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Bioassay for the Determination of Microbial Sensitivity to Nigerian Honey

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Abstract: The antibacterial activity of honey has been known for more than a century and has been well documented. The inhibitory activity has been attributed to several key properties of honey including production of hydrogen peroxide, osmotic effect and its naturally low pH. The current bioassays for determining this antibacterial effect employ the disc diffusion technique or well diffusion assay using zones of inhibition as indicator of bacterial susceptibility. Spectrophotometric assay proved to be more sensitive and more amenable to analysis than the other currently used assays. This simple and rapid assay permits extensive kinetic studies even in the presence of low honey concentration and it is able to detect inhibitory levels below those recorded for well and disc diffusion assays.

Key words: Nigerian honey · Antibacterial activity · Bioassay · *Escherichia coli* · *Staphylococcus aureus*

INTRODUCTION

Honey is a natural sweet substance produced by honeybees from the nectar of blossoms or from the secretions of living parts of plants or excretions of plants-sucking insects on the living parts of plants, which honeybees collect, transform and combine with specific substances of their own, store and leave in the honeycomb to ripen and mature [1]. Numerous studies demonstrate that honey possesses antimicrobial activity [2-6]. It destroys and/or inhibits the growth of some pathogenic vegetative microorganisms [7]. Mundo *et al*. [8] reported that honey inhibited bacteria due to a high sugar concentration (lowering water activity), hydrogen peroxide and proteinacious compounds present in honey.

Majority of studies on Nigerian honey used well and sometimes, disc diffusion methods to evaluate and study the antimicrobial activities [3, 6]. Diffusion bioassays are comparatively basic and robust, but have limitations in their detection and quantification abilities at low concentrations and are open to subjective interpretation [9]. One major difficulty in honey research and application is obtaining accurate and precise quantification using bioassays. The agar diffusion assay is still the preferred method for honey bactericidal quantifications, but the subjective nature of this assay limits the interpretation of results. It is also time-consuming and laborious, requiring preparation and cooling of plates, boring of test wells in agar and manual measurement of inhibition zones after 24 hours of incubation. Results depend largely on technique and judgment and the suggested precision cannot be obtained when the inhibition zone is unclear or not perfectly circular [10]. The possibility of using infra-red methods for quality analysis of honey has also been proposed [11].

The present study was undertaken to compare the use of spectrophotometric method with well/agar diffusion methods in the evaluation of the antimicrobial activities of Nigerian honey.

MATERIALS AND METHODS

Bacterial Growth Condition: The bacterial species used in this study were recent clinical isolates of *Escherichia coli* and *Staphylococcus aureus*. The isolates were grown to mid-exponential phase in nutrient broth at room temperature in a rotary incubator (150 rpm) after which the cells were harvested by centrifugation at 600 rpm for 10 minutes and washed thrice with phosphate buffer. The washed cells were re-suspended in the buffer and the turbidity was adjusted to an optical density of 0.85 at 500 nm. An aliquot (0.2 ml) of the cell suspension was inoculated into Mueller-Hinton agar and incubated at 37°C for 24 hours. The plate count was determined after the incubation period.

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Honey Preparation: The initial honey dilution of 50% (v/v) was prepared in nutrient broth. Double fold serial dilution of the initial dilution was made to obtain different concentrations of the honey dilution up to 0.01%.

Disc Diffusion: Nutrient agar plates seeded with 1 ml of the standard culture using spread plate technique was used. Plates were allowed to stand for 20 minutes before discs were applied. Sterile absorbent discs were placed into honey concentrations for 10 minutes before being applied directly onto inoculated agar plates. Control sterile discs were also applied. Plates were incubated at 37°C for 24 hours and zones of inhibition were measured.

Well Diffusion Assay: Nutrient agar plates were prepared and seeded with the test isolates as stated above. Wells, having the same diameter as the discs above, were bored into the surface of the agar. Aliquot (0.2 ml) of diluted honey was placed into each well. A hole filled with sterile water served as control. Plates were incubated at 37°C for 24 hours and the zones measured after the incubation period.

Spectrophotometric Assay: Up to 0.2 ml of the cell suspension was inoculated into 4 ml volume of honey concentration in a test tube while inoculation of 4 ml volume of nutrient broth with 0.2 ml of the cell suspension served as control. The optical density was determined in a spectrophotometer at 620 nm prior to incubation (T_0) and recorded after which, the cultures were incubated for 24 hours in the dark at 37°C with constant shaking to prevent adherence and clumping. After 24 hours of incubation, the optical densities were again determined (T_{24}) and recorded.

The optical density for each replicate at T_0 was subtracted from the optical density for each replicate at T_{24}. The growth inhibition for the test at each dilution was determined using the formula:

Percentage inhibition = 1 - (OD test/OD control) x 100

Where the resulting measurement recorded a negative inhibition value (growth promotion), this was reported as stimulation using the formula:

Percentage inhibition = (OD test/OD control) x 100

The antibacterial threshold concentrations were compared. This is reported as inhibitory concentrations, expressed as IC_0 (the highest concentration of the test material which results in no growth inhibition), IC_{50} (concentration of test material which results in 50% growth inhibition) and IC_{100} (the lowest concentration of test material which results in 100% growth inhibition). For each diffusion assay, 100% inhibition was taken at the highest concentration of the test material. Statistical analysis was performed using ANOVA at P=0.05.

RESULTS

The dose-response curves obtained from the plot of log of the concentration of honey against the percentage inhibition of the growth of the test organisms are presented in Figures 1 and 2. The inhibition activities correlated with the honey concentration with very high R^2 values ($0.94 \leq R^2 \geq 0.95$). The percentage inhibition model gave a good linearization of the dose-response data for both isolates. The equation of the curve for both *Staphylococcus aureus* and *Escherichia coli* were given as% inhibition = 64.918log10 honey concentration (% v/v) + 19.453 and% inhibition = 54.961log10 honey concentration (% v/v) + 25.307, respectively. These models were used to derive the threshold inhibitory concentrations for the spectrophotometric assay. The IC_0, IC_{50} and IC_{100} for *S. aureus* were given as 0.5, 2.95 and 17.41% respectively, while that of *E. coli* were given as 0.35, 2.18 and 22.86% respectively (Table 1). From the inhibitory concentration, it can be deduced that the Nigerian honey was more effective against *E. coli* at lower concentrations but more effective against *S. aureus* at higher concentrations.

In the well diffusion assay, the antibacterial activity was determined by measuring the zones of inhibition. From these data, a dose response curve was obtained by plotting the diameter of the zone squared versus the log of the honey concentration (Figure 3) and the plots were highly linearized with R^2 values greater than 0.96 ($0.968 \leq R^2 \geq 0.9759$). The linear regression models were given as inhibition zone (mm^2) = $351.75\log_{10}$ honey concentration (%v/v) - 154.42 and inhibition zone (mm^2) = $228.31\log_{10}$ honey concentration (% v/v) - 36.936 for *S. aureus* and *E. coli*, respectively. From these plots, the threshold inhibitory concentrations were calculated.

Table 1: Threshold inhibitory concentrations of the test organisms by Nigerian honey (%v/v)

	Staphylococcus aureus			*Escherichia coli*		
	Disc	well	spectophotometric	Disc	well	spectophotometric
IC_0	7.38	3.16	0.5	1.58	1.42	0.35
IC_{50}	28.69	26.58	2.95	25.79	25.71	2.81
IC_{100}	50	50	17.41	50	50	22.86

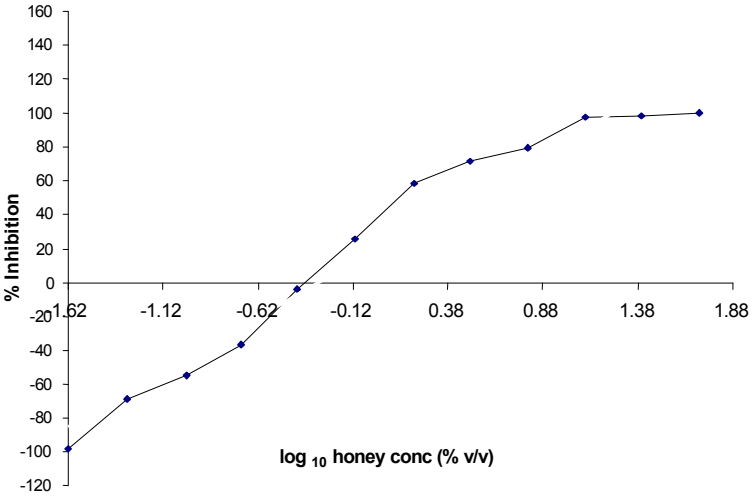

Fig. 1: Dose-response curve for the antibacterial activity of Nigerian honey agains *Staphylococcus aureus* determined spectrophotometrically

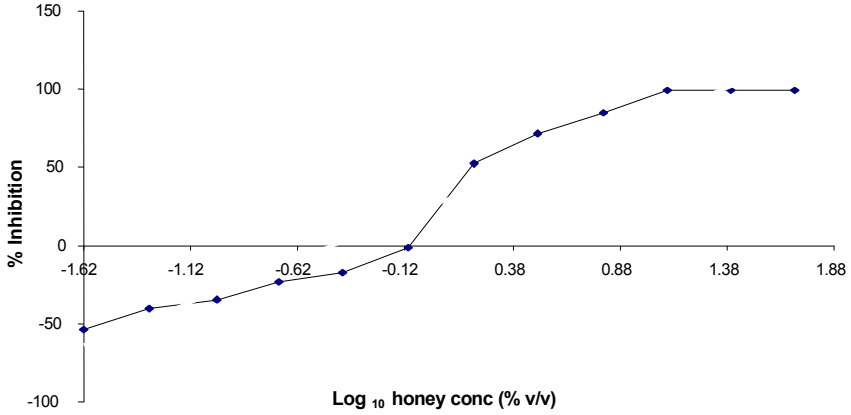

Fig. 2: Dose-response curve for the antibacterial activity of Nigerian honey against *Escherichia coli* determined spectrophotometrically

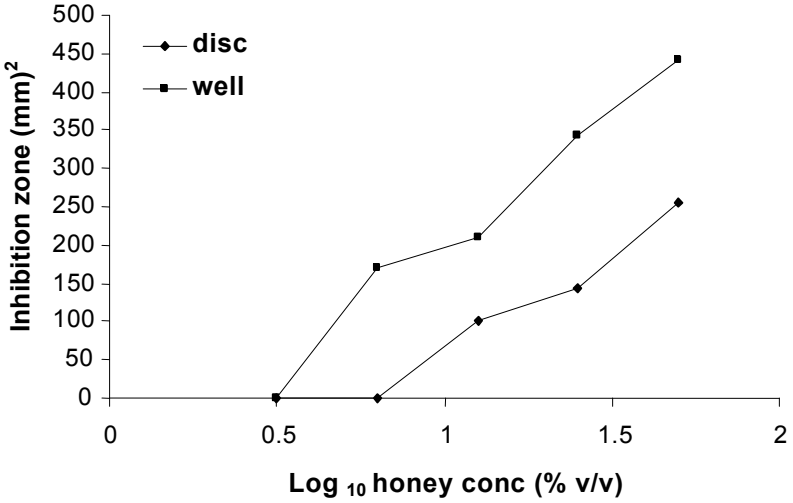

Fig. 3: Dose-response curve for antibacterial activity of Nigerian honey against *S. aureus* using well and disc diffusion assays

The IC_0 was obtained by extrapolation of the regression line, which intersects the squared diameter of the well plus 0.1 mm detection buffer (minimum detectable area of inhibition by the naked eye). The IC_0 for *S. aureus* was 3.16% (v/v) while that of *E. coli* was 1.42% (v/v). The IC_{100} was taken as the highest concentration of honey used for the assay. The IC_{50} was calculated as the median of the two values. They were given as 26.58% (v/v) and 25.71% (v/v) for *S. aureus* and *E. coli*, respectively (Table 1).

The antibacterial activity of the Nigerian honey using the disc diffusion assay was again determined by measuring the inhibition zones, as for the well diffusion assay. The values were plotted to produce the dose-response curves for each culture (Figures 3 and 4). There was also a good linearization of the curve with R^2 values being 0.965 and 0.9303 respectively for *S. aureus* and *E. coli*. The regression equation for *S. aureus* was given as inhibition zone (mm^2) = $218\log_{10}$ honey concentration (% v/v) - 140 while that of *E. coli* was given as inhibition zone (mm^2) = $144.98\log_0$ honey concentration (% v/v) - 22.269. The threshold inhibitory concentrations were obtained as for well diffusion assay. The IC_0 for *S. aureus* was 7.38% (v/v) while *E. coli* had the IC_0 of 1.58% (v/v). The IC_{50}, calculated as median of these two values, were given as 28.69 and 25.79% (v/v) for *S. aureus* and *E. coli*, respectively (Table 1).

Generally, it was observed that the spectrophotometric method was more sensitive in detecting the inhibition of the test organisms by the Nigerian honey than the well and disc diffusion methods as depicted by the threshold inhibitory concentrations. There was high significance difference between the values obtained by the spectrophotometric method and those obtained by the well and the disc diffusion methods ($P \leq 0.05$).

DISCUSSION

The high R^2 values obtained in all the regression plots indicated that honey concentration was a strong determinant of the bacterial inhibition. Thus, the organisms were at serious stress at high concentration of the Nigerian honey. Determination of microbial susceptibility to inhibitory compounds requires that a decision be made as to the type of method to be employed. The choice of methods includes agar diffusion, disc diffusion, broth diffusion, or variants of these methods. The agar/disc diffusion method is regarded as the method of choice for inhibition tests [12]. More recently, there has been an increased interest in spectrophotometric method [4; 10; 13; 14]. Both the agar and disc diffusion methods can be unreliable in certain situations due to the subjectivity associated with visual determination [15]. In addition, both of these methods have associated time, sample and cost implications.

The methods for disc and well diffusions and the results obtained are comparable to those used and obtained by previous studies [12; 16]. Developing the procedure for the spectrophotometric methodology was based on previous articles [10; 14; 17; 18]. The amount of bacterial growth occurring in the 24 hour period was measured (end point) rather than the rate of growth, due to reports that at low concentration of honey some species are suppressed in growth rate by having prolonged lag phase [19]. As small changes in OD were repeatedly detected, reporting the percentage inhibition using the formula enabled accurate measurement on normally undetectable changes in bioactivity. Reporting the minimum and median response concentrations was chosen along with 100% IC as this is derived from a series of determinations as opposed to a single isolated point [4].

Results from well, disc and spectrophotometric assays show that the isolates vary in their sensitivity to Nigerian honey. The variation was significant at P=0.05. This variation in sensitivity has been described previously [4; 8]. For the disc and well diffusion methods, the IC values obtained are similar to the MIC obtained by Cooper *et al*., [20]. The IC_{50} for the spectrophotometric method correlates with the work of Willix *et al*., [19] using a turbidometric method, albeit by a different technique of calculation.

Compared to the two diffusion assays, the spectrophotometric assay offers a number of advantages including: increased sensitivity; increased repeatability; ease of automation and reduced manipulation; and removal of subjective observations of inhibition zones.

The results obtained from the spectrophotometric method demonstrate an ability to determine inhibition. However, the method also permits the detection and quantification of stimulation. The stimulation of the cultures at low Nigerian honey concentrations was observed using this method.

From the results, limit of detection, sensitivity and ability to detect stimulation/inhibition, there is large disparity between the evaluated methods. The spectrophotometric method consistently demonstrated an improvement over current methods.

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