Print ISSN: 0375-9237
Online ISSN: 2357-0350

EGYPTIAN JOURNAL OF BOTANY (EJBO)

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PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Promising antimicrobial activity of Saudi honey against seventeen multidrug resistant *P. aeruginosa* strains

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Pseudomonas aeruginosa, a significant drug-resistant pathogen, is a major issue in hospitals, especially the wide range infection in intensive care units, due to its complicated antibiotic resistance mechanisms and protective biofilms formation. The study aimed at evaluation the potential antibacterial activity of Sidr, Talh, and Sommra honey obtained from Al-Bahha region, Saudi Arabia against 17 drug-resistant *P. aeruginosa* strains collected from patients of various ages at King Abdulaziz University Hospital. The identification and antimicrobial susceptibility testing were performed throughout disk diffusion and Vitek 2 automate system, and genetic manipulation. Our results showed that Talh, Sidr and Sommra honey was exhibited high antimicrobial efficiency against bacterial infection, also indicated the synergistic efficiency of honey supplementation in fighting a drug-resistant *P. aeruginosa* strain with synthetic antibiotics. This study outcomes pointed out the crucial role of natural honey supplementation in healthcare and bacterial infections control, which in turn is consistent with the Saudi Arabia's goals for improving public health and combating infectious diseases. These results need a further exploration into honey's potential as a natural approach to managing infections resistant to antibiotics and highlight its significance in developing novel healthcare solutions that align with the Kingdom of Saudi Arabia's objectives for advancing medical science.

Key words: Natural Approach, *Pseudomonas aeruginosa*, a drug-resistant bacterium, honey, Vitek 2, molecular identification 16S rDNA, phylogenetic tree

ARTICLE HISTORY

Submitted: November 15, 2024
Accepted: December 04, 2024

CORRESPONDANCE TO

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DOI: 10.21608/ejbo.2024.336703.3079

EDITED BY: S. Abd Ellatif

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that exhibits extensive antibiotic resistance and poses a considerable challenge in healthcare environments, particularly among individuals with underlying health conditions like cancer, cystic fibrosis, or diabetes (Shereen, et al. 2023; Hernando-Amado and Martínez 2023; Martínez-Solano et al. 2008; Wilson and Pandey 2020; Behzadi et al. 2021). This pathogen is recognized for its persistence on medical equipment and its ability to withstand antibiotic therapies. Classified as one of the ESKAPE pathogens, *P. aeruginosa* is notable for its virulence factors and diverse resistance strategies, which include forming biofilms and employing multi-drug resistance (MDR) mechanisms such as active efflux, decreased membrane permeability, and the production of β-lactamases (Wagih, et al. 2019; Metwally, et al. 2020; Hernando-Amado and Martínez 2023). The widespread occurrence of MDR P. aeruginosa globally, and especially in Saudi Arabia, has severely restricted therapeutic options, as the bacterium demonstrates strong resistance to a range of antibiotics, including carbapenems and cephalosporins. (Al-Agamy et al. 2016; Azim et al. 2019; Khan et al. 2016; Vijayakumar et al. 2020).

With the increase in antibiotic resistance, alternative therapeutic options such as honey, recognized for its medicinal properties, are being investigated for their antimicrobial potential (Majno 1975). Research indicates that honey, particularly Manuka honey, possesses antibacterial properties effective against multi-drug resistant (MDR) pathogens, including *P. aeruginosa* (Almasaudi 2021; Al-Nahari et al. 2015; Hamza et al. 2023). This research aims to assess the antibacterial effects of three honey varieties sourced from the Al-Bahah region of Saudi Arabia against MDR *P. aeruginosa* strains, using medically graded Manuka honey (MGO+300) as a benchmark. The study will involve isolating MDR *P. aeruginosa* strains, evaluating the antibacterial properties of the honey samples through diffusion and dilution methods, establishing the minimum inhibitory concentrations (MICs), and exploring the potential synergistic interactions between honey and antibiotics. The hypothesis proposes that Talh honey from Al-Bahah will show antibacterial activity against MDR *P. aeruginosa*, potentially serving as an alternative or supplementary treatment alongside traditional antibiotics.

Honey, a naturally derived substance celebrated for its bioactive components and healing properties, has been widely studied for its antibacterial capabilities through various laboratory assays. However, its effectiveness against multi-resistant bacteria, such as *P. aeruginosa*, remains insufficiently explored. This study aimed to investigate the antibacterial potential of three types of honey from the Al-Bahah region in Saudi Arabia—Sidr, Talh, and Sommra. The antibacterial effects were assessed using diffusion

and dilution techniques to determine which honey exhibited the most potent activity against these difficult-to-treat bacteria. For benchmarking purposes, medically certified Manuka honey (MGO+300) was used as a standard reference. In this study we highlight or open the door to return to nature and natural products for maintaining our health.

MATERIALS AND METHODS

Nutrient Agar (NA): Nutrient agar (NA) serves as a versatile nutrient solution capable of cultivating numerous microorganisms, following the pamphlet. It is produced by dissolving 28 grams of nutrient agar powder in one liter of distilled water through boiling until the powder is fully dissolved. Following this, the solution is sterilized through autoclaving at a temperature of 120 °C and a pressure of 15 pounds per square inch for a duration of 15 minutes.

Research Site: The purpose of this study was to evaluate the antibacterial efficacy of locally sourced Saudi honey against multidrug-resistant (MDR) strains of *P. aeruginosa* in the Department of Biological Sciences at the Faculty of Science, King Abdulaziz University. The experimental work was conducted between Sep 2022 and Feb 2023.

Sample Gathering: Beekeepers in Saudi Arabia's Al-Bahha region provided three samples of honey. *Apis mellifera jemenitica*, a native bee species that has adapted to the region's hot and dry climate, produced these samples (Alattal & AlGhamdi, 2015). Each honey sample's botanical origins were ascertained by considering its geographic location, nearby flowering plants, honey color and aroma, and established identification techniques (Allen et al., 1991; Yao et al., 2004, Al-Masaudi and Al-Maaqar 2020). Medically graded Manuka (300+ MGO) honey was purchased from a pharmacy in Jeddah and was used for comparison.

Honey's sterility: One gram of each honey sample was mixed with ten milliliters of sterile distilled water and diluted to check for the presence of harmful microbiological contaminants in honey. After inoculating blood agar and nutrient agar plates with a loopfull of the diluted honey, the plates were incubated at 37 degrees Celsius for 18 hours. After that, the agar plates were checked for bacterial or other microorganism growth. To preserve their antibacterial properties, the honey samples were kept at room temperature (20–30°C) in a dark location. Honey should be stored in a cool, dark place because heat and light can break down its antibacterial compounds (Allen et al. 1991).

Preparing the Sample

Ethanol extract preparation: In a glass flask, a 100 g sample of honey was incubated for two days with 500 mL of 80% ethanol. A Shaker SHO 1-D was used to shake the mixture continuously at 150 RPM for 48 hours while it was at room temperature. The extract was filtered after incubation, and the solvent was then removed using a rotary evaporator (Buchi Rotavapor R-114 and Water bath B-480) at 55°C and low pressure. An ADAM electronic balance (precision: 0.0001 g) was used to weigh the finished extract, and the extraction yield was computed. The extract was subsequently stored in a dark glass bottle at 4°C in a refrigerator until needed for further use.

Preparation of Honey Extract in Water: A 100 g portion of honey was mixed with 500 mL of sterile distilled water and incubated for 48 hours in a glass flask. The solution was agitated at 150 RPM for 48 hours with a Shaker SHO 1-D at room temperature. Subsequently, the extract underwent filtration and concentration utilizing a rotary evaporator (Buchi Rotavapor R-114 & Water bath B-480), where the solvent was evaporated at 55°C under lowered pressure. The last extract was measured using an ADAM electronic balance (precision: 0.0001 g), and the yield was determined. The extract was subsequently kept in a dark glass bottle at 4°C until it was needed again.

Bacterial Varieties: Between Sep 2022 and Feb 2023, 16 Multi-drug resistant *P. aeruginosa* (PA) isolates were obtained from King Abdulaziz University Hospital in Jeddah, Saudi Arabia. Isolates of *P. aeruginosa* were sourced from clinical samples of respiratory, deep wound, urine, and tissue, with *P. aeruginosa*–serving as the control. The hospital staff (phlebotomists and nurses) collected the samples and transported them to the microbiology laboratory as pure cultures on Blood Agar medium.

Preparation for Bacterial Suspension: A loop was used to scratch the surface of an agar slant with *P. aeruginosa*, and the slant was then placed in an incubator at 35±2°C for 24 hours. The next day, the microbe that had grown on the surface of the agar slant was gently removed using the loop and suspended in Mueller Hinton Broth (MHB), which was then incubated for a further 18-24 hours at 35 ± 2 °C. Subsequently, the bacterial suspension was diluted to achieve an absorbance between 0.08 and 0.13, as measured by a 0.5 McFarland standard. For the

synergistic antibiotic and honey test, the bacterial suspension was further diluted in MHB, and 1 mL of the diluted bacterial suspension was added to 9 mL of MHB with honey extract 50%, resulting in a final concentration of 10^6 colony-forming units (CFU)/ml.

Antibiotics susceptibility test of bacterial isolates: In compliance with the Clinical and Laboratory Standards Institute instructions, the Kirby-Bauer disk diffusion method was used to determine the antimicrobial profile of bacterial tested (Oxoid, Basingstoke, UK) (MA, 2006). Following antibiotics were used: amikacin (30µg), ampicillin (10µg) augmentin (30µg), gentamicin (10µg), cefotaxime (30µg), cefuroxime (30µg), ciprofloxacin (5µg), cefoperazone (75µg), Ceftazidime (30µg), netilmicin (30µg), ofloxacin (5µg), and norfloxacin.

Antibacterial Activity of Honey

Two common techniques to assess the antimicrobial effects of natural products like honey are the agar well diffusion and microbroth dilution methods.

Agar Well Diffusion (ATCC) Method: This method tests the ability of honey's antimicrobial compounds to diffuse into agar and inhibit bacterial growth. In this setup, *P. aeruginosa* is spread onto a Mueller Hinton Agar plate, where wells are created using a sterile cork borer. Each well is filled with 100 µL of honey at various concentrations (50%, 25%, 12.5%), with Manuka honey (300+ MGO) as a control. After 30 minutes of refrigeration for diffusion, the plates are incubated at 37 ± 1 ºC for 24 hours under aerobic conditions. The inhibition zone around each well is measured afterward to assess honey's antibacterial efficacy, the larger the inhibition zone, the more potent the honey's effect.

Microbroth Dilution Method

This method determines the minimum inhibitory concentrations (MICs) of honey against bacteria, using a 96-well microtiter plate with Mueller Hinton broth. After incubation at 37°C for 24 hours, a Resazurin sodium salt dye (Sigma-Aldrich R7017) is added to each well. Column 12, containing only media, acts as a contamination control, and Column 1 serves as a negative control with the cultured strain. Columns 2-6 contain serial dilutions of honey (50% to 1.56%). This method provides accurate MIC values while reducing interference from color or solubility (Al-Maaqar et al., 2022).

Bacterial Identification

Morphological Characterization: Isolates were grown on individual Acetamide Agar plates at 37ºC for 24 hours, and colony shape and color were documented.

Gram Staining: To differentiate between Gram-positive and Gram-negative bacteria, Gram staining was performed following Vincent and Humphrey (1970). A loopful of bacteria was placed on a glass slide, stained with crystal violet, rinsed, treated with iodine, ethanol, and safranin, and then viewed under a light microscope.

Molecular Identification through 16S rRNA Gene Sequencing and Phylogenetic Analysis

DNA Extraction: Genomic DNA extraction followed the protocol of Azcárat Peril and Raya (2001) with modifications. Bacterial pellets from overnight cultures were treated with 200 µL of TES buffer and 20 µL of lysozyme (10 mg/mL) and incubated at 37 °C for 20 min. Proteinase K (20 µL, 10 mg/mL) was added and incubated a second time at 37 °C for 20 min. After ice cooling, add 250 µL of 4M sodium acetate and 250 µL of chloroform: isoamyl ester (24:1), mix and centrifuge at 13,000 RPM for 2 min. Carefully transfer the supernatant to a new tube, mix with isopropyl alcohol and store at −20 °C overnight. Centrifuge again to remove the supernatant, dry the DNA and resuspend in 50 µL of distilled water. Load a 10 µL aliquot of the isolated DNA onto a 0.5% agarose gel in 1x TBE buffer, run at 100V for 60-90 minutes and stain with ethidium bromide for visualization. Polymerase Chain Reaction (PCR). The DNA from the bacterial isolates was amplified using 16S rDNA primers: 27F (AGAGTTTGATCMTGGCTCAG) and 1100R (GGGTTGCGCTCGTTG). Amplification was carried out using a PCR thermal cycler. Each 50 µL reaction mixture included 25 µL of Mastermix (containing 50 units/mL Taq polymerase, 400 µM dNTPs, and 3 mM $MgCl_2$), 19 µL of ultrapure PCR water, 2 µL of the forward primer, 2 µL of the reverse primer and 2 µL of the DNA template. The standard PCR conditions were applied: initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of 1 minute at 95 °C, annealing at 58 °C for 1 minute, and a 2-minute elongation at 72 °C, with a final extension at 72 °C for 5 minutes. The successful amplification was verified by observing ethidium bromide-stained bands in a 1% agarose gel.

Agarose Gel Electrophoresis: To separate and visualize the DNA fragments by size, agarose gel electrophoresis was performed. A 1% agarose solution was prepared by dissolving 1 g of agarose in 100 mL of 1X Tris/Borate/EDTA (TBE) buffer and microwaving it for 30 seconds. After cooling, 4 µL of ethidium bromide was added, and the mixture was

poured into a gel tray. The comb was carefully placed to avoid bubbles and left to set for 20 minutes. After removing the tape and comb, 5 µL of each DNA sample was mixed with 2.5 µL of loading dye, and the gel was run at 100 V for 40 minutes. Bands were visualized under UV light.

Analysis of Amplified PCR Product: A 3 µL portion of each PCR product was electrophoresed on a 1% agarose gel containing ethidium bromide in 1X Tris-acetate-EDTA (TAE) buffer at 120 V for 40 minutes, then visualized using a UV transilluminator (BioDoc-IT system, Japan).

Sequencing Amplified PCR Products: The amplified PCR products were purified using the QIA quick PCR purification kit (Promega, Madison,WI, USA) and sequenced with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis: The obtained sequences were edited manually using SnapGene Viewer software version 3.3.3, then aligned and compared with sequences in the GenBank database (NCBI). (http://www.ncbi.nlm.nih.gov) using the BLASTN search, and reference sequences were retrieved to perform phylogenetic analyses. Phylogenetic trees were constructed using the MEGA available on the NCBI website.

Analytic Statics

In this study, Excel was utilized for data analysis to calculate the mean and standard deviation.

RESULTS

The study aimed to characterize and analyze these MDR *P. aeruginosa* isolates, offering insights into their prevalence, clinical sources, and potential implications for patient management and treatment strategies. Primarily, the study used microbial and molecular identification methods to accurately identify MDR *P. aeruginosa* strains. Additionally, it examined the antibacterial effectiveness of locally produced Saudi honey against these MDR *P. aeruginosa* strains. The experimentation was conducted in the Jeddah region of Saudi Arabia from Sep 2022 to Feb 2023.

Study Population

In this study, a total of 16 bacterial isolates were collected from patients across various age groups (4-84 years). This age range covered a broad demographic spectrum. Among the isolates, four (25%, or 4/16) were obtained from children aged 3-16 years, indicating that *P. aeruginosa* infections affect pediatric populations and highlighting the importance of managing these infections in young patients. Additionally, one isolation (6.25%, or 1/16) was obtained from young adults aged 17-30 years, representing the transition from adolescence to early adulthood, a notable demographic for *P. aeruginosa* infections. Two isolates (12.5%, or 2/16) were from middle-aged adults (31-45 years), a group typically in their prime working years, indicating their susceptibility to these infections. Interestingly, most of the isolates (56.25%, or 9/16) were from elderly adults over 45 years old. This suggests that older individuals may be more prone to *P. aeruginosa* infections, possibly due to age-related factors such as weakened immune systems or underlying health conditions.

Description of the Samples

In this study, the researcher collected a total of 16 isolates of multidrug-resistant *P. aeruginosa* from King Abdulaziz University Hospital in Jeddah, Saudi Arabia. These isolates (n=16) were obtained from various clinical specimens, each representing a different source of infection. Most *P. aeruginosa* isolates, approximately 75% (12 out of 16), were obtained from respiratory samples, indicating that the respiratory system was the primary site of infection associated with these multidrug-resistant strains. Additionally, 13% (2 out of 16) of the isolates came from urine samples, suggesting urinary tract infections caused by *P. aeruginosa*. Deep wound samples accounted for 6% (1 out of 16) of the isolates, highlighting the presence of *P. aeruginosa* in wound infections. Lastly, 6% (1 out of 16) of the isolates were derived from tissue samples, indicating *P. aeruginosa's* involvement in tissue-related infections. For experimental comparisons and analyses, the researchers included a control strain of *P. aeruginosa* (ATCC 27853) obtained from the EPS laboratory. This control strain served as a standardized reference for evaluating the multidrug-resistant isolates collected from clinical specimens.

Antibiotic susceptibility of bacterial isolates Using Vitek 2

The Vitek 2 system is a standard tool in clinical microbiology for assessing the antibiotic susceptibility of bacterial isolates. In this research, it was utilized to determine how *P. aeruginosa* isolates respond to a variety of antibiotics. The findings were interpreted

based on established antimicrobial susceptibility test guidelines (Table 1). Antibiotics tested included Meropenem, Piperacillin/Tazobactam, Amikacin, Ciprofloxacin, Cefepime, Gentamicin, Imipenem, and Ceftazidime. These antibiotics were chosen due to their frequent use in treating *P. aeruginosa* infections. To ensure the assay's accuracy, a control strain of *P. aeruginosa* (designated as *P. aeruginosa* control or PC) was included in the study. The control strain was fully sensitive to all antibiotics tested (100% or 8/8), confirming that the assay was functioning correctly.

The analysis showed significant variations in antibiotic resistance among the *P. aeruginosa* strains. Strains PA9 and PA12 exhibited complete resistance to all eight antibiotics (100% or 8/8), indicating a very high level of antibiotic resistance. Strains PA1 and PA3 demonstrated resistance to seven out of the eight antibiotics (87.5% or 7/8), also indicating a high level of resistance. Eight strains— PA 2, PA 4, PA 5, PA 6, PA 7, PA 10, PA 14, and PA 15—were resistant to six out of the eight antibiotics (75% or 6/8), suggesting a moderate level of resistance. Strain PA 13 showed resistance to five antibiotics (62.5% or 5/8), indicating a lower resistance level compared to the previously mentioned strains. The least resistance was observed in strains PA 8, PA 11, and PA 16, which were resistant to only four antibiotics (50% or 4/8) (Figure 1).

Overall, the study evaluated the effectiveness of various antibiotics against seventeen *P. aeruginosa* strains and recorded the corresponding resistance rates. The findings indicated that Piperacillin/ Tazobactam and Imipenem were largely ineffective, with a resistance rate of 88.25% (15/17) Meropenem, Ceftazidime, and Cefepime also had limited efficacy, with an 82.4% (14/17) resistance rate. Amikacin and Ciprofloxacin showed inadequate activity, with 58.8% (10/17) of the strains being resistant. Conversely, Gentamicin had the lowest resistance rate, with only 35.3% (6/17) of strains showing resistance (Figure 2).

Antimicrobial Susceptibility Testing Using Antibiotic Multi-Discs

The study evaluated the phenotypic antimicrobial susceptibility of *P. aeruginosa* using a selection of 12 antibiotics. Seventeen *P. aeruginosa* isolates were analyzed, and their inhibition zone diameters were measured to establish the bacteria's susceptibility to each antibiotic. Resistance levels varied among the isolates, with four strains (PA12, PA13, PA14, and PA16) being resistant to all tested antibiotics. Notably, strains PA3 and PA6 had resistance rates exceeding 90%, while PA1, PA6, PA7, and PA8 showed resistance rates above 80%. Five isolates (PA2, PA4, PA5, PA11, and PA15) had a resistance rate of 75%, and PA9 had the lowest resistance rate at 67%. The *P. aeruginosa* PC strain served as a control and was sensitive to all 12 antibiotics. Ceftazidime, Augmentin, and Cefotaxime had the highest resistance rates at 94.1%, followed by Ofloxacin, Ampicillin, and Cefuroxime at 88.2%. Norfloxacin had a resistance rate of 82.3%, while Netilmicin and Gentamicin showed resistance rates of 76.4% and 70.5%, respectively. Cefoperazone had a resistance rate of 70.5%, Ciprofloxacin showed 52.9%, and Amikacin had the lowest resistance rate at 58.8% (Table 2).

Antimicrobial Susceptibility Testing Using Antibiotic Multi-Discs Combined with Honey

This research aimed to evaluate the phenotypic antimicrobial susceptibility profile of *P. aeruginosa* against a panel of 12 antibiotics and to determine the synergistic effects of honey on antibiotic sensitivity. When *P. aeruginosa* isolates were treated with honey before antibiotic testing, the results showed an increase in bacterial sensitivity to antibiotics, suggesting a synergistic interaction. Seventeen *P. aeruginosa* isolates were tested, and inhibition zone diameters were measured to assess their susceptibility to each antibiotic (Figure 3).

The study also explored the antibacterial properties of various types of honey, including Talh, Sidr, Sommra, and Manuka honey. Key findings indicated that Talh honey produced a larger inhibition zone than the Saudi honey types, Sidr and Sommra, though it was less effective than Manuka honey, which is well-known for its strong antibacterial properties. Additionally, the research examined each honey type's potential to enhance antibiotic effectiveness. Talh honey showed a significant ability to boost the efficacy of antibiotics compared to Sidr and Sommra honey, indicating its potential use in combination therapy. Resistance rates to the different types of honey were also assessed. Talh honey exhibited a resistance rate of 57%, while Sommra and Sidr honey showed resistance rates of 61.5% and 67%, respectively. Manuka honey had the lowest resistance rate at 56%, indicating a higher susceptibility to its antibacterial effects than the Saudi honey varieties (Figure 4). Overall, the findings highlight the promising antibacterial properties of Talh honey, particularly its larger inhibition zone and its ability to work synergistically with antibiotics (Figure 5).

Table 1. Antibiotic sensitivity and resistance of test (by VITEK® 2)

No	Bacteria	Antimicrobial									
		Meropenem	Piperacillin/Ta zobactam	Amikacin	Ciprofloxacin	Cefepime	Gentamicin	Imipenem	Ceftazidime	Resistance%	Sensitivity%
1	PC	S	S	S	S	S	S	S	S	0%	100%
2	PA1	R	R	S	R	R	R	R	R	87.5%	12.5%
3	PA 2	R	R	S	S	R	S	R	R	75%	25%
4	PA3	R	R	R	R	R	S	R	R	87.5%	12.5%
5	PA4	R	R	S	R	R	S	R	R	75%	25%
6	PA5	R	R	R	S	R	S	R	R	75%	25%
7	PA6	R	R	R	R	S	R	R	S	75%	25%
8	PA7	R	R	R	S	R	S	R	R	75%	25%
9	PA8	R	S	R	S	R	S	R	S	50%	50%
10	PA9	R	R	R	R	R	R	R	R	100 %	0%
11	PA10	S	R	R	R	S	R	R	R	75%	25%
12	PA11	S	R	S	R	R	S	S	R	50%	50%
13	PA 12	R	R	R	R	R	R	R	R	100 %	0%
14	PA 13	R	R	S	S	R	S	R	R	62.5%	37.5%
15	PA 14	R	R	R	S	R	S	R	R	75%	25%
16	PA 15	R	R	S	R	R	S	R	R	75%	25%
17	PA 16	R	R	S	R	S	S	R	S	50%	50%
Resistivity%		82%	88%	53%	59%	77%	29%	88%	77%		

PC: *Pseudomonas aeruginosa* Control, PA: *P. aeruginosa*, R: Resistant, S: Sensitive.

Table 2. Phenotypic antimicrobial susceptibility profile of *P. aeruginosa* by disks (mm)

No	Bacteria	Antibiotics													
		Norfloxacin	Ofloxacin	Netilmicin	Gentamicine	Ceftazidime	Cefoperazon	Cefuroxime	Ciprofloxacin	Cephotaxime	Augmentin	Amikacin	Ampicillin	Resistance%	Sensitive%
1	PC	23±0.5	27±0.5	25±0.5	12±0.5	25±0.5	17±0.5	23±0.5	27±0.5	27 ±0.5	23±0.5	28 ±0.5	25±0.5	0%	100%
2	PA1	R	R	R	17±0.5	R	R	R	R	R	R	30 ±0.5	R	83%	17 %
3	PA 2	R	R	R	R	R	21±0.5	R	25±0.5	R	R	25 ±0.5	R	75%	25 %
4	PA3	R	R	R	R	R	R	R	26±0.5	R	R	R	R	92%	8%
5	PA4	R	R	R	27.5 ±0.5	R	R	R	19±0.5	R	R	30 ±0.5	R	75%	25 %
6	PA5	R	R	R	R	R	28.±0.5	R	22±0.5	R	R	31 ±0.5	R	75%	25 %
7	PA6	R	R	R	R	R	R	33±0.5	23±0.5	R	R	R	R	83%	17 %
8	PA7	R	R	R	16.5 ±0.5	R	R	R	R	R	R	29 ±0.5	R	83%	17 %
9	PA8	R	R	R	R	R	28±0.5	R	23±0.5	R	R	R	R	83%	17 %
10	PA9	R	R	R	12±0.5	R	22±0.5	R	20±0.5	R	R	21 ±0.5	R	67 %	33 %
11	PA10	15±0.5	23±0.5	24±0.5	R	R	R	R	R	R	R	R	R	75%	25 %
12	PA11	R	R	R	R	R	R	R	R	R	R	R	R	100%	0%
13	PA 12	R	R	R	R	R	R	R	R	R	R	R	R	100%	0%
14	PA 13	R	R	R	R	R	R	R	R	R	R	R	R	100%	0%
15	PA 14	12±0.5	R	17±0.5	R	R	R	R	R	R	R	R	15±0.5	75%	25 %
16	PA 15	R	R	R	R	R	R	R	R	R	R	R	R	100%	0%
17	PA16	R	R	23.5 ±0.5	R	R	R	R	R	R	R	R	R	92%	8%
Resistance%		82.3%	88.2%	76.4%	70.5%	94.1%	70.5%	88.2%	52.9%	94.1%	94.1%	58.8%	88.2%		

PC. *P. aeruginosa* control, PA; *P. aeruginosa*, R: Resistant, S: Sensitive. From 1-16 bacterial strains

Nonetheless, Manuka honey remained the most potent, exhibiting superior antibacterial efficacy compared to all other honey types tested in this study.

The study investigated the resistance patterns of *P. aeruginosa* isolates to various antibiotics, with a focus on the effects of Talh honey. It was found that the isolates demonstrated varying degrees of resistance. Two isolates, Pa13 and Pa16, showed resistance to more than 83% of the antibiotics tested, indicating a high level of multidrug resistance. Six additional isolates (PA1, PA7, PA9, PA10, PA14, and PA15) had a resistance rate of 67%, reflecting a significant resistance level. Meanwhile, isolates PA3, PA4, PA5, PA6, PA8, and PA12 exhibited resistance rates between 50% and 58%, suggesting moderate resistance levels. Among the tested isolates, PA11 had the lowest resistance rate at 42%, indicating greater susceptibility to antibiotics. The control strain of *P. aeruginosa* used in the study remained sensitive to all 12 antibiotics, providing a baseline for antibiotic susceptibility.

The study also evaluated resistance patterns in *P. aeruginosa* isolates associated with Sommra honey. Outlines the distribution of resistance rates, three isolates (PA14, PA15, and PA16) demonstrated resistance to more than 83% of the antibiotics, indicating a high level of multidrug resistance. The remaining isolates showed resistance rates between 58% and 75%, indicating considerable antibiotic resistance. The control strain for Sommra honey-associated isolates was sensitive to 92% of the antibiotics, establishing a comparative reference.

For Sidr honey, the resistance levels among *P. aeruginosa* isolates were also variable. Four isolates were highly resistant, with resistance rates exceeding 83%. Additionally, ten isolates had resistance rates greater than 66%, signifying significant resistance. Two isolates demonstrated resistance rates of 50% to 58%, indicating moderate resistance. The control strain remained sensitive to 92% of the antibiotics, serving as a benchmark for comparison.

The study further analyzed the resistance patterns of *P. aeruginosa* isolates in the context of Manuka honey. It was shown that two isolates were highly resistant, with resistance rates over 83%. Five isolates displayed resistance rates above 66%, showing considerable resistance, while nine isolates had resistance rates ranging from 33% to 58%, indicating moderate resistance. Notably, the control strain was completely sensitive to all antibiotics tested, achieving a 100% sensitivity rate. This control strain served as a reference for comparing the susceptibility patterns of the Manuka honey-associated isolates.

Assessment of the minimum inhibitory concentrations (MIC) of honey

The microdilution assay was used to provide a more precise evaluation of the antibacterial properties of Talah honey against *P. aeruginosa*. This method determines the minimum inhibitory concentration (MIC), which is the lowest concentration needed to prevent visible bacterial growth. In this research, seventeen *P. aeruginosa* strains were analyzed to measure the MIC values for both Talah and Manuka honey. Manuka honey, recognized for its strong antibacterial effects, successfully inhibited the growth of all tested bacterial strains at concentrations ranging from 6.25% to 12.5% (v/v), demonstrating its effectiveness across all strains. In contrast, Talah honey exhibited MIC values ranging between 12.5% and 25%, indicating that varying concentrations were necessary to inhibit different *P. aeruginosa* strains. These results suggest that while Talah honey does have antibacterial activity against *P. aeruginosa*, it requires a higher concentration compared to Manuka honey to achieve similar levels of bacterial growth inhibition.

Identification of Bacterial Isolates

Phenotypic Identification: All isolates selected for this study demonstrated consistent morphological characteristics when cultured on Blood and acetamide agar. Microscopic examination revealed that the bacterial isolates belonged to the gram-negative group and exhibited a rod-shaped structure. Specifically, the pathogenic bacteria identified as *P. aeruginosa* were confirmed as gram-negative, rod-shaped organisms (Table 3). These consistent morphological traits provide further verification of the isolates' identity and align with the known properties of *P. aeruginosa*.

Molecular identification of bacterial isolates through 16S rRNA gene: The isolated strains were also detected as *Pseudomonas aeruginosa*. The 16S rRNA gene was amplified using the PCR tools after DNA was extracted. For sequencing, the PCR product was transported to Korea (Macrogen). Data has been submitted to the genetic sequence database at the National Center for Biotechnology Information (NCBI) following the sequencing of the 16S rRNA gene. The GenBank ID (http://www.ncbi.nlm.nih.gov) of these isolated strains is indicated in Table 3). BLAST analysis of each unambiguous sequence yielded very high

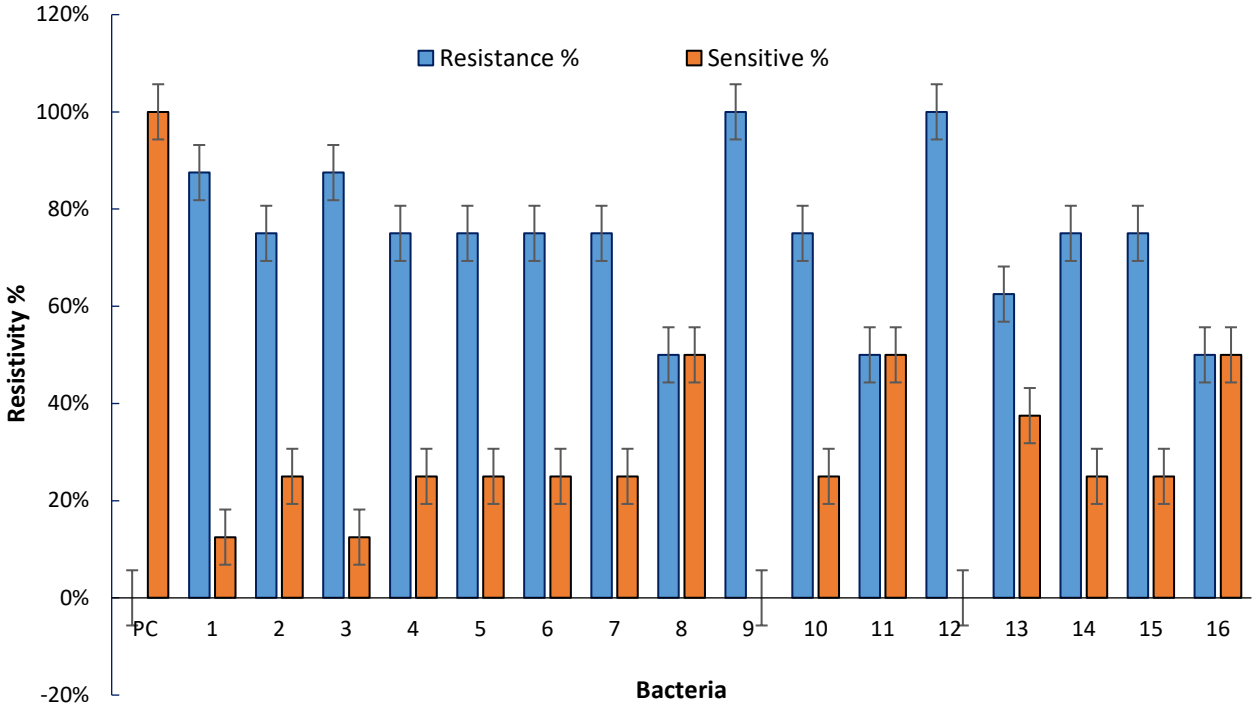

Figure 1. Antibiotic sensitivity and resistance of test (byVITEK®2) according to bacteria examined.

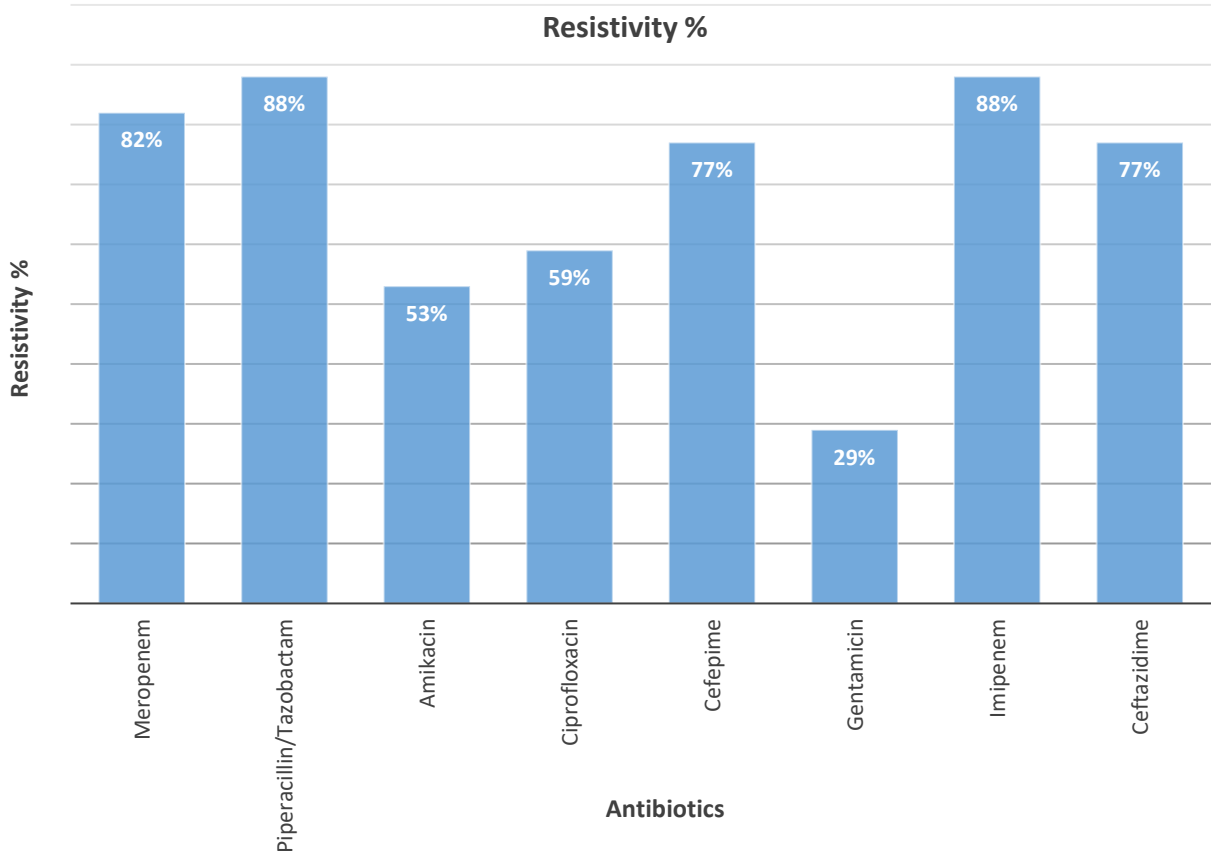

Figure 2. Antibiotic sensitivity and resistance of test (byVITEK®2) according to antibiotics examined

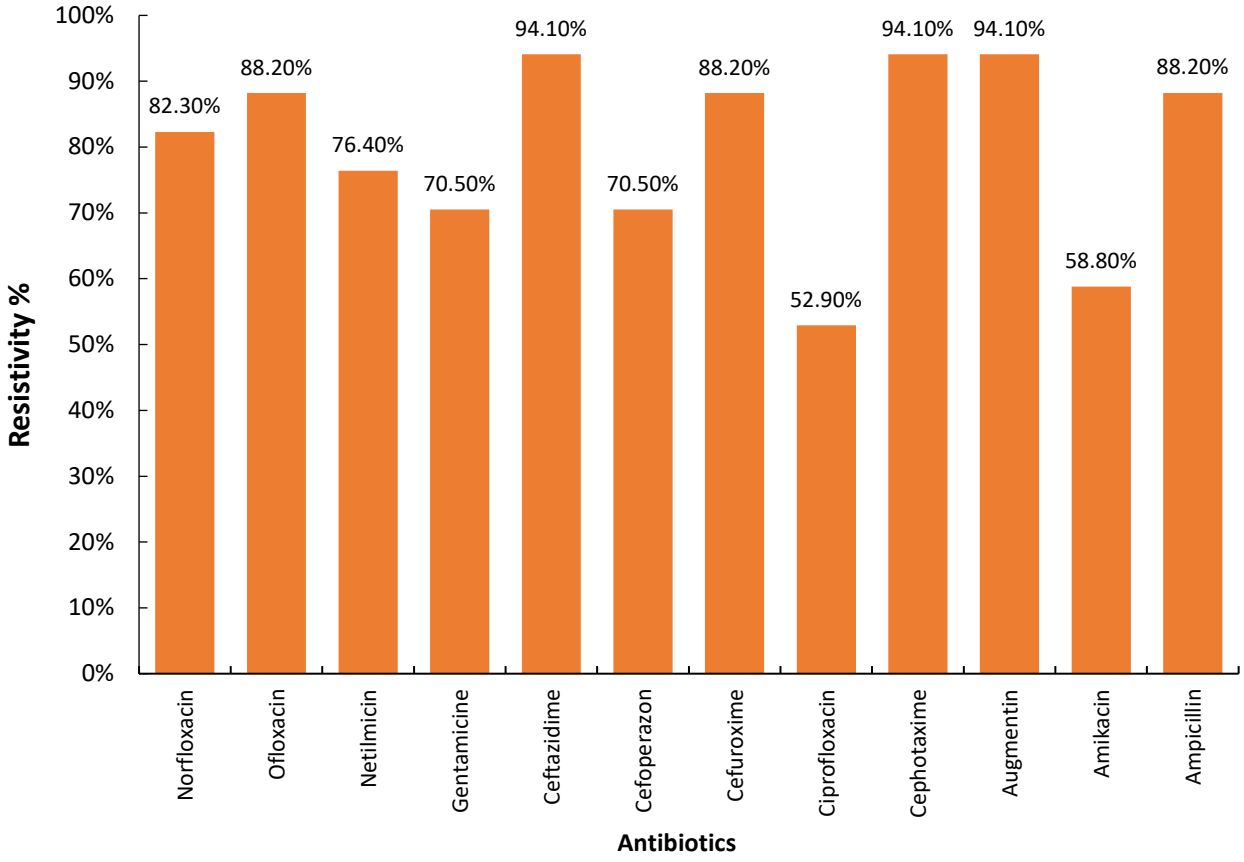

Figure 3. Antibiotic sensitivity and resistance of test (by multidisc) according to antibiotics examined.

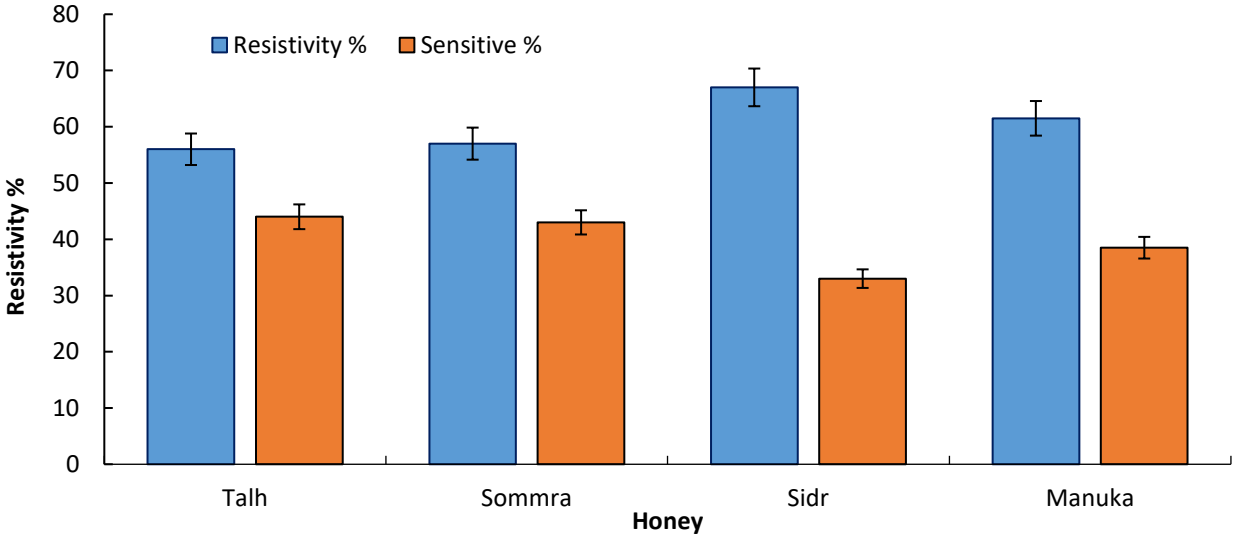

Figure 4. Phenotypic antimicrobial susceptibility profile of *P. aeruginosa* by disks treated by all honey.

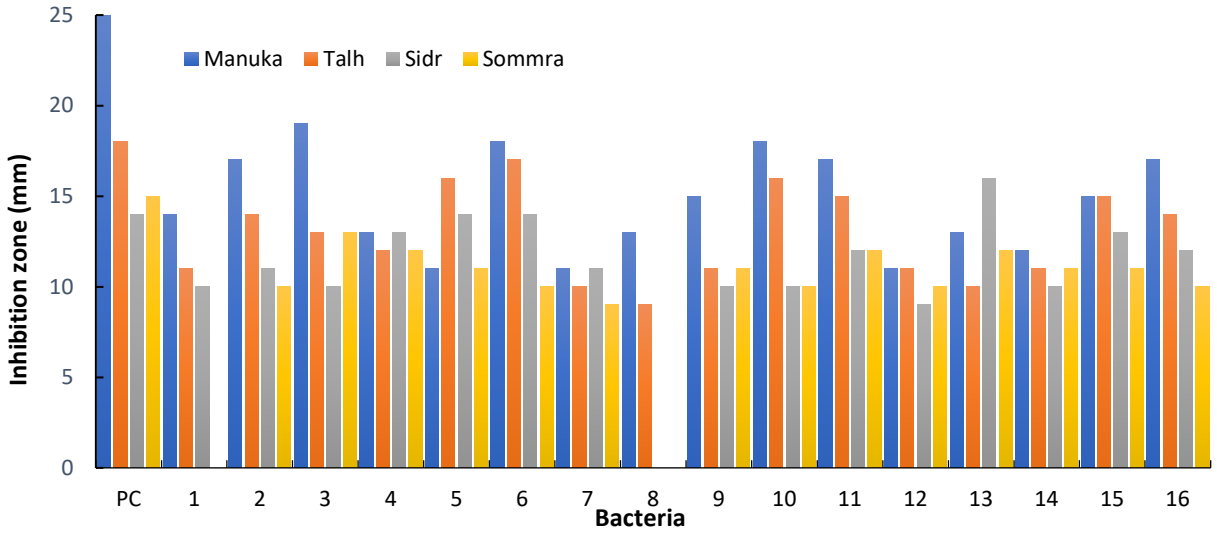

Figure 5. (mm) of Saudi honey samples at 50%(w/v) extracted by sterile distilled water by agar well diffusion assay against *Pseudomonas aeruginosa.*

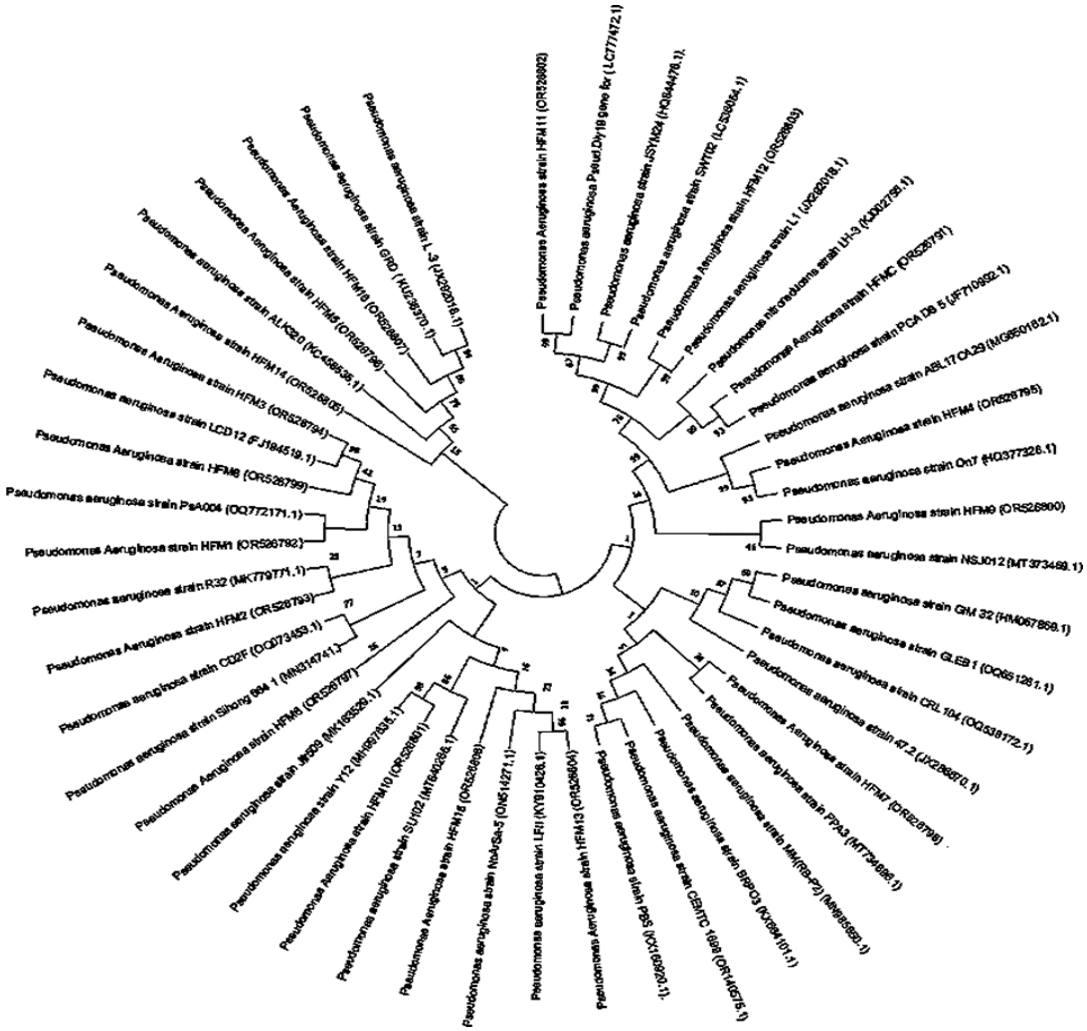

Figure 6. Phylogenetic analysis of bacteria isolates from the clinical specimens of respiratory, deep wound, urine, and tissue based on partial nucleotide sequences of the 16S rRNA gene. The tree was constructed using the neighbor-joining method.

Table 3. Molecular characterization analysis (based on 16S rDNA sequence).

No	Bacteria	Gram stain	Closest species from GenBank	Sequence Similarity (%)	Scientific name	Accession no. of the isolates
1	PC	Negative	*P. aeruginosa*	99.32%	*P. aeruginosa*strain HFMC	OR526791
2	PA1	Negative	*P. aeruginosa*	99.23%	*P. aeruginosa*strain HFMC1	OR526792
3	PA 2	Negative	*P. aeruginosa*	99.76%	*P. aeruginosa*strainHFMC2	OR526793
4	PA3	Negative	*P. aeruginosa*	99.23%	*P. aeruginosa*strainHFMC3	OR526794
5	PA4	Negative	*P. aeruginosa*	99.33%	*P. aeruginosa*strain HFMC4	OR526795
6	PA5	Negative	*P. aeruginosa*	99.32%	*P. aeruginosa*strainHFMC5	OR526796
7	PA6	Negative	*P. aeruginosa*	99.23%	*P. aeruginosa*strain HFMC6	OR526797
8	PA7	Negative	*P. aeruginosa*	99.42%	*P. aeruginosa*strainHFMC7	OR526798
9	PA8	Negative	*P. aeruginosa*	99.23%	*P. aeruginosa*strain HFMC8	OR526799
10	PA9	Negative	*P. aeruginosa*	99.13%	*P. aeruginosa*strainHFMC9	OR526800
11	PA10	Negative	*P. aeruginosa*	99.23%	*P. aeruginosa*strainHFMC10	OR526801
12	PA11	Negative	*P. aeruginosa*	99.68%	*P. aeruginosa*strainHFMC11	OR526802
13	PA 12	Negative	*P. aeruginosa*	99.32%	*P. aeruginosa*strainHFMC 12	OR526803
14	PA 13	Negative	*P. aeruginosa*	99.32%	*P. aeruginosa*strainHFMC 13	OR526804
15	PA 14	Negative	*P. aeruginosa*	99.23%	*P. aeruginosa*strainHFMC 14	OR526805
16	PA 15	Negative	*P. aeruginosa*	99.42%	*P. aeruginosa*strainHFMC 15	OR526806
17	PA16	Negative	*P. aeruginosa*	99.42%	*P. aeruginosa*strainHFMC 16	OR526807

From 1-16 bacterial strains

sequence similarities (98%) with GenBank submissions (Table 3).

Phylogenetic analysis: Multi-drug resistant *P. aeruginosa* (PA) isolates were isolated obtained from the clinical specimens of respiratory, deep wound, urine, and tissue. All isolates were closely related to *Pseudomonas aeruginosa* (Figure 6). Phylogenetic analysis of bacteria isolates based on partial nucleotide sequences of the 16S rRNA gene.

DISCUSSIONS

The study was carried out in the Department of Biological Sciences at King Abdulaziz University, aiming to assess the efficacy of native Saudi honey against multidrug-resistant (MDR) *P. aeruginosa*. This bacterium, which is on the rise in healthcare environments, especially impacts patients in intensive care units (ICUs) (MacVane, 2017). In this research, a total of 17 *P. aeruginosa* isolates were gathered from various clinical samples, including respiratory secretions, deep wound cultures, urine, and tissue samples, spanning a broad age range. The distribution revealed that infections can occur from childhood to older adulthood, highlighting the importance of tailoring management and prevention strategies to different age groups.

Specifically, 25% of the bacterial isolates were from children aged 3 to 16 years, underscoring the vulnerability of pediatric populations to these infections, possibly due to their underdeveloped immune systems or exposure to hospitals. Another isolation (6.25%) was isolated from young adults aged 17 to 30 years, showing that this age group is also at risk. Furthermore, 12.5% of the isolates were from adults aged 31 to 45 years, suggesting that individuals in this demographic may be susceptible, possibly due to their lifestyle or occupational exposures. Notably, 56.25% of the isolates were from patients over 45 years, indicating a higher susceptibility in older adults, likely due to weakened immune systems or pre-existing health conditions. These results align with previous research findings. (Mansoor et al., 2009; Nanvazadeh et al., 2013; Ameen et al., 2015).

The study identified 17 bacterial isolates from various infection sites. Most of these isolates (75%) were derived from respiratory samples, underscoring the respiratory system as a primary site of infection and aligning with previous research (Zupetic et al., 2021; Woo et al., 2018). Urine samples accounted for 13% of the isolates, indicating potential for urinary tract infections (Motbainor et al., 2020; Park & Koo, 2022). Meanwhile, deep wound and tissue samples each represented 6% of the isolates, suggesting the involvement of the bacterium in wound-related infections (Farahpour et al., 2020; Hattab et al., 2021; Puca et al., 2021). These findings offer valuable insights into the prevalence of MDR *Pseudomonas aeruginosa* across various clinical specimens (Elbehiry et al. 2022).

Interestingly, the study observed gender differences in infection rates, with males experiencing a higher prevalence (69%) compared to females (31%), which stands in contrast to previous research conducted at King Fahad Medical City in Riyadh (Hafiz et al., 2023).

This discrepancy emphasizes the importance of further exploring the role of gender in susceptibility to infections. Antibiotic susceptibility testing was performed using the Vitek2 system, revealing significant resistance to several antibiotics, including Piperacillin/Tazobactam, Imipenem, Meropenem, Ceftazidime, and Cefepime, with the highest resistance observed in these drugs. Conversely, Gentamicin demonstrated the lowest resistance levels. Resistance to Ceftazidime, Augmentin, and Cephotaxime was particularly alarming, while Amikacin was found to be the most effective antibiotic against *P. aeruginosa*. These results highlight the challenges associated with treating *P. aeruginosa* infections and underscore the necessity for strategic antibiotic use.

The study further investigated honey's potential as a complementary treatment. It examined the antibacterial activity of several indigenous honeys, including Talh, Sidr, and Soummra honey, finding that there was a variability in their effectiveness, as assessed through the agar well diffusion assay. Talh honey emerged as having the largest zone of inhibition among the local honeys, although it was less potent compared to Manuka honey. However, some bacterial strains exhibited resistance to these local honeys, underscoring the variability in their antibacterial effectiveness across different strains. Minimum inhibitory concentration (MIC) tests showed that Manuka honey effectively inhibited bacterial growth at concentrations ranging from 6.25% to 12.5%, whereas Talah honey required concentrations of 12.5% to 25% to achieve similar outcomes (AL-Maaqar, 2020; Al-Ghamdi & Ansari, 2021).

For bacterial identification, both phenotypic and molecular methods were utilized. The phenotypic analysis verified the rod-shaped morphology, confirming it as a gram-negative strain of *P. aeruginosa*. The molecular identification was based on 16S rRNA gene sequencing, which yielded high similarity scores (98%) with existing records in GenBank. Furthermore, phylogenetic analysis, performed using the UPGMA method, elucidated evolutionary relationships among isolates. Collectively, these approaches offered a thorough profile of the MDR *P. aeruginosa* strains, enhancing our understanding of their characteristics and genetic relatedness.

CONCLUSION

This study highlights the critical challenge posed by multidrug-resistant (MDR) *Pseudomonas aeruginosa*, particularly in healthcare settings where vulnerable populations, including pediatric and elderly patients, are at heightened risk. The prevalence of infections across various clinical specimens, with a significant predominance in respiratory samples, underscores the bacterium's adaptability and potential for severe health impacts. The age and gender distribution of isolates revealed important epidemiological trends, including the susceptibility of older adults and males, necessitating tailored prevention and management strategies. Antibiotic susceptibility testing confirmed alarming resistance to multiple commonly used antibiotics, with Amikacin emerging as the most effective treatment option. This finding emphasizes the urgency of implementing prudent antibiotic stewardship programs and exploring alternative or complementary therapies. The investigation into the antibacterial properties of indigenous Saudi honeys demonstrated promising, albeit variable, efficacy, with Talh honey showing notable inhibitory effects, although less potent than Manuka honey. The study provides valuable insights into the potential role of natural products like honey in combating MDR pathogens, especially in resource-limited settings. Finally, the integration of phenotypic and molecular analyses offered a comprehensive understanding of the genetic and phenotypic characteristics of *P. aeruginosa*, facilitating future research into its epidemiology and the development of targeted interventions. These findings underscore the pressing need for innovative strategies to address the global threat posed by MDR pathogens.

FUNDING

This Project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. (**GPIP: 45-130-2024**). The authors, therefore, acknowledge with thanks DSR for technical and financial support.

ACKNOWLEDGMENT

The author acknowledge with thanks DSR for technical and financial support.

AVAILABILITY OF DATA

This investigation offers all the data collected.

AUTHORS' CONTRIBUTION

The author constructed the framework, data collection, data representation, writing, reviewing, and editing.

CONFLICT OF INTEREST

The author proclaims that there is no conflict of interest.

ETHICAL APPROVAL

Not applicable

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