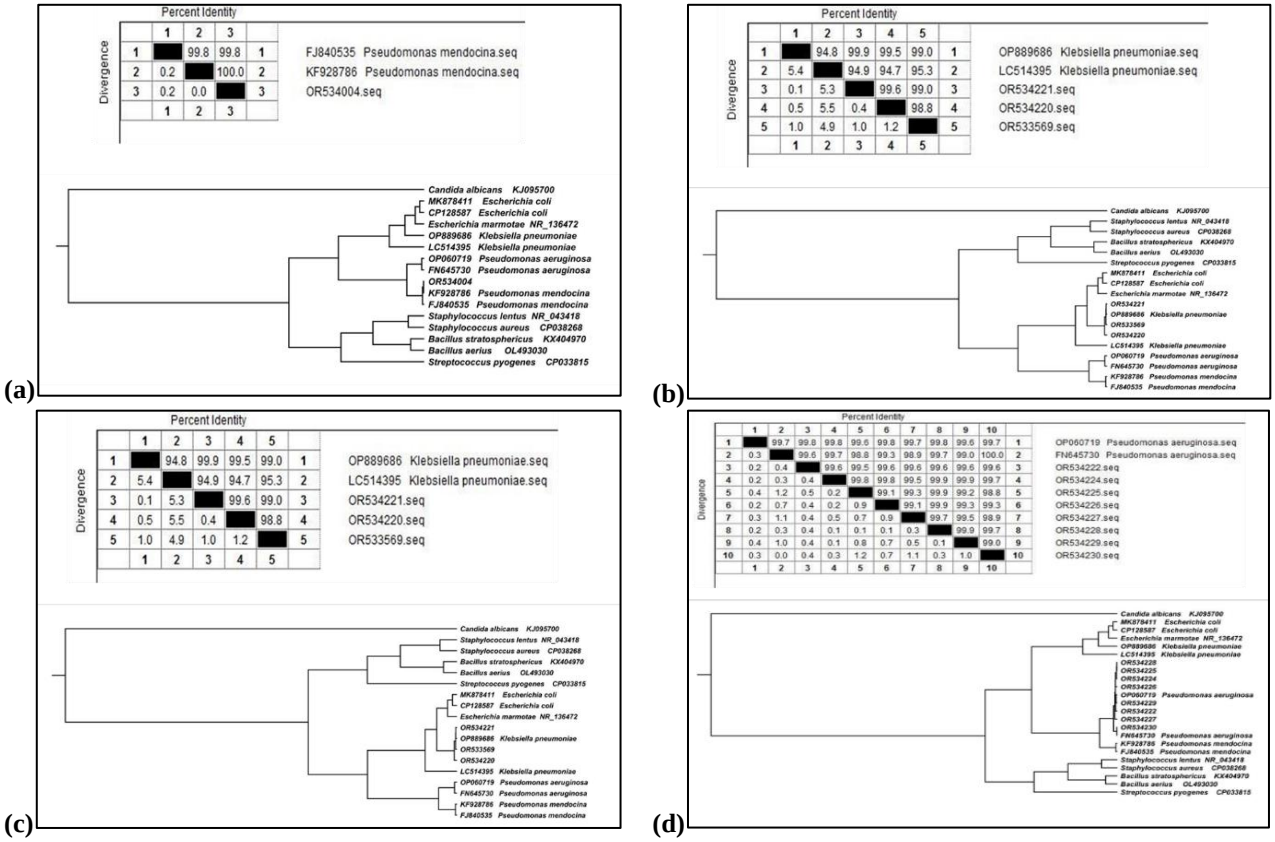

Fig. 1: a) Shows the phylogenetic tree and the accession number of isolate one (*Pseudomonas mendocina*). b) Shows the phylogenetic tree and the accession numbers of isolates two, twelve, and fourteen (*Klebsiella pneumonia).* C) Shows the phylogenetic tree and the accession numbers of isolates three and thirteen (*Escherichia coli*), d) Shows the phylogenetic tree and the accession numbers of isolates from four to eleven (*pseudomonas aeruginosa).*

Chemical composition of the bee venom

The GC/MS study of bee venom revealed 15 organic compounds with Tebuconazole and Azoxystrobin as the main constituents (Table 2).

Table (2) Chemical composition of bee venom using GC-MS

Compound	RT (min)	Peak area %	Molecular formula	Molecular weight (g/mol)	Medical Effects
3- (Methoxy methylene)2(3H) benzofuranone	38	0.94	$C_{10}H_8O_3$	176.17	Antitumor Antibacterial, antioxidant-Antiviral activity [15-16].
Hexadecanoic acid, methyl ester	48	2.18	$C_{17}H_{34}O_2$	270	Inhibit the growth of pathogenic bacteria especially multidrug resistant bacteria e.g., *K.penuomonia &Pseudomonas* Spp .[17-18].
Methyl stearate	54	2.42	$C_{19}H_{38}O_2$	298	Antibacterial & antifungal [18].
Kaur-16-en-18-oic acid methyl ester	57	0.68	$C_{21}H_{32}O_2$	316	Antifungal- anti-inflammatory- Antioxidant, has positive effect with MDR bacteria [19].
Tebuconazole	62	51.5	$C_{16}H_{22}CIN_3O$	307	Antifungal &decrease the bacterial motility [20].
3,3-Dimethylthienyl (thiophene derivatives)	63	0.52	$C_{12}H_{10}O_2S_2$	250	Antimicrobial -anti-inflammatory- antifungal- anticancer [21].
Belcomethasone-propionate	67	0.72	$C_{25}H_{33}ClO_6$	464	Fight pulmonary disease [22].
Docosenamide(z)	73	0.88	$C_{22}H_{43}NO$	337	Antibacterial activity against gram negative and gram positive [23].
Schisandrol B	74	1.22	$C_{23}H_{28}O_7$	416	Protect against cholestatic liver injury [24]. Antibacterial activity [25].
Cholesta-3,5-diene	76	1.09	$C_{27}H_{44}$	368	Antibacterial [26-27].
Azoxystrobin	79	30.41	$C_{22}H_{17}N_3O_5$	403	antifungal, antibacterial & make synergetic effect with antibiotics [28].

Inhibitory effect of bee venom

Table (3) showed the inhibitory effect of bee venom only against 14 bacterial isolates of MDR bacteria which revealed that the bee venom had high inhibitory effect with bacterial isolates 2,3,4,13 and14 and moderate activity with bacterial isolates 6,7,8,9,10,11, and 12 and weak activity with bacterial isolates 1 and 5.

Table 3: Inhibitory effect of bee venom against MDR bacteria (IZ=mm)

Bacterial isolates	Bee venom (IZ= mm)
Bac 1	6
Bac 2	10
Bac 3	12
Bac 4	15
Bac 5	6
Bac 6	9
Bac 7	7
Bac 8	7
Bac 9	8
Bac 10	8
Bac 11	8
Bac 12	9
Bac 13	10
Bac 14	10

In vitro antimicrobial combination assay

Figs 2- 7 showed the antimicrobial activity of bee venom against MDR species, *Pseudomonas mendocina*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* and revealed that, the bee venom had inhibitory effect on the growth of current bacteria. The inhibitory effect in some cases increased by the combination between the bee venom with antibiotics and in other cases an increase was observed in case of bee venom alone without combination. The results revealed that the aminoglycoside antibiotics group had resistant effect on all bacterial isolates except bacterial isolates 3 &12 that it had moderate inhibitory effect according to CLSI and the inhibitory effect increased when combined with the bee venom in all bacterial isolates. The macrolides antibiotics group had sensitive effect on isolates 5&11 and resistant effect on the remaining bacterial isolates according to CLSI and become more effective when combined with bee venom. The results also showed that, the tetracycline antibiotics groups had sensitive effect on bacterial isolate 14 only, moderate with 2,12 &13, and resistant with the remaining isolates and the inhibitory effect increased on all isolates when combined with bee venom. Fluroquinolones antibiotics group had no effect on all isolates and the inhibitory effect increased to be sensitive when combines with bee venom on all bacterial isolates. Cephalosporine antibiotics group has

no inhibitory effect on all bacterial isolates and it increased to be more effective when combined with bee venom. The results revealed that the penicillin antibiotics group had no inhibitory effect on all bacterial isolates and the bacterial isolates increased to be more sensitive when antibiotics combined with bee venom.

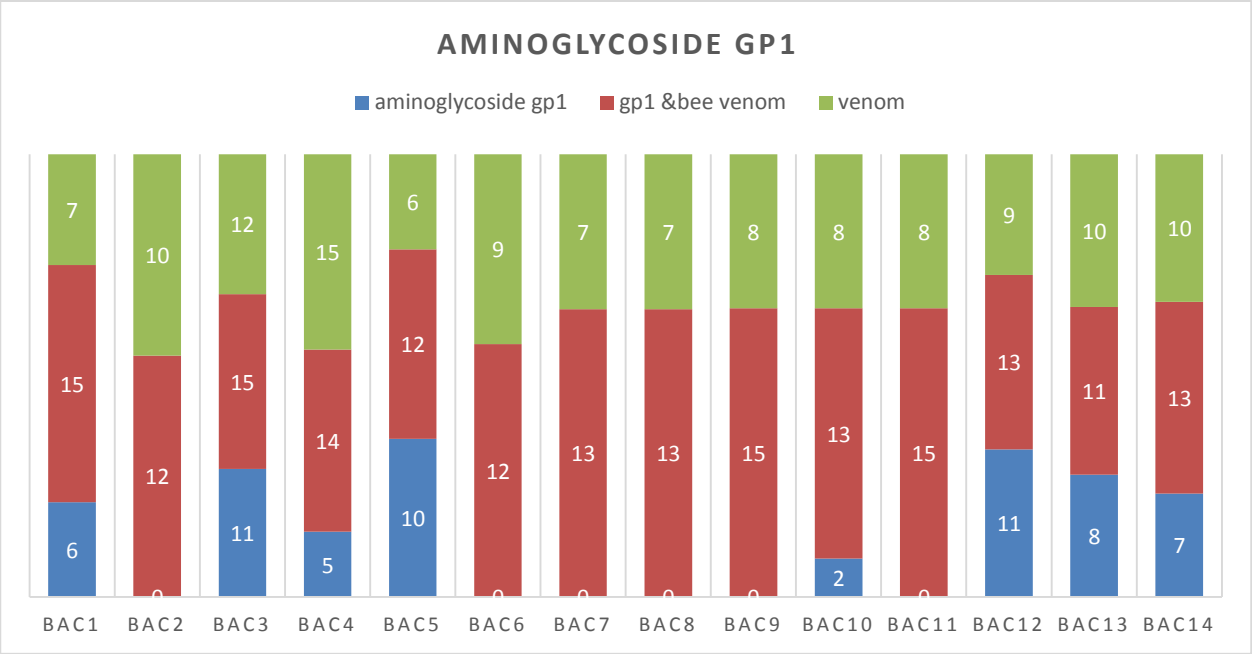

Fig. 2: The antibacterial effect (inhibition zones = mm) of aminoglycoside Gp1 antibiotics, bee venom, and combination between them (Gp1 and venom)

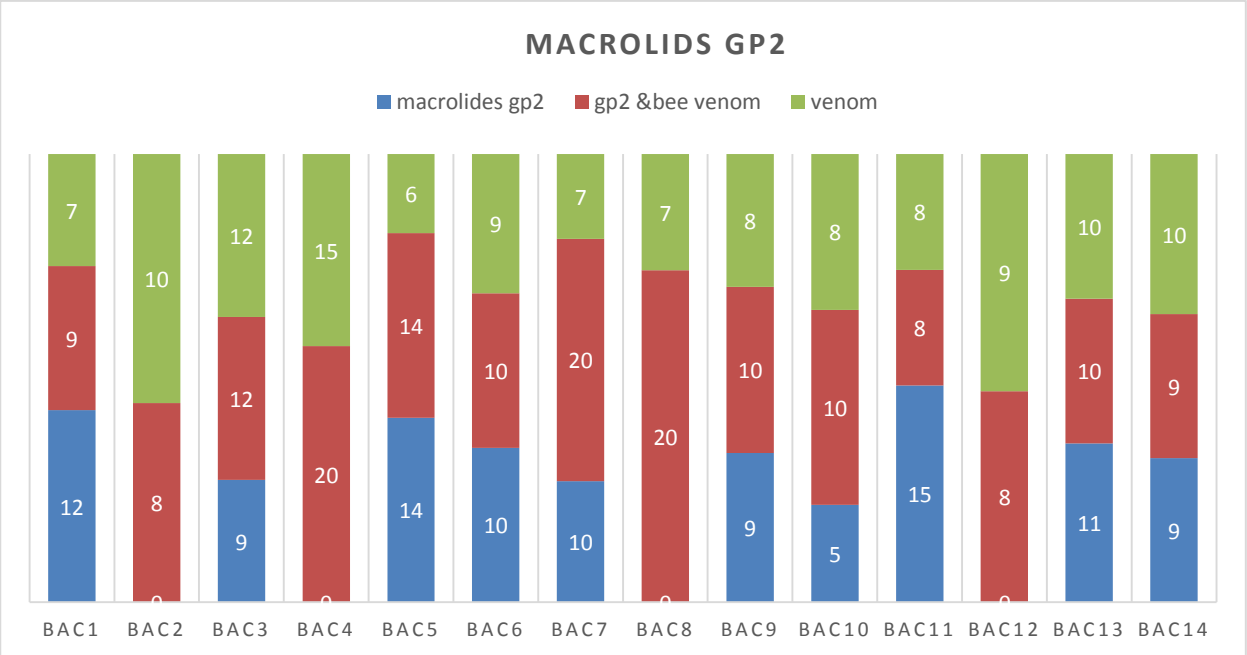

Fig. 3: The antibacterial effect (inhibition zones = mm) of Macrolides Gp2 antibiotics, bee venom, and combination between them (Gp2 and venom)

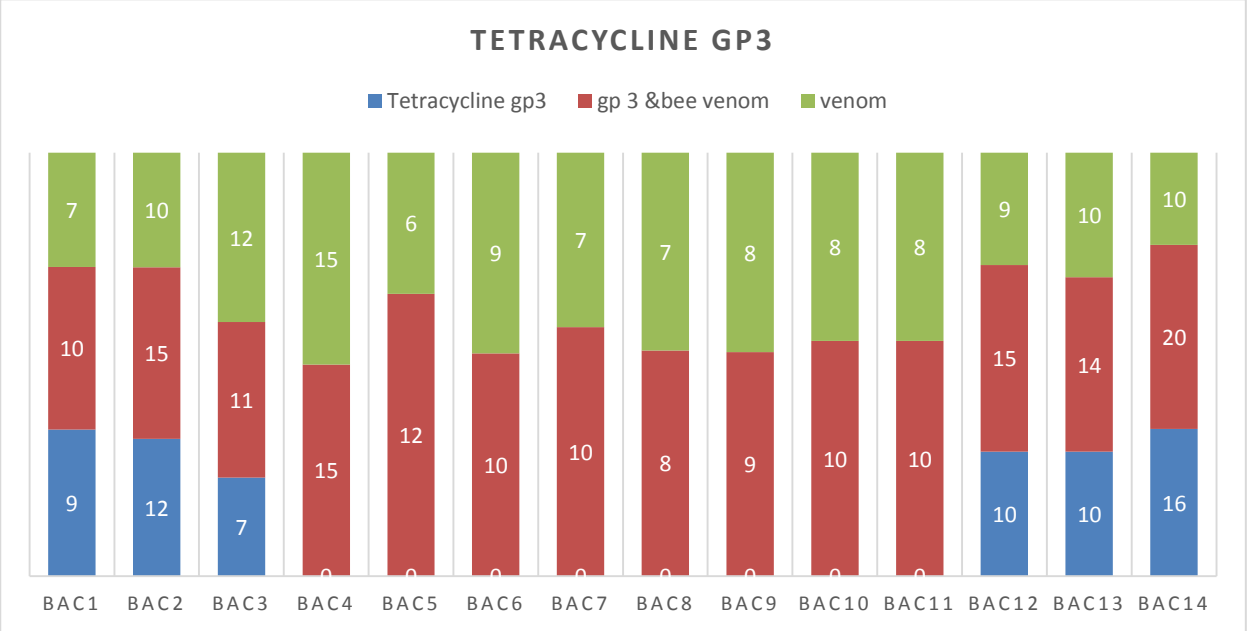

Fig. 4: The antibacterial effect (inhibition zones = mm) of Tetracycline Gp3 antibiotics, bee venom, and combination between them (Gp3 and venom)

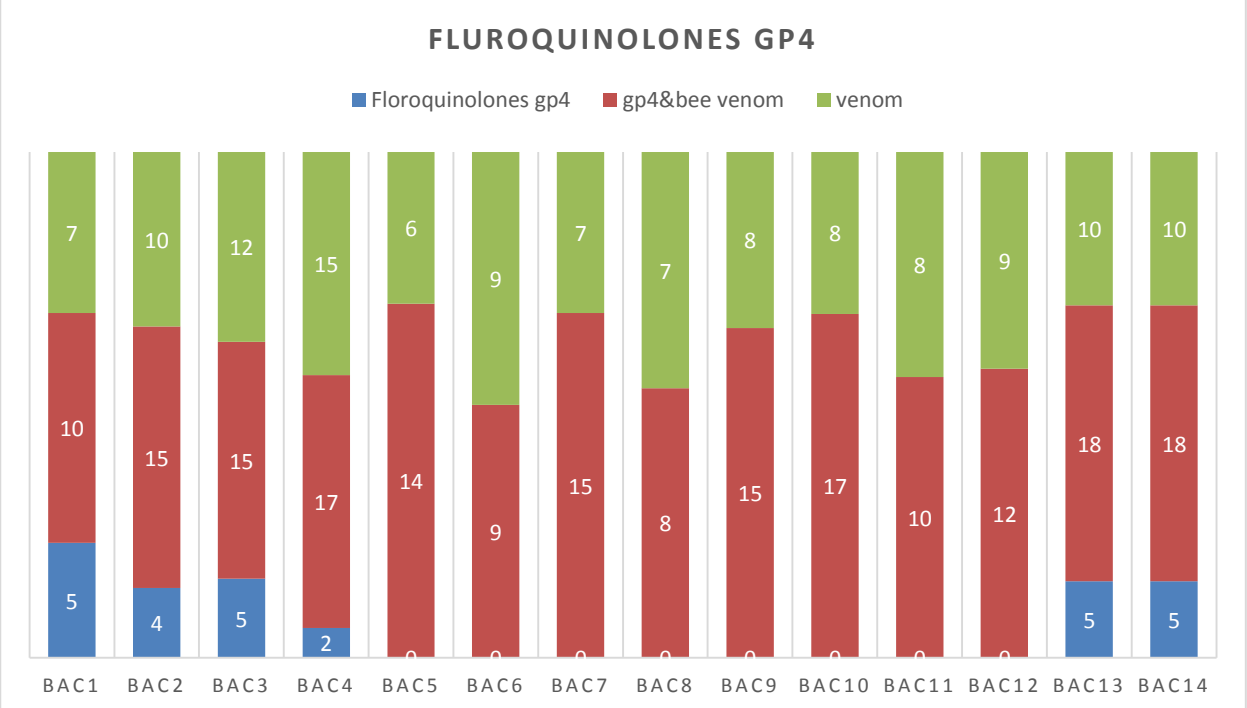

Fig. 5: The antibacterial effect (inhibition zones = mm) of Fluroquinolones Gp4 antibiotics, bee venom, and combination between them (Gp4 and venom)

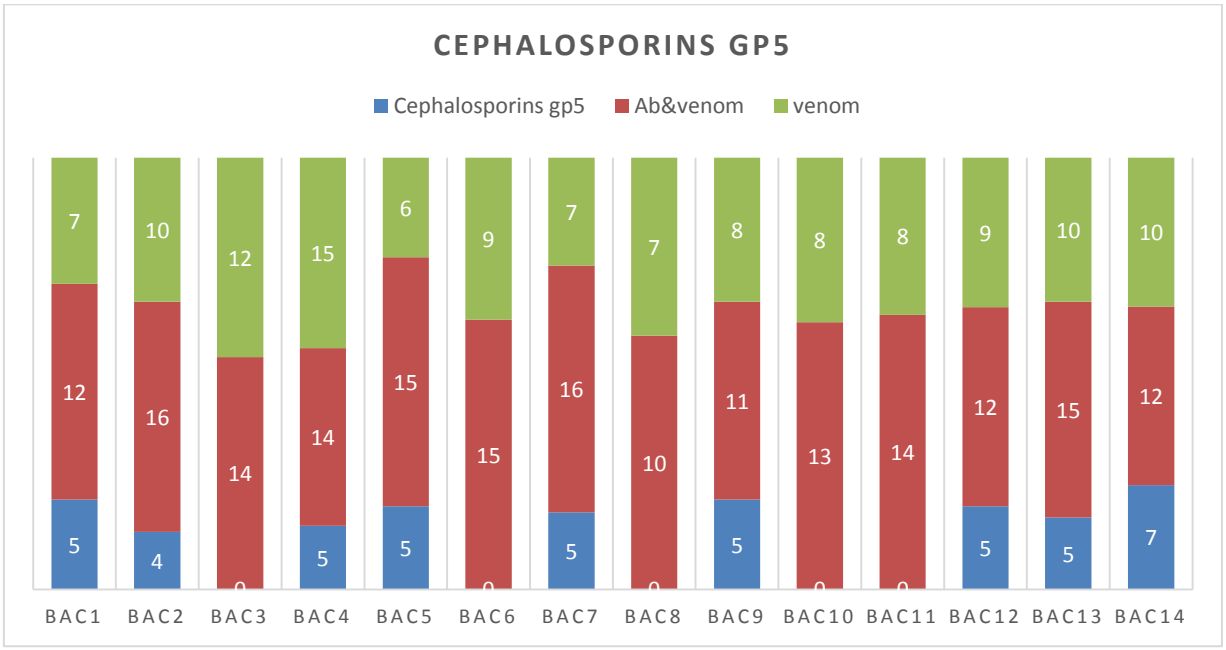

Fig. 6: The antibacterial effect (inhibition zones = mm) of Cephalosporins Gp5 antibiotics, bee venom, and combination between them (Gp5 and venom)

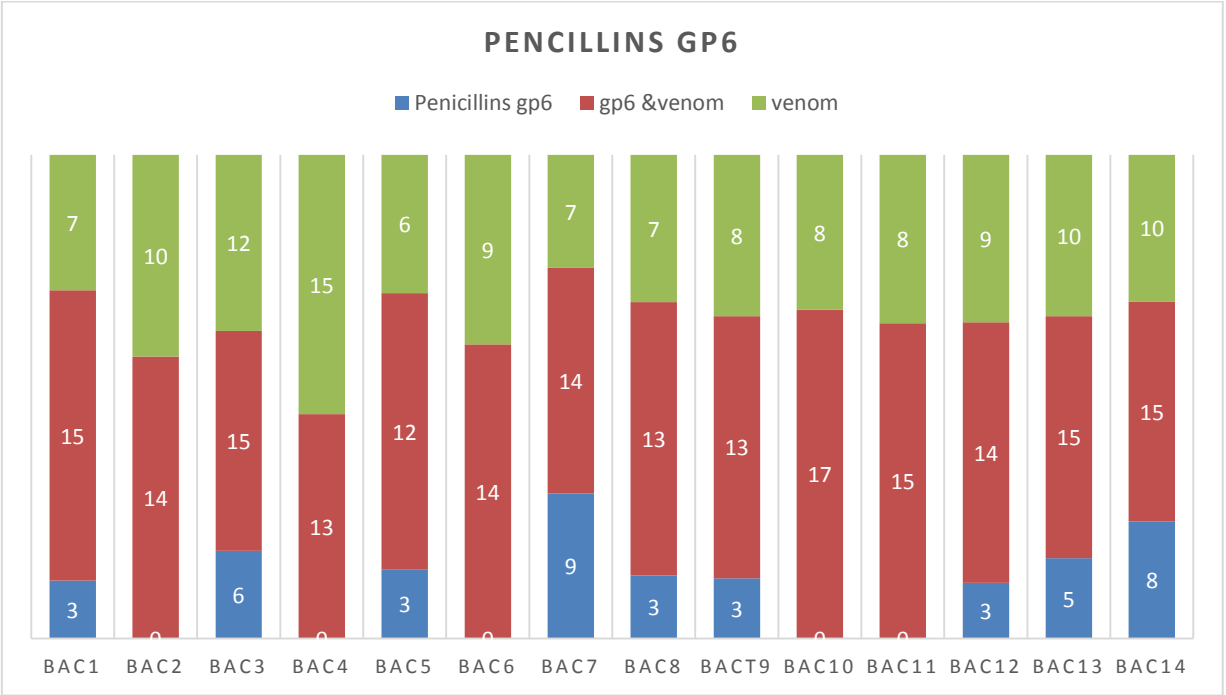

Fig. 7: The antibacterial effect (inhibition zones = mm) of Pencillins Gp6 antibiotics, bee venom, and combination between them (Gp6 and venom).

DISCUSSION

The most significant public health challenge facing all of humanity is still antimicrobial resistance (AMR), The World Health Organization (WHO), located in Geneva, Switzerland, reports that antibiotic resistance is increasing globally to dangerously high levels, which is increasing morbidity and mortality [12]. By 2050, it's predicted that there won't be any viable antibiotics accessible to treat these lethal resistant bacteria if, new innovative medications aren't found or developed, for this reason, we have to look for the formulation of some new novel drugs or other options or substitutes to treat

such multidrug-resistant microorganisms (MDR) [29]. Our target is to use natural products that has antimicrobial effect to improve the action of antibiotic, these natural products may be oils, plant extract, algae, bees and their products [30]. Worldwide, apitherapy is becoming a popular therapeutic approach with applications in numerous medical fields. Additionally, bee venom can be used as a raw material for drugs or disinfectants, bridging the gap in natural products that can be used to combat resistant bacteria [6]. In this study, 16S rRNA was used for the identification of fourteen bacterial isolates which detected that, bacterial isolate (1) was *Pseudomonas mendocino,* bacterial isolates (4-11) were *Pseudomonas aeruginosa*, bacterial isolates (2,12, and 14) were *Klebsiella pneumoniae and* bacterial isolates (3 and 13) were *Escherichia coli.* After the antimicrobial sensitivity tests using six groups of antibiotics (Aminoglycoside, Macrolides, Tetracycline, Fluroquinolones, Cephalosporine, and Penicillin), the results revealed that the fourteen isolates resisted at least three groups of antibiotics were considered as multidrug resistant bacteria (MDR) [31]. Our investigations showed that higher inhibitory effect of bee venom alone against our studied bacteria and the antibacterial activity of the venom against gram negative bacteria especially *E. coli & Pseudomonas* spp were observed. Varied results based on the condition of treatment, it might be affecting the membrane integrity and the plasma membrane potential of *E. coli* cells in association with significant loss of viability[33]. Our results are in compatible with Gavanji & Bakhtari. [33] who showed that BV had better inhibitory effects against *K. pneumoniae* and *P. aeruginosa*, even though both are Gram-negative bacteria. The inhibitory differences are probably due to the antimicrobial resistance pattern and also compatible with those obtained by Zolfagharian *et al*. [34] who evaluated the effect of BV on several Gram positive and Gram negative bacteria including *S. aureus*, *S. typhimurium*, and *E. coli*. The results indicated that BV had a better inhibitory effect on *E. coli* than on other Gram-positive bacteria. Although, in an earlier study Hegazi *et al*.[35] showed that bee products were less effective against *E. coli*, Hegazi *et al*.[8] provided an evidence that bee venom has antibacterial activity against both Gram-positive and Gram-negative bacteria with no significant differences between both groups. In contrast, Fennel *et al.* [36] reported that the bee venom inhibitory effect was more active against gram positive more than Gram negatives. These results agreed with Kondo and Kanai. [37] who found that *Mycobacteria* and *Staphylococci* were affected by the chemical mechanism of venom fraction (melittin), but not *E. coli*. Ortel and Markwrdt.[38] quantitatively determined the zones of inhibition. They found that Gram positive organisms were sensitive at lower concentrations of bee venom than Gram negative bacteria.

Our findings also demonstrated that the highest inhibitory effect was by the interaction between the bee venom and each group of the used six groups if compared to the bee venom, or antibiotics alone. Kamel *et al*. [39] have reported that the bee venom demonstrated better action when taken with antimicrobial medications, indicating synergism against variety of multi-drug resistant bacteria especially *P. aeruginosa*. Our results are compatible with results of Han *et al.* [40] who detected that the BV showed both antibiotic-enhancing and antibacterial activity against MRSA strains. MRSA treated by BV showed an increase in the (Atl) gene, (is a peptidoglycan hydrolase involved in bacterial cell wall degradation and cell separation during cell division) indicating that cell division was disrupted. As a synergistic combination of antibiotic potency and a natural antibacterial agent, also compatible with Gökmen *et al*.[6] who showed that the effective bacterial inhibition rate of bee venom with the antimicrobial drugs suggested that it could be a potential antibacterial agent for multi-drug resistant pathogens.

Our GC-MS results for bee venom showed that our venom is composed of several compounds some of them act as antimicrobial, anti-pulmonary diseases, and other protect against cholestatic liver injury compounds and more of them act as antibacterial agents as 3-(Methoxy methylene)2(3H) benzofuran, comparing benzofurans with a number of reference antibiotic medications, the antibacterial activity of benzofurans is comprehensively studied against a variety of bacterial infections (both Gram-positive and Gram-negative bacteria) and fungal pathogens and that agreed with Abbas & Dawood [16] who have reported that key component of many physiologically active natural and artificial heterocycles is the benzofuran molecule. These heterocycles are used in several medicinal treatments and have unique therapeutic potential. In order to explore the antibacterial potential of benzofuran-based heterocycles, a number of researches have addressed their synthesis and extraction derivatives of benzofurans are a significant class of heterocyclic chemicals with well-established biological functions [15].

Hexadecenoic acid methyl ester which inhibit multidrug resistant bacteria as previously reported by Abubacker & Deepalakshmi [17]; Shaaban *et al*. [18]. They showed the compound hexadecenoic acid methyl ester had a good inhibitory effect against Gram negative and Gram positive bacteria especially when tested against *S. aureus, P. aeruginosa,* and *K. pneumoniae*. Azoxystrobin which act as antibacterial and antifungal and decrease the motility of bacteria and act synergetic effect with antibiotics[41]. Tebuconazole from our GC-MS major compounds which act as an antimicrobial, tends to increase the Gram positive bacteria but decrease the Gram negative bacteria as it prevents the development of bacterium cells, but does not affect cell breakdown [42]. So, the main target of most of compounds

of bee venom is the cell membrane of bacteria, it also may interfere with cellular energy production or inhibition of enzyme activity and make direct lysis of bacterial cell as shown by Suresh *et al*.[43].

CONCLUSION

In the view of positive results of the effects of bee venom in controlling the multi-drug resistant bacteria and increasing their synergetic effect with the antibiotics. The impact of MDR on health may be minimized if safe medication combinations are developed with the help of a better knowledge of the mechanisms guiding synergism.

Declarations:
Consent for publication: Not applicable
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Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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