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To cite this article: PA Akinduti *et al* 2019 *J. Phys.: Conf. Ser.* **1299** 012131

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Suitability of spectrophotometric assay for determination of honey microbial inhibition

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ABSTRACT

Commonly used methods for determination of antibacterial potency of honey are usually the disc, agar well diffusion and dilution plate assay which had shown various demerit of inaccuracies and impreciseness. Therefore, the suitability of spectrophotometric assay for determination of honey inhibitory activity is evaluated in this study. Honeys from different sources in southwest states in Nigeria were assayed for antibacterial activity using 96-well micro-titre plate spectrophotometric methods to determine the minimum inhibitory concentration (MIC) against enteric bacteria strains. The honey inhibition assay of Nigerian honeys tested against enteric bacilli showed more than 90% inhibitory activity. Among all the honeys sample assayed, only two honeys reveal a very low MIC of 31.25 and 125mg/mL. The use of spectrophotometry is a precise method to determine honey inhibitory rate and it is proven to be suitable highly sensitive, reproducible, specific, reduced cost, fewer amounts of sample and reagent are required.

Keywords: honey, spectrophotometer, bacteria strains, inhibitory

1.0 INTRODUCTION

Increasing rate of resistant bacterial strains in the recent years had called for the development of alternative antimicrobial agents from natural products [1,2]. One of such natural product is honey. Honey has been known and use for medical purposes since over two thousand years ago as therapy against various infections [3,4]. As a result of this development, most commonly used methods for determination of antibacterial potency of natural products or extract are usually the disc or agar well diffusion methods and the dilution plate assay [5]. Each of these methods had been in use for several years [6].

1.1 Agar well and Disk Diffusion method: These two methods are based on the ability of the test substance molecules to move or diffuse from high gradient to lower gradient in order to produce antimicrobial activity. For agar well diffusion, a known sized diameter of well was bored into agar medium seeded with bacteria agent and placing known amount of natural products in it. Disc diffusion method depends on adsorption of known quantity of antimicrobial agents onto sterile disc made from filter paper. This is placed on agar plate seeded with known amount of bacteria inoculum. After incubation, the zone of inhibition found is estimated and interpreted according to regulated standards [7]. In spite of the acceptability, safe and reproducibility for susceptibility testing and satisfactory growth of most non-fastidious pathogens using agar or disk diffusion method, many flaws were associated with the use of these methods. There is usually poor diffusion of antimicrobial agents due to gradient and matrix network of the agar used for the assay. Secondly, quantity of natural product to be adsorbed onto disc could be over-estimated while rate of diffusion into the medium may not be satisfactory.

1.2 Dilution Methods: Above described diffusion tests are widely used to determine the susceptibility of organisms isolated from clinical specimens but have their limitations as a result of 'Susceptible' and 'Resistant' but a precise assessment is to determine the Minimum Inhibitory Concentration (MIC) of the natural product or antibiotic against the organisms concerned. Dilution methods are used to determine the minimal concentration of antimicrobial that can inhibit or kill the microorganism. This can be achieved by dilution of natural product or antimicrobial agent in either agar or broth media by making serial dilutions (usually in two folds).

The Broth Dilution method is a simple procedure for determining MIC with added advantage of knowing the minimum bacteriocidal concentration (MBC) when the MIC is sub-cultured. This assay is performed by serial dilution of natural products and known amount of bacteria broth is added. After incubation, MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. This method is simple, reproducible but very faint turbidity may be given as MIC and this may give inaccurate test result. Addition of colour reagent has been employed by several researchers to accurately determine the end point which is MIC. The use of colour indicator had been flawed, as a result of delay or prolongs timing of reading the end point which could be caused by ionic dissociation of colour reagent in the medium, thereby producing faded colouration

[8]. These techniques are subjected to various inaccuracies in determination of minimal inhibitory concentration (MIC) [9].

1.3 Spectrophotometric assay by Micro-broth dilution test: Considering all disadvantages attached to the use of disc, agar well diffusion methods and broth or agar dilution method, the use of spectrophotometry to detect the inhibition rate has the following advantage over the others;

- i. It shows acceptable reproducibility for susceptibility testing
- ii. It gives satisfactory growth of most non-fastidious pathogens
- iii. It is not expensive
- iv. Bacteria cells are well exposed to the antimicrobial molecules in the medium

The use of microplate method (spectrophotometric assay) for determination of antimicrobial activity of honey samples from different sources is based on the inhibition of bacteria cell by honey and the amount of the cell reduction is calculated. This assay method is based on the principle of Beers law which state that the amount of light absorbed in a given medium is directly proportional to the amount of the substance (bacteria) absorbing the light. It is convenient to consider that amount of light that passed through to the detector is usually determined and it is called Percent Transmittance which is defined mathematically as;

$$\%T = 100 \times \frac{I_t}{I_i} \dots \dots \dots 1$$

Where;

I_t is the light transmitted through the sample and reaches the detector

I_i is the incident light.

The mathematical relationship between absorbance (A) and percent transmittance (%T) is:

$$A = 2 - \text{Log } (\%T) \dots \dots \dots 2$$

It could be deduce from the equation 2 that, the more light that is absorbed, the higher the absorbance reading while the less light that pass through the sample to the detector, the lower the percent transmittance reading. Spectrophotometer as a versatile instrument, it can both read absorbance and transmittance of which both has no units. Therefore, the suitability of spectrophotometric assay for determination of honey inhibitory activity is evaluated in this study.

2.0 MATERIALS AND METHODS

2.1 Honey sampling: Honey from different wholesale stores in most patronized locations in southwest states in Nigeria were collected, labeled with alphabetic codes and information relating to its sources were verified for authenticity and originality.

2.2 Determination of antimicrobial activity: Standard micro-tube dilution bio-assay in a 96 wells micro-titre plate was used to determine the minimum inhibitory concentration (MIC) of the honey samples against the bacteria strains. To each of the well, 100 μ l of sterile 1% peptone water was placed in well 2 to well 10. To well 1 and well 2, 100 μ l of honey sample was placed and serial

doubling dilution was made separately from well 2 to well 10 while 100 μ l was discarded from well 10. Equal volume of 100 μ l broth culture of 0.5 MacFarland turbid identified bacteria was added to all the dilution ranges from well 1 to well 10. Overnight broth culture of 100 μ l was placed in well 11 and 100 μ l of 1% sterile peptone water was added to serve as control while 100 μ l of sterile 1% peptone was added to 100 μ l sterile water in well 12 (blank). The turbidity of each well (that is absorbance known as optical density) was measured at zero hour (T_0) using Jenway 6405UV/Vis spectrophotometer at 620nm wavelength. The plate was incubated at 37°C in ambient air for 24 hours on a shaker. After incubation for period of 24 hours, absorbance of the each well turbidity was again measured using Jenway 6405UV/Vis spectrophotometer at 620nm wavelength. The absorbance of each well which is proportional to the turbidity of the bacteria growth which was noted while the percentage rate of inhibition was calculated using equation 3;

$$\text{Inhibition rate (\%)} = 1 - \left(\frac{T_{24 \text{ test}} - T_0 \text{ test}}{T_{24 \text{ broth}} - T_0 \text{ broth}} \right) \times 100 \dots\dots\dots 3$$

Where;

$T_{24 \text{ test}}$ = Absorbance of test (mixture of honey + bacteria cells) at 24 hours

$T_0 \text{ test}$ = Absorbance of test (mixture of honey + bacteria cells) at 0hour

$T_{24 \text{ broth}}$ = Absorbance of broth culture (mixture of bacteria cells + 1% peptone) at 24 hours

$T_0 \text{ broth}$ = Absorbance of broth culture (mixture of bacteria cells + 1% peptone) at 0 hours

Therefore, the MIC is defined as the lowest dilution of honey showing more than 95% inhibition rate according to CLSI [7] and Berhanu [10].

2.3 Data analysis: Analysis of variance (ANOVA) was used to test for the significance of the antimicrobial activity of each of the honey samples against different bacteria strains taking p value >0.05 at 95% confidence interval.

3.0 RESULTS AND DISCUSSION

Considering the honey inhibition assay in a well containing of 6.25% honey dilution, the following absorbance were obtained;

$T_{24 \text{ TEST}} = 0.023$ (Absorbance of test (mixture of honey + bacteria cells) at 24hours

$T_0 \text{ TEST} = 0.106$ (Absorbance of test (mixture of honey + bacteria cells) at 0hour

$T_{24 \text{ BROTH}} = 0.209$ (Absorbance of Broth culture (mixture of bacteria cells + 1% peptone) at 24hours

$T_0 \text{ BROTH} = 0.052$ (Absorbance of Broth culture (mixture of bacteria cells + 1% peptone) at 0 hour

$$\text{Inhibition rate (\%)} = 1 - \left(\frac{0.023 - 0.106}{0.209 - 0.052} \right) \times 100$$

$$\text{Inhibition rate (\%)} = 1 - \left(\frac{-0.08}{0.15} \right) \times 100$$

$$\text{Inhibition rate (\%)} = 1 - (-0.53) \times 100$$

$$\text{Inhibition rate (\%)} = 1 - (-53)$$

$$\text{Inhibition rate (\%)} = 1 + 53$$

$$\text{Inhibition rate (\%)} = 54$$

It was calculated that honey dilution of 6.25% in one of the honey sample produced 54% inhibition rate which is very low to MIC of 90% inhibition rate. In Table 1, inhibition rate for in each well was calculated as done earlier and the MIC of the honey assay was determined at 125mg/ml which showed more than 90% inhibition rate (90.3%).

Table 1; Typical honey inhibition rate determined by spectrophotometric assay

Well	Honey concentration (mg/ml)	Absorbance		Inhibition rate
		T _{0TEST}	T _{24TEST}	%
1	2000	2.471	2.377	98.7
2	1000	1.959	1.866	98.4
3	500	0.951	0.858	98.0
4	250	0.530	0.438	97.8
5	125	0.302	0.216	90.3
6	62.5	0.191	0.107	88.5
7	31.25	0.123	0.070	56.1
8	15.63	0.097	0.074	24.0
9	7.81	0.072	0.062	10.1
0	3.91	0.032	0.031	0.03
11	Peptone+organism	0.094	0.189	Nil
12	Peptone+honey only	0.005	0.006	Nil

Among all honey used, honey of low MIC 31.25 and 125mg/ml (Table 2) showed more than 90% inhibition rate (that is 90.3%) which is regarded as MIC. This is very definitive and precise to give the concentration or dilution of honey that can effectively inhibit bacteria strains. In the screening for antimicrobial activity of honey, the microplate spectrophotometric method has provided a useful technique for determining MICs and also for a large numbers of test samples, requiring small amounts of substances; this can be particularly important if the natural product quantity is small [11]. This method can also be used for a wide variety of bacteria strains which honey of various varieties can be measured for its inhibitory concentration. The antibacterial activity of honey has been assayed using various methods across the globe with special attention devoted to agar diffusion assay. The use of 96-well micro-titre plate by means of a spectrophotometric endpoints evaluation has proven to be highly sensitive, reproducible and repeatable, less time consumption, reduced cost, fewer amounts of sample and reagent are required. Visual inspection of endpoint might not be accurate because of impurities in

the honeys which might cause error and unprecised determination of MIC levels but this is overcome by spectrophotometric reading.

Table 2; Minimum inhibitory concentration (MIC) of Nigerian honey samples against enteric bacteria strains

Honey samples	MIC (mg/mL)	Susceptibility rate (%)
SEJ	500	16.2
JO	1000	0.0
OM	500	24.3
CSO2	31.25	8.1
CSO1	125	10.8
R1	500	37.8
PAK	500	16.2
RR1	1000	0.0
RR2	500	8.1
RR4	500	8.1
RKL2	500	13.5
RKL1	250	8.1
RR3	500	16.2
MAK	500	10.8

(Key: N=total number of bacteria tested; n=number of bacteria isolates susceptible; %=percentage; F_{test}=3.743, p=0.003)

In conclusion, spectrophotometric assay is suitable, economic and accurate for determination of honey inhibitory activity.

Competing interest: Authors declare no competing interest

Acknowledgement: The authors profoundly appreciate Covenant University Center for Research, Innovation and Development (CUCRID) for publication funding.

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