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***In-Vitro* Antimicrobial Activities of Honey on Multi-Resistant Bacteria Isolates from Infected Wounds of Patients in Benin City.**

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**Abstract**

This study was done to show the antimicrobial activity of honey against isolated bacteria from infected wounds. In this study, a total of two hundred (200) bacterial isolates recovered from patients with different kinds of wound infections were used. Wound swab from patients were each processed using standard microbiological techniques and the recovered isolates identified accordingly. Antimicrobial susceptibility test with standard antibiotics and pure honey as well as the detection of Extended Spectrum  $\beta$ -lactamase (ESBL) was carried out on the recovered bacterial isolates using standard protocols. The minimum inhibitory concentration of the pure honey on the multi-resistant and ESBL producing isolates was demonstrated using agar diffusion methods. The organisms recovered from the infected wounds in order of prevalence were *Escherichia coli* (23%), *Staphylococcus aureus* (21%), *Pseudomonas aeruginosa* (15.5%) and *Proteus mirabilis* (14%), Coagulase Negative *Staphylococcus* (12.5%), *Providencia spp* (7%), *Proteus vulgaris* (4.5%) and *Klebsiella oxytoca* (2.5%). The antibiotic susceptibility profile of the isolates revealed that 75.5% of the isolates were susceptible to imipenem while most isolates were resistant to other antibiotics whereas, all the isolates were susceptible to the pure honey. Thirty percent (30%) of the clinical isolates were discovered to be ESBL producing bacteria with 46 out of the 60 ESBL producing bacteria being the ceftazidime-type while 20 of the ESBL producers were cefotaxime-type and 12 isolates produced both. The MIC of honey on the clinical isolates ranged between 10%v/v to

30% v/v since at 30% v/v all of the clinical isolates were susceptible to the honey whereas the MIC of honey on the ESBL producing bacteria ranged from 15% v/v to 30% v/v with the 30% v/v exerting 100% antibacterial activity on all ESBL producing bacterial. This study has shown that honey has *in-vitro* antimicrobial activity on bacteria isolates from infected wounds including multidrug resistant bacteria thereby justifying the use of honey for wound dressing for its antibacterial property.

**Keywords:** Antimicrobial, Extended Spectrum  $\beta$ -lactamase, honey, Multi-drug resistant isolates, wound infections.

**Introduction**

The application of traditional and indigenous natural products in the treatment of ailments and diseases has been practiced since the origin of mankind. Honey which is a pure, natural, dark viscous sweetener secreted by the bee, *Apis mellifera*, from flower nectars is one of such natural products considered to be very essential (Mandal and Mandal, 2011). To the ancient people, honey has been associated with strength, beauty and longevity. Honey has been used as an ancient remedy for the treatment of infected wounds and this dates back to about 2000 years before bacteria were discovered and its use for the treatment of microbial infections dates back to ancient times when it was described as "good for all rotten and hollow ulcers" (Molan, 1992). More recently, honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, gram-positives and gram-negatives. Antifungal action

has been observed for some yeasts and species of *Aspergillus* and *Penicillium*, as well as the common dermatophytes (Harris 1994).

The antibacterial property of honey was first reported by van Ketel (1891) and has recently been, 'rediscovered' by clinical practitioners as effective, especially where conventional modern therapeutic agents are failing. There are many published articles describing the effectiveness of honey in rapidly clearing infection from wounds, with no adverse effects (Efem, 1993).

Although honey has not been standardized as a cure for serious disorders such as plague, it is used today in hospitals and in specific circumstances, is a component of proprietary and dispensed medicines in the treatment of infected wounds, sores, skin ulcers and ragged wounds difficult to dress (Namias, 2003). However, there is paucity of data on its actual mechanism of action in these applications. Since micro-organisms are becoming resistant to most antibiotics (Levy and Marshall, 2004), there is a quest for more antibacterial agents. Following the traditional use of honey in healing wounds, this work was born out of a need to justify its antimicrobial properties. Also there has been a current emergence of Extended Spectrum Beta-Lactamase (ESBL) producing organisms as a cause of wound infection (Ogefere *et al.*, 2015; Omoregie *et al.*, 2010) and since ESBL is known to be the cause of bacterial resistance to third generation cephalosporins, there is the need to examine the effectiveness of honey, which has proved effective in wound dressing, against these group of bacteria.

The specific objectives of the study therefore were to:

1. Characterize the bacterial isolates from infected wounds of patients attending clinics within Benin Metropolis.
2. Determine the antimicrobial susceptibility profile of bacterial isolates from infected wounds to standard antibiotics.
3. Determine the antimicrobial activity of honey against bacterial isolates from infected wounds.
4. Determine the antimicrobial activity of honey against multi-resistant and extended

spectrum  $\beta$ -lactamase producing bacteria causing wound infections.

## **Material and Method**

### **Source of Test Organisms**

The organisms used in this study were isolates obtained from wound infection of patients in University of Benin Teaching Hospital, UBTH, (accident and Emergency ward, orthopaedic ward, burns ward and other surgical wards) in Benin City, Edo State between March and December, 2018. A total of Two hundred (200) bacterial isolates recovered from wound specimens were obtained from different patients having different types of wound infections which were processed in the Medical Microbiology Laboratory of the hospital using standard microbiological techniques as previously described (Barrow and Feltham, 2003).

### **Source of Honey**

The honey used during the experiment was common natural honey sourced from the open market in Benin City.

### **Tests for the Purity of Honey**

Two methods were used to determine the purity of the honey used in this study as described below:

#### **The flame test**

A lighter and a candle with a cotton wick were used. Cotton wick of the candle was dipped into a bit of honey and the excess shaken off. An attempt to light the wick was made. If it burns, then it is completely pure honey. If it doesn't, it could be an indication of presence of water or other impurities. The test was carried out with the honey used and the cotton wick was completely burnt.

#### **The dilution test**

Water was poured into a clear glass cup and a tablespoon of honey was added. If the honey is pure, the lump will sink to the bottom of the glass. If impure, it will readily mix up with the water in the glass cup. When the test was carried out, the honey sank to the bottom of the cup.

### **Sterility Testing of Honey**

The sterility of honey was tested by inoculating a

loopful of the honey into blood agar and MacConkey agar plates and incubated for 24 hours at 37°C, the absence of bacterial growth showed that the honey was sterile.

#### **Determination of susceptibility/resistance of the isolates to antibiotics and pure honey**

Disc susceptibility test was performed according to the modified method of Bauer *et al.* (1966). Briefly, the test isolates from overnight agar cultures were suspended in sterile water and the turbidity was matched with 0.5 McFarland standards. A sterile cotton wool swab stick was dipped into the organism suspension, the excess was drained off by pressing the swab stick against the inner walls of the test tube and the swab stick used to spread the inoculum evenly over the entire surface of the Mueller Hilton Agar plate. The commercially available antibacterial discs (Abbek, England) (ciprofloxacin (5µg), ofloxacin (5µg), gentamicin (10µg), erythromycin (5µg), amoxicillin and clavulanate acid (30µg), cefixime (5µg), ceftazidime (30µg), tetracycline (30µg), cloxacillin (5µg), cefuroxime (30µg), Imipenem (10µg) and Azithromycin (15 IU)) and disc impregnated with pure honey were placed on the surface of the seeded plate after 10 minutes of inoculation. The plates were incubated at 37°C for 18 hours. After incubation the zone diameter was measured in millimeters and a standard chart was used to interpret zones of inhibition to determine if test organisms were susceptible or resistant.

#### **Detection of extended spectrum beta lactamase**

The presence of ESBL was detected in bacterial isolates using the double-disc synergy test (Livermore and Brown, 2001). Briefly, test organisms were emulsified in sterile water and standardized using 0.5 McFarland solution (equivalent to  $1 \times 10^8$  colony forming units per ml). A sterile cotton wool swab was dipped in the organism suspension the entire surface of Mueller-Hinton agar plate was seeded by swabbing in three directions with the swab. A disc containing amoxicillin–clavulanate (30 µg) (Oxoid, England) was placed at the centre of the agar plate. A ceftazidime disc (30 µg) was placed 25 mm from the amoxicillin–clavulanate disc and another disc containing cefotaxime (30 µg) was

placed on the opposite side of the amoxicillin–clavulanate disc. The plates were incubated at 37 °C overnight and ESBL production was inferred as positive if there was an expansion of the zone of inhibition between the ceftazidime and amoxicillin–clavulanate disc, cefotaxime and amoxicillin–clavulanate disc or both.

#### **Minimum Inhibitory Concentration of Honey on the Multi-resistant and ESBL producing Isolates**

The minimum inhibitory concentration of honey on the clinical isolates was studied *in-vitro* using the agar dilution method as stated in CLSI guidelines (CLSI, 2012). The different concentrations of honey (v/v) were prepared in molten Nutrient agar medium at 56°C to give the final concentrations of 5%, 10%, 15%, 20%, 25% and 30%. The standard bacterial broth cultures of the isolates from the wounds were inoculated on the various concentration of honey in the Nutrient agar medium including control. The plates were incubated at 37°C for 24 hours overnight. After overnight incubation, the plates were observed for inhibition of growth. Bacterial cultures inoculated on Nutrient agar medium without honey served as control. Minimum Inhibitory Concentration (MIC) is the lowest concentration of the honey that yielded no growth.

#### **Results**

Of the 200 bacterial isolates recovered from wound swabs specimen used for this study, 46 (23%) were *Escherichia coli* with *Staphylococcus aureus* (21%) being the next in prevalence followed by *Pseudomonas aeruginosa* being 15.5% (Table 1).

The susceptibility of the clinical isolates to antibiotics and pure honey examined in this study revealed that all the test isolates were susceptible to the pure honey while 75.5% were susceptible to imipenem and most isolates were resistant to tetracycline, erythromycin, cefotaxime, gentamycin, ceftazidime and azithromycin and all isolates were resistant to cloxacillin (Table 2).

The distribution of the ESBL producing bacterial

isolates shows that 30% of the recovered isolates from wound infection produced ESBL. Nineteen (19) of the 46 *Escherichia coli* isolates were ESBL producers making the highest group, 55.5% of the *Proteus vulgaris* produced ESBL while none of the *Klebsiella oxytoca* produced ESBL (Table 3).

From the study, it was shown that 23% of the clinical isolates produced ESBL against ceftazidime while 10% produced against cefotaxime whereas only 6% produced against both (Table 4).

The ability of honey to inhibit bacterial growth at different concentration was examined in this study and it shows that at 5% v/v honey had no anti-bacterial activity whereas considerable activity was observed at 15% v/v against 22% of test organisms while 30% v/v honey had 100% activity against all test organisms (Table 5).

On the antibacterial activity of honey against ESBL producing bacteria the activity was seen in a progressive order from 15% v/v (46.7%) to 30% v/v achieving 100% activity against the 60 ESBL producing test organisms (Table 6).

**Table 1: Organisms Recovered from Infected Wounds and their Prevalence**

<b>Organisms</b>	<b>Number Isolated</b>	<b>% Prevalence</b>
<i>Escherichia coli</i>	46	23.0
<i>Klebsiella oxytoca</i>	5	2.5
<i>Pseudomonas aeruginosa</i>	31	15.5
<i>Proteus mirabilis</i>	28	14.0
<i>Proteus vulgaris</i>	9	4.5
<i>Providencia species</i>	14	7.0
<i>Staphylococcus aureus</i>	42	21.0
Coagulase Negative <i>Staphylococcus</i>	25	12.5
<b>Total</b>	<b>200</b>	<b>100</b>

**Table 2: Antibiotic Sensitivity Profile of clinical Isolates recovered from infected Wound**

Clinical Isolates	Antibiotics												
	TE	E	CTX	CN	OFX	AUG	CIP	CAZ	OCF	OB	IPM	AZM	HP
<i>Escherichia coli</i> (46)	0 (0.0)	0 (0.0)	33 (71.7)	38 (82.6)	24 (52.2)	30 (65.2)	30 (65.2)	25 (54.3)	41 (89.1)	0 (0.0)	31 (67.4)	15 (32.6)	46 (100)
<i>Klebsiella oxytoca</i> (5)	0 (0.0)	0 (0.0)	4 (80.0)	3 (60.0)	5 (100)	3 (60.0)	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (100)	2 (40.0)	5 (100)
<i>Pseudomonas aeruginosa</i> (31)	0 (0.0)	0 (0.0)	20 (64.5)	15 (48.4)	21 (67.7)	12 (38.7)	21 (67.7)	12 (38.7)	10 (32.3)	0 (0.0)	15 (48.4)	14 (45.2)	31 (100)
<i>Proteus mirabilis</i> (28)	1 (3.6)	3 (10.7)	13 (46.4)	12 (42.9)	20 (71.4)	12 (42.9)	20 (71.4)	0 (0.0)	14 (50.0)	0 (0.0)	14 (50.0)	2 (7.2)	28 (100)
<i>Proteus vulgaris</i> (9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.4)	2 (2.2)	3 (3.3)	1 (1.1)	1 (1.1)	0 (0.0)	9 (100.0)	1 (1.1)	9 (100)
<i>Providencia species</i> (14)	0 (0.0)	0 (0.0)	10 (71.4)	5 (35.7)	11 (78.6)	12 (85.7)	10 (71.4)	2 (14.3)	0 (0.0)	0 (0.0)	14 (100)	4 (28.6)	14 (100)
<i>Staphylococcus aureus</i> (42)	11 (26.2)	23 (54.8)	13 (31.0)	21 (50.0)	30 (71.4)	21 (50.0)	20 (47.6)	10 (23.8)	24 (57.1)	0 (0.0)	41 (97.6)	28 (66.7)	42 (100)
Coagulase Negative <i>Staphylococcus</i> (25)	0 (0.0)	10 (40.0)	6 (24.0)	0 (0.0)	16 (64.0)	12 (48.0)	20 (80.0)	11 (44.0)	14 (56.0)	0 (0.0)	23 (92.0)	11 (44.0)	25 (100)
TOTAL %	12 (6.0)	36 (18.0)	99 (49.5)	94 (47.0)	131 (65.5)	103 (51.5)	128 (64.0)	61 (30.5)	104 (52.0)	0 (0.0)	151 (75.5)	77 (38.5)	200 (100)

**Key:** TE: TETRACYCLINE, E: ERYTHROMYCIN, CTX: CEFOTAXIME, CN: GENTAMYCIN, OFX: OFLOXACIN, AUG: AMOXICILLIN-CLAVULANIC ACID, CIP: CIPROFLOXACIN, CAZ: CEFTAZIDIME, OCF: CEFUROXIME, OB: CLOXACILLIN, IPM: IMEPENEM, AZM: AZITHROMYCIN, HP: PURE HONEY

**Table 3: Distribution of ESBL Producing Bacterial Isolates Recovered from infected wounds**

Organisms	Number isolated	Number Positive for ESBL (%)
<i>Escherichia coli</i>	46	19(41.3)
<i>Klebsiella oxytoca</i>	5	0(0)
<i>Pseudomonas aeruginosa</i>	31	8(25.8)
<i>Proteus mirabilis</i>	28	13(46.4)
<i>Proteus vulgaris</i>	9	5(55.6)
<i>Providencia species</i>	14	3(21.4)
<i>Staphylococcus aureus</i>	42	10(23.8)
Coagulase Negative <i>Staphylococcus</i>	25	2(8.0)
<b>Total</b>	<b>200</b>	<b>60(30.0)</b>

**Table 4: Type of ESBL production by ESBL producing clinical Isolates recovered from infected Wound**

Antibiotics	Number of Recovered Bacterial isolates	Number positive for ESBL (%)
CAZ	200	46(23)
CTX	200	20(10)
CAZ+CTX	200	12(6)

**Table 5: Inhibition of Bacterial Growth at Different Concentration (V/V) Of Honey**

Clinical Isolates	Concentration of Honey					
	5%v/v	10%v/v	15%v/v	20%v/v	25%v/V	30%v/v
<i>Escherichia coli</i> (46)	0 (0.0)	0 (0.0)	13 (28.3)	38 (82.6)	24 (52.2)	46 (100)
<i>Klebsiella oxytoca</i> (5)	0 (0.0)	0 (0.0)	1 (20.0)	3 (60.0)	5 (100)	5 (100)
<i>Pseudomonas aeruginosa</i> (31)	0 (0.0)	0 (0.0)	11 (35.5)	15 (48.4)	21 (67.7)	31 (100)
<i>Proteus mirabilis</i> (28)	0 (0.0)	1 (3.6)	10 (35.7)	12 (42.9)	20 (71.4)	28 (100)
<i>Proteus vulgaris</i> (9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)	4 (44.4)	9 (100)
<i>Providencia species</i> (14)	0 (0.0)	0 (0.0)	1 (7.1)	5 (35.7)	11 (78.6)	14 (100)
<i>Staphylococcus aureus</i> (42)	0 (0.0)	2 (4.8)	3 (7.1)	20 (47.6)	30 (71.4)	42 (100)
Coagulase Negative <i>Staphylococcus</i> (25)	0 (0.0)	0 (0.0)	5 (20.0)	9 (36.0)	16 (64.0)	25 (100)
TOTAL	0	3	44	104	131	200
%	(0.0)	(1.5)	(22.0)	(52.0)	(65.5)	(100)

**Table 6: Inhibition of ESBL producing Bacterial Growth at Different Concentration (V/V) Of Honey**

<i>Clinical Isolates</i>	Concentration of Honey					
	5%v/v	10%v/v	15%v/v	20%v/v	25%v/V	30%v/v
<i>Escherichia coli</i> (19)	0 (0.0)	0 (0.0)	10 (52.6)	18 (94.7)	19 (100)	19 (100)
<i>Pseudomonas aeruginosa</i> (8)	0 (0.0)	0 (0.0)	6 (75.0)	6 (75.0)	8 (100)	8 (100)
<i>Proteus mirabilis</i> (13)	0 (0.0)	0 (0.0)	8 (61.5)	10 (76.9)	12 (92.3)	13 (100)
<i>Proteus vulgaris</i> (5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	4 (80.0)	5 (100)
<i>Providencia species</i> (3)	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	2 (66.7)	3 (100)
<i>Staphylococcus aureus</i> (10)	0 (0.0)	0 (0)	3 (30.0)	10 (100)	10 (100)	10 (100)
Coagulase Negative <i>Staphylococcus</i> (2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	2 (100)
Total	0 (0.0)	0 (0.0)	28 (46.7)	46 (76.7)	57 (95.0)	60 (100)

**Discussion**

The use of honey as a traditional remedy for microbial infection dates back to ancient times (Molan, 2001) and unheated honey have been reported to have broad spectrum antibacterial activity when tested against pathogenic bacteria, oral bacterial as well as food spoilage bacteria. It is known that various micro-organisms have the ability to infect open wounds thereby, aggravating wounds leading to gangrene, abscess, ulcerations and other severe conditions. In most of the cases, micro-organisms isolated from wound infections are usually resistant to most antibiotics both in-vivo and in-vitro (Efem, 1993). Since the current trend in wound care advocates the use of honey for dressing (Fox, 2000), it becomes imperative to ascertain the in vitro antimicrobial activity of honey against wound isolates as most researchers have asserted that the immunomodulatory property of honey is

relevant to wound healing (Okeniyi *et al.*, 2005; Stephen-Haynes, 2004; Tonks *et al.*, 2003; Moore *et al.*, 2001; Tonks *et al.*, 2001; Oryan and Zaker 1998; Tovey, 2000).

In this study, it was discovered that *Escherichia coli* was the most prevalent in wound infection (23%) followed closely by *Staphylococcus aureus* (21%) with the least being *Klebsiella oxytoca* (2.5%) and this finding is in-line with the finding of Omoregie *et al.* (2010). From the result presented above, it was observed that all bacterial isolates tested against honey at a concentration of 20%v/v of molten nutrient agar did not thrive substantially. However, at higher concentration of 30%v/v of molten nutrient agar, there was a complete inhibition of bacterial growth for all isolates. This is in consonance with the findings of Efem (1993) where there was a complete inhibition of bacterial growth at 30%

v/v. Therefore, it can be asserted that the speedy healing of wounds treated with honey is not only due to the immunomodulatory property of honey but also due to the antimicrobial activity of natural unheated honey as seen in this study. Various researchers have shown that honey exerts an antibacterial activity against various organisms, including gram-positive and gram-negative bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* *Proteus mirabilis*, *Streptococcus pyogenes* (Hegazi, 2011; Mandal and Mandal, 2011).

Pure unheated honey also demonstrated very good antibacterial activity against ESBL producing bacteria in this study. This findings agree with the findings of Pratibha and Manita (2015) who discovered that the MIC of honey against all the ESBL producing isolates of *Klebsiella pneumonia* recovered from wound infection was at concentrations ranging between 30%-40% v/v. Namias *et al.* (2001) has asserted that the antibacterial activity of honey is mainly due to inhibines whereas other researchers have attributed its antimicrobial activity to the osmotic effect of sugar content on microbial cells resulting in shrinkage and disruption of bacteria, its low water activity which induces an unfavorable environment for microbial growth, its low pH of 3.6 thus inhibiting the growth of acid sensitive organisms. In addition, it has a unique process of fermentation which is associated with the production of alcohol, an antimicrobial agent and the highly viscous nature of honey could act as a barrier to prevent bacteria penetration and colonization of the wound surface (Subrahmanyam, 2001).

### Conclusion

In conclusion, although the use of honey to treat infected wound is viewed with skepticism by some, it has been shown to be clinically useful in various settings involving soft tissue infections and non-healing wound. This work has shown that honey has an in-vitro antimicrobial activity on multi-drug resistant bacteria as well as ESBL producing bacteria. This activity in addition to immunomodulatory property and highly viscous nature justifies why honey prevents bacterial penetration and colonization of the wound surfaces as well as yields speedy wound healing.

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