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Physicochemical properties, antioxidant and antimicrobial activities of Nigerian Polyfloral honeys

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Abstract
Natural honey is widely applied as food sweetener, food preservative and in folk medicine for treatment of varying degree of human affliction and diseases. This study evaluates the antimicrobial activities, physicochemical, phytochemical and antioxidant properties of four Nigerian honey samples collected from Ekiti, Kogi, Osun and Ondo (coded as A, B, C and D respectively). Physicochemical determination of honey samples include: pH, free acidity, electrical conductivity and density. Determination of phytochemical was carried out quantitatively using phenolics and flavonoid; while the total antioxidant capacities was measured in an in-vitro system using inhibition of lipid peroxidation, thiobarbituric acid reactions and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Agar diffusion method was used to measure the antimicrobial activities of the honey samples against some selected microorganisms (*Staphylococcus aureus*, *Bacillus subtilis*, *Psuedomonas aeruginosa* and *Escherichia coli*). The results of the physicochemical characterization showed that the honey samples were polyflorals characterized by low pH (3.76 ± 0.02, 4.17 ± 0.15, 3.94 ± 0.10 and 3.77 ± 0.01 for A, B, C and D respectively) capable of inhibiting the growth of most microorganisms and density ranging between 1.38 and 1.45 g/ml. All the honey samples showed antimicrobial activity against the selected microorganisms at concentration greater than 60 % v/v. The antioxidant investigation also demonstrated ability to inhibit lipid peroxidation and thiobarbituric acid reactions and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity possibly due to the reasonable amount of flavonoids and phenolics therein.

Keywords: Antioxidant, honey, antimicrobial activity, ph, electrical conductivity, density, free acidity

1. Introduction
Honey, a natural sweet viscous substance is known to be a by-product of honey bees (*Apis melliferaI*) produced from the nectar of plants, secretions of living part of plants or excretions of plant sucking insects on the living part of plants. Honey samples can be classified into several groups and subgroups according to their origin (blossom origin) as follows: blossom honey (monofloral, multifloral or polyflora) with predominance of some plant(s), honeydew (forest honey) and compound honey (blend of honeydew and blossom honey) [1]. Electrical conductivity is the most widely applied technique for distinguishing between these various types of honey [2, 3]. However, honey can be characterized by its high density, viscosity, and low pH (acidic) [4, 5].
Honey is widely applied in folk medicines for the treatment of several diseases and ailments. The bioactive components of honey make it finds wide application in both ethnomedicine and ethnopharmacology. Honey is a remarkably complex natural liquid that is reported to contain at least 181 substances [6] including sugar (fructose, glucose and galactose) as its major constituent, and a wide range of minor constituents such as phenolic acids and flavonoids [7, 8], carotenoid-like substances certain enzymes (glucose oxidase, catalase) [9], Maillard reaction products [6], organic acids, amino acids and proteins [10]. Many researchers have shown that honey possesses antioxidant properties that could act as protector of the body from oxidative stress by stabilizing free radicals [7, 8, 11-13]. The phytochemical components of natural honey, in particular tannins, flavonoids, vitamins C and E, possess the ability to maintain β-cells performance and decrease glucose levels in the blood [14]; thus, honey can be used to inhibit enzymes, treat diabetes and other oxidative related stress diseases such as cancer [15].
Moreso, natural honey has been found to possess antimicrobial properties which could be explained by different mechanisms.

For instance, Morenike *et al*. [16] recently showed that honey from south-western Nigeria, could potentially inhibit some microorganisms studied the anti-microbial activity of honey samples from Nsukka and Ugwuaji in Enugu State (Eastern Nigeria) on three selected pathogenic bacteria (*Staphylococcus aureus, Escherichia coli and Streptococcus pyogenes*) and found that the organisms were susceptible to the honey samples [17]. Also, the result of their study showed that antimicrobial activities of the honey samples increases with increase in concentration. Hence, the antibacterial effects of honey are due to several factors that include not only due to osmilarity, visicosity, presence of hydrogen peroxide, low protein contents and organic acids [18, 19]. By studying the antibacterial potential of honey from different origins Alnimat *et al.*, [20] stated that the main antibacterial activity in honey is due to the presence of hydrogen peroxide, which is produced by glucose-oxidase action. Thus these properties certify the application of honey in folk medicine. This study is therefore aimed at validating the Nigerian honeys that have been used extensively in traditional medicine by evaluating their antioxidant properties, physicochemical parameters and antimicrobial activities in comparison to their respective geographical locations.

2. Materials and Methods

2.1 Sampling

Four honey samples (250 g each) were freshly acquired from local honey bee farms in different parts of Nigeria coded as samples A, B, C and D. The samples were sealed in glass bottles and stored at room temperature prior to analysis. The samples' analyses were carried out between the first three months of acquisition.

2.2 Determination of Physicochemical parameters

2.2.1 Free acidity and pH determination of the honey samples

The free acidity and pH of the honey samples were determined by titration according to the methods of [5]. The free acidity of honey from titration expressed in meq/kg of honey samples was calculated according to Equation (1)

$$\text{Free acidity (meq/kg)} = \frac{volume\ of\ 0.1M\ NaOH\ used \times 10}{mass\ of\ honey\ sampple\ (Kg)} \quad \text{(I)}$$

Where 10 indicates the dilution factor of honey sample during the analysis, while pH of the samples were determined by a calibrated pH meter in a solution containing 10 g of each honey sample in 100 ml distilled water. The experiments were conducted in triplicate.

2.2.2 Electrical conductivity

The electrical conductivity of the honey samples was carried out using solution containing 5 g of honey samples in 50 ml distilled water by a conductimeter at the temperature of 20 ^{o}C. The experiment was done in triplicate and the results expressed in milli Siemens per centimeter (mS/cm).

2.2.3 Density (g/ml)

The density of each honey sample was determined by estimating the weight of 50 ml of each sample in 100 ml volumetric cylinder with the aid of an analytical balance. The experiment was conducted in triplicate density of each honey sample was calculated by Equation II.

$$\text{Density of honey} = \frac{mass\ of\ honey\ (g)}{volume\ of\ honey (ml)} \quad \text{(II)}$$

2.3 Antimicrobial Activities

Test organisms

Test organisms used for antibacterial susceptibility test were reference isolates of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 29213) and *Bacillus subtilis* (NCTC 8253) obtained from the culture bank of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.

2.3.1 *In vitro* antimicrobial activities

The *in vitro* antimicrobial activities of the honey samples were carried out by well diffusion method using Nutrient agar as described by Cooper *et al.* [19] on the selected microorganisms (*S. aureus, E. coli, B. Subtilis and P. aeruginosa).* Different concentrations (10, 20, 40, 60 and 80 % v/v) of each honey sample were prepared by diluting in sterilized water; a net concentration (100 % v/v) of each honey sample was also used. Nutrient agar plates were prepared for culturing of the organisms and each plate was properly inoculated with each test organism using streaking method with the help of a sterile wire loop [17]. Wells with diameter of 20 mm were bored using a sterile cork borer and each well was filled with 1ml different concentrations of the honey. A distance was maintained from the edges of the plates to prevent overlapping of the inhibition zones and the plates were incubated for 24 hrs at 37 °C. The inhibition zones were measured in triplicate for each isolate after incubation.

2.4 Quantitative Phytochemical Analysis

2.4.1 Estimation of Total Phenol

The estimation of the phenol concentration in the honey sample was carried out according to the methods described by [21] with little modifications. This involved pipetting 0.5 ml each of 5 mg/ml aqueous honey into clean dry test tubes in triplicate. The volumes were adjusted to 1.0 ml with distilled water. To each of the test tubes was added 1.5 ml of Follin Ciocalteu's phenol reagent. The reaction mixture was incubated at room temperature for 15 minutes. To the mixture was added 1.5 ml of 10 % w/v $NaHCO_3$ solution to give a total of 4.0 ml. The reaction mixture was further incubated for additional one and half hours. The mean absorbance was read at 725 nm against the reagent blank on a SpectrumLab70 spectrophotometer and the result was expressed in terms of gallic acid in mg/ml of extract.

2.4.2 Determination of Flavonoid

Each of the honey sample, (0.1 g) was dissolved in 20 ml of 7 0% v/v ethanol. 0.5 ml of the sample was diluted with 4.5 ml of distilled water in clean and dry test tubes. To each test tube was added 0.3 ml of 5 % sodium nitrite ($NaNO_2$) solution, 0.30 ml of freshly prepared 10 % v/v aluminum chloride ($AlCl_3$) and 4ml of 4 % w/v NaOH solutions were also added. The mixture was allowed to stand for 15 min at room temperature and absorbance was read at 500 nm on a SpectrumLab70 spectrophotometer against reagent blank. The result was expressed as quercetin equivalents (QE).

2.5 Determination of Free Radical Scavenging Activities

2.5.1 Inhibition of Lipid Peroxidation and Thiobarbituric Acid Reactions

A modified thiobarbituric acid reactive species (TBARS) assay as described by [22] with little modification was used to measure the lipid peroxide formed using egg yolk

homogenate lipid rich media. Egg homogenate (1.0 ml of 10 % v/v) and 0.2 g each of the honey sample were added to a test tube (one test tube for each of the honey sample) and made up to 1ml with distilled water; 0.1ml of iron sulphate (0.07 M) was added to induce lipid peroxidation and the mixture was allowed to stand for 30 minutes. Then, 2 ml of 20 % acetic acid and 2 ml of 0.8 % w/v thiobarbituric acid in 1.1 % sodium dodecyl sulphate were added and the resulting mixture was vortexed and then heated at 95 ^{o}C for 60 minutes. After cooling, 5.0 ml of butanol was added to each tube and centrifuged for 10 minutes. The absorbance of the organic layer was measured at 532 nm. Inhibition of lipid peroxidation and thiobarbituric acid reaction by the honey was then calculated using the expression in Equation III

$$\% \text{ inhibition of lipid peroxidation } \textit{and thiobarbituric acid Reactions} = \frac{1 - E}{C} \times 100 \ldots\ldots\ldots \text{III}$$

Where C is the absorbance value of fully oxidized control and E is the absorbance in the presence of the honey sample.

2.5.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of the honey samples on the basis of their scavenging activity of the stable (DPPH) free radical, were determined by the method described by [23]. 0.1ml of each of the honey sample was added to 3 ml of a 0.004 % MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 minutes. Trolox was used as a standard to compare the free radical scavenging activity of the honey and the percentage DPPH inhibition activity was calculated using the expression in Equation IV;

$$\% \text{ DPPH scavenged} = \frac{A\ con - A\ test}{A\ con} \times 100 \ldots\ldots\ldots\ldots \text{IV}$$

Where *A con* is the absorbance of control and *A test* is the absorbance of tested samples.

3. Results

Table 1: Free acidity and pH of the honey samples (mean ± standard deviation (SD))

Sample	Free acidity (meq/kg) ± SD	pH ± SD
A	40 ± 0.20	3.76 ± 0.02
B	27 ± 0.20	4.17 ± 0.15
C	33 ± 0.10	3.94 ± 0.10
D	36 ± 0.15	3.77 ± 0.01

A = Honey samples obtained from Ekiti
B = Honey sample obtained from Kogi Central
C = Honey sample obtained from Osun Central
D = Honey sample obtained from Ondo Central

Table 2: Electrical conductivity of the honey samples (mean ± standard deviation (SD))

Samples	A	B	C	D
Mean electrical conductivity (mS/cm) ± S.D	0.47 ± 0.02	0.51 ± 0.01	0.38 ± 0.05	0.27 ± 0.01

Table 3: Density of the honey samples (mean ± standard deviation (SD))

Samples	A	B	C	D
Mean density (g/ml) ± S.D	1.38 ± 0.30	1.33 ± 0.02	1.42 ± 0.25	1.45 ± 0.10

Table 4: Antimicrobial activity of the honey samples

Test organism	Agar plate	Concentration of honey (%v/v)	Diameter zone of inhibition (mm) mean ± SD value			
			A	B	C	D
P. aeruginosa	1	20	0.0	0.0	0.0	0.0
	2	40	8.0 ± 0.05	0.0	0.0	0.0
	3	60	8.5 ± 0.20	0.0	8.5 ± 0.23	9.0 ± 0.03
	4	80	9.2 ± 0.15	12.0 ± 0.30	12.5 ± 0.18	12.5 ± 0.02
	5	100	10.0 ± 0.12	15.0± 0.10	15.0 ± 0.15	14.5 ± 0.05
S. aureus	1	20	0.0	0.0	2.0 ± 0.01	3.0 ± 0. 33
	2	40	7.3 ± 0.18	0.0	3.5 ± 0.28	5.5 ± 0.22
	3	60	8.5 ± 0.13	9.0 ± 0.32	6.0 ± 0.55	6.8 ± 0.20
	4	80	9.5 ± 0.15	10.0 ± 0.38	9.5 ± 0.50	10.5 ± 0.20
	5	100	12.2 ± 0.20	13.0 ± 0.05	12.0 ± 0.10	12.2 ± 0.08
B. subtilis	1	20	0.0	0.0	5.0 ±	6.5 ± 0.55
	2	40	0.0	0.0	7.5 ± 0.01	8.5 ± 0.30
	3	60	6.5 ± 0.32	9.0 ± 0.10	11.0 ± 0.03	12.2 ± 0.20
	4	80	7.0 ± 0.10	12.0 ± 0.56	14.5 ± 0.15	14.5 ± 0.30
	5	100	7.5 ± 0.12	16.0 ±	18.0 ± 0.12	19.0 ± 0.05
E. coli	1	20	0.0	0.0	0.0	0.0
	2	40	0.0	0.0	0.0	0.0
	3	60	6.0 ± 0.02	11.4 ± 0.14	6.0 ± 0.14	6.0 ± 0.01
	4	80	7.3 ± 0.10	11.2 ± 0.19	9.5 ± 0.18	11.0 ± 0.01
	5	100	8.5 ± 0.01	13.0 ± 0.22	12.0 ± 0.15	13.0 ± 0.50

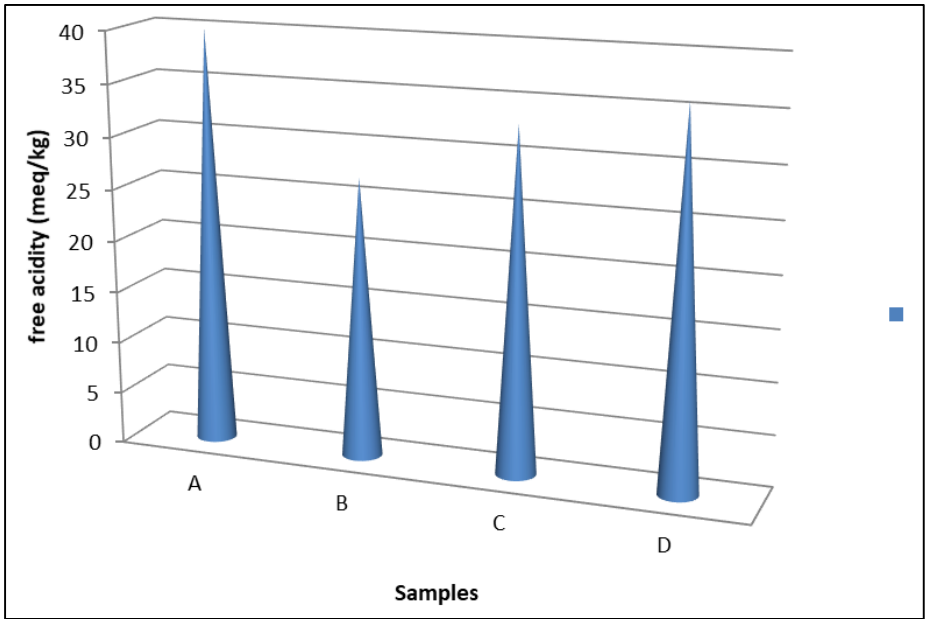

Fig 1: Free acidity of the honey samples

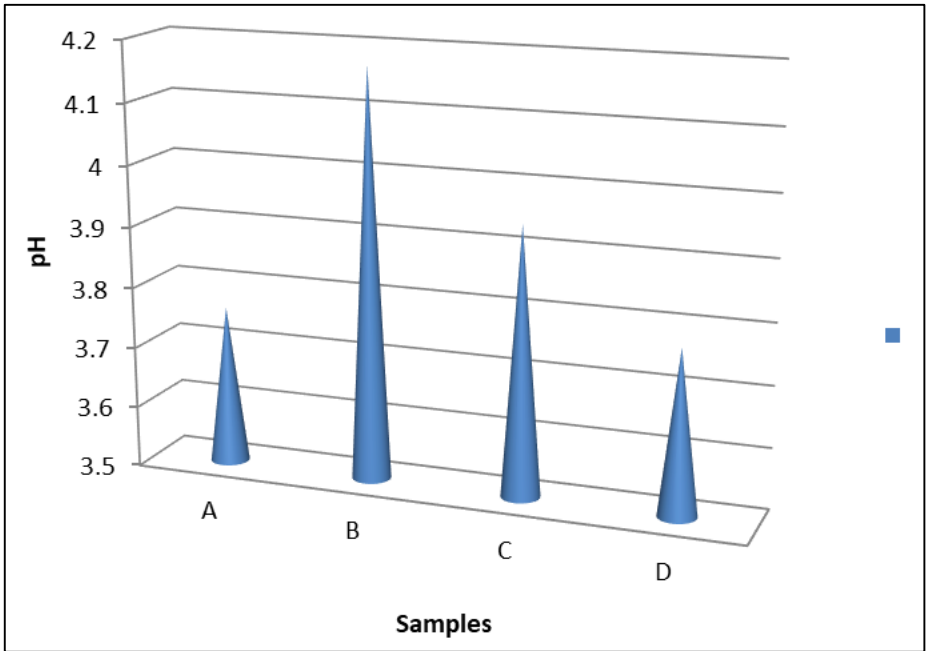

Fig 2: pH of the honey samples

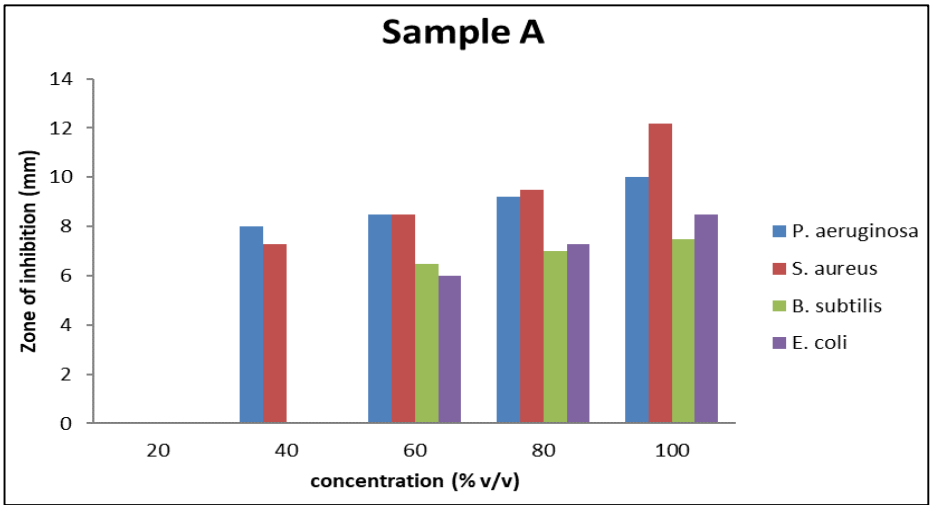

Fig 3: Plot of diameter zone of inhibition (mm) against concentration (% v/v) for Sample A

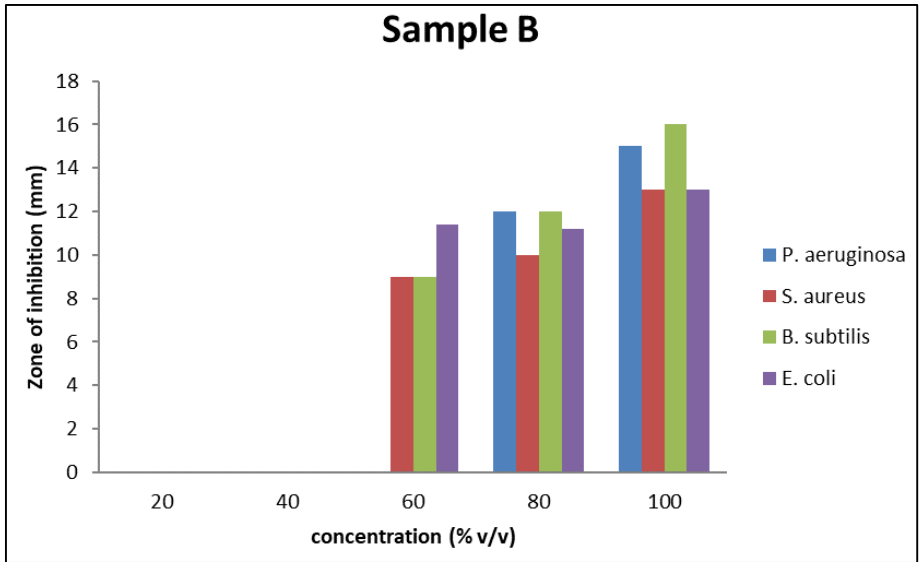

Fig 4: Plot of diameter zone of inhibition (mm) against concentration (% v/v) for Sample

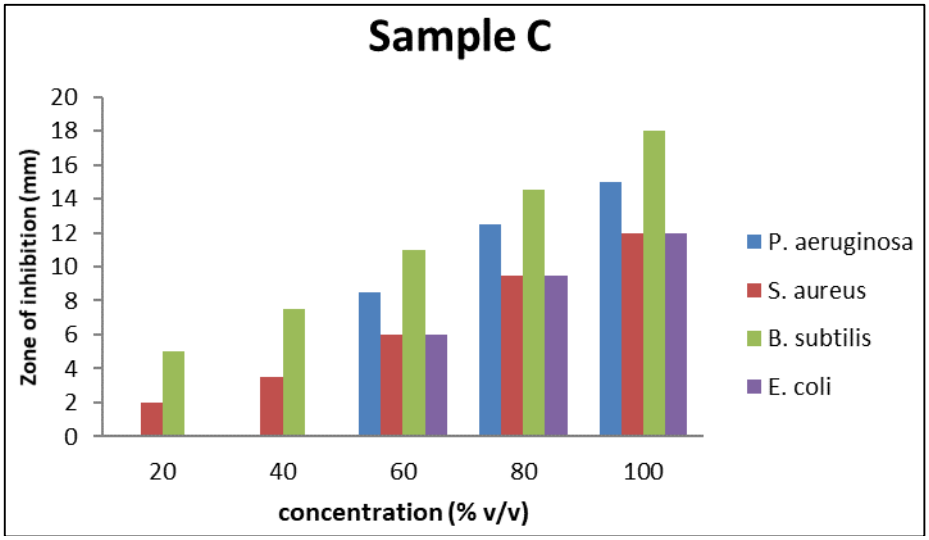

Fig 5: Plot of diameter zone of inhibition (mm) against concentration (%v/v) for Sample C

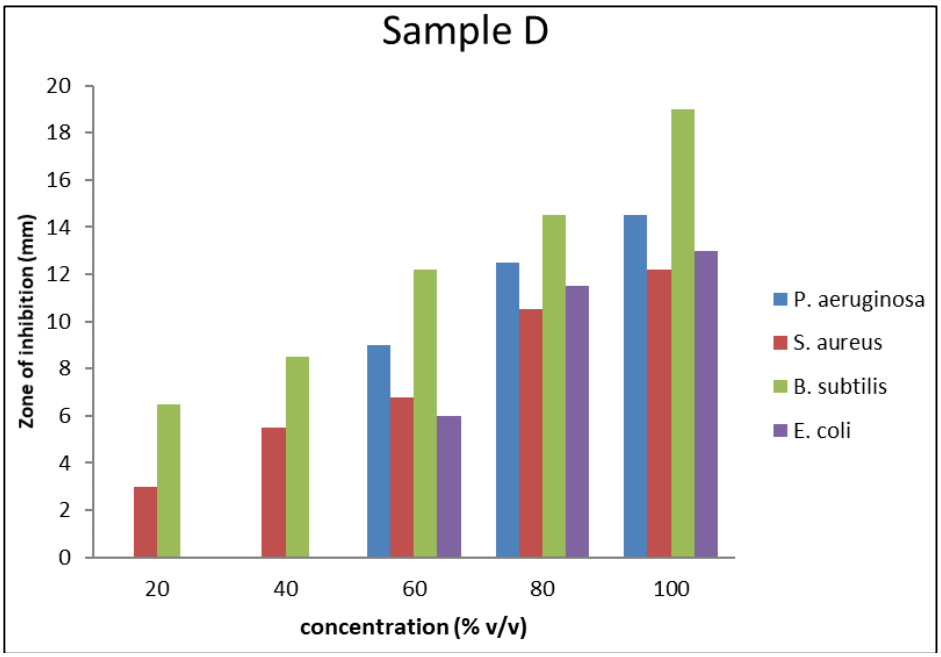

Fig 6: Plot of diameter zone of inhibition (mm) against concentration (%v/v) for Sample D

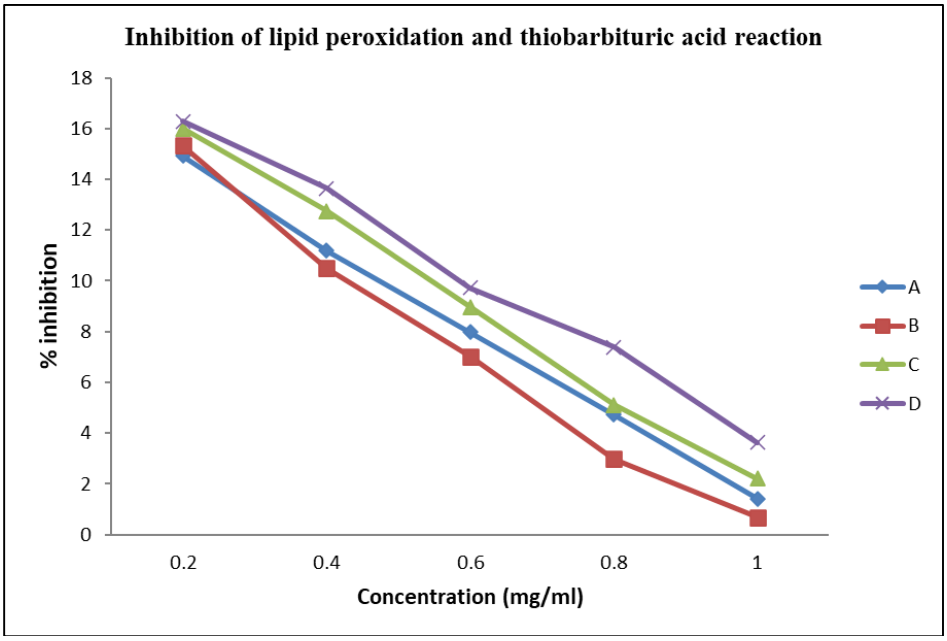

Fig 7: Plot of % inhibition of lipid peroxidation and thiobarbituric acid reaction against concentration of the honey samples

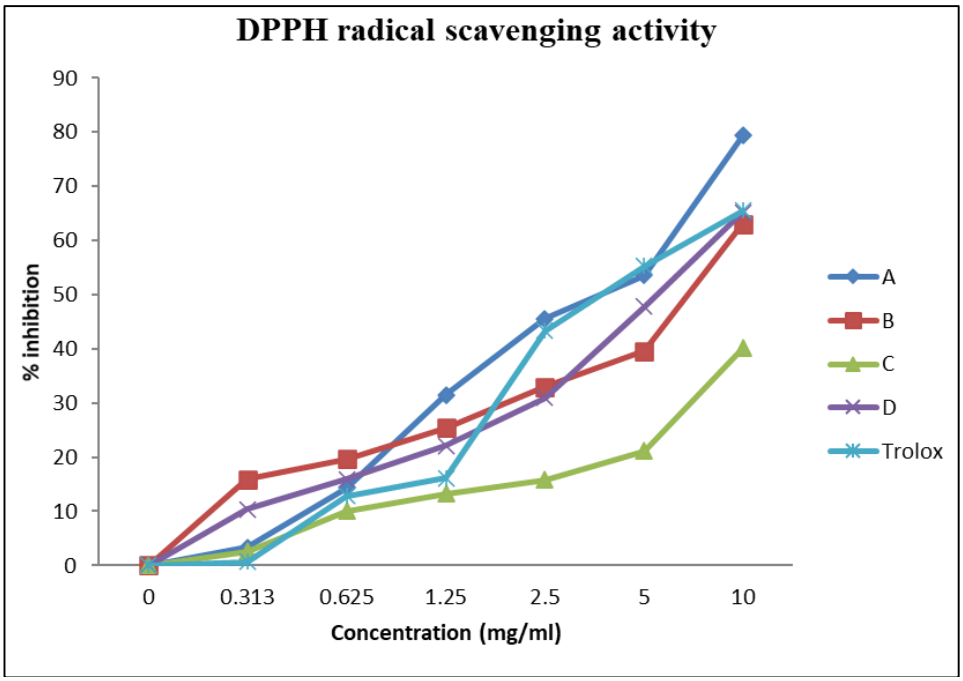

Fig 8: Plot of % inhibition of DPPH radical scavenging activities of the honey samples against their concentration

3. Discussion

3.1 Physicochemical Properties of the Honey Samples

3.1.1 Free acidity and pH

The free acidity of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate [24]. High acidity of honey can be indicative of fermentation of sugars (fructose, glucose and lactose) present in the honey. The aromatic acids of honey are important contributors to its flavour. The presence of the free acidity of the honey samples were estimated to be: 40 ± 0.2, 27 ± 0.2, 33 ± 0.1 and 36 ± 0.15 meq/kg for the samples A, B, C and D respectively (Table 1). Free acidity values for all the samples are within the limits (≤ 50 meq/kg) set for edible honey by [25]. Samples A and B values were above the limits (≤50 meq/kg) set for edible honey by [25]. The mean pH values of the honey samples A, B, C and D are 3.76 ± 0.02, 4.17 ± 0.15, 3.94 ± 0.10 and 3.77 ± 0.01 respectively. The values for the free acidity and pH of the honey samples are represented in Table 1, Figures 1 and 2. The results show that the honey sample A is the most acidic while the least acidic is honey sample B. The pH values are in agreement with the established fact that honey is characteristically acidic with pH being between 3.4 and 6.1 [26]. The degree of acidity may be attributed to the type and quantity of free acids present in the honey samples. The results obtained from this research work showed that the quantities of free acidity correlate to the degree of acidity (pH) of the honey samples. These results corroborate to the works previously reported by [27, 28].

3.1.2 Electrical conductivities

Electrical conductivity measurements of honey give an indication regarding its origin: either blossom (polyfloral) or forest (honeydew) [29] and the source of nectar. The electrical conductivity of blossom honey is generally below 0.80

mS/cm [31]. The mean electrical conductivities of the honey samples A, B, C and D are 0.47 ± 0.02, 0.51 ± 0.01, 0.38 ± 0.05, and 0.27 ± 0.01 mS/cm, respectively (Table 2). The results of this study therefore showed that the honey samples are polyflorals, that is, the honey types are produced by honey bees using nectar and pollens from many different plant/flower sources.

3.1.3 Density of honey

Density of honey is an important parameter to be determined to ensure its stability when used in pharmaceutical preparations and also to confirm the presence of contaminants and adulterants [32]. Density also influences the purity and quality of honey. The density (weight/ml) of the honey samples used in this research work were estimated 1.38 ± 0.30, 1.33 ± 0.02, 1.42 ± 0.25 and 1.45 ± 0.10 for honey samples A, B, C and D respectively (Table 3). These values agree with previously reported by [33, 34].

3.2 Antimicrobial activity of the honey samples

The antimicrobial activity of the honey samples at different concentrations against the selected microorganisms are shown in Table 4 and represented graphically as Figures 3, 4, 5 and 6. None of the honey samples showed an activity against *P. aeruginosa* at a concentration of 20 % v/v; only sample A showed an activity against the organism at concentration of 40 % v/v. At concentration of 80 % v/v and 100 % v/v, all the selected microorganisms were susceptible to the honey samples employed in the *in-vitro* antimicrobial evaluation. In like manner, all the honey samples employed in the evaluation showed an activity against *S. aureus* at a concentration ≥ 60 % v/v except sample B. The sample was also ineffective against *S. aureus* at a concentration below 60 % v/v. Samples C and D showed activity against *S. aureus* at all the concentrations used throughout the experiment. This showed that the activities of honey samples A and B were affected by dilution. The mostly pronounced activity of the honey samples was observed when they were tested against *Bacillus subtilis* at a concentration of 100 % v/v, that is, they have the largest diameter of zone of inhibition thus further reinforced the fact that *B. subtilis* is the most susceptible organism to the honey samples at a concentration of 100 % v/v. The honey samples were ineffective against *E. coli* at concentrations below 60 % v/v. The results also showed that antimicrobial activities of all the honey samples on the selected microorganisms increased with increase in concentration of the honey samples. The antimicrobial properties could be attributed to the level of free acids and pH of the honey samples. It is known that the growth of microorganisms is generally inhibited at low pH; thus, the low pH of the honey samples may account for the reasons the microorganisms were susceptible to the honey samples. More so, the results showed that their vulnerability was variably affected by dilution as this dilution has significant effects on concentration of the honey, pH and dissociation of the acids. In line with the present study, several researchers have shown that honeys possess antimicrobial properties [36-37, 17].

3.3 Phytochemical Content of the Honey Samples

3.3.1 Total phenolic and flavonoids

Evaluation of the antioxidant properties of plants extract used in folk medicine is important. This is partly due to roles of the antioxidant components of these plants as protective agents against reactive oxygen species related to pathophysiological disorders such as inflammation, arthritis, diabetes, cancer and genetoxicity [38-40]. In the current study, total flavonoids and phenolic of the honey samples were determined to show their antioxidant properties. The total flavonoids concentration of the honey samples determined were 1.15, 3.05, 1.21 and 1.15 mg/ml for honey samples A, B, C and D respectively while the total phenolic concentration of 1.09, 1.97, 1.08 and 1.11 mg/ml were recorded for the honey samples A, B, C and D respectively. The results showed that the honey samples contained flavonoids and phenolic compounds. This implies that they could exhibit antioxidant properties by mopping up reactive oxygen and nitrogen species in cells and tissues of organisms which in turn prevent oxidative damage. These results conform to the results from works previously reported by [41, 42].

3.4 Total Antioxidant Activities of the Honey Samples

3.4.1 Inhibition of lipid peroxidation and thiobarbituric acid reaction

Numerous plant and mineral constituents have proven to show free radical scavenging or antioxidant activity [43]. Among these are flavonoids and other phenolic compounds of plant origin have been reported as scavengers and inhibitors of lipid peroxidation [44]. Peroxidation of lipids has been shown to be a cumulative effect of reactive oxygen species, which disturbs the assembly of the membrane causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes [45]. From the results obtained, (Figure 7), it can be inferred that the higher the concentration of the honey sample used for the experiment, the lower is its percentage inhibition of lipid peroxidation and thiobarbituric acid reaction. This shows that the samples are able to inhibit lipid peroxidation and thiobarbituric acid reactions better at lower concentration in this order; D> C> B> A. Thus, the peroxidation of lipids inhibition activity of the honey samples may be due to their phenolics and flavonoid constituents.

3.4.2 2,2-diphenyl-1-1picrylhydrazyl (DPPH) radical scavenging activity

The DPPH free radical scavenging method has been widely applied for evaluating antioxidant activity in a number of studies [44, 46, 47]. High DPPH scavenging activity of a sample usually confers the superior antioxidant activity of such sample. Comparing the values gotten for the 50 % inhibitory concentration (IC_{50}), it shows that the honey sample A (IC_{50} = 5.147 mg/ml) has higher DPPH radical scavenging property at a lower concentration than the standard trolox (IC_{50} = 5.4495 mg/ml) while honey samples B, C and D showed lower DPPH radical scavenging activity than the trolox with the least activity exhibited by sample D (IC_{50} = 12.600 mg/ml) as shown in Figure 8. The results of the DPPH radical scavenging activities of the honey samples may have been greatly influenced by the presence and concentrations of phytochemicals in the honey samples.

4. Conclusion

The physicochemical properties, phytochemicals, antimicrobial and antioxidant activities of honey samples from different locations in Nigeria have been studied to validate their applications in folk medicine. The results from the present study show that all the honey samples are polyflorals that contain phenolics and flavonoids. The honey samples also showed good antioxidant and antimicrobial properties. This information therefore becomes an added value to existing information on honey and thus showing justification to application of Nigerian honey in treatment of

the acclaimed human diseases and ailments for which they have been reported. Comparison in physicochemical properties and biological activities of the honey samples were not significantly made and emphasized because only one sample of honey was acquired from each trial site and used for analysis rather than several of the honey samples. This implies that a sample from each trial site would not be enough statistically to establish comparative analysis in the physicochemical properties and biological activities of the honey samples. This study therefore, encourages improvement in the production and commercialization of honeys from Nigeria and their products for applications in ethnomedicine and ethnopharmacology.

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Conflict of Interest
The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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