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MULTIDRUG-RESISTANT *Pseudomonas aeruginosa* ISOLATES WITH VIRULENCE TRAITS FROM WOUND SAMPLES EXHIBITED LOW *In-vitro* SUSCEPTIBILITY TO HONEY, A VIABLE ALTERNATIVE FOR THE MANAGEMENT OF CHRONIC WOUNDS

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ABSTRACT

Pseudomonas aeruginosa can colonize the body when wounds disrupt the skin barrier, compromising immune status, and expressing virulence factors that aid its establishment in the host. Honey is highly medicinal and capable of promoting healing in infected wounds that defy conventional antibiotic therapy. This study assessed the antibacterial activities of honey against multi-drug resistant virulent *P. aeruginosa* isolates recovered from wounds. Sixty-one *P. aeruginosa* isolates were recovered, and their virulence factors were determined using phenotypic screening methods. Antibiotic susceptibility testing was evaluated with the Kirby-Bauer disc-diffusion method, while the antibacterial activities of honey were determined by agar-well and disc-diffusion techniques. The components of the honey samples were evaluated using Gas Chromatography-Mass Spectrometry (GC-MS). All isolates were multidrug-resistant (MDR) with 100% resistance to ticarcillin, tigecycline, and nitrofurantoin; the highest susceptibility to piperacillin, meropenem, and imipenem at 4.9%, 6.6%, and 9.8% respectively; and Multiple Antibiotic Resistance Indices (MARI) of ≥ 0.2. All isolates produced hemolysin, but none produced DNase. All the sixty-one isolates extruded multiple virulence factors via phenotypic screening and 96.7% possessed ≥ 50% of virulence determinants analyzed. GC-MS analyses revealed three variants of honey. The isolates exhibited resistance to the honey samples; the most effective honey sample types being H5 and H6. Possession of multiple virulence factors, multi-resistance to antibiotics, and to honey, which is well acclaimed for its wound healing characteristics by isolates raises the probability of invasion to deeper tissues and development of complications, underscoring the necessity to prevent contamination of wounds, a significant cause of morbidity and mortality.

Keywords: *Pseudomonas aeruginosa*, Honey, Wounds, Multidrug-resistance, Antibacterial, Virulence factors

INTRODUCTION

The development of a wound infection hangs on the integrity and protective role of the skin **(Anupurba *et al.*, 2010).** A wound is termed infected when invading microbes cause a marked impairment in wound healing, characterized by inflammation and purulence. Chronic wounds, otherwise called non-healing wounds, with rapidly rising prevalence **(Frykberg and Banks, 2015; Sen, 2019)** are characterized by symptoms such as low trans-cutaneous oxygen tension, necrotic tissues, foul odour and breakdown, discolouration of new connective tissue, and friability **(Al-Dabbagh *et al.*, 2017).** Wound contamination by bacteria is a significant cause of mortality **(Bach *et al.*, 2022).** Wound contamination may occur through endogenous sources like the surrounding skin, the gastrointestinal tract, and the buccal cavity **(Thaarup *et al.*, 2022)** or external sources such as the environment or the healthcare provider **(Sibbald *et al.*, 2003)**. However, the most common sources of contamination are endogenous sources **(Percival *et al.*, 2016).** *Pseudomonas aeruginosa* is an opportunistic pathogen often linked with nosocomial infections **(Amazian *et al.*, 2010; Kakupa *et al.*, 2016)**; causing life-threatening conditions when acquired by a patient during a hospital stay **(Khan *et al.*, 2015)**. It has been associated with respiratory, urinary, burn, diabetic ulcers, postsurgical sites **(Percival *et al.*, 2015),** and other chronic wound infections in hospital environments. *P. aeruginosa* is said to account for about 25.0% of chronic wound cases **(Wolcott *et al.*, 2016).** Wound infections due to *P. aeruginosa*, usually have a poor prognosis and delayed healing **(Zhao *et al.*, 2012; Watters *et al.*, 2013; Bach *et al.*, 2022).** As a result, such wounds usually have a larger surface area **(Gjødsbøl *et al.*, 2006; Bach *et al.*, 2022).**

Pseudomonas aeruginosa like other bacteria often colonizes the wound surface when there is a breach on the skin or when there is a burn on skin surfaces. Once it colonizes the wound surface, it establishes itself by forming biofilms on the skin surface making it difficult for the host to eliminate it naturally or through the activities of antibiotics **(Kirketerp-Møller *et al.*, 2008; Lu *et al.*, 2019; Fleming *et al.*, 2022).** Interestingly, biofilms have been reported to be implicated in roughly 80% of all human infections, and 90% of chronic wound infections **(Fleming *et al.*, 2022)**. *P. aeruginosa* is also known to produce rhamnolipids, a glycolipid surface-active biosurfactant capable of regulating epithelial function and stimulating cellular invasion, thereby further aggravating the situation by delaying wound healing **(Roberts *et al.*, 2015).**

P. aeruginosa's pathogenicity and survival in wounds are enhanced by the myriads of virulent genes present in its genome. These genes are often referred to as virulence factors and are responsible for *P. aeruginosa*'s adaptation to the host's harsh environment and their persistence in the host cells **(Neamah, 2017)**. In addition to the virulence factors, *P. aeruginosa* also possesses arsenals of other mechanisms through which they evade resistance posed by the host cell and other antimicrobial agents that may be used against them. Among such mechanisms, are genotypic and phenotypic alterations such as mucoid colony formation, loss of motility, and ability to form biofilms **(Mitov *et al.*, 2010; Khattab *et al.*, 2015; Neamah, 2017; Salih *et al.*, 2017; Fleming *et al.*, 2022)** as well as over-expression of resistance genes **(Salih *et al.*, 2017)**. Due to the myriads of defence mechanisms against antimicrobial agents by many bacterial species involved in wound infections, honey, a juice from bees has been used as a potent alternative to many antimicrobial agents **(Hazrati *et al.*, 2010; Mandal and Mandal, 2011; Cooper, 2016; Halstead *et al.*, 2016; Lu *et al.*, 2019)**.

Honey, a naturally sweet substance synthesized from the secretions of active parts or excretions of plants by bees (*Apis mellifera*), has been used in the treatment of several disorders in humans and is reported to be a good remedy for infected wounds because it is known to fast-track the wound healing process **(Molan, 2006; Simon *et al.*, 2009; Yaghoobi *et al.*, 2013)** as a result of its potent antimicrobial action **(Lu *et al.*, 2019).** Honey is acidic, with a low pH of between 3.2 – 4.5 **(Kwakman and Zaat, 2012; Mama *et al.*, 2019)**. It has high osmotic characteristics and slowly produces hydrogen peroxide (H_2O_2) due to the activity of the enzyme glucose oxidase from the bees **(Lu *et al.*, 2019).**

H_2O_2 is known to play a crucial role in its antimicrobial activity **(Kacaniova *et al.*, 2011)**. The antibacterial activities of honey have also been linked to some constituent enzymes in the honey. The antibacterial activities of honey have been demonstrated against a wide range of pathogenic bacteria including

Staphylococcus aureus, Escherichia coli, Helicobacter pylori, and *Pseudomonas aeruginosa*. The successes recorded against these bacteria make honey a promising alternative to conventional antibiotics in the treatment of wounds or stomach ulcers (**French *et al.*, 2005; Visavadia *et al*., 2008**), and wound dressing (including surgical wounds, bedsores and other skin infections resulting from burns and wounds) (**Cooper *et al.*, 2002a; b**). In addition to inhibition of vegetative cells, honey can also inhibit biofilm formation and help eradicate biofilm activities in different pathogens such as *P. aeruginosa*, *Staphylococcus* and *Streptococcus* species, *Escherichia coli, Enterobacter cloacae* and *Acinetobacter baumannii* **(Halstead *et al.*, 2016; Liu *et al.*, 2018; Lu *et al.*, 2019).** A study on Manuka honey showed that it exhibits antimicrobial activity against bacteria via cell lysis which is caused by induced conformational changes leading to the reduction in the expression of OprF **(Roberts *et al.*, 2015).** They also reported that it can impede adhesion to keratinocytes in humans and inhibit siderophore production **(Roberts *et al.*, 2015).**

Wound management poses serious public health challenges in third-world countries, especially when heightened by secondary bacterial infections (**Mama *et al.*, 2019**). As multidrug resistance escalates especially with *P. aeruginosa*, there is a continuous need to monitor existing alternative therapies and natural remedies for wound management for their continued efficacy. *Pseudomonas aeruginosa* infections have become critical clinical challenges whose eradication with conventional antibiotic therapy has remained difficult and may serve as a reservoir of resistance genes in the hospital environment. This research, therefore, aimed to determine the antibiotic resistance patterns, screen for the production of hydrolytic enzymes, and investigate the antibacterial activities of honey against *P. aeruginosa* isolated from wound swabs of patients from two selected hospitals in Osun State, Southwest Nigeria.

MATERIAL AND METHODS

Study Locations

This study was undertaken at two selected hospitals in Osogbo and Iwo in Osun State, Southwest Nigeria. Osogbo, the capital of Osun State, lies at coordinates 7.7827° N, 4.5418°E with a projected population of 749,750 by 2022 **(World Population Review, 2022)**; while Iwo lies at coordinates 7.6353° N, 4.1816° E **(Geodatos, 2020)** with 263,500 inhabitants **(National Population Commission of Nigeria, 2016)**. The two selected hospitals were established by the Osun State Government and are major referral centres for people living within a 40 km radius, covering both the urban and the adjoining rural communities. The Health Planning, Research and Statistics Department of the Ministry of Health, Osun State, Osogbo gave ethical approval for the study (approval number - OSHREC/PRS/569T/177).

Sample collection and processing

A total of 237 participants, both in and out-patients at the two hospitals, were enrolled in the study based on individual and parental consent for inclusion into the study. They included 133 patients from Osogbo and 104 participants from Iwo. Wound samples from surgical sites of caesarian sections (CS), trauma from automobile accidents, and other skin lesions were collected using sterile cotton-tipped applicators dipped in sterile Ringer solution, one swab per patient. The applicators were gently rubbed onto the surface of the wound, carefully rotated to sample the epithelial wall, withdrawn, and inoculated aseptically into Tryptone Soy Broth (TSB) (Oxoid). These were incubated at 37±2°C overnight. An uninoculated tube of TSB served as a control. Overnight growth from TSB was streaked out on Cetrimide agar and incubated at 37±2°C for 18-24 hours. The development of green, blue, or golden yellow colonies indicated the growth of *Pseudomonas aeruginosa*. The isolates were Gram-stained, and other conventional biochemical tests such as the oxidase test **(Vaseekaran *et al.*, 2010; Oseni & Ekperigin, 2013)** *were* used to identify distinct colonies. Molecular identification using species-specific primers (forward primer 5'-GGCGTGGGTGTGGAAGTC-3' and reverse primer 5'-GGTGGCGATCTTGAACTTCTT-3' [Inqaba Biotec, South Africa]) was done to confirm presumptive identification of isolates **(Adeyemi *et al.*, 2020)**; and pure cultures of isolates were preserved in freshly prepared TSB with 15% glycerol at -20ºC.

Antibiotic Susceptibility Testing

Recovered isolates were tested for antibiotic susceptibility with the Kirby-Bauer disc diffusion method. Isolates were screened against the following antibiotics (Oxoid, UK): Amikacin (30µ*g*), Aztreonam (30µg), Cefepime (30µ*g*)), Ceftazidime (10µ*g*), Ceftazidime/Avibactam (14µ*g*), Ciprofloxacin (5µ*g*), Colistin (10µg), Fosfomycin (50µg), Gentamycin (30µg), Imipenem (10µg), Levofloxacin (1µg), Meropenem (10µg), Nitrofurantoin (30µg), Piperacillin (100µg), Piperacillin/tazobactam (36µg), Compound sulphonamides (25µg), Ticarcillin (75µg) and Tigecycline (15µg). Mueller-Hinton agar (MHA) was inoculated with the test isolate using a sterile cotton-tipped applicator to create a lawn, the antibiotic discs were placed aseptically on the seeded agar using an 8-place disc dispenser (Oxoid) and incubated at 37±2°C for 18-24 hours. Visual observation of inhibition zones was done, and the diameter was recorded to the nearest millimetre. The results were interpreted as susceptible, intermediate, or resistant using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint table vs 11.0 **(EUCAST, 2021)**. *P. aeruginosa* ATCC 27853 served as the control organism to verify the methods and results. The criteria for classification as a multidrug-resistant isolate was resistance to ≥ one drug in ≥ 3 antibiotic classes. The Multiple Antibiotic Resistance Index (MARI) of each bacterial isolate was determined using established methods **(Krumpermam, 1983)**.

Determination of Virulence Factors:

Hemolysin production was determined by the pattern of hemolysis on blood agar using freshly prepared blood agar plates. Bacterial isolates were streaked on the blood agar and incubated at 37±2°C for 24 – 48 hours, and afterwards, visually observed for hemolytic patterns. Production of hemolysis was determined as β, α, or γ hemolysis indicated by clear/colourless zone, greenish zone, and complete absence of hemolysis, respectively. Gelatinase production was done by the Nutrient-gelatin stab method **(Pratiksha, 2015).** Alkaline protease production was determined by inoculating the test isolate onto skim milk agar and incubating it at 37±2°C for 24 hours. The development of clear zones surrounding the line of the streak was indicative of alkaline protease production. Coagulase production was assessed by the tube coagulase method. A volume of 0.5ml aliquot of nutrient broth was inoculated with test isolate and incubated at 37±2°C for 24 hours, after which 0.5ml of pooled human plasma was added. This was incubated at 37°C and visually observed at 4, 6, 18, and 24 hours for evidence of coagulation. The formation of a definite clot upon slanting the tube was taken as a positive reaction. DNase production was evaluated by streaking out 18 – 24-hour cultures of test isolates onto DNase Agar, incubated at 37±2°C for 18 – 24 hours, and flooded with 1N HCl. The plates were left to stand for 3 – 5 mins, and excess HCl was tipped off. The plates were examined within 5 minutes against a dark background. DNase production was indicated by clear zones around the line of the streak. Motility in each test isolate was assessed by stabbing the isolate into semi-solid nutrient agar in a test tube with a sterile inoculating needle and incubating at 37±2°C for 24 – 48 hours. Growth away from the line of the stab, complemented by turbidity throughout the medium, was regarded as a positive test **(Salih *et al.*, 2017)**. Pigment production was assessed by observing growth visually on cetrimide agar **(Sagar, 2020);** while biofilm production was screened using Congo Red Agar **(Oliveira *et al.*, 2010)** and the tube adherence method **(Christensen *et al.*, 1982)**.

Honey sample collection, processing, and *in-vitro* antibacterial activity screening

Six Nigerian honey samples were obtained from different local markets in sterile screw-cap bottles, immediately transported to the laboratory, and stored in a cool and dry place until processed. Each sample was initially filtered with sterile gauze to remove debris, and then stored at 2–8°C until further use. The antibacterial activity of the honey samples was evaluated by the Agar well diffusion method and the Kirby–Bauer disk diffusion technique.

Agar well diffusion method

An overnight culture of each test isolate was inoculated into 5ml sterile Ringer solution, and the turbidity was adjusted to 0.5 McFarland standard. Each inoculum was further diluted at 1:100 using sterile Ringer solution as a diluent to attain an inoculum density of 1×10^4 CFU/ml **(Kacaniova *et al*., 2011)**. Eight wells of 6mm diameter (at least 25mm from each other) and 4mm depth were bored on sterile MHA plates using an appropriately sized sterile cork borer, and the agar surface was swabbed uniformly with the prepared suspension of each test isolate using a sterile cotton-tipped applicator to create a lawn. A 50µl aliquot of each honey filtrate was carefully dispensed into each well using a micropipette and labelled. The plates were incubated in an upright position at 37°C for 18 - 24 hours and afterwards visually observed for clear zones around each well. The diameter of the zones of inhibition was measured to the nearest mm and recorded. Each test was performed in duplicate and the average measurement was taken. The absence of clear zones was recorded as resistant. Chloramphenicol (30μg) and sterile ringer solution served as positive and negative controls respectively.

Kirby–Bauer disk diffusion technique

Sterile MHA plates were inoculated as described above. Sterile blank discs of 6mm diameter impregnated with pure filtrates of the honey samples were then placed onto the seeded top layer of the MHA plates, and incubated overnight at 37°C. The presence of a zone of inhibition around each disc was observed, measured, and recorded. Chloramphenicol discs and blank discs impregnated with sterile Ringer solution served as positive and negative control discs respectively.

Gas Chromatography-Mass Spectrometry (GC-MS) analyses of honey samples

The GC-MS analysis of bioactive compounds in the honey samples was done using a Varian 3800 gas chromatograph equipped with an Agilent MS capillary column (30m × 0.25mm i.d.) connected to a Varian 4000 mass spectrometer operating in the electron impact (EI) mode of ionization (70eV; m/z 1 – 1000; source temperature 230°C and a quadruple temperature 150°C). An aliquot of 5ml of each honey sample in 15ml screw-top vials, along with PTFE/silicone septa using benzophenone as internal standard, NaCl and $MgSO_4$ salts were maintained in a water bath, partially submerged to maintain the liquid phase of the sample underwater during equilibration and extraction at 80°C for 30mins. The column temperature was maintained initially at 200°C for 2min, increased to 300°C at 4°C/min, and then held at 300°C for 20min. The flow rate of the carrier gas, Nitrogen was set at 1.0 mL/min. The inlet temperature was maintained at 300°C with a split ratio of 50:1. A sample volume of 1µL in chloroform was injected using a split mode, with a split ratio of 50:1. The analyses of the samples were done using computer searches on a NIST Ver.2.1 MS data library and compared with the spectrum obtained through GC-MS to identify compounds present in the samples. All the samples and replicates were continuously injected as one batch in random order to discriminate technical from biological variations. Additionally, the prepared pooled samples were used as quality controls (QCs), which were injected at regular intervals throughout the analytical run to provide a set of data from which the repeatability can be assessed.

RESULTS AND DISCUSSION

Information relating to participants

In total, we sampled 237 patients from the two selected hospitals. Their ages ranged from 10 to 92 years with a mean of 28.7 years. A large proportion (86.5%) were between 20 – 39 years, and more than half of the patients (59.4%) had been placed on antibiotics (Table 1). *Pseudomonas aeruginosa* isolates were recovered from 25.7% of the wound samples (61/237) (Table 2), correlating perfectly with the 25.0% prevalence rate reported by **Wolcott *et al.* (2016).** However, this value is lower than that reported by **Schaumburg *et al.* (2022)**, whose study reported a recovery rate of 46% of *P. aeruginosa* out of 163 wound samples but higher than the 9.23% reported by **Georgescu *et al.* (2016)**. These variations in prevalence are most probably due to differences in the locations and the types of wounds.
Although the highest number of isolates (23) was obtained from CS wounds, the recovery rate was the lowest as the incidence was 20.9% (23/110); the highest prevalence was however observed in motorcycle and automobile accident patients as 22 out of 72 (30.6%) of them were colonized with *P. aeruginosa*, closely followed by other wound types at 29.1% (16/55).

Table 1 Demographic Data of Participants

Criteria for Grouping	Categories	Frequency (%)	Osogbo (n=133)		Iwo (n=104)	
			Male (n=35)	Female (n=98)	Male (n=26)	Female (n=78)
Age (years)	≤19	16 (6.8)	0	2	7	7
	20-39	205 (86.5)	32	90	16	67
	40-59	14 (5.9)	3	6	1	4
	≥60	2 (0.84)	0	0	2	0
Education	No formal	48 (20.1)	10	20	4	14
	Primary	35 (14.8)	3	14	4	12
	Secondary	50 (21.1)	12	18	6	14
	Higher school	81 (34.1)	10	34	9	28
	Postgraduate	25 (10.5)	0	12	3	10
Marital Status	Single	97 (40.9)	15	39	8	35
	Married	140 (59.1)	20	59	18	43
Use of Antibiotics	On antibiotics	141 (59.4)	19	61	17	44
	Not on antibiotics	95 (40.1)	16	37	9	33

Table 2 The frequency of occurrence of *Pseudomonas aeruginosa* from wound samples based on wound type, study location, gender, and age of the participants

LOCATION	SAMPLE TYPE	Number of Samples (%)	Total no infected (%)	Frequency of occurrence of *Pseudomonas aeruginosa*					
				GENDER		AGE GROUPS			
				MALE	FEMALE	≤ 19	20-39	40-59	≥ 60
OSOGBO (n=26) (7.76958ºN; 4.54999ºE)	Automobile accidents	37	10	7	3	2	5	3	0
	Skin lesions/ abrasions	32	7	2	5	2	2	3	0
	Caesarian sections	64	9	0	9	1	6	2	0
	SUBTOTAL	**133 (56.1)**	**26 (42.6)**	**9**	**17**	**5**	**13**	**8**	**0**
IWO (n=35) (7.66686ºN; 4.19926ºE)	Automobile accidents	35	12	7	5	0	7	5	0
	Skin lesions/ abrasions	23	9	6	3	1	5	3	0
	Caesarian sections	46	14	0	14	2	8	4	0
	SUBTOTAL	**104 (43.9)**	**35 (57.4)**	**13**	**22**	**3**	**20**	**12**	**0**
GROSS TOTAL		**237**	**61 (25.7)**	**22**	**39**	**8**	**33**	**20**	**0**

Antibiotic resistance profile among isolates

All tested isolates (100%) were resistant to ticarcillin, tigecycline, and nitrofurantoin. Resistance rates of isolates to fosfomycin, levofloxacin, and aztreonam were also high at 96.3%, 86.9%, and 78.7%, respectively; and moderately high for amikacin at 52.5% (Table 3). Resistances to levofloxacin and amikacin were surprising as fluoroquinolones and aminoglycosides are usually employed in the treatment of *P. aeruginosa* infections **(Foulkes *et al.*, 2021).** The lowest resistance rates in the present study were 4.9%, 6.6%, and 9.8% for piperacillin, meropenem, and imipenem, respectively (Table 3). In line with our findings, **Georgescu *et al.* (2016)** reported high resistance rates to ticarcillin but reported that only 1 out of 12 strains exhibited resistance to meropenem and imipenem. **Mukerjee *et al.* (2012)** also observed that *P. aeruginosa* isolates showed multiple resistance to several antibiotics including β-lactams, cephalosporins, aminoglycosides, and fluoroquinolones; with lowered resistance to piperacillin, amikacin, and ciprofloxacin. The rate recorded in the present study for resistance to piperacillin/tazobactam was 34.4%, however, a recent study **(Schaumburg *et al.*, 2022)** recorded high resistance rates for piperacillin/tazobactam by Gram-negative organisms including *P. aeruginosa* (32.0%) amongst others. Another study reported absolute resistance (100%) to penicillin, moderate resistance to amikacin (35.5%), ciprofloxacin (31.26%), and colistin (40%), albeit sensitivity to piperacillin and ticarcillin in *P. aeruginosa* isolates from wound and burns **(Haleem *et al.*, 2011)**. This observation is similar to our findings of moderate resistance to ciprofloxacin (26.2%) and sensitivity to piperacillin (4.9%). The observed resistance to imipenem in *P. aeruginosa* could be due to several factors which may be molecular or phenotypic as had been previously described by other authors **(Livermore, 2002; Tomas *et al.*, 2010; Moore and Flaws, 2011; Matroş *et al.*, 2016; Fujitani *et al.*, 2017).** All (100%) of the isolates were multidrug-resistant (MDR) being resistant to three or more classes of drugs (Figure 1). A previous study reported that *P. aeruginosa* strains constituted nearly 50.0% of MDR isolates from 5 different hospitals **(Landman *et al.*, 2002).** Also, the profile of MAR indices revealed that all (100%) of the isolates were ≥ 0.2. This extreme value although worrisome, however, correlates well with the earlier observation that a high number of the participants in this study, about 59.4% had been previously exposed to antibiotics. This result denotes a high level

of antibiotic pressure in the study environment. Also, in most developing countries including Nigeria, the sales of antibiotics are not being properly regulated and allow over-the-counter purchases of drugs, contributing to the high rate of indiscriminate consumption of antibiotics at the slight incidence of any ailment.

Table 3 Antibiotic Resistance Profile of *Pseudomonas aeruginosa* isolates recovered from wound samples

Antibiotic Class	Antibiotics	Total[a]	Total[b] (%)	Frequency of occurrence of resistant isolates (%)	
				Osogbo (n = 26)	Iwo (n = 35)
Aminoglycoside	AMK	61	32 (52.5)	13 (40.6)	19 (59.4)
	GEN	48	8 (16.7)	4 (50.0)	4 (50.0)
β-lactam	PIP	61	3 (4.9)	2 (66.7)	1 (33.3)
	TIC	61	61 (100.0)	26 (42.6)	35 (57.4)
β-lactam/inhibitor	PTZ	61	21 (34.4)	10 (47.6)	11 (52.4)
Carbapenem	IMI	61	6 (9.8)	4 (66.7)	2 (33.3)
	MERO	61	4 (6.6)	2 (50.0)	2 (50.0)
Cephalosporins	CEFE	61	20 (32.8)	9 (45.0)	11 (55.0)
	CEFTA	61	14 (22.9)	8 (57.1)	6 (42.9)
Cephalosporin/inhibitor	CEF/AVI	61	20 (32.8)	11 (55.0)	9 (45.0)
Fluoroquinolone	CIP	61	16 (26.2)	7 (43.8)	9 (56.2)
	LEV	61	53 (86.9)	22 (41.5)	31 (58.5)
Glycylcycline	TIG	59	59 (100.0)	25 (42.4)	34 (57.6)
Monobactam	AZT	61	48 (78.7)	21 (43.8)	27 (56.2)
Nitrofuran	NIT	59	59 (100.0)	25 (42.4)	34 (57.6)
Polymyxin	COL	61	16 (26.2)	3 (18.8)	13 (81.2)
Phosphonic acid derivative	FOS	54	52 (96.3)	23 (44.2)	29 (55.8)
Sulfonamide	SUL	45	29 (64.4)	14 (48.3)	15 (51.7)

Legend: Amikacin (AMK), Aztreonam (AZT), Cefepime (CEFE), Ceftazidime (CEFTA), Ceftazidime/Avibactam (CEF/AVI), Ciprofloxacin (CIP), Colistin (COL), Fosfomycin (FOS), Gentamycin (GEN), Imipenem (IMI), Levofloxacin (LEV), Meropenem (MERO), Nitrofurantoin (NIT), Piperacillin (PIP), Piperacillin/Tazobactam (PTZ), Compound sulphonamides (SUL), Ticarcillin (TIC), Tigecycline (TIG). Total[a] = Total number of isolates tested; Total[b] = the Total number of resistant isolates.

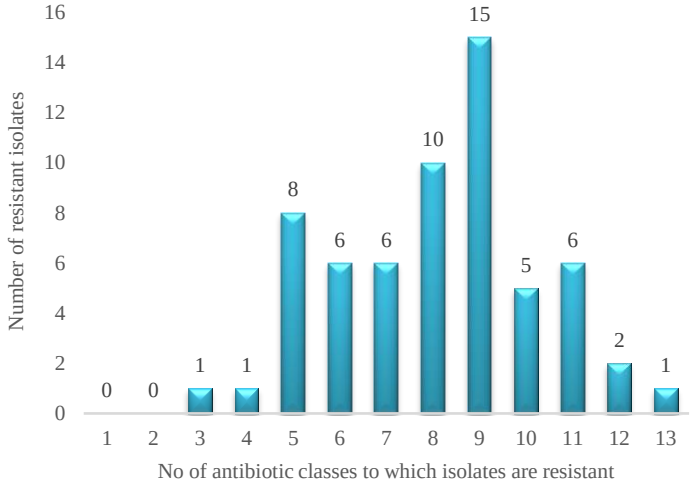


Figure 1 The Pattern of Multidrug Resistance in *Pseudomonas aeruginosa* isolates from various wound samples

Phenotypic detection of virulence determinants

The pathogenicity of *Pseudomonas aeruginosa* is multifaceted because of its invasive and toxigenic nature, regulated through various cell-associated and extracellular hydrolytic enzymes **(Feinbaum *et al.*, 2012)** associated with colonization and bacterial invasion **(Balasubramanian *et al.*, 2013)**. The following virulence determinants were phenotypically screened for in all the 61 *P. aeruginosa* isolates – production of hemolysin, gelatinase, alkaline-protease, coagulase, DNase, pigments, biofilm, as well as motility. Production of pigments, namely pyocyanin and pyorubin, was observed among 51 *P. aeruginosa* isolates; 50 (82.0%) isolates produced green to golden yellow pigments, indicative of pyocyanin production, while one isolate (1.6%) had brown pigment indicating pyorubin production (Figure 2). Pyocyanin is a redox-active, toxic, quorum sensing (QS) controlled phenazine that imparts the characteristic blue colour on *P. aeruginosa* and acts as a siderophore **(El-Fouly *et al.*, 2015).** It has been reported to aid the formation of Reactive Oxygen Species (ROS) **Castañeda-Tamez *et al.* (2018)**, leading to cellular damage and subsequent cell death **(Hall *et al.*, 2016; Castañeda-Tamez *et al.*, 2018). Gupta *et al.* (2011)** found pyocyanin in 57.1% of wound dressings from burn patients infected with *P. aeruginosa*, a rate lower than was observed in the present study. Our study detected hemolysin production in all isolates (100.0%). *P. aeruginosa* hemolysin has been reported to be its most potent toxigenic factor as it aids invasion through cytotoxic effects on eukaryotic cells **(Gupta *et al.*, 2011).** Siderophores and hemolysin act synergistically to promote virulence in *P. aeruginosa* by chelating bound tissue iron during infections, a fact documented in the pathogenesis of respiratory tract, corneal, burn wound, and urinary tract infections **(Gupta *et al.*, 2011).** The biofilm production ability of *P. aeruginosa* is another cause of increasing resistance **(Parasion *et al.*, 2014)**. The prevalence of biofilm producers in this study was 83.6%. A study by **Tahmasebi *et al.* (2022)** reported a rate of 69.0% biofilm production in *P. aeruginosa* isolates from wound infections. Biofilm-mediated infections make up almost 80% of clinical infections reported worldwide **(Lebeaux *et al.*, 2013)**, and biofilms are also implicated in roughly 80% of chronic wounds **(Malone *et al.*, 2017; Mendoza *et al.*, 2019; Thaarup *et al.*, 2022)** but a meagre 6% in acute wounds (**James *et al.*, 2008).** Mono- and polymicrobial biofilm infection has been reported to be a critical causal factor in the non-healing course of chronic wounds **(Rhoads *et al.*, 2012; Bjarnsholt, 2013; Thaarup *et al.*, 2022)**. Interestingly, reports state that *P. aeruginosa* increases the virulence potential of the biofilm by nourishing the growth of other microorganisms in polymicrobial biofilm infections **(Scales and Huffnagle, 2013; Birkenhauer *et al.*, 2014)**. Biofilm production has been reported to depend on several factors including but not limited to QS and the presence of siderophores **(Panayidou *et al.*, 2020; Tahmasebi *et al.*, 2022)**.

Various predisposing factors lead to a higher incidence of chronic wounds, resulting in recurrent hospitalizations and costly therapy **(Sen *et al.*, 2009)**. *Pseudomonas aeruginosa* secretes multiple proteases, including elastase and alkaline protease, that have been implicated in the adherence and invasion of host cells **(Osman *et al.*, 2015; Georgescu *et al.*, 2016).** This study reports that 91.8% of tested isolates were able to produce protease and 96.7% of isolates (59/61) had ≥ 50% of the virulence determinants assessed. Production of gelatinase was observed in 86.9% of the isolates. Gelatinases are protease exoenzymes that hydrolyze gelatin (to polypeptides, then amino acids), and other compounds, including pheromone, casein, collagen, and fibrinogen **(Balan *et al.*, 2012).** Protease production has been reported to stimulate injuries in host tissue and impede the healing of wounds. Again, 91.8% of tested isolates were motile. Motility is significant in pathogen dissemination in the burn wound infection model **(Arora *et al.*, 2005),** and assists microbial invasion and bacterial attachment. *P. aeruginosa* motility once again, is coordinated via the QS system and relayed on *las* and *rhl* systems **(Delden, 2004)**. The role of DNase in the pathogenesis of *Pseudomonas* infections is unclear (**Beenken *et al.*, 2012)**. DNase activity may disable the function of phagocytes once they have engulfed the producing bacteria. DNase may also conserve elastase production as a genetic marker for disseminating strains (**Beenken *et al.*, 2012)**. Varying values of DNase production by *P. aeruginosa* from wound infections have been reported in previous studies **(Holban *et al.*, 2013; Georgescu *et al.*, 2016)**, interestingly, none of the isolates screened in this study produced DNase (0.0%).

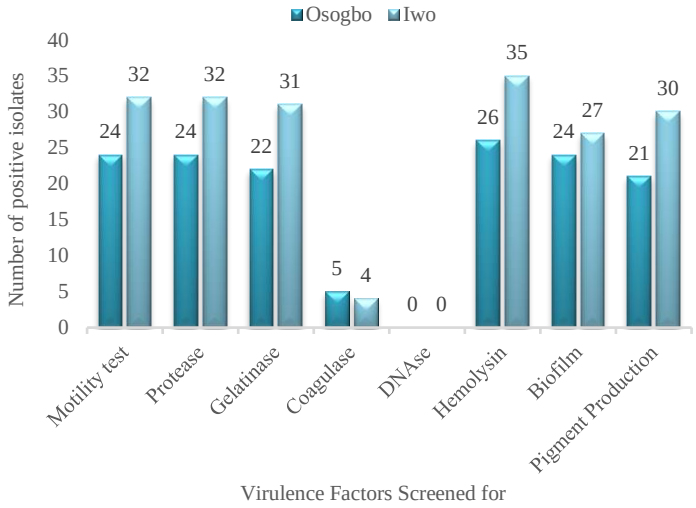


Figure 2 Frequency of occurrence of the virulence factors detected in *P. aeruginosa* isolates

***In-vitro* antibacterial activity screening of honey samples**

The *P. aeruginosa* isolates displayed a high level of resistance to the antibacterial effects of the six honey samples (H1 to H6). More isolates exhibited resistance to the honey samples with the disc diffusion method than with the agar well diffusion method. Fifty-seven out of 61 samples (93.4%) were resistant to H3, followed by 90.1% resistant to H1 and 88.5% resistant to H4. The most effective honey sample was H5 even though a high proportion of isolates were still resistant to it at 77.0%. With the agar well method, 72.1% of the isolates were resistant to H4 (44/61), closely followed by 70.5% and 65.5% resistance to H3 and H1 respectively. The highest activity was exhibited by H6 as only 30 isolates (49.2%) were resistant to it. The agar well diffusion method was observed to be more effective at inhibiting the growth of more strains of *P. aeruginosa* than the disc diffusion method (Figure 3). This could be a result of the fact that the honey samples used were applied directly into the wells and were probably able to diffuse faster into the agar than for disc diffusion. Eighteen *P. aeruginosa* isolates (29.5%) were completely resistant to all six honey samples while only one isolate was sensitive to all six honey samples using both the agar well method and the disc diffusion methods. Previous studies have reported the antibacterial effects of honey against *Staphylococcus aureus* and *Pseudomonas aeruginosa* from wounds **(Gambo *et al.*, 2018)**. Also, a study by **Roberts *et al.* (2015)** observed that the swarming and swimming action was significantly impeded in *P. aeruginosa* due to loss of flagella activity when treated with Manuka honey, and had reduced adhesive characteristics, translating to reduced invasiveness and virulence. In another relevant study, Manuka honey was declared to considerably lessen established *P. aeruginosa* biofilms and hinder the formation of new biofilms by the same pathogen even at low concentrations; and eliminated existing *P. aeruginosa* biofilms at concentrations obtainable in the clinic setting. This trait was correlated with the sugar component in the honey **(Lu *et al.*, 2019).**

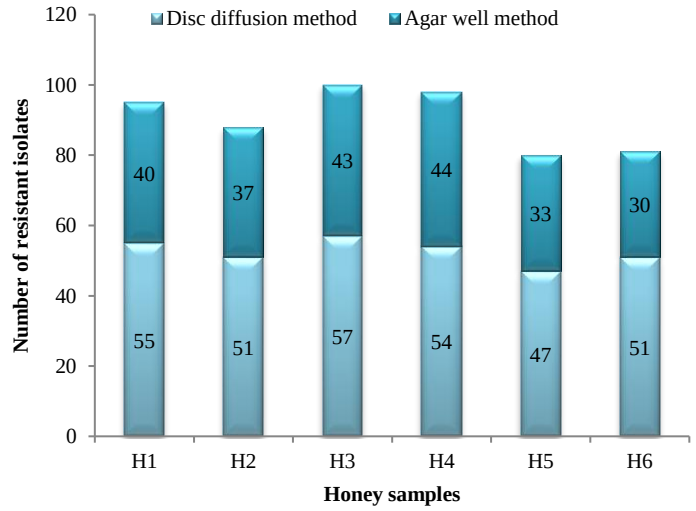


Figure 3 The frequency of isolates resistant to the different honey samples

Gas Chromatography-Mass Spectrometry (GC-MS) analyses of honey samples

Reports of bacterial resistance to honey are rare, hence the high level of resistance exhibited by the *P. aeruginosa* isolates screened in this study is of great concern. This could be adduced to the potency of their antibacterial activity, the concentration of honey used and the nature of the bacteria **(Adeleke *et al.*, 2006; Basualdo *et al.*, 2007).**

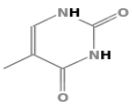
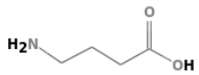
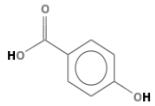
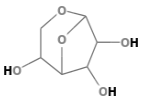
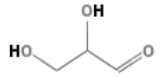
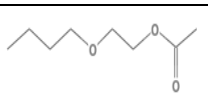
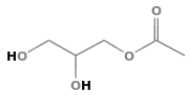
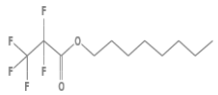
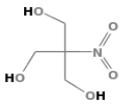
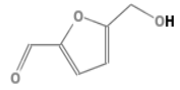
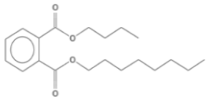
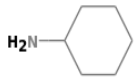
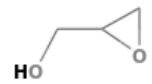
Gas chromatography-mass spectrometry (GC-MS) analyses of the six honey samples (H1 to H6) revealed a wide array of organic compounds present in each honey sample, including fatty acids, amino acids, amines, pyrimidines, monosaccharides, furans, alcohols, dihydroxyflavone, esters, phenolic acids and pyranones. Honey samples H1 and H5 (Type A) had the same organic composition, a total of 22 components; H2, H3, and H6 (Type B) had 20 similar organic components while H4 (Type C) was the only sample with a different organic composition from the others also with 20 phytoconstituents. A total of 14 constituents were found to be common to all six honey types. Two phytoconstituents each were common to honey types A and B (oleic acid and cyclohexylamine) and honey types B and C (glycidol and propanal,2,3-dihydroxy-, (S)-) respectively. However, three components (2,4-dimethyl-1-pentanol, chrysin, and N-Nitroso-N-methyl urea) were found in only types A and C (Table 4 and Figures 4a – 4c). Chrysin, a dihydroxyflavone, has been reported in different honey types such as Manuka, Sage, and Tualang amongst others **(Sun *et al.*, 2016; Deng *et al.*, 2018; Ranneh *et al.*, 2018; M˘arg˘aoan *et al.*, 2021)**, and has been postulated to contribute to the antioxidant property of honey as are other flavonoids. Also, 4-Hydroxybenzoic acid previously reported in various honey types **(Ranneh *et al.*, 2018; M˘arg˘aoan *et al.*, 2021)** was found in all six honey samples analysed in this present study. Other components also found in some (2-Dodecen-1-yl(-) succinic anhydride and maltol), or all of the honey samples in this study (5-Hydroxymethylfurfural, glycerol monoacetate, eicosene and glyceraldehyde), have been reported by another study (**Khan *et al.*, 2017)**

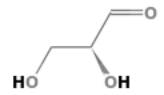
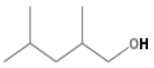
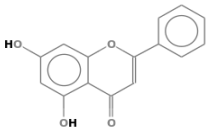
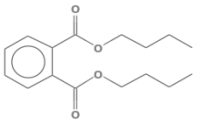
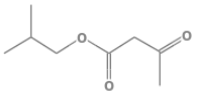
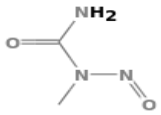
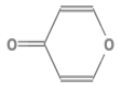
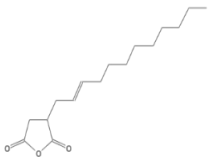
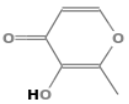
Unheated and unrefined honey has been reported by several authors to possess broad-spectrum antibacterial activity against pathogenic bacteria **(Vandamme *et al.*, 2013; Yaghoobi *et al.*, 2013).** This antibacterial trait is majorly attributed to its low pH, H_2O_2 production, high sugar concentration and high osmolarity, and the presence of various compounds such as acids, phenolic acids, flavonoids, proteins, and carbohydrates alongside a host of yet-to-be-identified compounds **(Al-Waili *et al.*, 2011; Rodriguez *et al.*, 2012; Vandamme *et al.*, 2013).** These compounds can affect the antibacterial activity of honey either by their singular action or through synergistic effects with each other. H_2O_2 is the predominant antimicrobial agent in honey and its concentration depends on the level of glucose oxidase - responsible for the production of H_2O_2 from the conversion of glucose to gluconolactone (**Mama *et al.*, 2019)**, catalase, and the presence of other substances including lysozymes, phenolic acids, and flavonoids **(Kwakman and Zaat, 2012)**. Enzyme levels in bees are said to fluctuate based on the bees' diet and health (**Alaux *et al.*, 2010)**. The level of H_2O_2 in the honey may be also subject to temperature, light, and amount of oxygen all of which change as a result of the processing and storage conditions of the honey (**Gambo *et al.*, 2018)**.

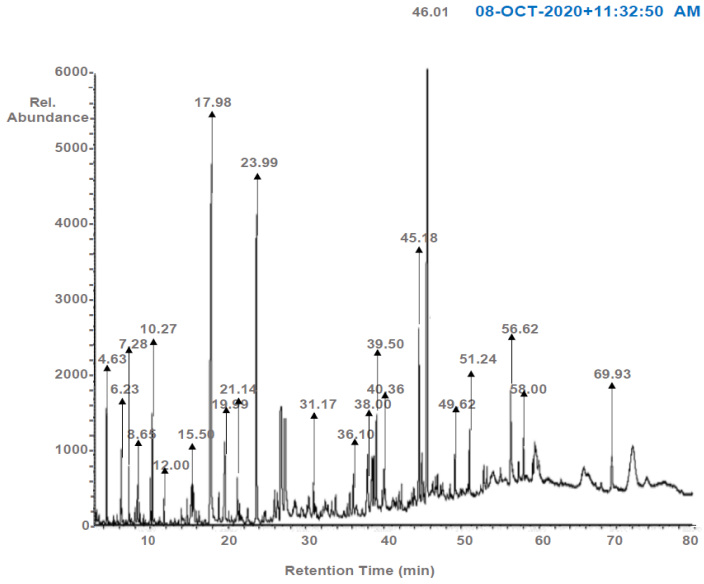
Polyphenolic compounds such as flavonoids and phenolic acids not only have antioxidant activity but also contribute to the antibacterial activity in honey. These compounds have been shown to inhibit the growth of a broad range of Gram-negative and Gram-positive bacteria **(Silici *et al.*, 2010).**

The antibacterial activity of honey against *P. aeruginosa* has been widely reported across many countries (**Wilkinson and Cavanagh, 2005; Sherlock *et al.*, 2010; Shenoy *et al.*, 2012; Anthimidou and Mossialos, 2013)**. Reports of bacterial resistance to honey are rare, hence the high level of resistance exhibited by the *P. aeruginosa* isolates screened in this study is of great concern. This could be adduced to the potency of their antibacterial activity, the concentration of honey used and the intrinsic or acquired resistance characteristics of the bacterial strain **(Adeleke *et al.*, 2006; Basualdo *et al.*, 2007)**. Other factors may include the variations in the phyto-components in each honey type as a function of the environment and floral source or parts from which the honey is produced as well as the bee specie.

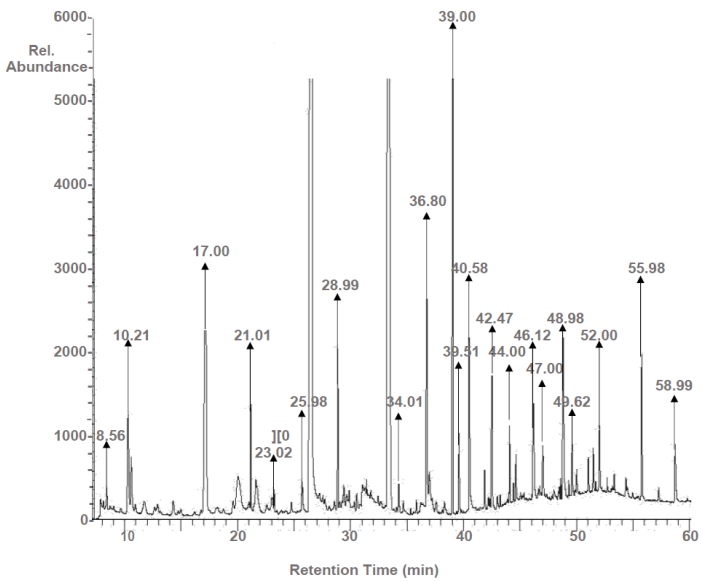
Table 4 Gas Chromatography-Mass Spectrometry (GC-MS) result showing organic components in honey samples

HONEY TYPES	Honey 1	Honey 2	Honey 3	Honey 4	Honey 5	Honey 6	Molecular formula	STRUCTURE
Organic compounds detected in all samples	Thymine	Thymine	Thymine	Thymine	Thymine	Thymine	$C_5H_6N_2O_2$	
	4-Aminobutanoic acid	4-Aminobutanoic acid	4-Aminobutanoic acid	4-Aminobutanoic acid	4-Aminobutanoic acid	4-Aminobutanoic acid	$C_4H_9NO_2$	
	Benzoic acid,4-hydroxy-	Benzoic acid,4-hydroxy-	Benzoic acid,4-hydroxy-	Benzoic acid,4-hydroxy-	Benzoic acid,4-hydroxy-	Benzoic acid,4-hydroxy-	$C_7H_6O_3$	
	1,6-Anhydro-.beta.-d-glucofuranose	1,6-Anhydro-.beta.-d-glucofuranose	1,6-Anhydro-.beta.-d-glucofuranose	1,6-Anhydro-.beta.-d-glucofuranose	1,6-Anhydro-.beta.-d-glucofuranose	1,6-Anhydro-.beta.-d-glucofuranose	$C_6H_{10}O_5$	
	Glyceraldehyde	Glyceraldehyde	Glyceraldehyde	Glyceraldehyde	Glyceraldehyde	Glyceraldehyde	$C_3H_6O_3$	
	2-Butoxyethyl acetate	2-Butoxyethyl acetate	2-Butoxyethyl acetate	2-Butoxyethyl acetate	2-Butoxyethyl acetate	2-Butoxyethyl acetate	$C_8H_{16}O_3$	
	Eicosene	Eicosene	Eicosene	Eicosene	Eicosene	Eicosene	$C_{20}H_{40}$	
	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	
	Glycerol monoacetate	Glycerol monoacetate	Glycerol monoacetate	Glycerol monoacetate	Glycerol monoacetate	Glycerol monoacetate	$C_5H_{10}O_4$	
	Pentafluoropropionic acid, octyl ester	Pentafluoropropionic acid, octyl ester	Pentafluoropropionic acid, octyl ester	Pentafluoropropionic acid, octyl ester	Pentafluoropropionic acid, octyl ester	Pentafluoropropionic acid, octyl ester	$C_{11}H_{17}F_5O_2$	
	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	$C_4H_9NO_5$	
	5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	5-Hydroxymethylfurfural	$C_6H_6O_3$	
	11-Octadecenoic acid, methyl ester	11-Octadecenoic acid, methyl ester	11-Octadecenoic acid, methyl ester	11-Octadecenoic acid, methyl ester	11-Octadecenoic acid, methyl ester	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	
	1,2-Benzenedicarboxylic acid, butyl octyl ester	1,2-Benzenedicarboxylic acid, butyl octyl ester	1,2-Benzenedicarboxylic acid, butyl octyl ester	1,2-Benzenedicarboxylic acid, butyl octyl ester	1,2-Benzenedicarboxylic acid, butyl octyl ester	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	
Other organic compounds present in each sample	Oleic acid	Oleic acid	Oleic acid	--Nil--	Oleic acid	Oleic acid	$C_{18}H_{34}O_2$	
	Cyclohexanamine	Cyclohexanamine	Cyclohexanamine	--Nil—	Cyclohexanamine	Cyclohexanamine	$C_6H_{13}N$	
	--Nil--	Glycidol	Glycidol	Glycidol	--Nil--	Glycidol	$C_3H_6O_2$	

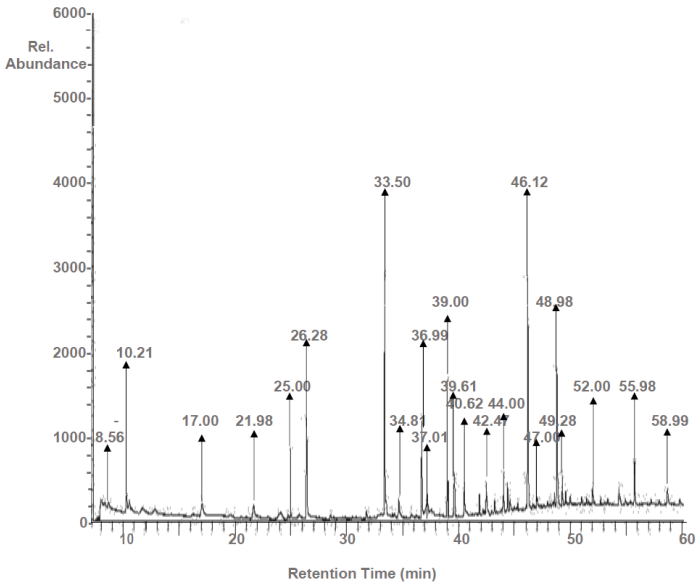
HONEY TYPES	Honey 1	Honey 2	Honey 3	Honey 4	Honey 5	Honey 6	Molecular formula	STRUCTURE
	--Nil--	Propanal,2,3-dihydroxy-, (S)-	Propanal, 2,3-dihydroxy-, (S)-	Propanal, 2,3-dihydroxy-, (S)-	--Nil--	Propanal, 2,3-dihydroxy-, (S)-	$C_7H_{16}O$	
	2,4-dimethyl-1-pentanol	--Nil--	--Nil--	2,4-dimethyl-1-pentanol	2,4-dimethyl-1-pentanol	--Nil--	$C_7H_{16}O$	
	Chrysin	--Nil--	--Nil--	Chrysin	Chrysin	--Nil--	$C_{15}H_{10}O_4$	
	--Nil--	Dibutyl phthalate	Dibutyl phthalate	--Nil--	--Nil--	Dibutyl phthalate	$C_{16}H_{22}O_4$	
	--Nil--	Butanoic acid, 3-oxo-, 2-methyl propyl ester	Butanoic acid, 3-oxo-, 2-methyl propyl ester	--Nil--	--Nil--	Butanoic acid, 3-oxo-, 2-methyl propyl ester	$C_2H_5N_3O_2$	
	N-Nitroso-N-methyl urea	--Nil--	--Nil--	N-Nitroso-N-methyl urea	N-Nitroso-N-methyl urea	--Nil--	$C_2H_5N_3O_2$	
	4H-pyran-4-one	--Nil--	--Nil--	--Nil--	4H-pyran-4-one	--Nil--	$C_5H_4O_2$	
	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	--Nil--	--Nil--	--Nil--	Octadecanoic acid 2-hydroxy-1-(hydroxymethyl)ethyl ester	--Nil--	$C_{21}H_{42}O_4$	
	2-Dodecen-1-yl(-) succinic anhydride	--Nil--	--Nil--	--Nil--	2-Dodecen-1-yl(-) succinic anhydride	--Nil--	$C_{16}H_{26}O_3$	
	--Nil--	--Nil--	--Nil--	Maltol	--Nil--	--Nil--	$C_6H_6O_3$	



(4a)



(4b)



(4c)

Figure 4a – 4c GC-MS Chromatograph of honey samples (a) Honey samples 1 and 5; (b) Honey samples 2, 3, and 6; (c)) Honey sample 4.

CONCLUSION

All the isolates recovered in this study were multidrug-resistant and extruded many virulence factors. More significantly, many of these isolates were able to resist the antibacterial effects of honey, which is reported widely to aid wound healing and inhibit the growth of both Gram-positive and -negative bacteria. The presence of such virulent and multidrug-resistant strains in open wounds emphasizes the need to prevent contamination of injuries, which is a significant cause of mortality. Healthcare practitioners must implement sound management practices. Appropriate hand hygiene, wearing gloves, and gowns, avoidance of direct contact between infected patients, sterilization, and disinfection of patient-care equipment is necessary for effective control of *P. aeruginosa* infections. Given the very scarce reports of resistance to honey by various bacterial species, the low activity of honey against *P. aeruginosa* with multidrug resistance and virulence traits portends serious complications, especially in patients with debilitating and chronic wounds, and underscores the necessity for the continued hunt for natural compounds with antibacterial properties against these virulent strains.

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