In vitro Assessment of the Antimicrobial Activity of Honey and Some Standard Antibiotics on *Staphylococcus aureus, Pseudomonas aeruginosa* and *Proteus mirabilis* Isolated from Wounds

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Abstract: This study assessed and compared the antimicrobial activity of honey to that of some commonly used antibiotics in Ebonyi State, against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolated from wounds. Fifty two (52) wound samples, were collected from patients at General Hospital Uburu, Ebonyi State. Wound samples were collected using sterile swab sticks. The bacteria species were isolated and identified by standard microbiological methods. Antibiotics Susceptibility test was performed by Kirby-Bauer-CLSI modified Disc Agar Diffusion method, while agar well diffusion technique was used to assess the *in vitro* activity of the honey against the test bacteria. Results, showed that the three (3) bacteria species were prevalent in the wound samples as follows; *Pseudomonas aeruginosa* 16(30.8%), *Staphylococcus aureus* 11(21.2%) and *Proteus mirabilis* 2(3.9%). Result of the antibiotics susceptibility tests, showed that all (100%) of the bacteria species were susceptibility to imipenem. Some *Staphylococcus aureus* isolates were susceptible to the other antibiotics while being totally (100%) resistant to ceftriaxone. *Pseudomonas aeruginosa* showed degrees of susceptibility to the other antibiotics. *Proteus mirabilis* was found to show 50% susceptibility to meropenem and ofloxacine, while being totally (100%) resistant to the other antibiotics. On the other hand, the honey sample inhibited the growth of all the test organisms producing inhibitory zone diameters in the range of > 10mm to 29mm. This suggests that the honey sample used was active against the test bacteria, and can be used as an alternative way of treating wounds infected, with the tested organisms in the locality.

Key words: Antibiotics, Honey, Infection, Honey, Patients, Wound.

INTRODUCTION

Wound infections arise from the contamination of injuries sustained on the human body. These injuries results from the breach in the normal continuum of tissues. Wounds provide moist, warm and nutritious environment that is conducive for microbial colonization, proliferation and growth (Serra *et al*., 2015; Tom *et al*., 2018). Therefore, chronic wounds harbour the presence of many microorganisms such as multi-drug resistant bacteria, polymicrobic flora, bacteria, fungi and viruses (Adeyinka *et al*., 2018).

The common tradition is to treat wound infections using antibiotics. Antibiotics exist as natural, semi-synthetic or synthetic substances. Antimicrobial agents are naturally-occurring, semi-synthetic and synthetic substances produced by microorganisms that have the capacity to kill or inhibit the growth of another microorganism's .The application of antibiotics to treat and prevent diseases have played a critical role in reducing the burden of infectious diseases all over the world (Street and Bogor, 2013).

The treatment of wound infections using antibiotics due to the of multi-drug resistance bacteria. The emergence of antibiotic-resistant bacteria causes enzymatic inactivation of antibiotics, alteration of target sites of the antibiotics, reduced cellular uptake, decreasing absorption or increasing efflux of the antibiotics and acquisition of the ability to break or modify the antibiotics leading to the depression of the activity of standard antibiotics on these multi-drug resistant bacteria. R Antimicrobial resistance of microorganisms to antimicrobial agents is a global public healthcare problem Ohalete *et al.* (2019), as a result many antibiotics microbial medicines are losing their ability to treat wound infections leading to many deaths (Jenkins and Cooper, 2012).

Given the difficulty in treating these infections, the interest in using other alternative therapies and natural remedies in wound management has evolved; one of such natural remedies is honey (Manisha and Shyamapada, 2011; Fyfe *et al*., 2017). Honey is a sweet food substance produced by bees (*Apis mellifera*) using nectar from flowers. It has been acknowledge that a wide range of antimicrobial properties is found in honey (Manjunatha and Lee, 2014). The potential of honey to assist with wound healing has been demonstrated repeatedly (Albaridi, 2019). There is no doubt that the antimicrobial mechanisms of honey has the ability to withstand the activities of microorganisms in wound infections due largely to its high osmolarity, acidity (PH and free acidity), low water activity, high content of reducing sugar, high viscosity, low protein content, hydrogen peroxide production and the presence of other phytochemical components such as methyl glyoxal, flavonoids, phenolic compounds and lysozyme (Kwakman and Zaat, 2012; Alvarez-Suarez *et al*., 2018).

Honey is used to enhanced wound-healing in humans as well boost the immune system (Oryan *et al*. 2016). Moreover, it can be used on skin grafts and infected skin graft donor sites successfully (Maghsoudi and Moradi, 2014). The routine surveillance for pathogens and their susceptibility to antibiotics is of paramount importance not only to reinforce strategies for successful wound infection control and management but in the control of antibiotic usage to stem the emergence and spread of resistant bacteria (Ohalete *et al*., 2019). The current prevalence of antibiotic resistance pathogens has led to the re-evaluation of ancient remedies, which include the use of honey. Therefore, this study set out to investigate and compare the antimicrobial activities of honey and some standard antibiotics on three bacteria species isolated from wounds.

MATERIALS AND METHODS

Collection of honey sample:

The unadulterated honey sample was collected in sterile universal bottles from Ebonyi State Ministry of Agriculture, Abakaliki. About 200 mls of the honey was collected.

Test organisms

The test organisms; namely *Stapylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* for the study were isolated from wound samples, from patients at General Hospital Uburu. Identification and authentication of the isolates were done using standard microbiological methods of cultural, morphological and biochemical tests. Biochemical tests conducted, included; catalase production, coagulase, oxidase production, motility, indole, urease production, carbohydrate breakdown, O-Nitrophenyl-β-D-galactoside (ONPG) breakdown, citrate utilization, nitrate reduction (Cheesbrough, 2010).

Standardization of inocula

The test organisms were standardized by inoculating 5ml normal saline in sterile test tubes with loop full of overnight cultures from nutrient agar slants. The mixtures were shaken to obtain homogenous suspensions. The homogenous suspensions were adjusted to 0.5 McFarland's standard corresponding to 10^8cells/ml.

Antibacterial susceptibility testing

The antibacterial susceptibility testing was performed using the modified Kirby Bauer disc diffusion method and interpreted according to guidelines of clinical laboratory standards institute (CLSI, 2015). Using a Pasteur pipette, 0.5ml of the standardized inoculum were transferred onto sterile plates of Mueller Hinton agar and spread with a glass spreader. Antibacterial discs used were nitrofurantoin (300µg), nalidixic acid (30µg), meropenem (10µg), imipenem (10µg), aztreonam (30µg), ceftriaxone (30µg), gentamicin (30µg), ofloxacin (5µg), ciprofloxacin (5µg) and sulphamethoxazole-trimethoprim (25µg).

They were placed equidistance to each other using sterile forceps on the Mueller Hinton agar, seeded with each test organism. They were then incubated at 37^0C for 24 hours. After incubation, the diameter of the zone of inhibition against each of the test organisms were measured in mm (Vineetha *et al,* 2015) The selection of the drugs was based on their availability and prescription frequency in the study area.

Antimicrobial susceptibility testing of honey
The antimicrobial activity of honey was assayed using agar well diffusion technique (Cheesbrough, 2010; Adeshina *et al.*, 2010). Using a Pasteur pipette, 0.5ml of the standardized inoculum were transferred onto sterile plates of Mueller Hinton agar and spread with a glass spreader. Five radial wells of 6mm diameter were punched equidistance at different places on the Mueller Hinton agar on each plate using a sterile cork borer. The honey was prepared by serially, diluting with sterile water to get different concentrations of 100mg/ml, 50, 25, 12.50 and 6.25mg/ml and added to the agar wells and labeled respectively. The plates were incubated at 37^{o}C for 24hours in upright position. After incubation, the diameter zone of inhibition for the different honey concentrations were measured in mm (Vineetha *et al,* 2015).

RESULTS

The result of the isolation of the test organisms showed that eleven (11) *Staphylococcus aureus* were isolated, sixteen (16) *Pseudomonas aeruginosa* isolated and two (2) *Proteus mirabilis* were isolated (Table 1)

Table 1: Percentage Occurrence of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolated from the 52 wound samples.

Bacteria specie	No (n) isolated	% Prevalence
Staphylococcus aureus,	11	21.2%
Pseudomonas aeruginosa	16	30.8%
Proteus mirabilis	2	3.9%

The antibiotic susceptibility test of the isolated bacteria species; *S. aureus, P. aeruginosa* and *P. mirabilis* are shown (Table 1). All the twenty nine (29) bacteria species; eleven (11) *S. aureus* isolated, sixteen (16) *P. aeruginosa* and two (2) *Proteus mirabilis* were all susceptible to imipenem (100%). and ofloxacin 8(72.7%). The susceptibility profile of *P. aeruginosa* revealed that out of 16 isolates; highest susceptibility was seen with imipenem 16(100%), gentamicin 13(81.3%), ofloxacin 11(68.7%) and ciprofloxacin 9(56.3%). On the other hand, out of 2 isolates of *Proteus mirabilis*; highest susceptibility was seen with imipenem 2(100%), meropenem 1(50%) and ofloxacin 1(50%).

Table 2: Antibiotics Susceptibility Pattern of the Bacteria species to the different antibiotics used.

Antibiotics	Conc (µg)	*S.aureus* (n=11)		*P.aeruginosa* (n=16)		*P.mirabilis* (n=2)	
		R. n(%)	S. n(%)	R. n (%)	S. n(%)	R. n(%)	S. n(%)
Nitrofurantoin	300	10(90.9)	1(9.1)	10(62.5)	6(37.5)	2(100)	0(0.0)
Nalidixic Acid	30	9(81.8)	2(18.2)	14(87.5)	2(12.5)	2(100)	0(0.0)
Meropenem	10	7(63.6)	4(36.4)	12(75.0)	4(25.0)	1(50.0)	1(50.0)
Imipenem	10	0(0.00)	**11(100)**	0(0.0)	**16(100)**	0(0.0)	**2(100)**
Aztreonam	30	9(81.8)	2(18.2)	9(56.3)	7(43.8)	2(100)	0(0.0)
Ceftriaxone	30	11(100)	0(0.0)	11(68.7)	5(31.3)	2(100)	0(0.0)
Gentamicin	30	6(54.5)	5(45.5)	3(18.7)	13(81.3)	2(100)	0(0.0)
Ofloxacin	5	3(27.3)	8(72.7)	5(31.3)	11(68.7)	1(50.0)	1(50.0)
Ciprofloxacin	5	8(72.7)	3(27.3)	7(43.8)	9(56.3)	2(100)	0(0.0)
Sulphamethoxazole-Trimethoprim	25	8(72.7)	3(27.3)	12(75.0)	4(25.0)	2(100)	0(0.0)

Key: Conc=Antibiotic Concentration, R=resistance, S=Susceptibility, Sa=*Staphylococcus aureus*, Pa=*Pseudomonas aeruginosa*, Pm=*Proteus mirabilis.*

The result of the antimicrobial activity of honey on the test organisms is presented in Figure 1. The chart shows that the bacteria species were all susceptible to the honey sample at the different concentrations except *Proteus mirabilis* that showed no zone of inhibition at the lowest concentration of 6.25mg/ml. The zones of inhibition for the species ranged from more than 10mm to 29mm across the different concentrations of the honey. Honey concentration of 100mg/ml produced the widest inhibition zone diameter (IZD) of 29mm against *Pseudomonas aureginosa* (Figure 1).

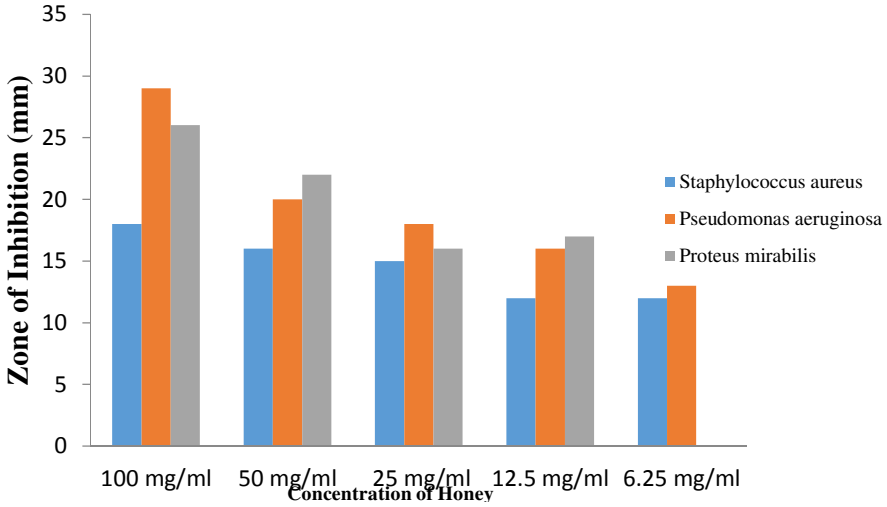

Fig 1: Inhibition Zone Diameters produced by the different Concentrations of Honey, against the test Bacteria species.

Figures 2 to 4 showed the graphs of the 100mg/ml of the honey concentration that produced the best activity against the tested organisms and imipenem, the antibiotic that gave the best activity against the tested organism. For *Staphylococcus aureus*, the inhibition zone diameter (IZD) produced by the honey for all the species of *S aureus* ranged from 12mm to 18mm. Imipenem produced inhibition zone diameter ranged from 25mm to 27mm (Fig 2). 100mg/ml honey concentration produced IZD against *Pseudomonas aeruginosa*, in the range of 14mm to 29mm while Imipenem produced IZD in the range of 19mm to 32mm (Fig 3). Also 100mg/ml of honey produced IZD of 22mm and 26mm, on the two species of *Proteus mirabilis* while imipenem produced IZD of 22mm and 25mm respectively.

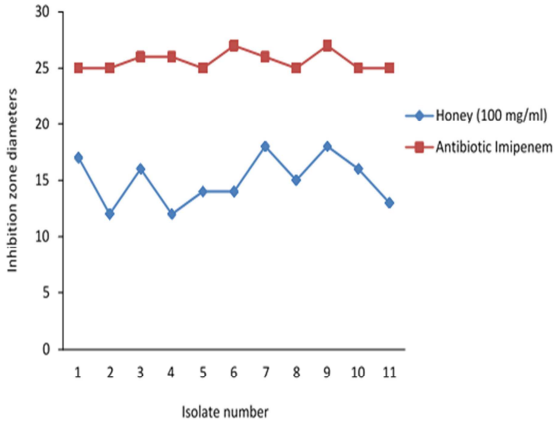

Figure 2: Inhibition zone diameters of honey and imipenem against *Staphylococcus aureus*

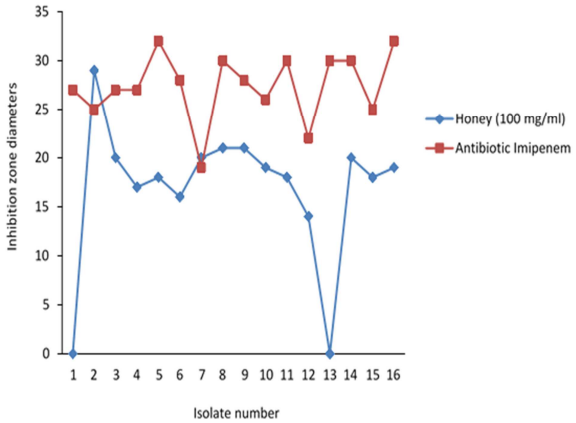

Figure 3: Inhibition zone diameters of honey and imipenem against *Pseudomonas aeruginosa*

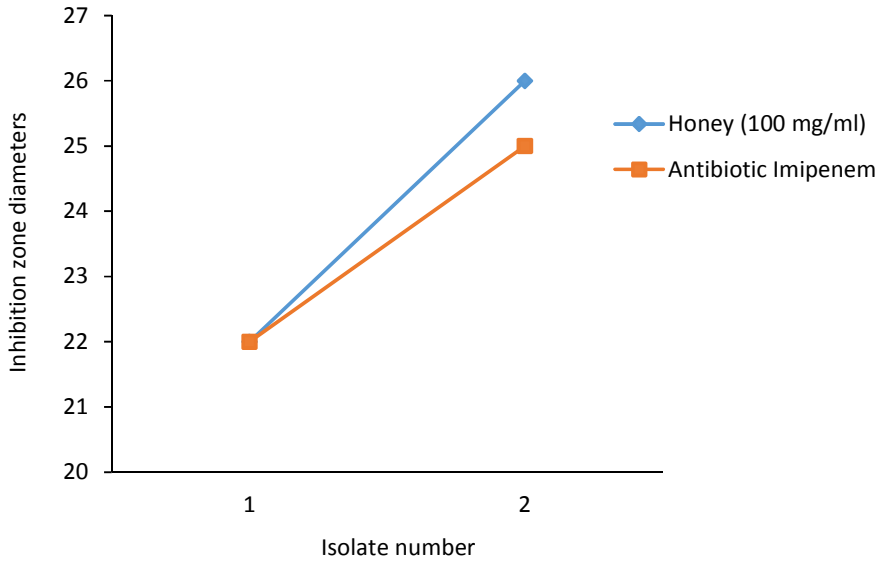

Figure 4: Inhibition zone diameters (IZD) of honey and imipenem against *Proteus mirabilis*.

Result of the T-Test for Honey and Imipenem at α=0.05 (95% confidence limit) for the organisms showed that the P-value for *Staphylococcus aureus,* was 0.000, for *Pseudomonas aeruginosa,* it was 0.000 and for *Proteus mirabilis,* it was 0.860 (Table 3)

Table 3: T-Test result for Honey and Imipenem for the three (3) test organisms at α=0.05 (95% confidence limit).

Organism	N	Antimicrobial	Mean±Sem	P-value	Decision
Staphylococcus aureus	11	Honey Imipenem	15.00±0.661 25.64±0.244	0.000	Significant
Pseudomonas aeruginosa	16	Honey Imipenem	16.88±1.828 27.38±0.875	0.000	Significant
Proteus mirabilis	2	Honey Imipenem	24.00±2.000 23.50±1.500	0.860	Not Significant

Key: N= no of isolates, Sem= Standard Error of Mean, P-value= Significance Probability value

DISCUSSION

The prevalence of the isolated bacteria species from the wound samples showed that *Pseudomonas aeruginosa* had the highest prevalent rate of 16 (30.8%) followed by *S. aureus* 11 (21.2) and *Proteus mirabilis*. The presence of *Pseudomonas aeruginosa* and *S. aureus*

Proteus mirabilis is also known to produce focal lesions in debilitated patients and those

In the wound samples is not surprising because these organisms are implicated in wound infections. *S. aureus* is a major cause of skin and soft tissue diseases (Geo *et al*, 2013). *Pseudomonas aeruginosa* for example is frequently implicated in burn wounds where they produces systemic diseases (Geo *et al*, 2013). receiving contaminated intravenous infusions (Geo *et al*, 2013). The findings of

this work agrees with the work of Pandukar *et al* (2020) and Oguntibeju and Nwobu (2004), that reported that *Pseudomonas aeruginosa* was more prevalent in wounds than *S. aureus.* But it is in disagreement with the works of Ogba *et al*. (2014) and Mulugeta and Beyene (2014) that reported higher prevalent rate of *S. aureus* to *Pseudomonas aeruginosa* in wounds. The higher prevalent rate of *Pseudomonas aeruginosa* over *S. aureus* in this study may be attributed to the kind and nature of the wound samples. If the wound samples came mostly from burn patients, it may explain the higher prevalent rate of *Pseudomonas aeruginosa* over *S. aureus* (Geo *et al*, 2013).

Antibiotics susceptibility profile of the test bacteria is presented in Table 2. From the result *S aureus* was susceptible to imipenem (100%), and Ofloxacin (72.7%) while *P aeruginosa* was susceptible to imipenem (100%), Gentamicin (81.3%), Ofloxacin (68.7%) and Ciprofloxacin (56.3%) This agrees with the work of Agbakoba *et al* (2020) that imipenem was the most active antibacterial agent against *S. aureus*, among the antibiotics used in their study. In this present time, it was discovered that the oral drug ciprofloxacin and injection of gentamicin are the most effective antibiotics against *P aeruginosa.* This findings was in tandem with the work done by Magiorakos *et al*, (2012). *Proteus mirabilis* showed susceptibility to imipenem (100%), Ofloxacin (50%) and meropenem (50%). Generally, imipenem and Ofloxacin was the most potent antimibacterial agent recorded in this study. Similar observation has been reported in the study conducted by Agbagwa and Peterside-Frank, (2010) and El-mahmood *et al.,* (2010) who stated that their effectiveness was as a result of it not been excessively used or abused.

The antimicrobial activity of the honey against the test bacteria, showed that the honey was more active against *P aeruginosa* This also showed that imipenem was more active against *P aeruginosa* than 100mg/ml of honey. For *Proteus mirabilis*, 100mg/ml conc of honey produced IZD of 22mm and and *P mirabilis* than *S. aureus* at the concentrations of 100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml. The greatest activity of the honey indicated by the widest inhibition zone diameter (IZD) of 29mm was against *P aeruginosa* at a concentration of 100mg/ml (Fig 1). Also at the honey concentration of 100mg/ml it produced an IZD of 26mm against *P mirabilis* and an IZD of 18mm against *S. aureus*. Both *P aeruginosa* and *P mirabilis* are Gram negative bacteria, while *S. aureus* is a Gram positive bacterium. This result was in tandem with the work of Agbagwa and Peterside (2010) who stated that Gram negative bacteria such as *P aeruginosa* and *P mirabilis* were more susceptible to honey when compared to the Gram positive. Adetuyi *et al.* (2009) in a bid to ascertain the total phenol, tocopherol and antibacterial quality of honey in Ondo State found out that honey samples exhibited antimicrobial activity against *Proteus mirabilis*. However, this findings contradicts the work of Molan (2016), who stated that Gram positive bacteria (*S. aureus*) were more susceptible to honey sample than Gram negative bacteria. The difference in susceptibility of Gram positive and Gram negative bacteria species to honey may not be unconnected to their cell wall.

In comparing the activity of the 100 mg/ml concentration of honey that produced the greatest activity against the test bacteria, to imipenem as the antibacterial agent that all the test bacteria, were susceptible to, showed that the IZD produced by 100mg/ml honey ranged from 12mm to 18mm, while imipenem produced IZD between 25mm to 27mm for *S. aureus*. This showed that imipenem was more active against *S. aureus* than 100mg/ml honey. For *P aeruginosa* 100mg/ml conc. of honey produced IZD in the range of 0mm to 29mm, while Imipenem produced IZD in the range of 19mm to 32mm.

26mm, while imipenem produced IZD of 22mm and 25mm respectively. The findings was in agreement with the work done by Molan (2016) in their study where they

reported that honey sample were found to have less comparable advantage over antibiotics they used. However, John-Isa *et al.*, (2019), reported that the mechanism of inhibition of antibiotic resistant bacteria by honey has not been fully documented.

The result of the T-Test for Honey and Imipenem in comparing their efficacy to the test organisms showed that the P-value for *S. aureus* was 0.000, which is less than 0.05 indicating that there is significant difference between the activities of imipenem compared to honey. In the same vein, the P-value for *P aeruginosa* was 0.000 also indicating that there is significant difference between the activities of Imipenem to that of honey. For *P mirabilis* the P-value was 0.860, indicating that there is no significant difference between the activities of imipenem and honey against *P mirabilis* (Table 3).

CONCLUSION

This study has demonstrated that honey have potential in inhibiting the *in vitro* growth of *Pseudomonas aeruginosa, S. aureus* and *Proteus mirabilis* isolated from wounds. Also this work reveals that honey activity is comparable to imipenem activity against *P mirabilis*. By being active against these organisms, honey can be used as an alternative method of treating patients with wound infected with the tested bacteria.

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