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Comparison of Antimicrobial Potential of Honey Samples from *Apis mellifera* and Two Stingless Bees from Nsukka, Nigeria

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Abstract

The antimicrobial activity of honey depends on many factors, including its botanical origin, geographical and entomological source. The aim of this study was to evaluate and compare the antimicrobial potential of honey varieties from *Apis mellifera, Hypotrigona* sp. and *Melipona* sp. against MDR *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* ATCC 25783*, Candida tropicalis, Candida albicans* SC 5314 and *Cryptococcus neoformans*. By using standard microbiological procedure, the agar-well diffusion and broth microdilution methods were used to evaluate honey samples for their antimicrobial and non-peroxidase activity. Different concentrations of the honey samples showed inhibition zones diameter (mm) against the test isolates. The Minimum Inhibitory Concentrations (MICs) of the honey varieties from *A. mellifera, Hypotrigona* sp. and *Melipona* sp. ranged from 6.3–25.0%, 3.1–12.5% and 6.3–25.0% (v/v) respectively. There were no statistically significant differences between the mean MICs of honey varieties against *E. coli, P. aeruginosa* (ATCC 25783) and *C. neoformans*. *Hypotrigona* sp. honey had the least mean MICs (4.15 ± 1.58–11.11 ± 2.76 % v/v) against most of the test organisms. The Minimum Biocidal Concentration (MBC) of the honey varieties from *A. mellifera, Hypotrigona* sp. and *Melipona* sp. against the test organisms varied from 6.3–50%, 3.1–25% and 12–50% (v/v) respectively. There were no significant differences between the mean MBCs of the honeys against MDR *S. aureus* (p=0.179), *E. coli* (p=0.564), *P. aeruginosa* (ATCC 25783) (p=0.846), and *C. albicans* (SC5314) (p=0.264). The honeys had some levels of non-peroxidase activity against *E. coli, P. aeruginosa* (ATCC 25783) and *C. neoformans.* This study has scientifically authenticated the potential use of stingless bee honeys from “Okotobo and Ifufu” as complementary therapeutic agents.

Keywords: Antimicrobial activity; MIC; MBC; Honey; *Hypotrigona* sp.; *Apis mellifera*; *Melipona sp.*; Stingless bee honey; Non-peroxidase activity

Abbreviations: MDR: Multi-Drug Resistant; *S. aureus*: *Staphylococcus aureus*; *B. cereus*: *Bacillus cereus*; *E. coli*: *Escherichia coli*; *P. aeruginosa*: *Pseudomonas aeruginosa* ATCC 25783; *S. enterica*: *Salmonella enterica*; *C. tropicalis*: *Candida tropicalis*; *C. albicans*: *Candida albicans* SC 5314; *C. neoformans*: *Cryptococcus neoformans*; MIC: Minimum Inhibitory Concentration; MBC: Minimum Biocidal Concentration; ANOVA: Analysis of Variance

Introduction

Antimicrobial agents are for now the world’s only hope of getting rid of infectious diseases. However, the change in pattern of resistance of pathogenic microbes to essential antibiotics, especially multidrug resistant once has diminished the effectiveness of known antibiotics [1]. As the frequencies of resistance are increasing worldwide, this poses a very serious danger to promotion of good health and all kinds of antibiotics, including the major last-ditch drug [2].

Therefore, there is need for evaluating alternative potential therapeutic agents with antimicrobial properties. Honey is bees’ natural product, made up of complex mixture of sugars such as, fructose and glucose. It has been used as a medicine in many cultures for centuries. In more recent times, the insight in the use of honey as a therapeutic substance has increased and it is gaining acceptance as a remedy for treatment of a wide variety of ailments caused by pathogenic microbes [3-5]. It is widely used as a topical antibacterial agent for treatment of wounds, burns and skin ulcers as reported in a review by Lusby [6]. The ability of honey to kill microorganisms has been attributed to factors such as high osmotic effect, acidity, hydrogen peroxide (produced enzymatically in especially diluted honey), phytochemical components, antimicrobial peptide (defensin-1), and the induction of increased lymphocyte and phagocytic activity [7-9]. There are many reports of biocidal as well as biostatic activity of honey against broad spectrum of bacterial and fungal species, which have developed resistance to antibiotics [10-13].

The hydrogen peroxide, especially in diluted form of honey, has been reported to help tissue growth and has the potential for wound healing. In the presence of catalase and/or heat, the activity of most of honeys can be destroyed. However, there are reports on non-peroxidase antimicrobial activity of catalase-treated honeys. This is important especially in topical antimicrobial and wound dressing’s fluids [14,15].

There are numerous species of honey bees and the chemical composition of their honeys may vary according to the habitat and sources of nectar of each species. *Apis mellifera* is a well-known honeybee, and there are more than 500 stingless bees’ species (from the Meliponini and Apidae family) of which are classified into five genera: *Meliponula, Melipona*, *Dectylurina, Lestrimelitta* and *Trigona* [16,17].

In traditional communities in Nigeria, stingless bee honeys are used extensively as sweeteners and natural home remedies for ailments,

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despite that, majority of previous studies have been conducted using honey from the *Apis* species. As there are no studies that have evaluated the antimicrobial activity of honeys from these species of stingless bees, therefore the aim of this study was to compare the antimicrobial and non-peroxidase activity of honeys collected in Nsukka, Nigeria from *Melipona* sp. (locally called ifufu in South East Nigeria), *Hypotrigona* sp. (Okotobo) and *A. mellifera* against eight different human pathogenic microorganisms.

Materials and Methods

Collection of honey samples

Three honey samples each from *Hypotrigona* spp. (Okotobo) and *Melipona* spp. (Ifufu) including *Apis mellifera* honey (widely known honey) were collected from keepers at Olido, Enugu Ezike, Igbo Eze North Local Government Area of Enugu State, between April and May, 2015. The matured combs, laden with honey, were harvested and aseptically collected in sterile screwed cups, and kept in a cool and dry place before transporting to the laboratory.

Test organisms

The test organisms were obtained from the Department of Microbiology, University of Nigeria, Nsukkka. They are: MDR *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* ATCC 25783, MDR *Staphylococcus enterica, Candida tropicalis, Candida albicans* SC 5314 and *Cryptococcus neoformans*. The cultures were maintained in their appropriate agar slants at 4˚C throughout the study and used as stock cultures.

Preparation of standard inocula

The inocula were prepared and standardized according to Clinical and Laboratory Standards Institute Approved Standard for bacteria [18]. Stock inoculum suspensions were prepared by taking five colonies (>1 mm in diameter) from 24 h cultures (37˚C) into 5 mL sterile saline). Each suspension was shaken for 15 s and density adjusted visually to 0.5 McFarland turbidity standards. The turbidity of each suspension was compared by holding both the standard and the inoculums tubes side by side in front of a white paper with black lines. The colony forming unit per mL (cfu/mL) of each standardized culture was also determined [19].

Antimicrobial activity

Agar well diffusion method: The agar diffusion technique was employed according to method used by Allen et al. [20]. The honey samples were first inoculated separately on standard nutrient media (Oxoid Ltd., UK), to test for sterility. A micropipette was used to introduce 30 µL of the standard inoculum of the previously prepared bacterial and yeast isolates onto Nutrient Agar (NA) and Sabouraud Dextrose Agar (SDA) plates respectively, and spread with a sterile glass spreader. The plates were allowed to dry for 20-30 minutes. With the aid of sterile cork borer, 6 radial wells of 6 mm diameter were punched equidistantly at different sites on the plates (three wells per plate). Fifty microlitre of each of the honey concentrations (100% (undiluted honey), 80%, 60%, 40%, 20% and 10%, (v/v) were placed onto the bored wells. Sterile distilled water and different concentrations of commercial antibiotics (500–31.3 µg/mL of ciprofloxacin and 400–12.5 µg/mL of ketoconazole) served as negative and positive controls respectively against *Pseudomonas aeruginosa* ATCC 25783 and *Candida albicans* SC 5314 respectively. The plates were left on the bench for 30 minutes for pre-diffusion to take place followed by an overnight incubation that lasted between 18-24 h at 37˚C. The assay was carried out in triplicate and the diameter of zones was recorded as mean ± standard deviation.

Determination of Minimum Inhibitory Concentration (MIC): Following the initial antimicrobial screening tests, the minimum inhibitory concentration of each honey was determined by using the broth tube microdilution method, a modified method of Andrews [21]. Serial dilutions of each honey sample were made in eppendorf tubes containing 700 µL of Mueller Hinton Broth (MHB) (Oxoid Ltd., UK) and Sabouraud Dextrose Broth (SDB) for bacteria and yeast respectively, to give a final concentrations of 50%, 25%, 12.5%, 6.3%, 3.1% and 1.6% (achieved by adding 700 µL of honey to 700 µL of MHB or SDB and then serially transferring 700 µL from it to the next tube and so on). 700 µL was removed from the last tube. About 10 µL of the standardized test organisms were dispensed into the tubes. Negative control tubes (for MHB or SDA) prepared as described above with different concentrations of each honey samples, were not inoculated with test organism. Positive control tubes contained only 700 µL broth medium and each of the organisms but no honey.

Also, different concentrations of ciprofloxacin and ketoconazole as above, for *Pseudomonas aeroginosa* ATCC 25783 and *Candida albicans* SC 5314 respectively were used as positive control drugs. The tubes were incubated in the dark at 37˚C for 24 h with constant shaking (at 250 rpm), to prevent adherence and clumping. The MIC was determined by visually inspecting the tubes for turbidity post-incubation (matching the mueller hinton broth and sabouraud dextrose broth respectively with the corresponding negative control tube of the same concentration). The MIC was reported as the lowest concentration of test material which results in 100% inhibition of growth of the test organism (the lowest concentration that has the same turbidity with the corresponding negative control). The MIC was determined in triplicates and the values were expressed in % (vol/vol).

Determination of Minimum Biocidal Concentration (MBC)

The Minimum Biocidal Concentration (MBC) of the honey varieties were determined by further sub-culturing from the tubes which showed no visible growth in the MIC assay onto fresh sterile nutrient agar and sabouraud dextrose agar plates respectively. The culture plates were incubated at 37ºC for 24 h. The MBC was therefore taken as the lowest concentration or highest dilution of honey that did not show any visible growth on the sub-cultured NA and SDA plates [20].

Determination of non-peroxide antimicrobial activities

In order to determine non-peroxidase antimicrobial activities of the honey varieties, honey dilutions (50-1.6% v/v) were prepared in MHB/SDB containing catalase solution (Sigma, C-40) at a final concentration of 0.2% (w/v) (2 mg of catalase in 10 mL of MHB/SDB). The assay was conducted similar to the MIC determination as previously described. Control tube received broth, catalase only and containing corresponding honey concentrations (negative control), and bacteria, broth and catalase (positive control) [20]. After incubation, MBCs were also determined as described previously.

Statistical analyses

Results were reported as the mean ± standard deviation of triplicate experiments. One-way ANOVA-Games-Howell Post Hoc Multiple Comparisons and Kruskal Wallis (KW) and Mann Whitney U-test were used for comparison of means using a significant level of $p<0.05$ (SPSS version 23).

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Results

Antimicrobial activity screening of the honey varieties

It was observed that all organisms tested showed clear zones of inhibition in response to different concentration of the honey varieties. Ten percent (v/v) and above of the honey samples showed inhibition zones against *E. coli* (Figure 1a). Twenty percent (v/v) and above showed inhibition zones against *B. cereus* (Figure 1b), *C. albicans* SC5314 (Figure 1c), *C. tropicalis* (Figure 1d), and *C. neoformans* (Figure 2a). While 40% and above showed inhibition zones against MDR *S. aureus* (Figure 2b) *P. aeruginosa* (ATCC 25783) (Figure 2c) and MDR *S. enterica* (Figure 2d).

All the three *Hypotrigona* sp. honey samples showed antimicrobial activity against the tested organisms at a concentration range of 10–40% (v/v). Except for *C. albicans* SC5314, the three honey samples inhibited all the test organisms at a concentration of 10% (v/v) and above (Figures 1a, 1b, 1d and 2a-2d). *Hypotrigona* sp. honey samples showed inhibition zones against *C. albicans* SC5314 at concentrations range of 20-40% (Figure 1c).

The *Melipona* sp. honey samples showed activity against all the tested organisms at a concentration range of 10-40% (v/v). The honey samples at 10% and above showed inhibition zones against *B. cereus* (Figure 1b) and *C. neoformans* (Figure 2a). While 20% (v/v) of the honey samples showed inhibition zones against *E. coli* (Figure 1a), MDR *S. enterica* (Figure 2d), *C. albicans* SC5314 (Figure 1c), and *C. tropicalis* (Figure 1d). MDR *S. aureus* (Figure 2b) and *P. aeruginosa* ATCC 25783 (Figure 2c) were both inhibited at concentration range between 40 and 100% (v/v).

As shown in Table 1, there were statistically significant differences between the mean inhibition zone diameters (mm) of *Apis Mellifera*,

Test organism	*Apis mellifera* Honey (n=3)	*Hypotrigona* sp. Honey (n=3)	*Melipona* sp. Honey (n=3)	*p*-value
Bacillus cereus	10.01 ± 6.58^{b}	8.37 ± 4.05^{ab}	5.71 ± 3.64^{a}	0.038
MDR *Staphylococcus aureus*	3.37 ± 3.16^{a}	7.14 ± 4.11^{b}	3.89 ± 3.74^{a}	0.007
Escherichia coli	12.13 ± 5.88^{b}	8.19 ± 4.41^{ab}	5.37 ± 4.30^{a}	0.001
Pseudomonas aeruginosa ATCC 25783	5.49 ± 4.64^{a}	9.77 ± 4.58^{b}	4.04 ± 3.60^{a}	0.001
MDR Staphylococcus enterica	3.95 ± 3.94^{a}	6.96 ± 4.03^{b}	4.09 ± 3.22^{a}	0.032
Candida albicans SC 5314	6.31 ± 4.64^{a}	5.09 ± 4.40^{a}	4.86 ± 3.53^{a}	0.548
Candida tropicalis	7.38 ± 5.46^{a}	6.76 ± 3.66^{a}	5.61 ± 3.86^{a}	0.480
Candida neoformans	7.37 ± 4.81^{a}	8.10 ± 4.42^{a}	6.09 ± 4.25^{a}	0.405

Mean zones of inhibition diameter (mm) ± Standard deviation. Means were compared by using one-way ANOVA and Games-Howell Post Hoc Multiple Comparisons. In each row, values with different letters (superscripts) indicate significant differences ($p<0.05$)

Table 1: Comparison of mean zones of inhibition diameter (mm) of *Apis mellifera, Hypotrigona* sp. and *Melipona* sp. honey samples against the test microorganisms.

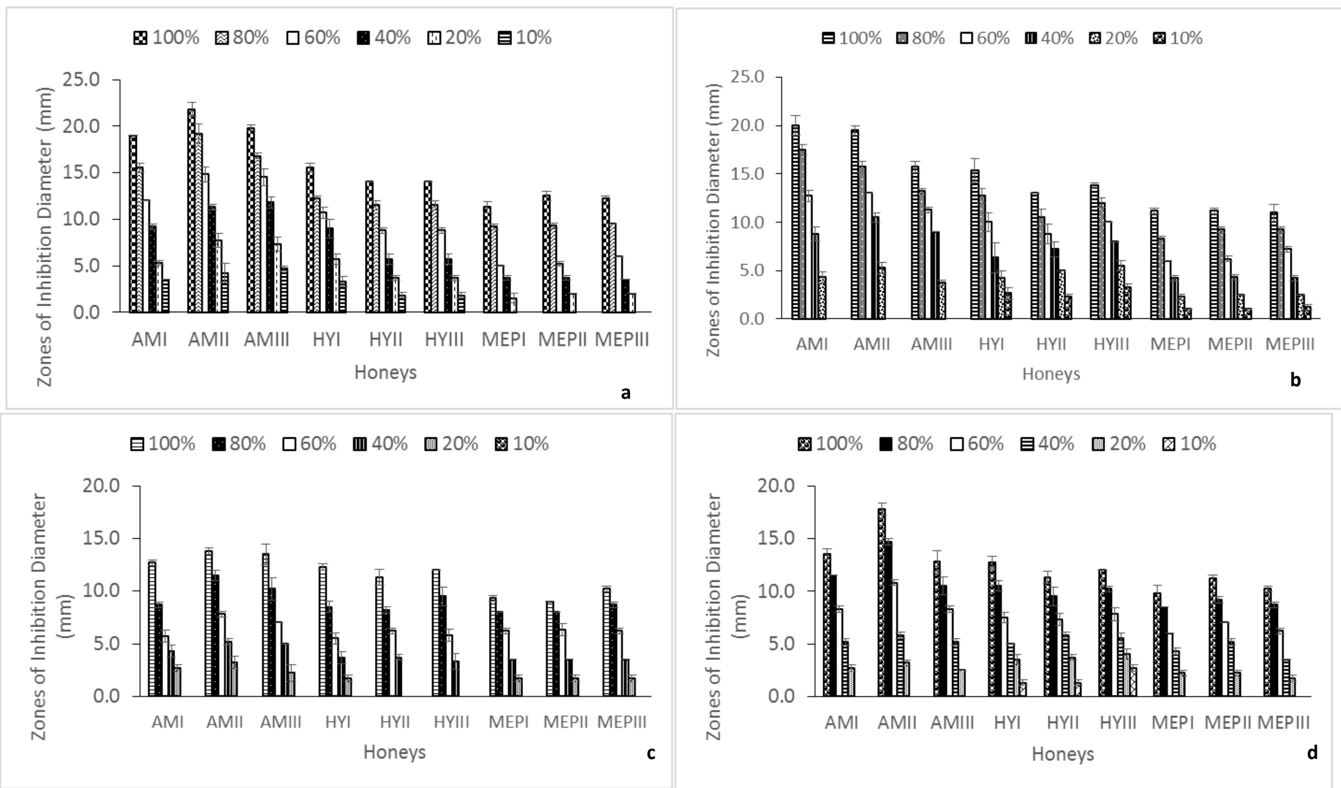

Figure 1: Zones of inhibition diameter (mm) of the honey samples against: a) *Escherichia coli;* b) *Bacillus cereus;* c) *Candida albicans*; and d) *Candida tropicalis* (Mean ± SE) (AM I–III, HY I–III and MEP I-III stand for *Apis mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively).

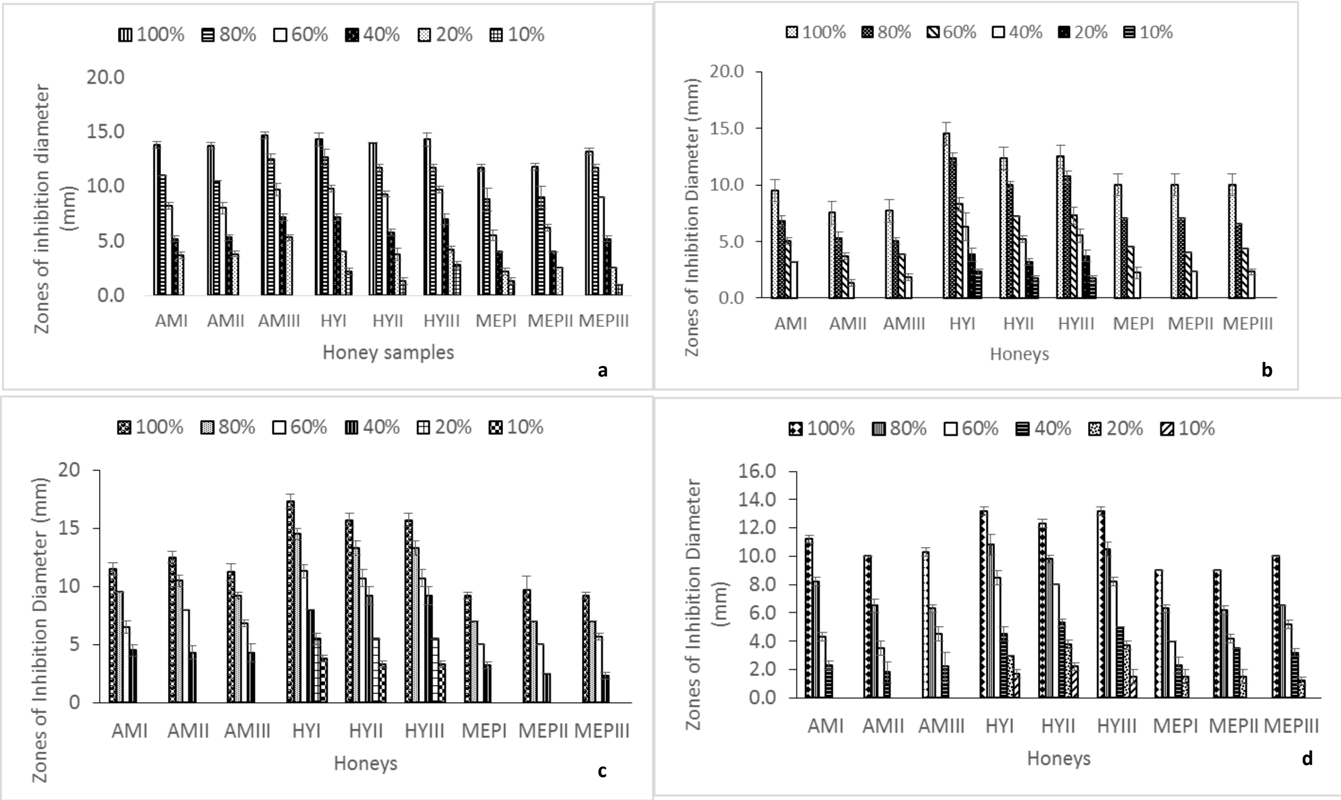

Figure 2: Zones of inhibition diameter (mm) of the honey samples against: a) *Candida neoformans;* b) MDR *Staphylococcus aureus;* c: *Pseudomonas aeruginosa* (ATCC 25783); d: MDR *Staphylococcus enterica* (Mean ± SE) (AM I–III, HY I–III and MEP I-III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively).

Hypotrigona sp. and *Melipona* sp. honeys against *B. cereus* ($F_{(2,51)}$=3.494, *p*=0.038), *S. aureus* ($F_{(2,51)}$=5.523, *p*=0.007), *E. coli* ($F_{(2,51)}$=8.609, *p*=0.001), *P. aeruginosa* ATCC 25783 ($F_{(2,51)}$=8.621, *p*=0.001), and MDR *S. enterica* ($F_{(2,51)}$=3.691, *p*=0.032). There were no significant differences between the mean zones of inhibition of the honeys against *C. albicans* SC5314 ($F_{(2,51)}$=0.609, *p*=0.548), *C. tropicalis* ($F_{(2,51)}$=0.746, *p*=0.480), and *C. neoformans* ($F_{(2,51)}$= 0.920, *p*=0.405).

In addition, positive control drugs i.e., ciprofloxacin (500–15.6 µg/mL) and ketoconazole (400–12.56 µg/mL) produced respectively 20 ± 0.88-10 ± 0.29 and 22 ± 0.87 - 9 ± 0.87 mm mean inhibition zone against reference strains respectively.

Minimum inhibitory concentration of investigated honey samples

The Minimum Inhibitory Concentrations (MICs) of the honey varieties were determined using micro-dilution methods. *Apis Mellifera* honey samples (I–III) inhibited all isolates tested at MIC range between 12.5 and 25.0% (v/v) (Table 2). Honey sample I had MIC of 12.5% (v/v) against *B. cereus*, MDR *S. aureus,* and *C. neoformans,* while *E. coli* and *P. aeruginosa* (ATCC 25783) were both inhibited at MIC of 6.3% (v/v). The MIC of 25.0% (v/v) inhibited MDR *S. enterica, C. albicans* SC5314 and *C. tropicalis.* The honey sample II and III had MICs similar to sample I except that *P. aeruginosa* (ATCC 25783), *C. albicans* (SC5314) and *C. tropicalis* were inhibited at MIC of 12.5% (v/v). *C. neoformans* was inhibited by honey sample II and III at MIC of 6.3 and 3.1% (v/v) respectively.

Hypotrigona sp. honey samples (I–III) inhibited all isolates tested at MIC range from 12.5 to 25.0% (v/v) (Table 2). Honey sample I had MIC of 3.1% (v/v) against *B. cereus, P. aeruginosa* (ATCC 25783), *C. tropicalis* and *C. neoformans,* while the rest of the test isolates were inhibited at MIC of 6.3% (v/v). In honey sample II, all the test isolates were inhibited at MIC of 6.3% (v/v) except for *C. tropicalis* and *C. neoformans* that were inhibited at MIC of 3.1% (v/v). *Hypotrigona* sp. honey sample III had similar MICs with honey sample I.

Melipona sp. honey samples (I-III) also inhibited all the tested isolates at concentration range of 6.3–25.0% (v/v) (Table 2). The three honey samples have MIC of 6.3% against *B. cereus, C. tropicalis,* and *C. neoformans.* Except for *P. aeruginosa* (ATCC 25783) and *E. coli* that were inhibited at MIC of 6.3%, the rest of the test isolates were inhibited at MIC of 12.5% (v/v).

In comparing the MICs as shown in Table 3, Kruskal-Wallis (KW) test revealed that there were statistically significant differences between the mean MICs of the honey varieties against *B. cereus* (*p*=0.029), *S. aureus* (*p*=0.018), MDR *S. enterica* (*p*=0.018), *C. albicans* SC5314 (*p*=0.030) and *C. tropicalis* (*p*=0.032). *Hypotrigona* sp. honey had the least mean MICs against *B. cereus, S. aureus,* MDR *S. enterica*, *C. albicans* SC5314 and *C. tropicalis.* There were no significant differences between the mean MIC of the honeys against *E. coli* (*p*=0.102), *P. aeruginosa* ATCC 25783 (*p*=0.846) and *C. neoformans* (*p*=0.102) (Table 3).

Minimum Biocidal Concentration (MBC) of investigated honey samples

Apis Mellifera honey samples were biocidal to most of the isolates tested at MBC range of 6.3–50.0% (v/v) (Table 2). The honey samples were biocidal to *B. cereus* and *P. aeruginosa* ATCC 25783

Test Organisms	*Apis mellifera* honey samples						*Hypotrigona* sp. honey samples						*Melipona* sp. honey samples					
	I		II		III		I		II		III		I		II		III	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	12.5	25.0	12.5	25.0	12.5	25.0	3.1	3.1	6.3	12.5	3.1	3.1	6.3	12.5	6.3	25.0	6.3	12.5
MDR *Staphylococcus aureus*	12.5	50.0	12.5	50.0	12.5	25.0	6.3	12.5	6.3	25.0	6.3	25.0	12.5	25.0	12.5	25.0	12.5	50.0
Escherichia coli	6.3	6.3	6.3	12.5	6.3	6.3	6.3	6.3	6.3	12.5	6.3	12.5	12.5	12.5	12.5	25.0	6.3	6.3
Pseudomonas aeruginosa ATCC 25783	6.3	25.0	12.5	25.0	6.3	12.5	3.1	12.5	6.3	25.0	12.5	25.0	12.5	50.0	6.3	25.0	6.3	12.5
MDR *Staphylococcus enterica*	25.0	>50.0	25.0	>50.0	25.0	>50.0	6.3	25.0	6.3	50.0	6.3	25.0	12.5	50.0	12.5	25.0	12.5	25.0
Candida albicans SC 5314	25.0	50.0	12.5	50.0	12.5	25.0	6.3	25.0	6.3	25.0	6.3	25.0	12.5	50.0	12.5	25.0	12.5	25.0
Candida tropicalis	25.0	50.0	12.5	25.0	12.5	25.0	3.1	6.3	3.1	12.5	6.3	12.5	6.3	12.5	6.3	25.0	6.3	12.5
Candida neoformans	12.5	25.0	6.3	12.5	3.1	12.5	3.1	3.1	3.1	6.3	3.1	3.1	6.3	12.5	6.3	12.5	6.3	12.5
Median of triplicate experiments. MIC in % (v/v)																		

Table 2: Minimum Inhibitory Concentration (MIC) of *Apis mellifera, Hypotrigona* sp. and *Melipona* sp. honey samples without addition of catalase.

Honey samples	Catalase	MIC/ MBC	Test organisms: *Bacillus cereus*	MDR *Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa* ATCC 25783	MDR *Staphylococcus enterica*	*Candida albicans* SC 5314	*Candida tropicalis*	*Candida neoformans*
Apis mellifera honey	Without	MIC	12.5 ± 0.0^{b}	12.5 ± 0.0^{b}	6.3 ± 0.0^{a}	8.4 ± 3.6^{a}	25.0 ± 0.0^{c}	16.7 ± 7.2^{b}	16.7 ± 7.2^{b}	7.3 ± 4.8^{a}
		MBC	25.0 ± 0.0^{b}	41.7 ± 14.4^{a}	8.4 ± 3.6^{a}	20.8 ± 7.2^{a}	>50.0	41.7 ± 14.4^{a}	33.3 ± 14.4^{b}	16.7 ± 7.2^{b}
	With	MIC	25.0 ± 0.0^{a}	25 ± 0.0^{a}	16.7 ± 7.2^{a}	16.7 ± 7.2	>50.0	33.3 ± 14.4^{a}	33.3 ± 14.4^{a}	20.8 ± 7.2^{a}
		MBC	>50.0	>50.0	41.7 ± 14.4^{a}	33.3 ± 14.4^{a}	>50.0	>50.0	>50.0	>50.0
Hypotrigona sp. honey	Without	MIC	4.2 ± 1.8^{a}	6.3 ± 0.0^{a}	6.3 ± 0.0^{a}	7.3 ± 4.8^{a}	6.3 ± 0.0^{a}	6.3 ± 0.0^{a}	4.2 ± 1.8^{a}	3.1 ± 0.0^{a}
		MBC	6.2 ± 5.4^{a}	20.8 ± 7.2^{a}	10.4 ± 3.6^{a}	20.8 ± 7.2^{a}	33.3 ± 14.4^{a}	25.0 ± 0.0^{a}	10.4 ± 3.6^{a}	4.2 ± 1.8^{a}
	With	MIC	16.7 ± 7.2^{a}	25.0 ± 0.0^{a}	16.7 ± 7.2^{a}	14.6 ± 9.5	20.8 ± 7.2^{a}	25.0 ± 0.0^{a}	25.0 ± 0.0^{a}	10.4 ± 3.6^{a}
		MBC	37.5 ± 17.0^{a}	>50.0	29.2 ± 19.1^{a}	37.5 ± 17.7^{a}	>50.0	>50.0	>50.0	20.8 ± 7.2^{a}
Melipona sp. honey	Without	MIC	6.3 ± 0.0^{a}	12.5 ± 0.0^{b}	10.4 ± 3.6^{a}	8.4 ± 3.6^{a}	12.5 ± 0.0^{b}	12.5 ± 0.0^{b}	6.3 ± 0.0^{a}	6.3 ± 0.0^{a}
		MBC	16.7 ± 7.2^{ab}	33.3 ± 14.4^{a}	14.6 ± 9.5^{a}	29.2 ± 19.1^{a}	33.3 ± 14.4^{a}	33.3 ± 14.4^{a}	16.7 ± 7.2^{ab}	12.5 ± 0.0^{b}
	With	MIC	16.7 ± 7.2^{a}	>50.0	20.8 ± 7.2^{a}	20.8 ± 7.2	33.3 ± 14.4^{a}	25.0 ± 0.0^{a}	16.7 ± 7.2^{a}	12.5 ± 0.0^{a}
		MBC	41.7 ± 14.4^{a}	>50.0	37.5 ± 17.7^{a}	>50.0	>50.0	>50.0	41.7 ± 14.4	33.3 ± 14.4^{a}
P-values	Without	MIC	0.029	0.018	0.102	0.846	0.018	0.030	0.032	0.102
		MBC	0.047	0.179	0.564	0.846	0.046	0.264	0.049	0.034
	With	MIC	0.202	0.651	0.670	0.564	0.437	0.368	0.110	0.110
		MBC	0.080	1.000	0.540	0.641	0.368	1.000	0.021	0.437

Mean ± SD (a>b>c in potency); MIC: Minimum Inhibitory Concentration; MB: Minimum Biocidal Concentration; Means were compared using Kruskal Wallis (KW) test and Mann Whitney U-test. In each column, values with different letters (superscripts) indicate significant differences (p<0.05) for MIC and MBC in with and without catalase respectively.

Table 3: Comparison of the mean MIC and MBC (% v/v) of honey varieties from *Apis mellifera, Hypotrigona* sp. and *Melipona* sp.

at concentration of 25% (v/v). The honey sample I and III had MBC values (6.3%) similar to MIC values against *E. coli.* There was no MBC against MDR *S. enterica.*

Hypotrigona sp. honey samples were biocidal to all isolates tested at MBC range of 3.1–25%, (v/v) (Table 2). The honey sample I had MBC of 6.3% similar to MIC against *E. coli.* While a 3.1% of honey samples I and III was biocidal to *B. cereus* and *C. neoformans,* similar to MIC values.

A concentration range of 6.3–50.0% (v/v) of *Melipona* sp. honey samples were biocidal to all the isolates tested. A lower concentration of the honey samples was biocidal to *B. cereus, E. coli, C. tropicalis* and *C. neoformans.* A MBC of 6.3% similar to MIC was observed in honey sample III against *E. coli.*

There were statistically significant differences between the mean MBCs of the honey varieties against *B. cereus* (p=0.047), MDR *S. enterica* (p=0.046), *C. tropicalis* (p=0.049) and *C. neoformans* (p=0.034) (Table 3). There were no significant differences between the mean MBCs of the honeys against MDR *S. aureus* (p=0.179), *E. coli* (p=0.564), *P. aeruginosa* ATCC 25783 (p=0.846), and *C. albicans* (SC5314) (p=0.264).

The MICs for the control drugs were 15.63 and 12.5 (μg/mL) against the *P. aeruginosa* (ATCC 25783) and *C. albicans* (SC5314) respectively. While the MBCs for the control drugs were 125 and 200 (μg/mL) against the *P. aeruginosa* (ATCC 25783) and *C. albicans* (SC5314) respectively.

Non-peroxidase activities of the honey varieties

The antimicrobial activity of the honey samples generally decrease after treatment with catalase. The MICs and MBCs of catalase treated *Apis mellifera* honey samples were within the range of 12.5–50.0% (v/v) and 25–50% (v/v) respectively (Table 4). The three honey samples were biocidal to *E. coli* and *P. aeruginosa* (ATCC 25783). The honey samples at the concentration used were biostatic to *B. cereus,* MDR *S. aureus,* MDR *S. enterica, C. albicans* and *C. tropicalis.*

The *Hypotrigona* sp. had non-peroxidase MIC and MBC range of 6.3–25% and 12.5–50% (v/v) respectively (Table 4). The catalase treated

Test Organisms	*Apis mellifera* honey samples						*Hypotrigona* sp. honey samples						*Melipona* sp. honey samples					
	I		II		III		I		II		III		I		II		III	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus	25.0	>50.0	25.0	>50.0	25.0	>50.0	12.5	25.0	25.0	>50.0	12.5	50.0	12.5	50.0	25.0	50.0	12.5	25.0
MDR *Staphylococcus aureus*	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	>50.0	>50.0	>50.0	>50.0	50.0	>50.0
Escherichia coli	25.0	50.0	12.5	50.0	12.5	25.0	12.5	12.5	12.5	25.0	25.0	50.0	25.0	50.0	25.0	>50.0	12.5	25.0
Pseudomonas aeruginosa ATCC 25783	12.5	25.0	25	50.0	12.5	25.0	6.3	25.0	12.5	50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	12.5	50.0
MDR *Staphylococcus enterica*	50.0	>50.0	>50.0	>50.0	>50.0	>50.0	25.0	>50.0	25.0	>50.0	12.5	50.0	50.0	>50.0	25.0	>50.0	25.0	>50.0
Candida albicans SC 5314	50.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0
Candida tropicalis	50.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	25.0	>50.0	12.5	50.0	25.0	50.0	12.5	25.0
Candida neoformans	25.0	50.0	25.0	>50.0	12.5	>50.0	12.5	12.5	6.3	25.0	12.5	25.0	12.5	25.0	12.5	50.0	12.5	25.0
Median of triplicate experiments. MIC in % (v/v)																		

Table 4: Minimum inhibitory concentration (MIC) of *Apis mellifera, Hypotrigona* sp. and *Melipona* sp. honey samples with addition of catalase.

honey samples were biocidal to *E. coli, P. aeruginosa* (ATCC 25783) and *C. neoformans*. The honey samples at the concentration used were biostatic to MDR *S. aureus,* MDR *S. enterica, C. albicans* and *C. tropicalis.*

Honey samples from *Melipona* sp. had non-peroxidase MIC and MBC range of 12.5–50% (v/v) and 25-50% against the test isolates respectively (Table 4). The catalase treated honey samples were biocidal to *B. cereus, C. tropicalis* and *C. neoformans.* The honey samples at the concentration used were biostatic to MDR *S. aureus,* MDR *S. enterica,* and *C. albicans.*

Discussion

All organisms tested showed clear zones of inhibition in response to different concentration of the honey varieties. *Hypotrigona* sp. honey samples showed comparatively higher activity than other honey varieties against MDR *S. aureus, P. aeruginosa* ATCC 25783, and MDR *S. enterica*. *A. mellifera* honey showed higher zones of inhibition diameter than *Hypotrigona* sp. and *Melipona* sp. honey samples against *B. cereus,* and *E. coli*. While the three honey varieties had comparatively similar activities against *Candida tropicalis* and *Candida albicans* SC 5314. There reports on inhibition diameters of Nigerian honey samples against *B. cereus* (9–15 mm), *E. coli* (13–20 mm), *P. aeruginosa* (ATCC 25783) (8–16 mm), *S. aureus* (11-55) and *Salmonella* sp. (8–18 mm) [22-25]. There are similar reports on the antifungal activity of *A. mellifera* honey from Nigeria against *C. albicans* (4–16 mm) [26]. This is the first report on antimicrobial activity of Nigerian stingless bee honeys. Through well diffusion assay, the antimicrobial activities of stingless bee honeys especially from *Melipona* sp. and *Trigona* sp. (3–22 mm) have been reported in Ethiopia [27], Australia [28], Germany [29], Thailand [30] and Brazil [31].

Almost all the honey varieties used in this study especially *Hypotrigona* sp. honey, inhibited most of the test isolates at a lower MIC. The honey varieties had similar inhibitory effects against *E. coli, P. aeruginosa* (ATCC 25783) and *C. neoformans.* Recently, similar findings were reported by Ewnetu et al. [27], Boorn et al. [28] and Fahim et al. [32], who showed that MIC of *A. mellifera* honey against some isolates did not exceed 40%. There are reports on MIC values for *Melipona* sp honeys (MIC range of 11.1–50%) [31] and *Trigona* sp. honeys (MIC range of 4->16%) [28] against bacterial and fungal isolates.

All tested honey samples were biocidal to all test isolates, except against MDR *S. enterica.* The MBC of the investigated honey samples corroborated with the findings of Oyeleke et al. [33], who also reported MBC range between 6.25% and >50%. The present findings are supported by Othman [34] who showed that MBC values of Yemeni honey samples were in the range of 20-40% and that *E. coli* was the most susceptible to antimicrobial activity of honey. Zainol et al. [35] also reported the MBC of selected Malaysian honey to range between 6.25 and 50% similar to our findings. Anwanwu [26] reported that the minimum fungicidal concentration of Nigerian honeys ranged between 12.5 and 50% (v/v) against *Candida albicans*. Similarly, Ewnetu et al. reported stingless bee honeys to be more effective than *A. mellifera* honey against all isolates they tested (MBC of 12.5%) [27]. On the contrary, there are reports on MBCs of *Melipona* sp. honeys (≥ 50%) [31] and *Trigon asp* honeys (1 ≥ 32%) [28] against some bacterial and fungal isolates.

When the honey samples were treated with catalase to eliminate the effects of hydrogen peroxide, the results showed that MIC and MBC values generally increased. In the absence of hydrogen peroxide, some of honey sample varieties were effective against *B. cereus, E. coli, P. aeruginosa* (ATCC 25783) and *C. neoformans.* This is the first report on non-peroxidase antimicrobial activity of Nigerian honey. These results were similar to findings of Fahim et al., who investigated the non-peroxidase activity of honeys indigenous to Pakistan against similar organisms (MBC range between 15% and >50%) [32]. Brudzynski reported similar results against some isolates, in which he showed that residual hydrogen peroxide was responsible for the antimicrobial activity of honey [15]. Even in the absence of hydrogen peroxide, other physicochemical properties of the honey maybe responsible for the antimicrobial activity of honey.

Conclusion

This research has shown that the honey varieties varied significantly in their antimicrobial potentials. *Hypotrigona* sp. and *Melipona* sp. honey varieties have shown to possess antimicrobial properties similar to widely used *A. mellifera* honey. This study scientifically authenticates the potentials use of these stingless bee honeys as an alternative therapeutic agent.

Hypotrigona sp. (Okotobo) and *Melipona* sp. (Ifufu) honeys that are not consumed as widely as regular bee honey have shown to have antimicrobial properties similar to those of regular bee honey.

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