Antimicrobial activity and time kill kinetics of Nigerian Honeys on multi-resistant Enteric Bacilli

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ABSTRACT
Antimicrobial activities of some Nigeria honeys were tested on multi-antibiotic resistant enteric bacilli strains (MAREBS) that are becoming dreadful among the populace. Enteric bacilli isolated from fecal samples randomly collected from community populace were biotyped and profiled for antibiotic susceptibility by micro-broth dilution assay. Honey physico-chemical and phyto-chemical metabolites were analysed and tested for antimicrobial susceptibility to MAREBS while its time kill kinetics was evaluated. Significant rate of 31.3% *Escherichia coli*, *Klebsiella oxytoca*, (19.5%), *Pseudomonas aeruginosa* (15.3%) were found with only 62.6% showed significant resistance to cefotaxime (30µg) and 61.6% to ampicillin (10µg). more than 40% showed significant resistance to Cotrimoxazole, ciprofloxacin and tetracycline with MIC $\geq$16 µg/ml ($p<0.05$). Physico-chemical parameters vary significantly with high phenol and alkaloids contents. Few honey samples showed antimicrobial activity of more than 37% inhibition rate while 8.1% MAREBS were further inhibited at lower MIC 31.25mg/mL, 10.8% at MIC 125mg/mL and 8.1% MIC 250mg/mL, while cidal rate of 8.1% was recorded. Significant reduction in average count of different MAREBS was recorded at honey dilutions of 1:2 and 1:4 to less than 2.10Log_{10}CFU/mL. Amidst global burden of enteric infection with persistence antibiotic resistance, Nigerian honeys showed a reliable bacteriostatic and cidal activity as prospective novel alternative therapy for MAREBS infections.

Keywords: *antibiotics, resistant, honey, phytochemicals, physico-chemicals*

1. INTRODUCTION

Continuous emergence of antibiotic resistant bacterial strains is a growing problem in both developed and developing countries of the world as it causes increasing poor health and economic drain. Treatment

of enteric infectious diseases continues to be problematic in recent time as its often leads to increasing morbidity mostly in developing countries of the worlds (Iruka *et al.,* 2007).
In spite of this dreadful trend, there is need to search for alternative natural source with high efficacy, economical and easily available for common man (Giske *et al.,* 2008; Akinduti *et al.,* 2012). According to the WHO estimation, approximately 80% of the world's population relies mainly on traditional medicine for their primary health with the use of natural product such as honey which is vital in controlling the threats posed by multi-antibiotics resistant enteric bacilli strain (MAREBS), having lesser side effects and lower cost (WHO, 1998; Akoh, 1991). Due to availability and multi-factorial antimicrobial mechanism of honey, honey has lately been rediscovered as a therapy of several applications (Zumla and Lulat, 1989; WHO, 1998).
Recently, the potent activity of several honeys against antibiotic-resistant bacteria has further increased the interest for its application against MAREBS (Cooper *et al.,* 2002; Cooper *et al.,* 2010). According to report of Molan and Cooper (2000), antimicrobial potency among different honeys depend on their geographical, seasonal and botanical source as well as harvesting, processing and storage conditions which could influence its cidal activity. However, honey antimicrobial was attributed to either singularly or synergistic activity of several physico-chemical parameters and phytochemical metabolites (Wilkinson *et al.,* 2005).
Moreover, use of medical-grade honey such as manuka (*Leptospermum*) honey, Tualang (*Koompassia excelsa*) honey, revamil and medi-honey has been reported to have shown variation in their *in vitro* antibacterial activity (Wilkinson *et al.,* 2005).
Therefore, this study was carried out to assess the antimicrobial activity of locally produced honeys in Nigeria against multi-resistant enteric bacilli in Abeokuta, Nigeria.

2. METHODS

Sampling and culture: Faecal samples of 251 community residents of Abeokuta township located within 7.15^0N and 3.35^0E in moderately hot tropical climatic zone of southwestern Nigeria (NPC, 2007; Shittu *et al.,* 2010) were randomly collected with permission of individual informed consent and ethical clearance for research study. All faecal samples collected were cultured for enteric bacilli.
Biotyping: Each isolate obtained was further characterized for enteric bacilli using Analytical Profile Index (API) and interpreted according to World Health Organization (WHO) manual for laboratory investigation of acute enteric infections (Bauer and Kirby, 1996).
Antimicrobial susceptibility test: Susceptibility profile of each isolates was performed by Kirby Bauer disc diffusion method on Mueller Hinton agar (CLSI, 2014); using Tetracycline (30µg), Cefuroxime (30µg), Clavulanic acid (10µg)+Amoxycillin (20µg), ceftazidime (30µg), gentamycin (10µg), Trimethoprim (5µg) + Sulphamethaxazole (25µg), ofloxacin (10µg), Ampicillin (10µg), and Ciprofloxacin (10µg). Briefly, pure isolates of 0.5McFarlan turbidity was spread on Mueller-Hinton Agar and each antibiotic disc was placed on the inoculated agar and incubated at 37^0C for 18 to 24 hours. The inhibition zone of each antibiotic was measured using graduated metre rule to determine diameter of the inhibition zones and interpreted according to CLSI guidelines (2014). Resistance isolates were selected for further assay.
Minimum inhibitory concentration determination (MIC): Broth micro-dilution method was used to determine the MIC of the resistant strains while minimum bactericidal concentration (MBC) of each antibiotic was also determined.
Honey samples: A total of 14 different honey samples were locally purchased from different sources in Nigeria. All the samples were transferred to the laboratory, stored in amber flasks, and kept at 4°-5°C until analysis. Sterility check was performed on all the honey by culturing on blood agar.
Determination of physico-chemical parameters: Colour of each honey sample was determined by absorbance of homogenized 50% (w/v) honey solution at wavelength 635nm and colour Pfund was

calculated (IHC, 2002; Aazza *et al*., 2012). pH, Electrical conductivity and Total dissolved solids were determined using pH electrodes (HANNA Instruments pH 210 microprocessor meter) immersed in 50% (w/v) honey solution while moisture content by digital refractometer at 20^{o}C (Lawal *et al.,* 2009). Ostwald U-tube viscometer was used for viscosity (Bang *et al.,* 2003) while hydrogen peroxide was determined by iodometric assay levels (Sturgeon, 1990). Estimation of Reducing sugar was done using phenol-sulphuric acid method (Adeniyi *et al.,* 2009).

Analysis of Phytochemical metabolite: The following metabolites in the honey were determined by spectrophotometric assay; alkaloids (Edeoga *et al.,* 2005), tannins (Dey *et al.,* 2012), phenol (Soladoyeet al., 2012), saponin (Humadi and Istudor,2008), flavonoids, glycoside, phlobatanin and terpenoids (Berhanu, 2003).

Honey antimicrobial susceptibility testing:

Agar well diffusion: Well of 6mm diameter, 4mm depth was made in Muller Hinton agar at 4 cm distance apart and overnight broth culture of 0.5McFarland turbid MAREBS was prepared and allowed to dry. Later, 100µL of honey samples were placed in the wells with sterile water as negative control and incubated at 37^{0}C for 18 to 24 hours. Inhibition zones produced were measured accordingly (Patton *et al.,* 2006).

Honey inhibition assay by spectrophotometric: Standard micro-tube dilution bio-assay was used to determine susceptibility of MAREBS to honey samples (Patton *et al.,* 2006; Nsofor and Iroegbu, 2013). Briefly, 10ml of sterile distilled water was added 10mg honey sample and thoroughly homogenized. To sterile microtitre plate, first and second well was added with 100µL of honey solution and serially diluted while other serves as control. To each well, 100µL of 0.5 MacFarland turbid MAREBS broth was added and incubated at 37°C in ambient air for 24hours on shaker. Absorbance of each well was measured at wavelength 590nm before incubation and after incubation. Turbidity of bacteria growth which is proportional to the absorbance was determined and calculated for MIC at more than 95% inhibition after 24 hours.

Minimum bactericidal Concentration (MBC) assay: Sub-culturing was made from each well showing no growth including the MIC well onto Nutrient agar plates. Following overnight incubation at 37°C in ambient air for 24hours, the plates were examined for growth of bacteria colonies.

Time kill assay: Time kill kinetics of honey samples showing significant antimicrobial activity were further evaluated for their activity against MAREBS (Vargas *et al*., 2004) and interpreted accordingly (Sreela *et al*., 2011). To a sterile 1ml volume of different honey dilutions, was added 100µl of 10^{5}CFU/ml broth culture of the MAREBS isolate and incubated in a shaking incubator at 37°C in ambient air. Aliquots of 100µl were removed at 0, 6, 12, 18 and 24 hours post-inoculation. At each 6hours interval, 100µl broth culture challenged with honey was serially diluted in sterile 0.85% sodium chloride solution, plated and incubated for 24hours at 37°C.The assay was performed in triplicate and the total viable colony count was estimated at every 6 hours interval for a period of 24 hours in Log_{10} CFU/ml. repeated four times.

Data analysis: The significance of resistant bacteria strains was determined using Chi square (χ^2) at p value < 0.05 while mean and respective standard error of mean and significance of physico-chemical and phytochemical metabolite was determined by t-test at p<0.05 taking confidence interval of 95%. Significance of the antimicrobial activity of each honey samples against different susceptibility of MAREBS was estimated with ANVA at p>0.05. The proportion total viable count were estimated in mean and Student's 2-tailed *t*-test to determine its significant at $P < 0{\cdot}05$.

3. RESULT AND DISCUSSION

Distribution of the enteric bacteria isolates and antinbiotic susceptibility profile: Among enteric isolates (Figure 1), 31.3% were *Escherichia coli*, *Klebsiella oxytoca*,(19.5%), *Pseudomonas aeruginosa* (15.3%), *Enterobacter aerogene* (9.4%), and lowest *Shigella specie* (2.0%). Only 62.6% show

significant resistant to cefotaxime (30µg); 61.6% to ampicillin (10µg) and 54.2% resistance to Clavulanic acid (10µg)+Amoxycillin (20µg) combination while 44.7%, 38.9% and 33.9% shown significant resistant to Cotrimoxazole (Trimethoprim + Sulphamethaxazole), ciprofloxacin and tetracycline with MIC ≥16 µg/ml (p=0.004). *Citrobacter freundii*, *Escherichia coli* and *Proteus mirabilis s*hown MBC ≥ 64 µg/ml (Table 1).

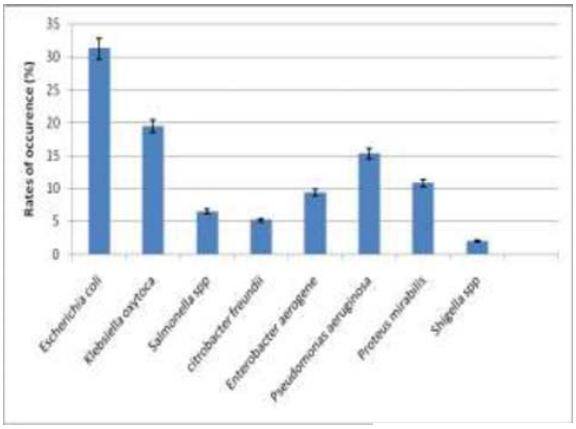

Figure 1: Distribution of enteric bacilli from community populace

Table 1: Susceptibility profile of enteric bacilli to commonly used antibiotics.

	Antibiotic susceptibility pattern (N=406)			
Antibiotic(ug/mL)	S n(%)	I n(%)	R n(%)	***MIC* *(≥*16* µg*/ml)* *(n)***
Tetracycline	170(41.9)	174(42.9)	62(15.3)	21(33.9)
Cefuroxime	62(15.3)	90(22.2)	254(62.6)	35(13.8)
Augmentin	125(30.8)	61(15.0)	220(54.2)	32(14.6)
Ceftazidime	181(44.6)	110(27.1)	115(28.3)	29(25.2)
Gentamycin	143(35.2)	176(43.3)	87(21.4)	18(20.7)
Cotrimoxazole	165(40.6)	147(36.2)	94(23.2)	42(44.7)
Ofloxacin	285(70.2)	102(25.1)	79(19.5)	17(21.5)
Ampicillin	30(7.4)	126(31.0)	250(61.6)	43(17.2)
Ciprofloxacin	188(46.3)	128(31.5)	90(22.2)	35(38.9)

Key: S=Susceptible I=Intermediate, R= Resistant, **N**= Total number of enteric bacteria isolates obtained; **n**=number of bacteria isolates; **%**=Percentage of bacteria; ***=CLSI (2012),** (10µg). (χ^2=31.094; p=0.009).

In the present study, the antimicrobial activities of Nigeria honeys were determined against MAREBS among the community residents. The rate of enteric bacterial pathogen found in this study was lower to 65.8% reported in Abuja, Nigeria (Estupinon and Sanjuan, 1999) and 50-60% in other developing countries (Bang *et al.,* 2003). Antibiotic resistant has been the major challenge to residents and health care provider in this locality and it was obvious with more than 50% resistant to cefotaxime, ampicillin and Clavulanic acid (10µg)+Amoxycillin (20µg) combination. Significant resistance to Cotrimoxazole, ciprofloxacin and tetracycline with MIC ≥16 µg/ml portrays an impending threat to the community. In spite of this, antimicrobial drug resistance among enteric bacteria pathogen continue to be a growing problem among the people with its attendant severe diarrheal (Nwankwo *et al.,* 2014). It was amazing to note that most of the enteric bacilli showed significant resistance to many commonly used antibiotics

(with MBC ≥ 64 µg/mL). This observation reveals an imminent danger to the health status of the community.

Physico-chemical parameters and phytochemical metabolites: Average quantity of physico-chemical parameters in Nigeria honeys significantly varies as shown in Table 2. Phytochemical compounds represented by box plot reveal high level phenol and alkaloids in Nigeria honey while low quantity of tannins, glycosides and phlobatanin was recorded.

Table 2: Variation of physico-chemical parameters of Nigerian honey sample

Physico-chemical	Mean±SD	T-test	P value
Viscosity (cp)	2892.58±134	93.169	0.001
pH	3.96±0.28	56.89	0.001
Moisture (%)	14.71±1.96	31.633	0.001
Electrical	265.31±185.	9.895	0.001
Total dissolved solids	62.60±19.79	10.337	0.001
Reducing sugar	25.79±6.20	23.697	0.001
Hydrogen peroxide	5.26±0.62	35.628	0.001

(T-Test= 23.5, p=0.001)

In spite of the use of honey for enteric infections, honeys from Nigeria reveal high level content of phenol and alkaloids but low in tannins, glycosides and phlobatanin level. This could be attributed to variation in the floral origin, which has been reported to affect its antibacterial activity that depends on plant species, environmental conditions, diversity and floristic characteristics (Agbaje *et al.,* 2006; Gomashe *et al.,* 2014).

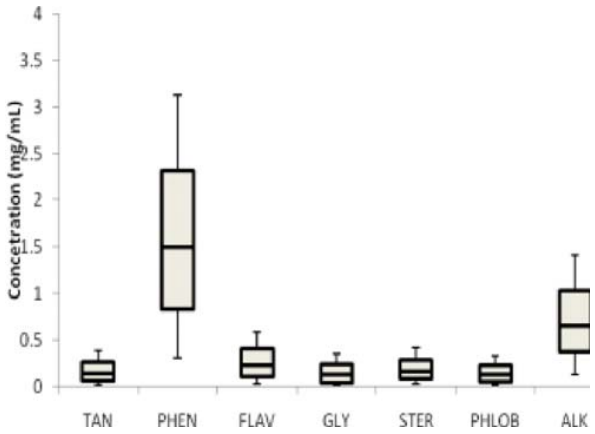

Figure 2: Box plots displaying the variation in phytochemical compounds in honey samples. (TAN=Tannins, PHEN=Phenol, FLAV=Flavonoids, GLY=Glycosides, STER=Steroids, PHLOB=Phlobatanin, ALK=Alkaloids)

Antimicrobial activity of Nigerian honey samples tested against MAREBS: Nigerian honey samples CSO2, R1 and RKL1 showed high antimicrobial activity of 46.0%, 43.2% and 37.8% inhibition rate respectively while 8.1% MAREBS were further inhibited by honey CSO2 at lower MIC 31.25mg/mL, 10.8% rate at MIC 125mg/mL by honey CSO1 and RKL1 showing 8.1% inhibition rate at MIC

250mg/mL ($p<0.005$). At lower MBC 125mg/mL, Nigerian honeys CSO2 and CSO1 show significant cidal rate of 8.1% against MAREBS respectively.

Table 3: Susceptibility pattern of MAREBS to Nigerian honey

Honey	Susceptibility		MIC		MBC	
	S n(%)	R n(%)	Conc (mg/mL)	n(%)	Conc (mg/mL)	n(%)
SEJ	7(18.9)	30(81.1)	500	6(16.2)	1000	0(0.0)
JO	2(5.4)	35(94.6)	1000	0(0.0)	1000	0(0.0)
OM	10(27.0)	27(73.0)	500	9(24.3)	1000	3(8.1)
CSO2	9(24.3)	28(75.7)	31.25	3(8.1)	125	3(8.1)
CSO1	17(46.0)	20(54.0)	125	4(10.8)	125	2(5.4)
R1	16(43.2)	21(56.8)	500	14(37.8)	1000	0(0.0)
PAK	7(18.9)	30(81.1)	500	6(16.2)	1000	2(5.4)
RR1	2(5.4)	35(94.6)	1000	0(0.0)	1000	0(0.0)
RR2	3(8.1)	34(91.9)	500	3(8.1)	1000	2(5.4)
RR4	3(8.1)	34(91.9)	500	3(8.1)	1000	1(2.7)
RKL2	6(16.2)	31(83.8)	500	5(13.5)	1000	2(5.4)
RKL1	14(37.8)	23(62.2)	250	3(8.1)	500	3(8.1)
RR3	6(16.2)	31(83.8)	500	6(16.2)	1000	3(8.1)
MAK	4(10.8)	33(89.2)	500	4(10.8)	500	1(2.7)

(Key: S=susceptible, R=Resistant; n=Number of bacteria isolates tested; %=percentage; F test=1.806 p=0.097)

Furthermore, significant inhibition of MAREBS at MIC 31.25mg/mL and MIC 125mg/mL with high cidal rate at MBC 125mg/mL, shows that most Nigerian honeys are more effective against resistant bacilli (Anjali and Sheetal, 2013; Omojate *et al.,* 2014). High phenol and alkaloids could enhance anti-enteric activity of these honey samples as a result of effective scavenging activity for reactive oxygen species including superoxide anions and hydroxyl radicals causing damage to bacteria cellular functions/ (Anjali and Sheetal, 2013). Presence of saponin couls as well inhibit lipopolysaccharides component of the bacilli cell membrane, thereby causing hydrolysis and alter the permeability of cell walls and hence exert toxicity on organelles leading to bacteria cell lysis (Stermitz *et al.,* 2000). A combine activity of high reducing sugar with acidic property, low moisture content and antiseptic properties of hydrogen peroxide in honey usually induce osmotic effect on bacteria cytosol with resultant inhibition of cell wall synthesis. This further confirms the broad-spectrum anti-enteric activity of these honey samples for treatment of gastro-intestinal infections. Deprivation of bacteria cell of metabolised iron or hydrogen bonding with vital proteins such as microbial enzymes by phenol and flavonoid could result to inhibition of bacteria cell replication while inhibition of efflux pump by glycoside render resistance mechanism of many Gram negative pathogen ineffective (Stermitz *et al.,* 2000).

Increase permeability of solute and loss of osmotic activity of the cell membrane resulting to cell death by saponin (Thakur *et al.,* 2011) and inactivation of tannin could leads to loss of microbial adhesions, cell envelope transport proteins, and mineral uptake (Min *et al.,* 2003). Similarly, high alkaloids in Nigerian honey could act similarly as DNA intercalating agents or like topoisomerase inhibitors that adversely inhibit DNA replication and supercoiling (Bonjean *et al.,* 1998).

Time kill rate of honey samples: Significant reduction in average count of different bacilli of MAREBS was recorded when tested against 1:2 and 1:4 honey dilutions from initial count of 5.0

Log_{10}CFU/mL to range of 2.10 and 2.79 Log_{10}CFU/mL while low reduction rate of 3.0 to 3.30 Log_{10}CFU/mL was recorded at honey dilutions of 1:8 and 1:16 as shown in Figure 3,4,5,6,7, and 8.

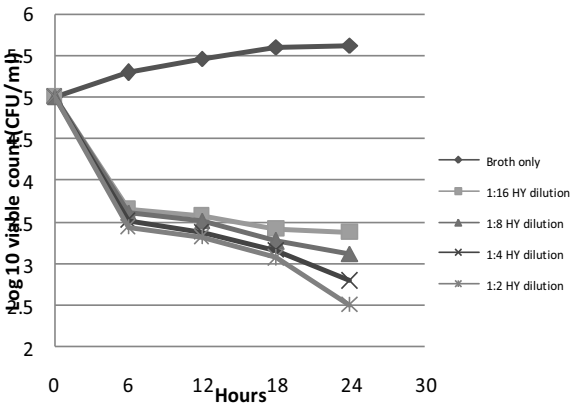

Figure 3: Rate of killing of multi-antibiotics resistant *Escherichia coli* to different dilutions of honey sample (HY).

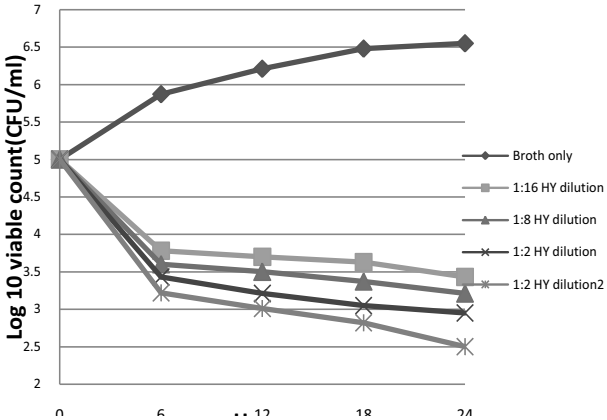

Figure 5: Time kill assay of multi-antibiotics resistant *Citrobacter species* isolates to different dilutions of honey (HY).

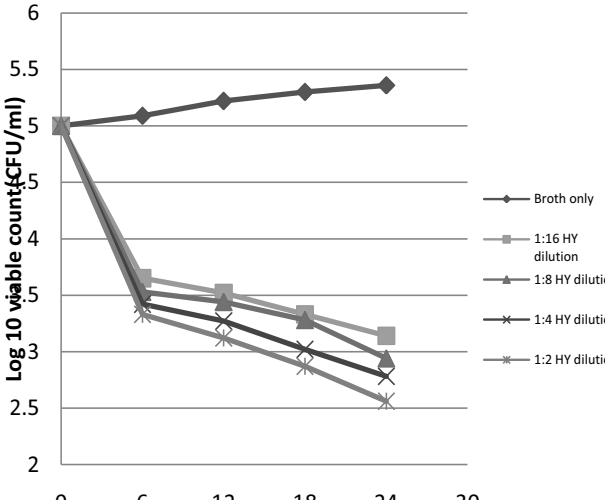

Figure 4: Time kill assay rate of multi-antibiotics resistant *Salmonella specie* isolates to different dilutions of honey (HY).

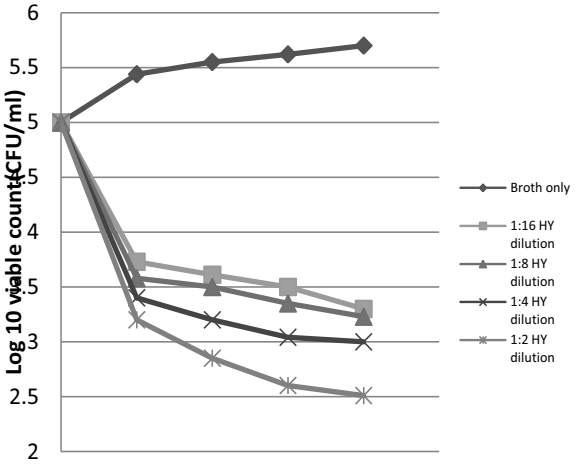

Figure 6: Time kill assay of multi-antibiotics resistant *Pseudomonas species* isolates to different dilutions of honey (HY).

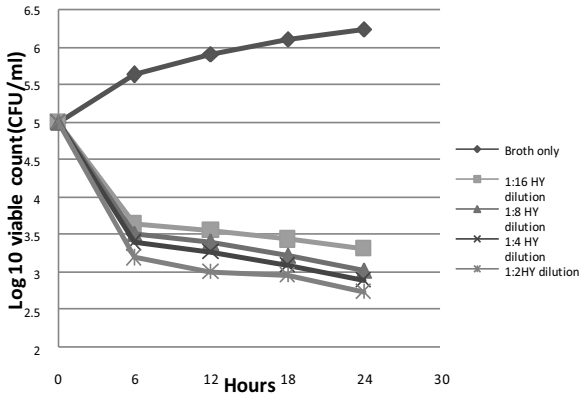

Figure 7: Time kill assay of multi-antibiotics resistant *Proteus specie* to different dilutions of honey (HY).

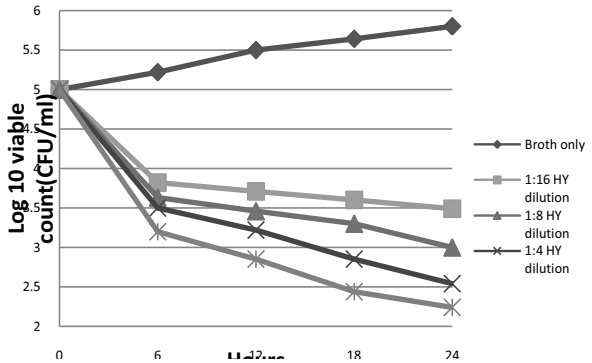

Figure 8: Time kill assay of multi-antibiotics resistant *Shigella specie* isolates to different dilutions of honey (HY).

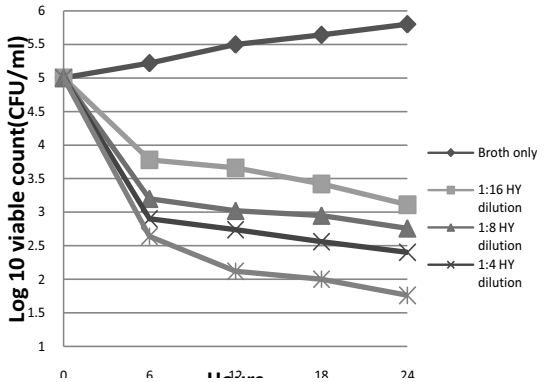

Figure 9: Time kill assay of multi-antibiotics resistant *Enterobacter specie* to different dilutions of honey (HY).

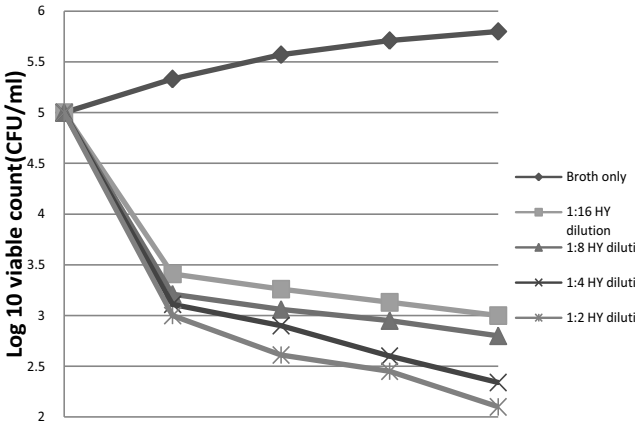

Figure 10: Time kill assay of resistant *Klebsiella species* isolates to different dilutions of honey (HY).

Evaluation of the kinetics of the honeys' antibacterial activity against the resistant bacilli being defined as bactericidal activity when viable count less than $3Log_{10}$CFU/mL relative to the initial inoculums (Scheetz *et al*., 2007). Significant reduction in average count ranging between 2.10 and 2.79 Log_{10}CFU/mL confirm high cidal activity of Nigeria honeys at dilutions of 1:2 and 1:4 suggesting a potential anti-enteric natural product with dependable efficacy. These cidal activities inform the suitable dilution of honey that could effectively produce significant anti-enteric activity in infection relating to gastro-intestinal diseases. Furthermore, the resultant reduction of viable count higher than 3.0Log CFU/mL recorded from 1:8 and 1:16 dilutions indicate a bacteriostatic action (Scheetz *et al*., 2007

Akinduti *et al.,* 2013].The growth inhibition and efficacy of these natural products were dose and time dependent to produce effective time-kill profiles against the resistant bacilli. The anti-enteric effect obtained from agar diffusion and microbroth dilution assay are complementary to these activities. The degree of killing and inhibitory activity may be accounted for by different mode of activity of individual phytochemical compounds which could be acting synergistically to produce potent anti-enteric effects (Eloff, 1998). The overall variation in anti-enteric bacteria activity of Nigerian honey could largely depend on its source, physico-chemical characteristics and its phytochemical metabolites which are major factors that determine its efficacy against many bacteria pathogens Akinduti *et al.,* 2016).

4. CONCLUSION

Amidst global burden of infectious enteric diseases with persistence emerging antibiotic resistance that are threat to the populace, Nigerian honeys have shown a valid evidence and reliable bacteriostatic and cidal activity with prospective antimicrobial activity due to multi-factorial mechanism of actions. The use of honey as anti-enteric bacteria agents against MAREBS has shown to be efficacious and potent natural products that could provide alternative therapy for enteric infections.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any financial obligations to any individual or group.

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