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Original Research Article

Evaluating the Biological Activity of *Apis mellifera* Extract as an Antibacterial Agent against Pathogenic Bacteria

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Abstract: ***Objective***: In the present study, the biological activity of the hexane extract of the honey bee *Apis mellifera* was evaluated against different pathogenic bacteria, as an alternative to synthetic antibiotics to prevent side effects and bacterial resistance. ***Methods of Work***: Bacterial samples were collected from medical laboratories and outpatient clinics in the city of Tikrit; eight isolates of pathogenic bacteria previously identified using the VITEK system were collected, comprising *K. pneumoniae* , *P.auroginosa* , *P.fluorescence* , *Staph. haemolyticus* , *E. aerogense* , *S. aureus* , *P. vulgaris* ,*E. coli* . Samples of the honeybee *Apis mellifera* were collected from the Tarmiyah area, north of Baghdad Governorate, using standard insect collection methods. Extracts were prepared at four concentrations (25, 50, 100, and 200 mg/mL) using hexane as the solvent. The tests were conducted using the agar well diffusion method. ***Results*:** The results showed that the 200 mg/ml concentration exhibited the highest inhibitory efficacy, with a mean inhibition zone diameter of 17.1 mm, significantly outperforming the other concentrations, while the lowest concentration (25 mg/ml) showed the least efficacy, with a mean inhibition zone diameter of 9.7 mm. The Gram-positive bacterium *S. haemolyticus* demonstrated the highest sensitivity, with an inhibition zone diameter of 18.0 mm. In contrast, *E. coli* (Gram-negative) exhibited the weakest response, with an inhibition zone diameter of 8.25 mm. The inhibitory activity of the extract against the studied bacterial isolates, including multidrug-resistant (MDR) strains, is attributed to the specific biochemical composition of the honeybee extract. The superior performance of the hexane extract is attributed to its high capacity for extracting non-polar, hydrophobic organic compounds such as free fatty acids, their esters, and lipophilic substances, which possess a strong ability to penetrate the lipid layers of microbial cell membranes; this highlights the importance of solvent selection in determining the extract's biological activity.

Keywords: Biological Activity, Antibacterial, *Apis Mellifera, Pathogenic Bacteria*.

INTRODUCTION

Bacterial resistance to antibiotics constitutes one of the greatest challenges facing humans today in the world [1, 2], as it is considered one of the worst threats to public health in our century due to the huge loss of life [3]. The emergence of new strains of pathogens (bacteria, viruses, fungi, and protozoa) that are resistant to most current antibiotics has led to the necessity of finding alternatives to antibiotics that are effective in resisting pathogenic microorganisms [4]. Insects are one of the most widespread and successful creatures on earth. They exist in different environments and spread throughout the world, Insects represent mechanical vectors or intermediate or final hosts for many pathogens without being affected by these pathogens [5]. Extensive antibiotic utilization is critically linked to the emergence of multidrug-resistant bacteria, thereby escalating risks to human health [6]. This phenomenon underscores the necessity of establishing efficacious therapeutic strategies designed to abrogate pathogen replication while ensuring a favorable safety profile [7]. Insects can have promising results in the field of eliminating microbes [8]. The fact that they are resistant to microorganisms has been known for a long time through the production of antimicrobial peptides, which represents one of the mechanisms of resistance to microorganisms [9]. The body of the *Apis mellifera* possesses a complex and diverse biochemical composition that confers potent natural defensive

properties against pathogenic microorganisms, as an array of biologically active compounds within the bee's tissues and glands work synergistically to eradicate microbial threats Antimicrobial peptides (AMPs) occupy a primary position in this defensive hierarchy, with melittin the major component of bee venom standing out for its remarkable ability to penetrate bacterial cell membranes. This peptide induces physical and chemical instabilities in the membrane, leading to cell lysis, while other peptides, such as apidecin and apisin, interfere with intracellular vital processes, effectively hindering bacterial growth and proliferation [10]. Body extract of black soldier fly inhibited growth of human pathogenic bacteria, The prepared chitosan from grasshopper, *Calliptamus barbarus* chitin have antimicrobial activity against human pathogenic microorganisms with IC50 about 10 mg/ml. Also, extract of the eusocial insects *Apis mellifera*, *Monomorium pharoanis*, *Cremotogastor auberti* and *camponotus xerxes* had significant inhibition of four pathogenic bacteria in comparison with standard drugs, Ceftriaxone, Gentamycin and Tetracycline antibiotics [11]. Therefore, this study aims to evaluate the antimicrobial efficacy of honeybee extracts against various clinical bacterial pathogens. Furthermore, it seeks to explore potential bio-derived alternatives to conventional antibiotics, thereby addressing the growing challenges of antimicrobial resistance and the adverse side effects associated with synthetic therapeutic agents.

MATERIAL AND METHOD

1- Materials and Reagents

The chemical reagents used in this study were: Hexane, Chloroform, Dimethyl sulfoxide (DMSO, $SO(CH_3)_2$, M_W=78.13 g moL^{-1}), and Glycerol, were purchased from BDH, West Yorkshire, England. The media used for antibacterial applications were: Nutrient Agar, Nutrient broth Muller - Hinton Agar, obtained from Biolab, Samut Prakan, Thailand.

2- Organic Solvents Used:

Two organic solvents were tested using the sequential solvent extraction method based on polarity. The following solvents were selected:

Hexane:

A hydrocarbon with the chemical formula CH3(CH2)4CH3. The prefix "hex-" indicates the presence of six carbon atoms, while the suffix "-ane" denotes single bonds between the carbon atoms. It is used as a solvent in organic reactions; it has a molecular weight of 86.18 g/mol, a melting point of -95°C, and a boiling point of 69°C. It is non-polar.

3- Collect Insect Samples

Samples of (*Apis mellifera*) bees were collected from apiaries in the Tarmiyah district, north of Baghdad, between May 1, 2026, and June 1, 2026, at temperatures 38°C to 43°C, The samples were kept in special tubes until they were studied. After collecting the samples, they were dried in the shade, crushed, and stored in containers in the refrigerator at 4°C temperature.

4- Bacterial Isolates

The Gram-positive and Gram-negative bacterial isolates (*Klebsiella pneumonia, Pseudomonas auroginosa, pseudomonas fluorescence, Staphylococcus haemolyticus*, *Entrobacter aerogense, Entrobacter aerogense, Proteus vulgaris,* and *Escherichia coli*) were collected from the medical laboratories in Tikrit city, from people suffered from various medical conditions, such as wounds, burns, and UTI, for both sexes and different age groups. The isolated bacteria were grown on culture media specific to each species, and diagnosed using Vitek 2 system (VITEK, Biomérieux, Marcy-l'Etoile, France).

5- Preparation of Insect Extracts

Crushed dried insects were extracted using organic solvents (absolute methanol and ethanol) of different polarities to allow the extraction of various active substances, according to previous method (12), as follows:

1. Absolute methanol and ethanol solvent (100 ml of each alone) was mixed with 15g of powder (crude) of each insect, left for 24 hours under laboratory conditions with continuous stirring using a magnetic stirrer.
2. The extract is transferred into sterile glass tubes with 10 ml capacity and placed in a centrifuge at 1000 rpm for 10min.
3. The upper layer is pulled out using a fine pipette and transferred to new sterile tubes. Then, poured into glass dishes with 15 cm diameter, and left in front of an electric fan until it dries.
4. Collect the insect dry extract, the weight of which varies according to the type of insect and the solvent used in its extraction. A specific volume of DMSO is added to it according to the required concentration, and it is stored at 4°C until use.

6-Antibacterial Activity

The effect of insects extracts on the growth of bacterial isolates were evaluated using Mueller-Hinton agar medium and bacterial suspensions. The McFarland standard was used to obtain suspensions at $1.5*10^8$ CFU/ml. Mueller–Hinton agar medium plates were swabbed with each bacterial suspension, then four wells at 8mm diameter were punched out of each agar plate using a sterilized well cutter. The wells were loaded with 100 µL of insects extracts at four concentrations (200,100,50,25) mg/ml for each tested bacterium. Deionized water was used as the control. After incubation at 37 °C for 24 h, the inhibition zones were observed around each well and measured (to the nearest mm) to determine the antibacterial activity of insect's extracts [13].

7- Antibiotic Activity

Bacterial susceptibility was tested using the Kirby-Bauer disk diffusion method against five types of antibiotics: Ciprofloxacin, Vancomycin, Amoxicillin, Azithromycin, and Penicillin.

8- Statistical Analysis

Data for the current study were analyzed using Version 9 (SAS statistical software JMP, 2000). Following the Anova test for all types according to the extract and the solvent. The deviation rate of the inhibition zone was calculated using Dunkin's multiple ranges test at a probability level (P-value) ≤ 0.01 [3].

RESULTS

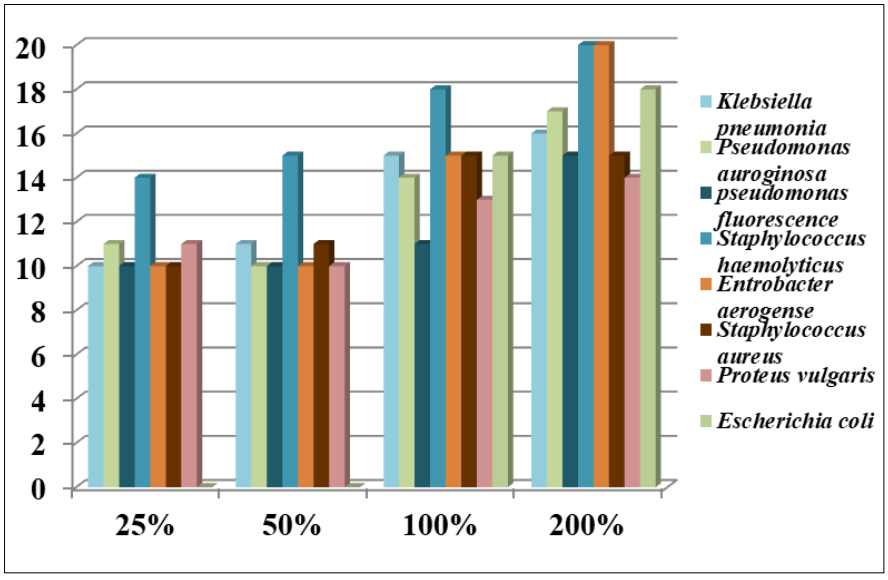
1- Antibacterial Activity of Bee Extract

The results of the current study, illustrated in Table (1) and the graph (1), demonstrated that the hexane extract of *Apis mellifera* exhibited significant inhibitory activity against the tested bacterial isolates. The highest concentration (200 mg/ml) showed the greatest inhibitory efficacy, with a mean inhibition zone diameter of 17.3 mm significantly outperforming the other concentrations whereas the lowest concentration (25 mg/ml) showed the least efficacy, with a mean inhibition zone of 9.7 mm. Concentrations of 50 and 100 mg/ml yielded comparable results (15.0 mm and 14.8 mm, respectively). The results also revealed a clear variation in the bacterial species' response to the extract; the Gram-positive bacterium *S. haemolyticus* exhibited the highest sensitivity, with an inhibition zone diameter of 18.0 mm, while the Gram-negative bacterium *E. coli* showed the lowest response, with an inhibition zone diameter of 9.0 mm.

Table 1: Inhibitory effect of *Apis mellifera* Hexan extract

Bacteria	Concentration mg/ml					
	25	**50**	**100**	**200**	**Average**	**Cont**
Klebsiella pneumonia	11	12	13	17	bc 14.0	0.0
Pseudomonas auroginosa	11	10	14	17	bc 13.0	0.0
pseudomonas fluorescence	12	11	12	15	e 12.5	0.0
S. haemolyticus	15	15	18	22	a 18.0	0.0
Entrobacter aerogense	10	10	15	20	b 13.75	0.0
Staphylococcus aureus	10	11	15	15	cd 12.75	0.0
Proteus vulgaris	11	10	13	14	de 12.0	0.0
Escherichia coli	0	0	15	18	f 9.0	0.0
	c 9.7	**c 9.7**	**b 14.8**	**a 17.3**	Average	

- Bacteria are considered resistant if the diameter of the inhibition zone is 0.0 mm.
- Letters sharing the same value horizontally or vertically indicate no significant differences at the P ≤ 0.01 probability level.



Curve 1: Inhibitory effect of the hexane extract of *Apis mellifera* against the growth of several pathogenic bacterial species

2- Antibacterial Activity of Antibiotics against Pathogenic Bacteria

Statistical analysis of the antibiotic test results presented in Table (2) showed that the antibiotic cip10 exhibited the highest inhibitory potency across all samples, with a mean inhibition zone diameter of 21.6 mm. The antibiotic Amc recorded a mean inhibition zone diameter of 12.6 mm, whereas the antibiotics VA30 and p10 demonstrated the lowest inhibitory potency against all isolates, with mean inhibition zone diameters of 0.6 mm and 0.75 mm, respectively.

Regarding the susceptibility of the bacterial species to antibiotics, *E. aerogenes* exhibited the highest sensitivity, with a mean inhibition zone diameter of 11.4 mm. The effects of the antibiotics varied across the species: *P. fluorescens* showed a mean inhibition zone diameter of 11.2 mm, while *P. aeruginosa* and P. *vulgaris* recorded 10.0 mm and 10.2 mm, respectively. In contrast, *S. aureus* demonstrated the highest resistance to all the antibiotics tested, with a mean inhibition zone diameter of 5.4 mm.

Table 2: Results of antibiotic tests on bacterial species

Bacteria	**Antibiotics**					
	Amc	**Azm15**	**VA30**	**Cip10**	**P10**	**Average**
Klebsiella pneumonia	10	12	0	25	0	**c 9.4**
Pseudomonas auroginosa	22	11	0	17	0	**ab 10**
pseudomonas fluorescence	10	10	6	25	5	**a 11.2**
Staph.haemolyticus	6	11	0	22	0	**d 7.8**
Entrobacter aerogense	22	13	0	22	0	**a 11.4**
Staphylococcus aureus	5	10	0	12	0	**e 5.4**
Proteus vulgaris	17	9	0	25	0	**ab10.2**
Escherichia coli	9	15	0	25	0	**c 9.8**
	b 12.6	**11.3**	**c 0.75**	**a 21.6**	**c 0.6**	**Average**

DISCUSSION

Regarding the strength of the inhibitory effect, the highest concentration (200 mg/ml) demonstrated the greatest inhibitory potency compared to the other concentrations; this aligns with the finding [14], that an antibiotic's inhibitory power increases with concentration. Additionally, the concentrations of 50 and 100 mg/ml yielded similar results (15.0 mm and 14.8 mm, respectively), suggesting that the extract reached a point of saturation or effect stabilization within this concentration range, such that further increases in concentration did not produce a significant, perceptible difference. The results revealed a clear variation in the responsiveness of the bacterial species to the extract; *S. haemolyticus* (Gram-positive) exhibited higher sensitivity, whereas *E. coli* showed lower sensitivity. This variation is primarily attributed to structural differences in the cell wall; Gram-positive bacteria (*S. haemolyticus*) possess a relatively simple cell wall composed of multiple layers of peptidoglycan, which allows for greater permeability to the active compounds within the extract. As for Gram-negative bacteria (such as *E. coli*), they possess an additional outer membrane composed of lipopolysaccharides; this membrane acts as a selective barrier that prevents the penetration of many hydrophobic compounds found in the hexane extract, thereby explaining their lower sensitivity compared to Gram-positive species a finding confirmed by [13]. A comparison of the extract's results with antibiotic susceptibility testing reveals statistically significant differences; *S. haemolyticus* showed greater susceptibility to the extract, with a mean inhibition zone diameter of 18.0 mm, whereas it exhibited lower susceptibility to the antibiotic, with a mean inhibition zone of 7.8 mm. *E. coli* demonstrated high resistance to the extract compared to the other species, with a mean inhibition zone of 8.0 mm, while its mean inhibition zone for the antibiotic was 5.4 mm. Statistically, the extract outperformed the antibiotics. The inhibitory efficacy of the extract against the studied bacterial isolates including multidrug-resistant (MDR) strains is attributed to its specific biochemical composition, particularly the compound hexadecanoic acid ethyl ester. Scientific research indicates that this compound exhibits antimicrobial activity due to its hydrophobic alkyl chain, which enables it to integrate into and dissolve within the bacterial lipid membrane, thereby disrupting its permeability. These findings align with the results reported in [15], regarding the inhibitory potential of this compound. Furthermore, the fatty acid methyl ester contributes to this effect by inhibiting the fatty acid biosynthesis pathway through the suppression of FabI gene expression a pathway also highlighted in study [16]. On the other hand, part of this efficacy may be attributed to the presence of melittin peptides derived from honeybees, which are highly efficient antimicrobial agents. These peptides operate via a mechanism based on electrostatic attraction, wherein the positively charged melittin is drawn to the negatively charged surfaces of microbial lipid membranes; this facilitates penetration of the lipid bilayer and the formation of membrane pores that disrupt osmotic balance, ultimately leading to cell death. In this context, study [17], highlighted that bacterial response varies according to cell wall composition: melittin penetrates the peptidoglycan layer of Gram-positive bacteria more easily than that of Gram-negative bacteria, as the latter possess a lipopolysaccharide (LPS) layer that provides additional protection and enhances resistance to penetration. The

superior performance of the hexane extract is attributed to its high capacity for extracting non-polar, hydrophobic organic compounds such as free fatty acids, their esters, and lipophilic substances which possess a strong ability to penetrate the lipid layers of microbial cell membranes; this highlights the importance of solvent selection in determining the extract's biological activity. Given that the crude extract consists of a complex mixture of ester compounds and peptides such as melittin its efficacy cannot be attributed to a single compound; rather, it arises from the synergistic effect among the various components of the honeybee extract. Hexadecanoic acid and its ester derivatives work alongside antimicrobial peptides (like melittin) to disrupt the cell wall and cytoplasmic membrane through simultaneous, complementary mechanisms that prevent bacteria from rapidly developing resistance. These results confirm the potential of *Apis mellifera* extracts as promising natural alternatives in medical and biological applications for combating bacterial pathogens.

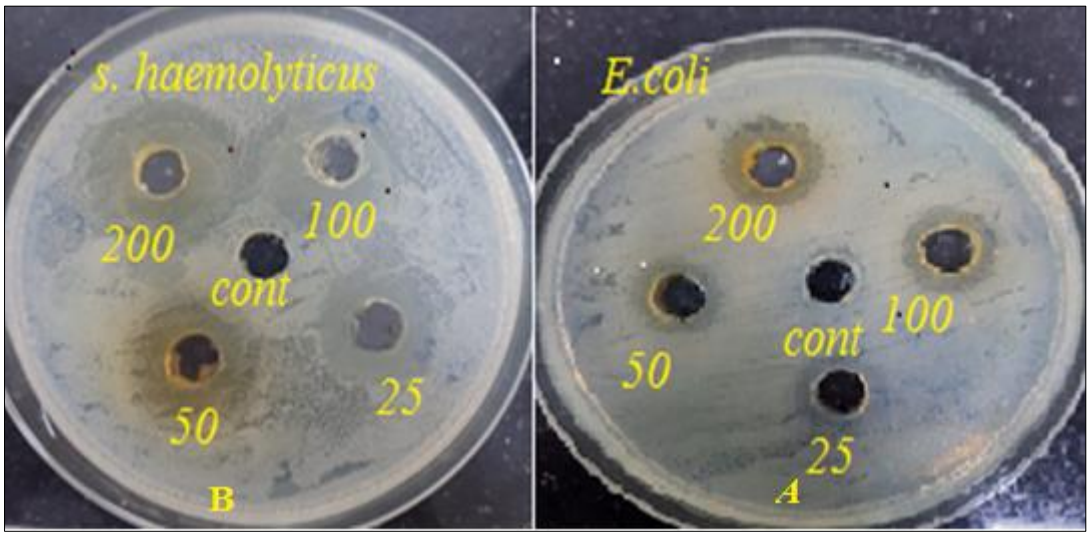


Figure 1: Inhibitory effect of the Hexan honeybee extract against *E. coli* and *S. haemolyticus*.

CONCLUSION

This study concludes the high efficacy of the honeybee *Apis mellifera* hexane extract as a broad-spectrum natural antimicrobial agent, particularly at the highest concentration (200 mg/ml). The research revealed that the extract's effectiveness is influenced by the structural composition of the bacterial cell wall; Gram-positive isolates *S. haemolyticus* exhibited greater sensitivity compared to Gram-negative ones *E. coli*. This inhibitory effect is primarily attributed to the extract's bioactive constituents specifically hexadecanoic acid ethyl ester and the peptide melittin which operate via molecular mechanisms targeting the microbial cell wall and associated biological pathways. Consequently, these extracts hold great promise as sustainable natural alternatives in medical and biological applications for curbing the spread of multidrug-resistant (MDR) pathogens.

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