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Research Article

Antimicrobial Activity of Honey against Bacterial Isolates Associated with Wound Infections

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Abstract

The emergence of multidrug resistant bacteria strains constitutes a global public health challenge. Alternative antimicrobial sources like plants and plant based products such as honey have currently gained considerable attention. Due to the varieties of honey that exist and major classifications being used on geographical location and floral source, it was observed that only few varieties of honey with popular sources have been tested and affirmed to possess antimicrobial activities. This study therefore investigated the antibacterial potentials of honey from five different sources against selected clinical bacterial isolates associated with wound infections and their corresponding typed isolates, viz: *Staphylococcus aureus,* Methicillin Resistant *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Citrobacter freundii* and *Acinetobacter baumannii.* The organisms were cultured on appropriate media and subjected to various biochemical tests to confirm their identities. The honey samples were screened for phytochemical constituents using standard chemical methods. The antibacterial activity of honey was assayed using Agar well diffusion technique. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by standard microbiological techniques. Determination of killing rate on the bacteria was performed by time-kill kinetic assay. Determination of sodium and potassium ions leakage was evaluated using flame photometry. The qualitative phytochemical screening of the honey samples revealed the presence of various phyto constituents, including: Tannins, Alkaloids, Flavonoids, Saponin, Phlobatannins and Terpenoid. The isolates demonstrated varying resistance to the commercially available antibiotics employed in this study. The mean zone of inhibition of the honey against the typed isolates ranged between 4.00 ± 0.00^{a} and 20.00 ± 0.3^{c}; While that for the clinical isolates ranged between 2.00 ± 01.4^{b} and 14.00 ± 0.8^{c}. Amongst the five honeys evaluated in this study, only honey from apiaries in Oyo State and Kwara State showed inhibitory effect on the growth of the studied pathogens. The honeys exhibited concentration dependent inhibition against the test isolates, with honey from Oyo State showing the highest efficacy with zone of inhibition 20.00 ± 0.3^{c} against typed isolate of *Proteus mirabilis* ATCC 29906. The honey used in this study compared favorably with Amoxicillin, Chloramphenicol, Augumentin, Amplicox and Zinnacef antibiotics. Investigation of the rate of killing of the honey revealed that the honey had an appreciable killing rate against the test bacterial isolates. Findings from this study proved two varieties of honey to be effective against wound associated antibiotic resistant bacteria; being able to inhibit the growth of all test organisms at varying concentrations. Natural products like honey hold a great potential in combating the challenge of antibiotics resistance. Further exploration into the effect of honey in living systems by *in vivo* experiments should be pursued.

Keywords: *Inhibitory; Antibiotics; Minimum Bactericidal Concentration; Multidrug Resistance; Phytochemical; Photometry*

Introduction

The primary function of intact skin is to control microbial populations that live on the skin surface and to prevent underlying tissue from becoming colonized and invaded by potential pathogens [24]. Exposure of subcutaneous tissues, following loss of skin integrity by wounds provides a moist, warm and nutritious environment that is conducive to microbial colonization and proliferation. Since wound colonization is mostly caused by mixed microbial populations, that are potentially pathogenic, any wound is at some risk of becoming infected [9]. The indiscriminate use of antibiotics has led to the emergence of multidrug resistant bacterial strains, a global public health problem [18].

To solve this challenge, alternative antimicrobial strategies like plants and plant-based products such as honey have currently gotten more attention [3,22].

Honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature. To date, there are many publications on studies performed both *in vitro* and *in vivo* on the therapeutic properties of honey and these recent findings have confirmed its activity against a wide range of medically important bacteria [20]. Honey is produced from many floral sources and its antimicrobial activity varies greatly with its origin and processing. Due to the high cost of importation, production and resistance to antibiotics, the need to look inward for a natural approach and less expensive way of handling wound infections arises. The findings of this study would be important as it would provide alternative antimicrobial agent to overcome the problems of increasing bacterial resistance to synthetic antimicrobial agents and the high cost of production and importation of these drugs. Knowledge of honey and its antimicrobial activity is known and has been deployed, but due to varieties of honey that exists and major classification being based on geographical location and floral source, it was discovered that only few varieties of honey with popular origins have been tested and affirmed to have antimicrobial activity. This study therefore investigated the antimicrobial activities of honey from five different sources.

Materials and Methods

Culture media used in this study include: Nutrient agar (NA), MacConkey Agar (MCA), Eosin Methylene Blue Agar (EMB), Blood Agar (BA) and Mueller Hinton Agar (MHA). Chemicals used include: staining reagents, alcohols, acids, distilled water, normal saline solution, barium chloride ($BaCl_2$), neutralization buffer and Dimethyl sulfoxide (DMSO).

Test organisms

The test organisms used were both typed and clinical isolates of *Staphylococcus aureus,* Methicillin Resistant *Staphylococcus aureus* and *Bacillus subtilis, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Citrobacter freundii* and *Acinetobacter baumannii*.

The clinical isolates associated with infected wounds were obtained from University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria; while the typed isolates were obtained from bioscience laboratory at International Institute of Tropical Agriculture (IITA), Ibadan, Oyo state, Nigeria.

Preparation and sterilization of culture media

All the culture media were prepared according to manufacturer's specifications and standard techniques.

Identification and characterization of isolates

Colony morphology

The obtained isolates were identified by using their cultural and morphological characteristics on media. This was followed by microscopic examination of the bacterial isolates and cultural features examined include shape, elevation, surface, edge and consistency [6].

Gram's staining technique

To carry out gram stain, a discrete colony was picked from 24 hour old culture with a sterile inoculating loop and made into a smear on a clean grease free slide. The smear was air dried and heat-fixed by quickly passing the slide over Bunsen flame. The slide was then placed on staining rack over a sink and flooded with crystal violet solution for 1 minute, after which it was rinsed with running water. It was again flooded with mordant lugol's iodine for 30 seconds and then rinsed with water. It was decolorized with 95% alcohol for about 30 seconds and washed immediately with water. The slide was counter stained with Safranin solution for 1 minute and washed with distill water, blot dried and examined under the microscope with oil immersion objective [10]. Bacteria that showed blue-purple colour after counter staining are Gram positive, while those that appeared pink-red in colour are Gram negative.

Catalase test

Catalase test was carried out to know the specific species of bacteria related with hydrogen peroxide to produce oxygen and water. The presence of the enzyme catalase is evident in most aerobic organism. A sterile wire loop was used to pick a colony of the organism of 24 hours old culture and placed in a drop of 3% hydrogen peroxide on a clean glass slide. Effervescence caused by the liberation of oxygen as a gas bubble indicates the production of catalase by the bacterium while a negative result indicates the absence of catalase [10].

Coagulase test

A colony from the isolate was emulsified in normal saline on clean grease free slide and an equal volume of plasma were added and mixed together aseptically. Clumps or precipitate in the mixture indicates a positive coagulase test, this shows that the organism produces coagulase enzyme while the absence of clumps gives a negative result [10].

Citrate utilization test

Each bacterium was inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. The medium also contained inorganic ammonium salts, which were utilized as sole source of nitrogen. This results in a change in the medium's colour from green to blue. Bacteria colonies are picked up from a straight wire and inoculated into slope of simmons citrate agar and incubated for 24 hours at 37°C. If the microorganism has the ability to use citrate, the medium changes its colour from green to blue [6].

Acid production from carbohydrate (Sugar test)

Sugar fermentation test was performed to detect the ability of the bacteria isolate to ferment sugars either with the production or non-production of gas. The sugars used includes: lactose, glucose, and sucrose. Each sugar solution was prepared by dissolving 0.5g of the various sugars in 5 ml of distilled water in test tubes. Nutrient broth was prepared according to manufacturer's specification and two drops of phenyl red indicator was dispensed into the conical flask containing the broth. The broth media was then dispensed in equal proportion (10 ml) into the test tubes. Durham tubes were inserted in an inverted form into the mixture and sterilized in an autoclave at 15 psi-120°C for 15 minutes. Each tube was labeled and inoculated with the test organisms and incubated at 37°C for 2 - 7 days. Uninoculated bottles were used as control. Fermented sugar solution gives yellow colouration indicating a positive reaction while unfermented sugar solution retains its original colour, indicating a negative reaction. Acid production in some cases is accompanied with carbon dioxide evolution which was made visible in the Durham tube [27].

Urease test

A little of the culture of the test bacteria was streaked over the surface of the agar slant of urease test medium with phenol red as indicator and incubated at 37°C for 7 days. A control of the basal medium containing no added urea was equally inoculated. A colour change of the medium from yellow to pink or red was an indication of a positive result and no colour change indicate a negative result [10].

Methyl red test

Glucose phosphate broth (1g glucose, 0.5% KH_2PO_4, 0.5% peptone and 100 ml distill H_2O) was dispensed in clean test tubes and sterilized at 121°C for 15minutes. The tubes were inoculated with the test organism and incubated at 37°C for 48 hours. At the end of incubation, five drops of methyl red solution was added to each test tube. The appearance of red colour indicates positive reaction [27].

Motility test

This test was performed by making a bacterial suspension with a loopful of bacteria in normal saline solution on a clean grease-free glass side. The suspension was covered with a cover slip and observed under x10 and x40 objective lenses. Motile bacteria possess motility structures and move freely in the suspension while the non-motile bacteria show no movement [27].

Oxidase test

A piece of filter paper was placed in a sterile petri dish and 2 - 3 drops of freshly prepared oxidase reagent was added. Using a sterile glass rod, a colony of the test bacterium was picked and smeared on the filter paper and was observed for 10 seconds. The presence of blue-purple colour indicates a positive oxidase while no blue-purple colour indicates a negative oxidase test [6].

Preservation of pure cultures

The identified isolates were transferred onto sterile Nutrient agar after identification and kept refrigerated at 4°C until the antimicrobial susceptibility tests were performed.

Standardization of inoculum

In order to ensure equal distribution of inoculum for the Antimicrobial susceptibility testing, 0.5 McFarland Standard was prepared. A solution of 1% (w/v) anhydrous Barium Chloride ($BaCl_2$) was prepared by suspending 0.5g of $BaCl_2$ in 50 ml of distilled water. After which 1% (v/v) cold pure sulfuric acid (H_2SO_4) was prepared by dispensing 1 ml of H_2SO_4 in 99 ml of distilled water. Thereafter, 0.5 ml of 1.0% $BaCl_2$ was added to 99.5 mL of 1% H_2SO_4 solution. The resulting solution was thoroughly mixed to maintain a suspension. Afterwards, about 5 mL of the 0.5 McFarland Standard was distributed into bijou bottles. The solution was kept at 4°C in the refrigerator until it is required for use [8]. When these standards are thoroughly shaken the turbidity equals that of a culture containing about 1.5×10^8 cells [8].

Collection of honey

Honey samples were obtained from local apiarists in Federal University of Technology Akure (FUTA), Oja oba market Akure, Oyo state, Kwara state, Akure supermarket, all in Nigeria.

Preparation of honey

The honey was aseptically filtered with sterile Seitz filter with a pore size of 0.02 mm connected to an electrically operated vacuum pump. This was done to remove particles and impurities in the honey. The honey was protected from bright light to prevent photo degra-

dation of the glucose oxidase enzyme. The honey filtrates were stored at room temperature in amber bottle for protection against bright light to prevent photo degradation of the glucose oxidase enzyme.

Phytochemical properties of honey

The sample was screened for the following compounds: alkaloids, flavonoids, saponins, tannins, phlobatannin and terpenoid using standard laboratory techniques [26].

Preparation of diluent

Dimethyl sulfoxide was used to dissolve honey to make different concentrations. The 50% of Dimethyl sulfoxide was prepared by adding 5 ml of sterile distilled water to 5 ml of Dimethyl sulfoxide.

Susceptibility test of organisms

The antimicrobial activity of honey was assayed using Agar well diffusion technique [6]. Mueller-Hinton agar plates (in triplicates) were prepared according to manufacturer's specification. A 0.1 ml of the test organisms was spread onto the solidified agar surface with a sterile glass spreader; the plates were allowed to fully adsorb the inoculum before a sterile cork-borer of (5 mm) was used to dig wells on the seeded plates. The holes were sufficiently spaced and labeled accordingly with numbers. A 0.1 ml of the honey samples which includes dilutions of 20%, 50% 70%, dimethyl sulfoxide as diluent and undiluted honey were introduced into the holes, the plates were allowed to stand for 30 minutes to ensure proper diffusion of the honey into the medium before incubating un-inverted at 37˚C for 24 hours. The sensitivity of the test organisms is indicated by a halo around the wells and the diameter of the halo were taken as an index of the degree of susceptibility of isolates to honey.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration that is able to inhibit any visible bacterial growth on the culture tube [25]. The MIC of the honey against isolates was evaluated by the broth dilution method. Decreased concentration of the honey sample with Dimethyl sulfoxide as diluent was prepared (20 - 70% v/v). In test tubes containing 8 ml of sterile nutrient broth, a 1 ml of each concentration and 1 ml of broth culture of test organism approximately 10^7 - 10^8 cells were introduced separately. The test tubes were gently homogenized for even mix and were incubated at 37°C for 24 hours. The test tubes were examined for turbidity and clarity by measuring the absorbance and transmittance using spectrophotometer after 24 hours incubation.

Determination of minimum bacteriocidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of the sample required to kill the bacteria [12]. The MBC was determined by plating a 1 ml of the MIC positive tubes for 24 hours on nutrient agar to evaluate the bacteriostatic and bactericidal activities of honey.

Determination of killing kinetics on bacteria

The method of [26] was used to determine the killing rate of honey against isolates.

Viable count of the microbial cells of 3log cfu to be used was first determined and then 0.5 ml volume of known concentration from each 18 hours broth culture suspension was added to 4.5 ml of the test fraction such that the final concentration gave 0.5, 0.25, 0.12, 0.06 and 0.03 percent (v/v). The suspension was thoroughly mixed and held at room temperature of 28°C and the killing rate after ½h, 1h, 1½h

and 2 h was determined. A 0.5 ml volume of each suspension was withdrawn at appropriate interval and transferred to 4.5 ml neutralization buffer to neutralize the effect of any antimicrobial compound carrying over the suspension. The resulting mixture was diluted serially at 10 fold with sterile distilled water. Exactly 0.5ml of the final dilution was pour plated into pre-sterilized nutrient agar at 45°C. The plates were allowed to set and incubated upside down at 37°C for 72 hours and controls were set up alongside after which microbial population was enumerated. The resulting data was graphically represented by plotting Log cfu was against time for construction of the bactericidal kinetic curves. Gentamicin was used as a positive control. Triplicate reading were made for each suspension.

Determination of sodium and potassium ions leakage

The methods of [13] were adopted for the determination of the potassium and sodium ions leakage from susceptible clinical isolates. An 18 hour old nutrient broth culture was used; the cells were washed in physiological saline and centrifuged before use. A 0.5 ml of the cell suspension of each organism was added to 4.5 ml of the prepared concentrations of honey. The supernatant solutions obtained after centrifugation at 6000 rpm were analyzed for potassium and sodium ion leakage using flame photometer. Triplicate reading were made for each suspension.

Haemolytic assay

The haemolytic activity of honey extracts was determined using the disc diffusion methods as described by [5]. Blood agar was prepared and wells measuring 5.0 mm were made on the agar using sterile cork borer and wells were filled with 0.5 ml of the honey. The plates were then incubated at 37°C for 24 hours. Clear zones of haemolysis indicated positive result. The tests were carried out in triplicates and the means recorded; control was made for the experiment using chloramphenicol as positive control and water as negative control.

Statistical analysis

All experiments were carried out in triplicates, and data obtained were subjected to one way analysis of variance, while the means were compared by Duncan's New Multiple Range Test at 95% confidence interval using Statistical Package for Social Sciences version 23.0. Differences were considered significant at $p \leq 0.05$.

Results

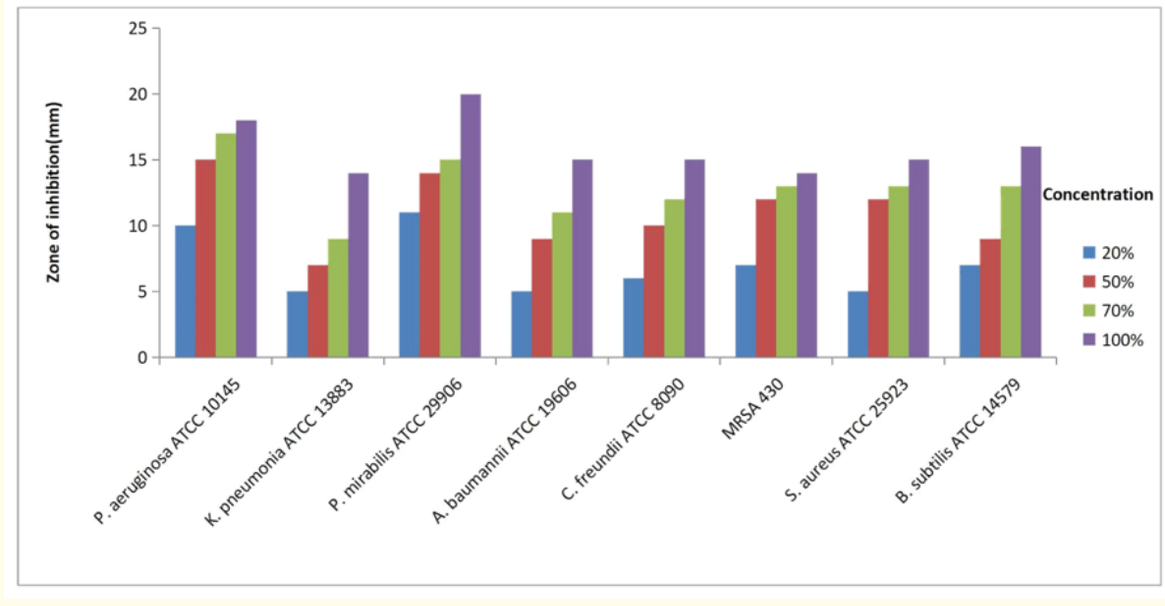

Figure 1: *Zone of inhibition produced by Honey A at varying Concentrations on typed strain bacteria isolates. A represents Honey obtained from Ibadan, Oyo state local apiarists, mm: milimeter.*

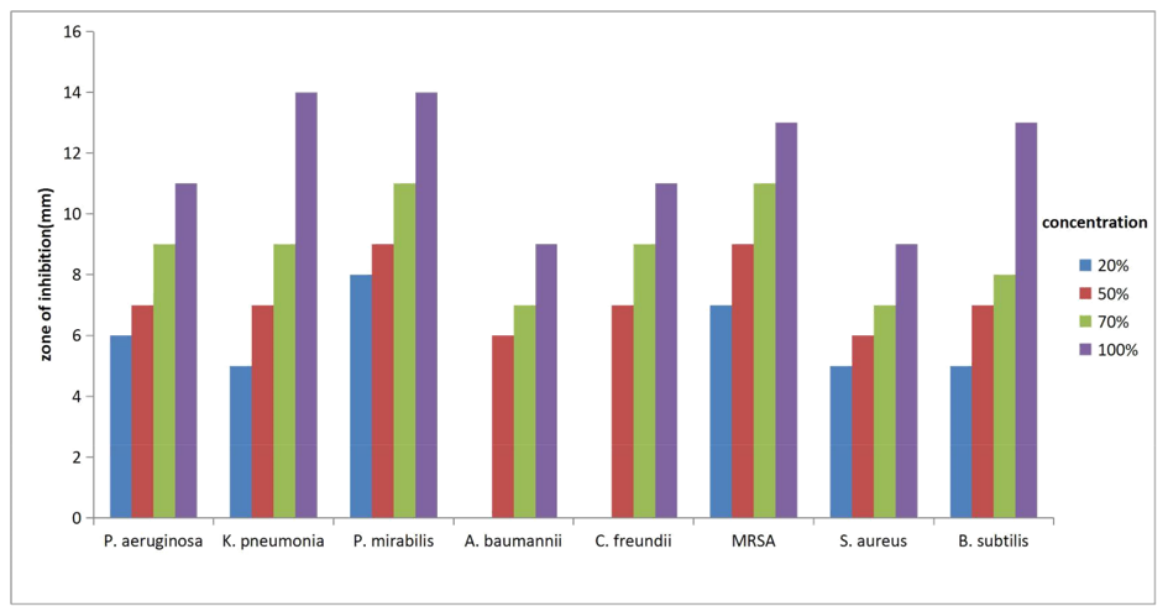

Figure 2: *Zone of inhibition produced by honey A at varying Concentrations on clinical bacteria isolates. A represents Honey obtained from Ibadan, Oyo state local apiarists, mm: Millimeter.*

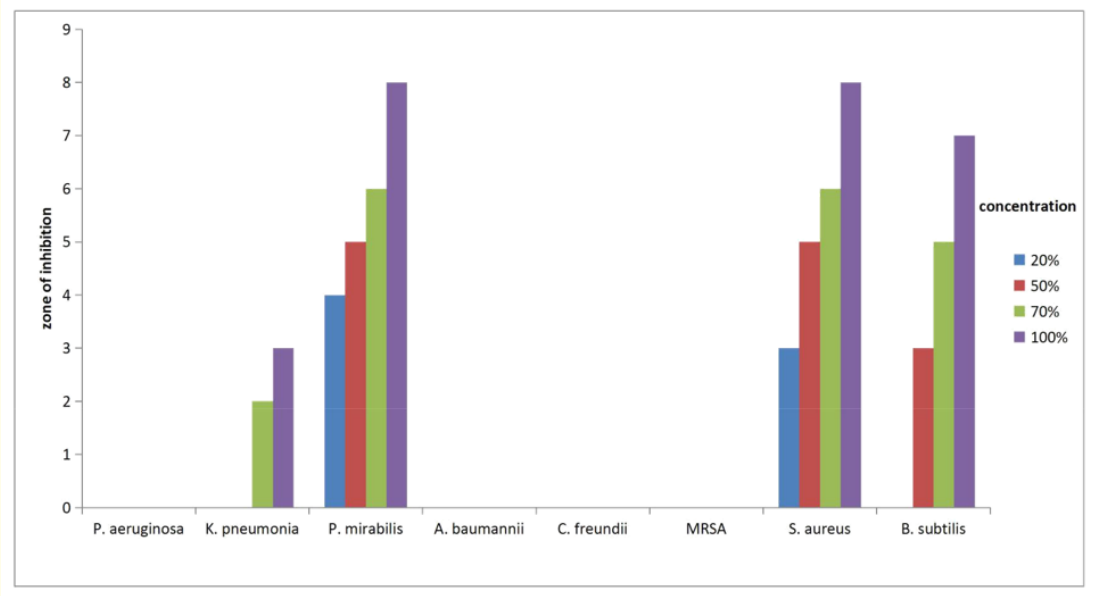

Figure 3: *Zone of inhibition produced by honey C at varying Concentrations on clinical bacteria isolates. C represents honey obtained from Ilorin, Kwara state, mm: Millimeter.*

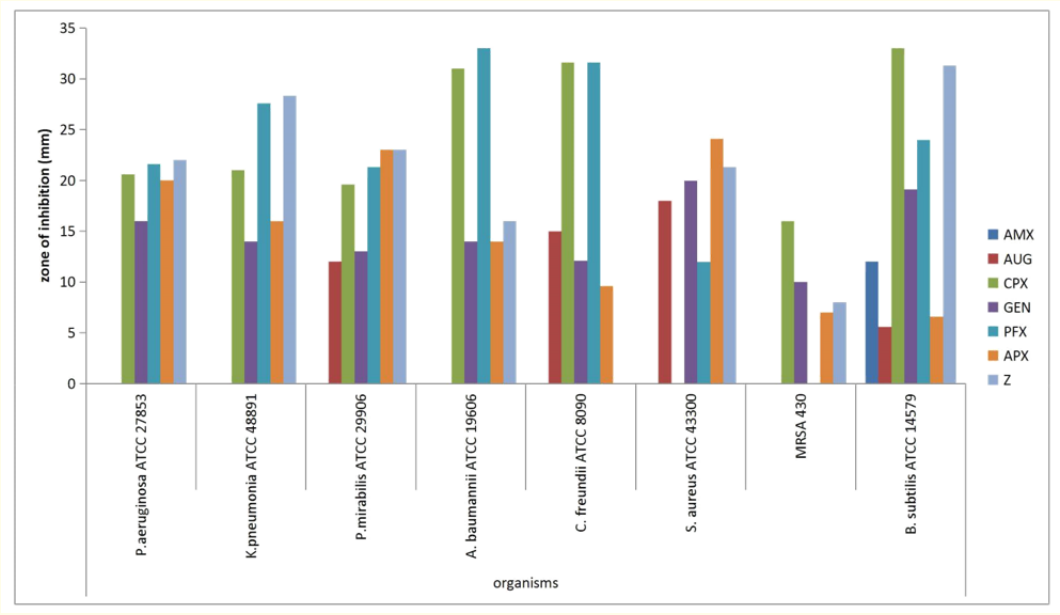

Figure 4: *Antibiotics susceptibility test of Typed bacteria isolates.*

AMX: Amoxicillin; AUG: Augumentin; CPX: Ciprofloxacin; GEN: Gentamicin; PFX: Pefloxacin; APX: Amplicox, Z: Zinnacef.

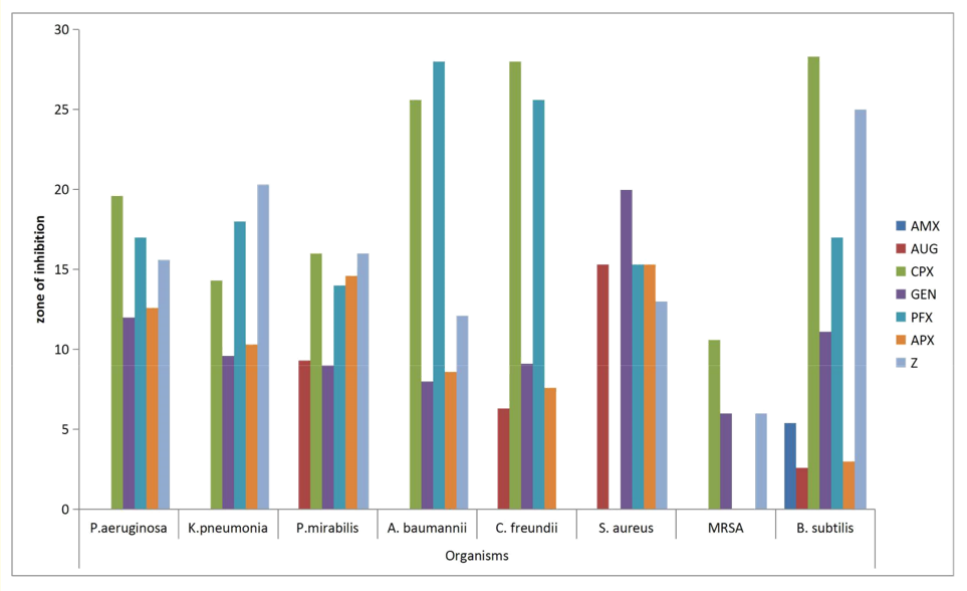

Figure 5: *Antibiotics susceptibility test of clinical bacteria isolates.*

AMX: Amoxicillin; AUG: Augumentin; CPX: Ciprofloxacin; GEN: Gentamicin; PFX: Pefloxacin; APX: Amplicox, Z: Zinnacef.

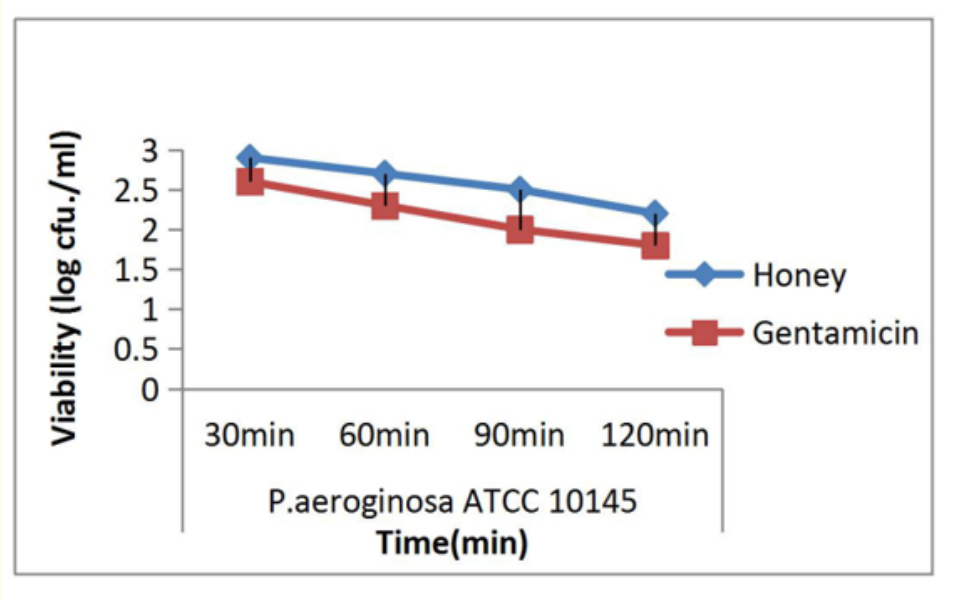

Figure 6: *Kinetic killing time of honey and K. pneumoniae ATCC 27853.*

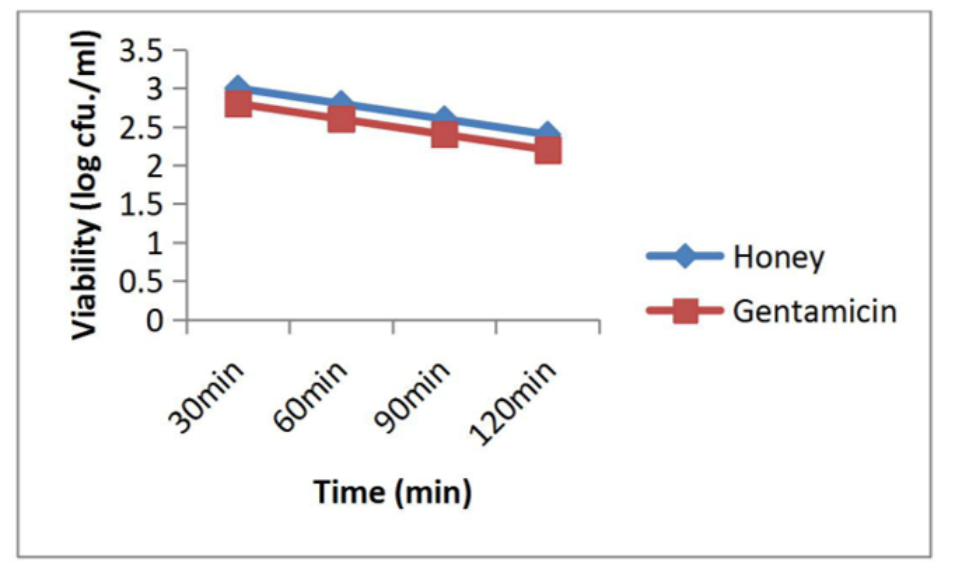

Figure 7: *Kinetic killing time of honey and gentamicin against Gentamicin against P. aeruginosa ATCC 48891.*

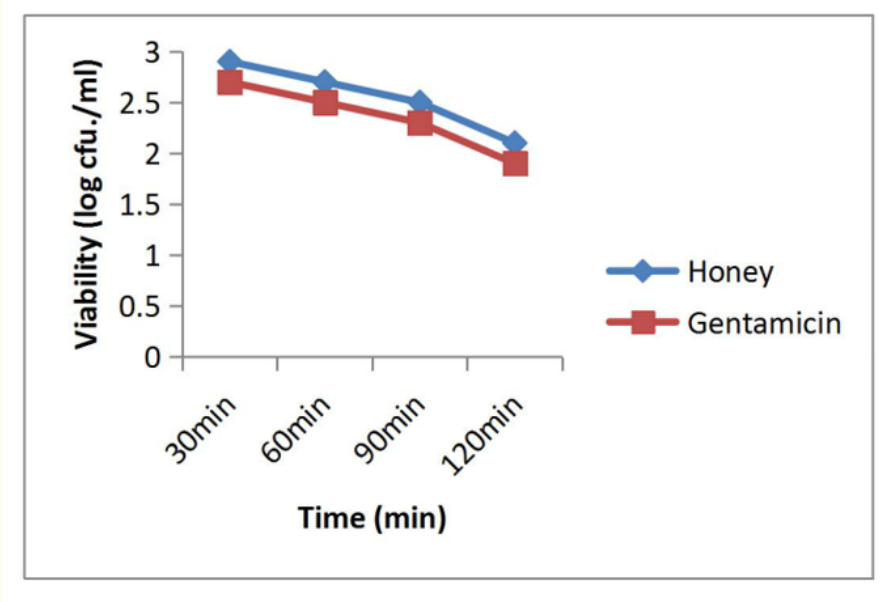

Figure 8: *Kinetic killing time of honey and Gentamicin against gentamicin against P. mirabilis 29906.*

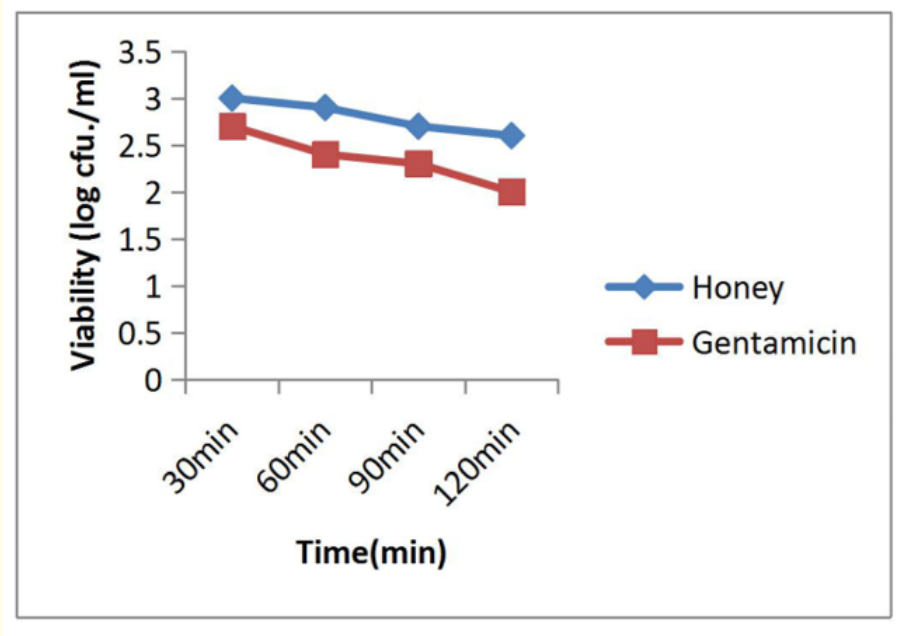

Figure 9: *Kinetic killing time of honey and A. baumannii 19606.*

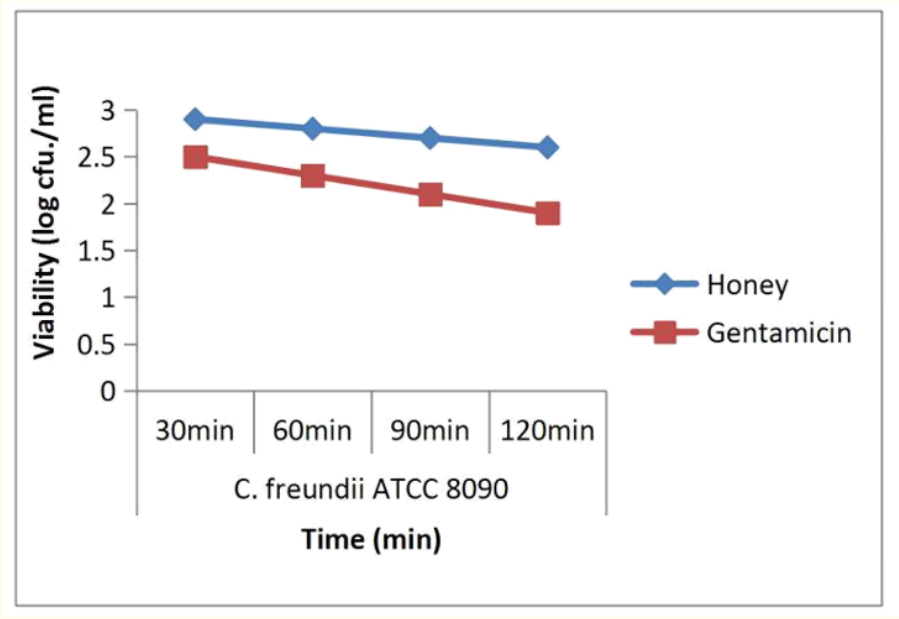

Figure 10: *Kinetic killing time of honey and Gentamicin against C. freundii 8090.*

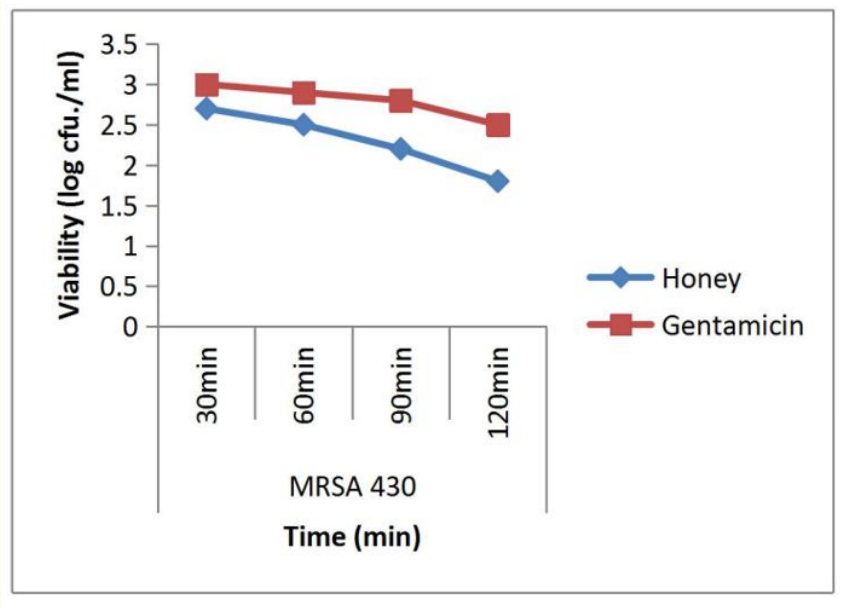

Figure 11: *Kinetic killing time of honey and Gentamicin against MRSA 430.*

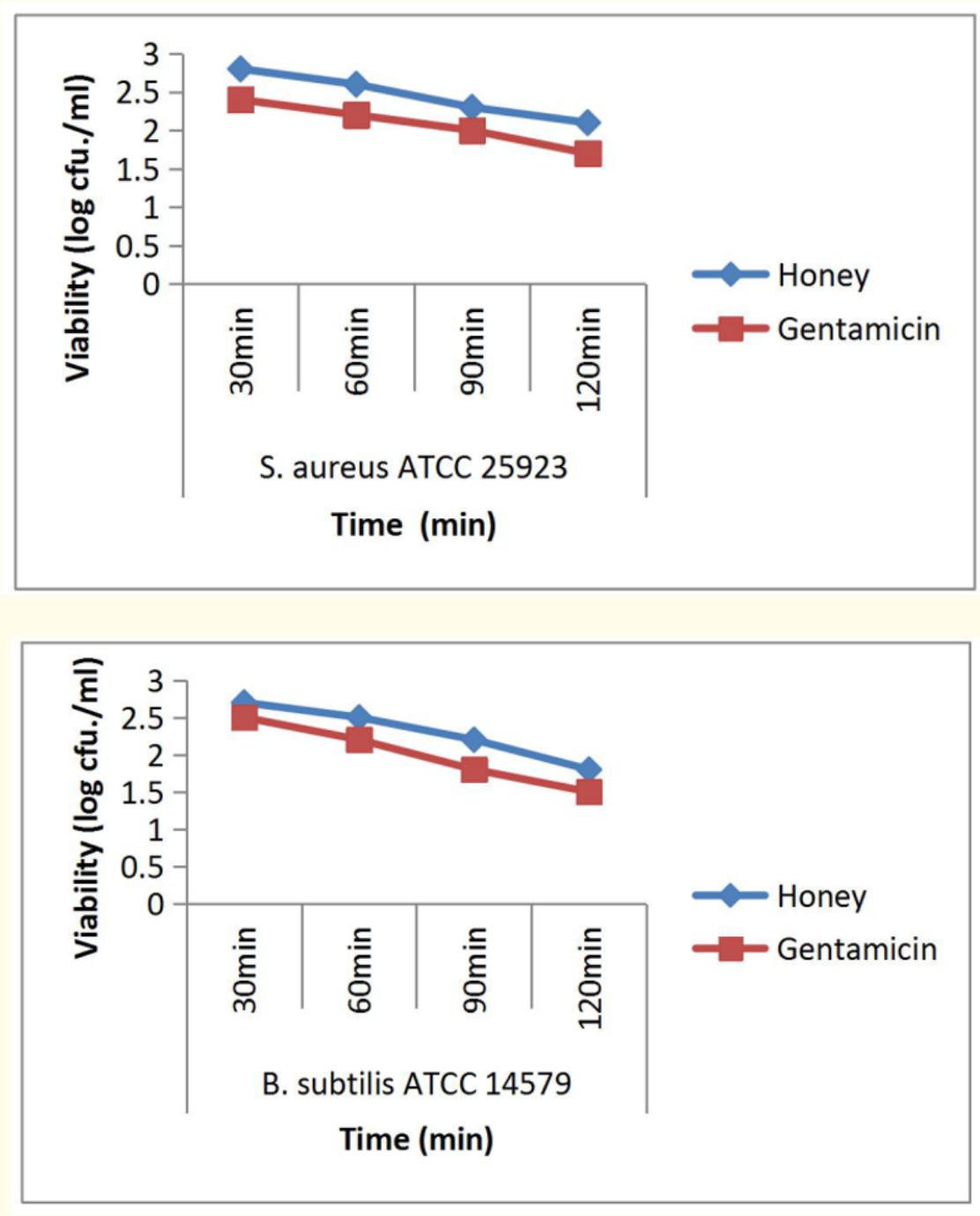

Figure 12: *Kinetic killing time of honey and Gentamicin against S. aureus ATCC 43300 and B. subtilis ATCC 14579*

Isolates	Cultural properties	Morphological Characteristics	Special medium used	Gram reaction	H_2S	Coagulase	Oxidase	Catalase	Methyl red	Citrate	Mannitol	Urease	Motility	Sucrose	Lactose	Glucose	Presumptive identification
A	Blue-Greenish fluorescent colonies on NA with slimy surface	Rod shaped, flagellated, red colour	NA	-	-	-	+	+	-	+	+	-	+	-	-	-	*Pseudomonas aeruginosa*
B	Mucoid pink-red white on MCA	Rod shaped	MCA	-	-	-	-	+	-	+	+	+	-	+	+	+	*Klebsiella Pneumoniae*
C	Swarming growth on BA	Short straight rods	BA	-	+	-	-	+	+	+	-	+	+	-	-	+	*Proteus mirabilis*
D	Smooth rounded mucoid colonies	Cocco-bacilli in pairs	NA	-	-	-	-	+	-	+	-	-	-	-	+	+	*Acinetobacter baumannii*
E	Gray shiny surface on NA	Straight rods singly or in pairs	NA	-	+	-	-	+	+	+	+	V	+	+	+	+	*Citrobacter freundii*
F	Beta-haemolysis on BA from pinkish to yellow	Cocci in clusters, deep blue colour	MSA, BA	+	-	+	-	+	+	+	-	-	-	+	+	+	
G	Grey- white granular colonies	Straight rods	NA	+	-	-	V	V	-	+	+	-	+	+	V	+	*Bacillus subtilis*
H	Beta-haemolysis	Cocci in clusters, deep blue colour	MSA, BA	+	-	-	-	+	+	+	+	+	-	+	+	+	*Staphylococcus aureus*

Table 1: *Morphological and Biochemical Characteristics of Isolates.*

Keys: *BA: Blood Agar; EMBA: Eosin Methylene Blues Agar; V: Variable; MSA: Mannitol Salt Agar; MAC: MacConkey Agar; NA: Nutrient Agar; +: Positive Result for Test; -: Negative Result for Test.*

	Honey				
Component	**A**	**B**	**C**	**D**	**E**
Alkaloid	+	-	+	+	+
Tannin	+	-	+	-	-
Phlobatannin	-	-	+	-	-
Flavonoids	+	+	-	-	+
Saponins	+	-	+	-	-
Terpenoid	+	+	+	+	-

Table 2: *Qualitative phytochemical screening of various honey samples.*

Keys: *A represents Honey obtained from Ibadan, Oyo state local apiarists; B represents Honey obtained from Oja Oba, Akure local apiarist, Ondo state; C represents honey obtained from Ilorin, Kwara state; D represents honey obtained from FUTA, Akure, Ondo state and E represents foreign, Honey obtained from Akure supermarket, Ondo state. + indicates positive and – indicates negative.*

Organisms	Mean zone of inhibition (mm)						
	Concentrations (%)						
	20	50	70	100	Water	DMSO	Chloram
P. aeruginosa ATCC 27853	10.00 ± 0.50^{a}	15.00 ± 0.6^{b}	17.00 ± 0.60^{bc}	18. ± 0.800^{c}	-	-	10.00 ± 0.6^{a}
K. pneumoniae ATCC 48891	5.00 ± 0.03^{a}	7.00 ± 1.2^{ab}	9.00 ± 1.0^{b}	14.00 ± 0.8^{c}	-	-	12.00 ± 0.6^{b}
P. mirabilis ATCC 29906	11.00 ± 0.5^{a}	14.00 ± 0.6^{b}	15.00 ± 1.0^{b}	20.00 ± 0.3^{c}	-	-	17.00 ± 0.6^{c}
A. baumannii ATCC 19606	5.00 ± 0.3^{a}	9.00 ± 0.5^{a}	11.00 ± 0.0^{b}	15.00 ± 0.3^{c}	-	-	11.00 ± 0.9^{b}
C. freundii ATCC 8090	6.00 ± 0.00^{a}	10.00 ± 0.0^{b}	12.00 ± 0.3^{c}	15.00 ± 0.3^{d}	-	-	9.00 ± 0.9^{a}
MRSA430	7.00 ± 0.3^{a}	12.00 ± 1.2^{ab}	13.00 ± 1.0^{ab}	14.00 ± 1.2^{c}	-	-	7.00 ± 0.5^{a}
S. aureus ATCC 43300	5.00 ± 0.5^{a}	12.00 ± 0.6^{b}	13.00 ± 0.6^{b}	15.00 ± 0.3^{c}	-	-	10.00 ± 0.6^{a}
B. subtilis ATCC 14579	7.00 ± 0.5^{a}	9.00 ± 1.2^{b}	13.00 ± 0.3^{c}	16.00 ± 0.5^{d}	-	-	10.00 ± 0.6^{a}

Table 3: *Susceptibility of typed isolates to honey A at different concentrations.*

Keys: *Values were determined on triplicate sets, inhibition zones are reported as "mean ± standard error of mean, (n = 3)", values with the same superscript letter(s) along the same column are not significantly different (p < 0.05), water was used as negative control, chloram-chloramphenicol and 50% Dimethyl sulfoxide was used as positive control, mm- millimeter, %- percentage; A represents Honey obtained from Ibadan, Oyo state local apiarists.*

Organisms	Mean zone of inhibition (mm)						
	Concentrations (%)						
	20	50	70	100	Water	DMSO	Chloram
P. aeruginosa ATCC 27853	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-	10.00 ± 0.6^{b}
K. pneumoniae ATCC 48891	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.00 ± 1.2^{b}	6.00 ± 1.2^{b}	-	-	12.00 ± 0.6^{c}
P. mirabilis ATCC 29906	9.00 ± 0.8^{a}	12.00 ± 0.9^{b}	13.00 ± 1.4^{b}	15.00 ± 0.5^{c}	-	-	17.00 ± 0.6^{d}
A. baumannii ATCC 19606	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-	11.00 ± 0.9^{b}
C. freundii ATCC 8090	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-	9.00 ± 0.9^{b}
MRSA 430	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	-	-	7.00 ± 0.5^{b}
S. aureus ATCC 43300	4.00 ± 0.00^{a}	7.00 ± 0.9^{b}	8.00 ± 1.8^{b}	9.00 ± 0.00^{c}	-	-	10.00 ± 0^{c}
B. subtilis ATCC 14579	6.00 ± 0.70^{a}	9.00 ± 1.7^{b}	11.00 ± 0.5^{c}	13.00 ± 0.8^{d}	-	-	10.00 ± 0.6^{b}

Table 4: *Susceptibility of typed isolates to honey C at different concentrations.*

Keys: Values were determined on triplicate sets, inhibition zones are reported as "≈mean ± standard error of mean, (n = 3)", values with the same superscript letter(s) along the same column are not significantly different (p < 0.05), water was used as negative control, chloramphenicol and 50% Dimethyl sulfoxide was used as positive control, mm- millimeter, chloram-chloramphenicol, %-percentage; C represents honey obtained from Ilorin, Kwara state.

Organisms	Mean zone of inhibition (mm)						
	Concentrations (%)						
	20	50	70	100	Water	DMSO	Chloram
P. aeruginosa	6.00 ± 0.5^{a}	7.00 ± 0.3^{b}	9.00 ± 0.0^{c}	11.00 ± 0.3^{c}	-	-	8.00 ± 0.5^{b}
K. pneumoniae	5.00 ± 0.3^{a}	7.00 ± 0.0^{a}	9.00 ± 0.8^{b}	14.00 ± 0.8^{c}	-	-	9.00 ± 0.0^{b}
P. mirabilis	8.00 ± 0.5^{a}	9.00 ± 1.0^{b}	11.00 ± 0.6^{c}	14.00 ± 0.5^{c}	-	-	12.00 ± 0.5^{c}
A. baumannii	0.00 ± 0.0^{a}	6.00 ± 0.3^{b}	7.00 ± 0.6^{b}	9.00 ± 0.5^{c}	-	-	5.00 ± 0.9^{a}
C. freundii	0.00 ± 0.0^{a}	7.00 ± 0.3^{b}	9.00 ± 0.3^{c}	11.00 ± 0.5^{d}	-	-	6.00 ± 0.5^{a}
MRSA	7.00 ± 1.0^{a}	9.00 ± 1.0^{ab}	11.00 ± 1.2^{bc}	13.00 ± 0.0^{c}	-	-	5.00 ± 1.4^{a}
S. aureus	5.00 ± 0.3^{a}	6.00 ± 0.0^{a}	7.00 ± 0.3^{b}	9.00 ± 0.3^{c}	-	-	6.00 ± 1.0^{a}
B. subtilis	5.00 ± 0.3^{a}	7.00 ± 0.3^{b}	8.00 ± 0.5^{b}	13.00 ± 0.0^{c}	-	-	7.00 ± 0.9^{b}

Table 5*: Susceptibility of clinical isolates to Honey A at different concentrations.*

Keys: Values were determined on triplicate sets, inhibition zones are reported as "≈mean± standard error of mean, (n = 3)", values with the same superscript letter(s) along the same column are not significantly different (p < 0.05), Negative control – water, Positive control- 50% Dimethyl sulfoxide and chloram- chloramphenicol, mm- millimeter, %- percentage, A represents Honey obtained from Ibadan, Oyo state local apiarists.

Organisms	Mean zone of inhibition (mm)						
	Concentrations (%)						
	20	50	70	100	Water	DMSO	Chloram
P. aeruginosa ATCC 27853	6.00 ± 0.5^{a}	7.00 ± 0.3^{b}	9.00 ± 0.0^{c}	11.00 ± 0.3^{c}	_	_	8.00 ± 0.5^{b}
K. pneumonia ATCC 48891	5.00 ± 0.3^{a}	7.00 ± 0.0^{a}	9.00 ± 0.8^{b}	14.00 ± 0.8^{c}	_	_	9.00 ± 0.0^{b}
P. mirabilis ATCC 29906	8.00 ± 0.5^{a}	9.00 ± 1.0^{b}	11.00 ± 0.6^{c}	14.00 ± 0.5^{c}	_	_	12.00 ± 0.5^{c}
A. baumannii ATCC 19606	0.00 ± 0.0^{a}	6.00 ± 0.3^{b}	7.00 ± 0.6^{b}	9.00 ± 0.5^{c}	_	_	5.00 ± 0.9^{a}
C. freundii ATCC 8090	0.00 ± 0.00^{a}	7.00 ± 0.3^{b}	9.00 ± 0.3^{c}	11.00 ± 0.5^{d}	_	_	6.00 ± 0.5^{a}
MRSA 430	7.00 ± 1.0^{a}	9.00 ± 1.0^{ab}	11.00 ± 1.2^{bc}	13.00 ± 0.0^{c}	_	_	5.00 ± 1.4^{a}
S. aureus ATCC 43300	5.00 ± 0.3^{a}	6.00 ± 0.0^{a}	7.00 ± 0.3^{b}	9.00 ± 0.3^{c}	_	_	6.00 ± 1.0^{a}
B. subtilis ATCC 14579	5.00 ± 0.3^{a}	7.00 ± 0.3^{b}	8.00 ± 0.5^{b}	13.00 ± 0.0^{c}	_	_	7.00 ± 0.9^{b}

Table 6: *Susceptibility of clinical isolates to Honey C at different Concentrations.*

Keys: Values were determined on triplicate sets, inhibition zones are reported as "≈mean ± standard error of mean, (n = 3)", values with the same superscript letter(s) along the same column are not significantly different (p < 0.05), Negative control – water, Positive control- 50% Dimethyl sulfoxide and chloram: Chloramphenicol; mm: millimeter; %: Percentage, C represents honey obtained from Ilorin, Kwara state.

Organisms	Mean zone of inhibition (nearest mm)						
	Antibiotics						
	AMX	AUG	CPX	GEN	PFX	APX	Z
P. aeruginosa ATCC 27853	00.00 ± 0.00^{a}	00.00 ± 0.00^{a}	19.60 ± 0.90^{c}	15.60 ± 0.25^{b}	20.30 ± 1.60^{cd}	20.30 ± 0.00^{cd}	22.00 ± 0.00^{d}
K. *pneumoniae* ATCC 48891	00.00 ± 0.00^{a}	00.00 ± 0.00^{a}	21.00 ± 1.60^{d}	14.00 ± 0.27^{b}	27.33 ± 2.0^{e}	16.00 ± 0.00^{c}	28.66 ± 0.58^{f}
P. mirabilis ATCC 29906	00.00 ± 0.00^{a}	12.33 ± 0.84^{b}	19.33 ± 0.90^{c}	12.66 ± 0.32^{b}	21.33 ± 1.2^{d}	23.00 ± 0.00^{e}	23.00 ± 0.00^{e}
A. baumannii ATCC 19606	00.00 ± 0.00^{a}	00.00 ± 0.00^{a}	31.16 ± 1.20^{d}	14.00 ± 0.25^{b}	33.30 ± 1.40^{e}	14.66 ± 0.14^{b}	16.00 ± 0.00^{c}
C. freundii ATCC 8090	00.00 ± 0.00^{a}	15.00 ± 0.80^{d}	31.16 ± 0.40^{e}	12.16 ± 0.50^{c}	31.50 ± 1.20^{e}	8.60 ± 1.20^{b}	00.00 ± 0.00^{a}
S. aureus ATCC 43300	00.00 ± 0.00^{a}	18.00 ± 0.00^{c}	00.00 ± 0.00^{a}	20.30 ± 0.56^{d}	11.33 ± 0.49^{b}	24.33 ± 0.00^{e}	21.30 ± 0.50^{d}
MRSA430	00.00 ± 0.00^{a}	00.00 ± 0.00^{a}	16.00 ± 0.00^{c}	10.00 ± 0.00^{b}	0.00 ± 0.00^{a}	7.00 ± 0.00^{b}	8.00 ± 0.00^{b}
B. subtilis ATCC 14579	12.00 ± 0.00^{b}	5.50 ± 0.50^{a}	33.00 ± 0.80^{f}	20.00 ± 0.50^{c}	23.67 ± 0.80^{d}	6.33 ± 0.50^{a}	31.33 ± 0.50^{f}

Table 7: *Antibiotics susceptibility test for typed isolates.*

Key: *AUG: Augumentin; CPX: Ciprofloxacin; AMX: Amoxicillin; PEF: Pefloxacin; GEN: Gentamicin; APX: Amplicox; Z: Zinnacef. Values were determined on triplicate sets, inhibition zones are reported as "mean ± standard error of mean, (n = 3)", values with the same superscript letter(s) along the same column are not significantly different (p < 0.05).*

Organism	Mean zone of inhibition (mm)						
	Antibiotics						
	AMX	AUG	CPX	GEN	PFX	APX	Z
P. aeruginosa	0.00 ± 0.00[a]	0.00 ± 0.00[a]	19.6 ± 0.30[d]	12.3 ± 0.66[b]	17.00 ± 1.1[d]	12.66 ± 0.33[b]	15.33 ± 0.33[c]
K. pneumoniae	0.00 ± 0.00[a]	00.00 ± 0.00[a]	14.33 ± 0.33[d]	9.00 ± 0.57[b]	17.3 ± 0.66[e]	10.33 ± 0.33[c]	20.66 ± 0.33[f]
P. mirabilis	00.00 ± 0.00[a]	9.33 ± 0.33[b]	16.10 ± 0.16[d]	9.00 ± 0.57[b]	13.33 ± 0.33[c]	13.33 ± 0.33[c]	15.66 ± 0.33[a]
A. baumannii	00.00 ± 0.00[a]	00.00 ± 0.00[a]	24.60 ± 0.33[d]	8.00 ± 0.25[b]	28.00 ± 1.15[e]	8.33 ± 0.33[b]	11.66 ± 0.33[c]
C. freundii	00.00 ± 0.00[a]	6.50 ± 0.50[b]	27.66 ± 0.33[e]	8.66 ± 0.33[c]	25.66 ± 0.88[d]	6.66 ± 0.66[b]	0.00 ± 0.00[a]
S. aureus	0.00 ± 0.00[a]	14.66 ± 0.33[c]	0.00 ± 0.00[a]	20.33 ± 0.88[d]	14.66 ± 0.33[c]	15.33 ± 1.60[c]	13.00 ± 0.00[b]
MRSA	0.00 ± 0.00[a]	0.00 ± 0.00[a]	10.66 ± 0.33[c]	5.66 ± 0.33[b]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	5.33 ± 0.33[b]
B. subtilis	5.66 ± 0.33[b]	2.66 ± 0.33[a]	28.33 ± 0.33[f]	11.33 ± 0.33[c]	17.00 ± 0.00[d]	3.0 ± 0.0[a]	25.00 ± 0.57[e]

Table 8: *Antibiotics susceptibility test for clinical isolates.*

Key: AUG: Augumentin; CPX: Ciprofloxacin; AMX: Amoxicillin; PEF: Pefloxacin; GEN: Gentamicin; APX: Amplicox; Z: Zinnacef. Values were determined on triplicate sets, inhibition zones are reported as "mean ± standard error of mean, (n = 3)", Values with the same superscript letter(s) along the same column are not significantly different ($p < 0.05$).

Concentrations (%)	Organisms															
	***P. aeruginosa* ATCC27853**	***P. aeruginosa* (C)**	***Klebsiella* ATCC48891**	***K. pneumoniae* (C)**	***P. mirabilis* ATCC29906**	***P. mirabilis* (C)**	***A. baumannii* ATCC19606**	***A. baumannii* (C)**	***C. freundii* ATCC8090**	***C. freundii* (C)**	**MRSA 430**	**MRSA (C)**	***S. aureus* ATCC43300**	**S. aureus (C)**	***B. subtilis* ATCC14579**	***B. subtilis* (C)**
20%	+	-	-	-	+	+	-	-	-	-	+	+	-	-	+	-
50%	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
70%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Stock	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 9: *Minimum inhibitory concentration of honey A against clinical and typed isolates.*

Key: *C: Clinical; +: Indicates Positive; -: Indicates Negative.*

Organism	***P. aeruginosa* ATCC 27853**	***P. aeruginosa* (C)**	***Klebsiella* ATCC 48891**	***K. pneumoniae* (C)**	***P. mirabilis* ATCC 29906**	***P. mirabilis* (C)**	***A. baumannii* ATCC 19606**	***A. baumannii* (C)**	***C. freundii* ATCC 8090**	***C. freundii* (C)**	**MRSA 430**	**MRSA (C)**	***S. aureus* ATCC 43300**	***S. aureus* (C)**	***B. subtilis* ATCC 14579**	***B. subtilis* (C)**
MIC (%)	20	20	20	50	20	20	20	50	20	50	20	20	20	20	20	50
MBC (%)	20	50	50	50	20	50	50	70	50	50	20	20	50	50	50	50
Action	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 10: *Minimum inhibitory concentration and minimum bacteriocidal concentration of honey A against clinical and typed isolates.*

Keys: *C: Clinical; MBC: Minimum Bacteriocidal Concentration; MIC: Minimum Inhibitory Concentration; A: Action and S: Bacteriostatic.*

Organism	Zones of inhibition	Type of haemolysis
P. aeruginosa ATCC 27853	50	α-Haemolysis
K. pneumoniae ATCC 48891	30	α-Haemolysis
P. mirabilis ATCC 29906	40	α-Haemolysis
A. baumannii ATCC 19606	25	α-Haemolysis
C. freundii ATCC 8090	35	α-Haemolysis
S. aureus ATCC 43300	40	α-Haemolysis
MRSA 430	50	α-Haemolysis
B. subtilis ATCC 14579	50	α-Haemolysis

Table 11: *Haemolytic assay.*

Keys: α: Alpha; mm: Millimeter.

Organisms		P. aeruginosa ATCC 27853	Klebsiella ATCC 48891	P. mirabilis ATCC 29906	A. baumannii ATCC 19606	C. freundii ATCC 8090	MRSA 430	S. aureus ATCC 43300	B. subtilis ATCC 14579
Time	**Log (cfu/ml)**								
30 min	Honey	2.9 ± 0.0[b]	3.0 ± 0.5[b]	2.9 ± 2.1[b]	3.0 ± 0.9[b]	2.9 ± 0.8[b]	2.7 ± 1.2[a]	2.8 ± 0.8[a]	2.7 ± 0.5 [a]
	Gentamicin	2.6 ± 1.2[b]	2.8 ± 0.5 [c]	2.7 ± 0.9[b]	2.7 ± 0.9 [a]	2.5 ± 1.6[a]	3.0 ± 0.5	2.4 ± 1.2[a]	2.5 ± 1.6[a]
60 min	Honey	2.7 ± 0.5[b]	2.8 ± 0.8 [b]	2.7 ± 0.4[b]	2.9 ± 1.2 [c]	2.8 ± 0.5 [b]	2.5 ± 1.6 [a]	2.6 ± 0.0 [a]	2.5 ± 1.4 [a]
	Gentamicin	2.3 ± 1.6 [a]	2.6 ± 1.2 [c]	2.5 ± 1.2 [b]	2.4 ± 0.9 [b]	2.3 ± 0.5 [a]	2.9 ± 0.9[d]	2.2 ± 1.4 [a]	2.2 ± 0.0 [a]
90 min	Honey	2.5 ± 0.9 [b]	2.6 ± 0.0[b]	2.5 ± 0.5[b]	2.7 ± 0.0 [c]	2.7 ± 1.6 [c]	2.2 ± 0.4[a]	2.3 ± 0.9[a]	2.2 ± 1.6[a]
	Gentamicin	2.0 ± 0.9[b]	2.4 ± 0.0[c]	2.3 ± 1.6[c]	2.3 ± 1.6[c]	2.1 ± 0.8[b]	2.8 ± 0.8[d]	2.0 ± 1.4[b]	1.8 ± 0.5[a]
120 min	Honey	2.2 ± 1.4[b]	2.4 ± 1.4[c]	2.1 ± 0.0[b]	2.6 ± 1.4[d]	2.6 ± 2.1[d]	1.8 ± 1.4[a]	2.1 ± 0.5[b]	1.8 ± 1.6[a]
	Gentamicin	1.8 ± 1.6[a]	2.2 ± 0.9[d]	1.9 ± 0.0[b]	2.0 ± 0.0[c]	1.9 ± 0.9[b]	2.5 ± 0.0[e]	1.7 ± 1.4[b]	1.5 ± 1.2[a]

Table 12: *Rate of killing of honey A against typed bacterial isolates.*

Key: Values were determined on triplicate sets, results are reported as mean ± standard deviation, values with the same superscript letter(s) along the same column are not significantly different ($p < 0.05$), log: Logarithm; min: Minute; cfu/ml: Colony Forming Units Per ml.

Organism	Test Agent			
	Honey		Chloramphenicol	
	Na^+ (ppm)	K^+ (ppm)	Na^+ (ppm)	K^+ (ppm)
Pseudomonas aeruginosa	13.30	42.10	9.84	**8.89**
Klebsiella pneumonia	10.00	35.10	9.67	9.69
Proteus mirabilis	11.30	41.200	9.60	9.00
Acinetobacter baumannii	19.00	67.50	7.50	8.32
Citrobacter freundii	11.10	39.50	7.90	6.30
Staphylococcus aureus	12.30	40.00	10.08	9.42
MRSA	21.90	51.70	6.74	7.26
Bacillus subtilis	3.30	4.42	9.74	10.00

Table 13: *Amount of sodium and potassium ions released from clinical bacterial isolates by the action of honey.*

Key: *Ppm: Parts Per Million; Na^+: Sodium Ion, K^+: Potassium Ion, MRSA: Methicillin Resistant Staphylococcus aureus.*

Discussion

Honey was shown to have antibacterial effect on the growth of selected pathogens. Results of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values obtained from this study revealed that honey possess bacteriostatic activity against the pathogens; this is in coherence with findings of [11]. Comparison of susceptibility of typed and clinical bacterial isolates to honey at different concentrations showed that honey from Ibadan, Oyo State was more efficient in inhibiting the growth of the studied pathogens than those from other locations which had lesser inhibitory effect on clinical and typed isolates of *Proteus mirabilis, Staphylococcus aureus, Klebsiella pneumonia* and *Bacillus subtilis.* Honey samples from FUTA obanla, Southgate and Oja Oba had no inhibitory effect on test pathogens. This variation in antimicrobial effect of honey samples aligns with the findings of the study done by [1] who stated that different honey types have varied efficacies against the same type of bacteria. Hence, the antibacterial effects of honey are not only due to osmolarity, viscosity, presence of hydrogen peroxide and low protein contents but due to other important factors that affect the composition of honey [7]. According to [17], such factors depend to a great extent on the bee's source, the location of the flowers and related weather conditions, the storage time and conditions and the method of preservative treatment.

The honey used in this study compared favorably with Amoxicillin, Chloramphenicol, Augumentin, Amplicox and Zinnacef antibiotics; this agrees with the findings of [28]. However, when compared with Gentamicin, Ciprofloxacin and Pefloxacin antibiotics, honey had less effectiveness; a drawback highlighted to an earlier study by [4], this drawback also explains why different types of honey differ in their efficacies [1,15-17,19].

The minimum inhibitory concentration and minimum bactericidal concentration values obtained from this study reveals that at 20% (v/v) concentration, honey had bacteriostatic effect against typed isolates of *Pseudomonas aeruginosa, Proteus mirabilis,* methicillin resistant *Staphylococcus aureus* and clinical isolates of methicillin resistant *Staphylococcus aureus* which is in tandem with [4] who did a study on manuka honey. At 50% (v/v) concentration, honey had bacteriostatic effect against all test organisms used with the exception of *Klebsiella pneumoniae* clinical isolates which remained resistant. This suggests a good support of the work reported in [29] who stated that *Klebsiella pneumoniae* presents an irregular pattern in the response to the different concentrations of honey. Although the reason(s) for this fluctuation is not yet clear, the capsule and other determinant factors such as an extracellular enzyme produced by *Klebsiella pneumoniae* may influence this erratic behavior.

The most effective concentration with bacteriostatic effect was 70% (v/v) and the undiluted honey, this was in line with the report of studies that found out that phytochemicals present in honey do have antimicrobial effect, and the efficacy of honey antimicrobial activity depends on the concentration of honey administered [30]. In this study, clinical isolates of bacteria were found to be more resistant to honey at different concentration than typed isolates, this may be due to their exposure to administered antibiotics in patients whose samples were obtained from and had conferred on them a form of resistance. The mechanism of action of honey revealed that broad spectrum Gentamicin had a faster killing rate than the honey sample used in this study, also the effect on sodium and potassium leakage revealed more leakage of sodium and potassium from clinical isolates exposed to honey than chloramphenicol; this agrees with findings of [14]. This study also proved honey was effective against antibiotic-resistant bacteria that colonize burn wounds, such as MRSA and multiple-resistant Gram-negative rods, including members of the Enterobacteriaceae family as stated by [2,31].

Conclusion

Findings from this study proved honey to be an effective antimicrobial agent; being able to inhibit the growth of all test organisms at varying concentrations. The challenges of antibiotic resistance, which for long posed difficult to the world at large, might be solved by the use of natural products such as honey.

Recommendation

More research is required to determine the antimicrobial effect of other existing bee products and the effectiveness of honey on other disease conditions. Also, the antimicrobial activity of many varieties of honey existing in different locations of the world needs to be determined to ascertain the most effective and to investigate the peculiar composition of such honey variety. Finally, further exploration into the effect of honey in living systems by *in vivo* experiments can also be pursued.

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