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EVALUATION OF ANTIBACTERIAL POTENCY OF RAW HONEY ON WOUND PATHOGENS

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Abstract

Honey has been used in ancient times in management of various health conditions and has equally been rediscovered in recent times in wound treatment. The aim of this study is to evaluate the antibacterial potency of Honey on wound pathogens. The experimented honey was obtained from Eha-Alumona in Nsukka L.G.A and was tested for quality using the thumb test, water test and heat test. The physicochemical properties of the honey was analysed at National soil, plant, fertilizer and water laboratory in Umuahia where various methods such as spectronic method, automatic smart 3 colorimetric method and gravimetric oven drying method were used to obtain the values. Different concentration of honey 25-100% v/v were tested against each type of clinical isolates obtained from wound infection. A total of 80 bacterial isolates were collected from Federal Medical Centre Owerri among 100 subjects with a slant bottle. An antibiotic sensitivity test was done to all types of honey concentration using Agar Well diffusion method while minimum inhibitory concentration and minimum bactericidal concentration were determined for the most potent honey concentration by the broth dilution technique. All statistical analysis was performed using (ANOVA). The wound pathogens showed certain degrees of susceptibility to honey but the level of susceptibility is dependent on honey concentration and the bacteria in question. Among the three studies wound pathogens, *Staphylococcus aureus* was the most sensitive to honey at a zone diameter of 22.17mm. The MIC of honey on *Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa* was 25%,

75% and 50% respectively while their MBC is 50%, 100% and 75% respectively. The experimented honey has both bacteriostatic and bactericidal activity, although result obtained from Ciprofloxacin used as control showed higher level of susceptibility on the tested bacteria than honey. Therefore, honey cannot be used to replace conventional antibiotics in tackling the problem of resistant bacteria pathogens, but should be used in line with conventional antibiotics as a suppository application.

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INTRODUCTION

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished. This type of bacterial resistance to the antimicrobial agents possess a very serious threat to public health, and for all kinds of antibiotics, including the major last-resort drugs, the frequencies of resistance are increasing worldwide [1]. Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including honey [2].

Honey is derived from nectar gathered and modified by the honeybee. *Apismellifera*. It is a carbohydrate-rich syrup derived from floral and other plants nectars and secretions. Honey has been used in folk medicine since ancient times and has more recently been rediscovered by medical researchers for its use in dressing acute and chronic wounds [3-7].Traditionally, honey has been used to treat burns, infected and non-healing wounds and ulcers, boils, pilonidal sinus, venous and diabetic foot ulcer [8].

Recent studies confirm the efficacy of honey in treating venous ulcers [9]. In patients suffering from malignant wounds, improvement with respect to wound size and cleanliness was seen after treatment with honey [10-12]. Coated bandages [13]. Similarly, honey dressing quickened rates of healing in pressure wounds. Honey has also been used to lessen foul odours emanating from wounds which cause a social barrier for patients and may lead to isolation.

The antibacterial action is due to its acidity, hydrogen peroxide content, osmotic effects, nutritional and antioxidant content, stimulation of immunity, and unidentified compounds [14]. Different kinds of honey like Gelam, Medihoney, Tualang and Manuka; have been tested and found to have similar properties. Some forms of medically certified honeys have been licensed as medical products for professional wound care in Europe and Australia [15].

Quick effect on different kinds of bacteria and different fungi Acceleration of the physiological process of wound healing (debridement and granulation). No local or systematic adverse effects (allergy or toxicity).Cost effectiveness. Honey meets all but the first criterion ([15].Honey stimulates leukocytes to release cytokines which is what initiates the tissue repair process. It also stimulates immune response to infection. The stimulation of other aspects of the immune system by honey is also evident (proliferation of B- and T- lymphocytes and the action of phagocytes). Honey stimulates the production of antibodies. It is suggested that this is due to honey effect of enhancement of the immune system and antibacterial activity. The antibacterial activity of honey is in a broad spectrum as evidence by many studies.

MATERIALS AND METHODS

Study Design

This is an experimental study to demonstrate the effect of crude honey on microbial organisms collected from wounds of patients.

Study Area

This study was conducted on patients attending clinic inFederal Medical Centre Owerri, Imo State. Owerri is the capital of Imo State in Nigeria.

Sample size of determination and sampling technique

Taro Yamane's formula (1967) would be used to obtain the sample size 80 from the population of 100 using the formula

$$\mathbf{n}=\frac{N}{1+N(e)^2}$$

Where n= sample size

N= the population size

e= the acceptable sample to be 0.05

$$n=\frac{100}{1+100\ (0.05)^2}$$

$$n=\frac{100}{1+100(0.0025)}$$

$$n=\frac{100}{1+0.25}$$

$$n=\frac{100}{1.25}$$

∴ n=80

Then sample size =80

Sampling technique

Random sampling was used to select sample size.

SampleCollection Procedure

Wound pathogens were collectedfrom Federal Medical Center Owerri (FMC) using a slant bottle. The pathogens of interest were *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* A total of 80 slant bottles were used for sample collection. At the site of sample collection, the work area were properly disinfected using 1% hypochlorite solution. After which, the hands werewashed using soap and water. The gas burner was put on, the stock agar plate and the slant bottle were held in one hand. The other hand is used to pick the wire loop. The inoculation loop was held above the flame vertically until it glows red. The wire loop was allowed to cool by avoiding contact with other surfaces. The cover of the stock plate and the slant were removed using the loop hand. The mouths of slant bottle were sterilized by passing through flame several times. The wire loop was inserted into the slant bottle to cool. After which, it was inserted into the stock plate to pick 2-3 colonies of bacterial cell. The wire loop was carefully removed from the stock plate and the bacteria transferred to the slant bottle. The loop was streaked back and forth from the bottom to the top of the slant.

Subculture and Re-identification

Bacteria colonies were picked from the slant bottle and were streaked on blood agar, chocolate agar and CLED agar (Cystein lactose electrolyte deficient) agar by using a sterile inoculation loop. The plates were incubated at 37℃ for 24hours. Preliminary identification of bacteria was based on colony characteristics of the organisms, such as size, shape, colour, and elevation, hemolysis on blood agar, odour, and changes in physical appearance in differential media. Isolates were re-identified based on their gram reaction, catalase, coaglulase, indole and oxidase test results.

Catalase Test

This test is used to differentiate those bacteria that produce the enzyme catalase such as *Staphylococci*, from non-catalase producing bacteria such as *Streptococci*.

Method

2-3mls of hydrogen peroxide solution was put in a test tube, several colonies of the test organism was immerse in the hydrogen peroxide solution with the help of a sterile wooden stick. The solution was observed for immediate bubbling. Bubbles of oxygen given off is indicative of a positive catalase test while absence of bubbles is indicative of a negative catalase test. Proper care was ensured while picking the organism to avoid false positive reaction which can occur when picking colonies from a blood culture plate [16].

Coagulase Test

This test is used to identify *Staphylococcusaureus* which produces the enzyme coagulase.

Method

A drop of distilled water was placed at each end of a glass slide using a sterile pasture pipette. Two thick bacteria suspensions were made on each end of the glass slide by emulsifying a bacteria colony against each drop of the distilled water. Sterile wire loop was then used to add a loop full of plasma to one of the bacteria suspensions, while the other suspension serves as control. The suspension with plasma was gently mixed with the wire loop and was examined for clumping within 10 seconds. Clumping observed within 10 seconds is indicative of *Staphylococcus aureus* while absence of dumps within 10 seconds is indicative of no bound coagulate [16].

Indole Test

Testing for indole production is important in the identification of enterobacteria. Most stains of *E.coli, P. vulgaris, P. rettgeri, M. morganii* and providencia species break down the amino acid tryptophan with the release of indole.

Method

3mls of sterile tryptone water inside a bijou bottle was inoculated with the test organism using a sterile wireloop. The solution was incubated at 35-37^{o}cfor 48 hours. 0.5ml of Kovac's reagent was added to the solution and was shaked gently. Appearance of red colour was examined at the surface layer of the solution within 10 minutes presence of red colour at the surface layer of the bottle is indicative of positive indole test while absence of red colour on the surface layer of the bottle is indicative of Negative indole test [16].

Oxidase Test

The oxidase test is used to assist in the identification of *pseudomonas, Neisseria, vibrio, Brucella, and pasteurella species;* all of which produce the enzyme cytochrome oxidase.

Method of using an oxidase regent strip

An oxidase strip was moistened with a drop of sterile water. Colonies of the test bacteria was then rubbed on the strip using a sterile piece of stick. A red purple colour was examined within 20 seconds on the strip. Presence of a red purple colour showed a positive oxidase test while absence of a red purple colour on the strip showed a negative oxidase test [16].

Test Organisms

All test organisms used for this study where isolated from wound.

They include *Staphylococcusaureus*, *Escherichiacoli* and *Pseudomonasaeruginosa*. They are all bacteria and were confirmed with morphological and biochemical test using Cheesbrough [16]guideline.

Honey Collection and Quality Testing

Honey sample was collected from Eha-alumona in Nsukka and was tested for quality by the following methods thumb test, water test and heat test.In the thumb test, small quantity of honey was placed on the thumb and was examined for rate of spilling. Honey that spills quickly is indicative of fake honey.For the water test, a spoon of honey was introduced in a glass of water and the rate of dissolution in water is

assessed. Rapid mixing of the honey with the glass of water is a sign of low quality honey. However, the heat test was performed by dipping a stick of matches inside the honey. If the honey refuse to light when lighted is an indication of low quality or fake honey. The honey sample was filtered with a synthetic mesh to remove debris and then streaked on blood agar plate to check sterility.

Preparation of Honey Solutions

Hundred percent pure honey (100%.v/v) was obtained after filtered using sterile gauze. To get 75% honey solutions (v/v) 0.75ml of honey was diluted in 0.25ml sterilized distilled water. For 50% honey solution (v/v), 0.5ml of the honey was diluted with 0.5ml of sterile distilled water while 25% honey solution (v/v) was prepared by diluting 0.25ml of honey with 0.75ml of sterile distilled water.

Susceptibility testingof Honey

Susceptibility testing was performed by Kirby-bauer disk diffusion technique according to criteria set by CLSI, 2016. The inoculawere prepared by picking three colonies of similar test organisms with a sterile wire loop and suspended in sterile normal saline. The density of suspension was opacity standard on McFarland 0.5 barium sulphate solution. A sterile swab was dipped into the suspension of the isolate squeezed free from excess fluid against the side of the tube, and then spread over the agar plate. The test organism was uniformly seeded over the Mueller-Hinton agar (oxoid) surface, and the plates were left on the bench for the excess fluid being absorbed. Using a sterile cork borer (6mm diameter, 4mm deep, and about 2cm apart), wells were made in the agar medium. Using a micropipette, 50µl of honey with the concentration of 100%, 75%, 50%, and 25% was added to the wells in the plate. The plates were incubated at 37^0c for 24hours. The mean diameters of inhibition zones were measured in mm, and the results were recorded. A positive control well was equally filled with ciprofloxacin (5µg), while sterile distilled water used as negative control. The experiment was repeated 3times for each bacteria specie.

Determination of Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) of the antimicrobial agents were determined for each isolate by the tube dilution method. Briefly, ten sterile test tube were placed in the rack, labeled each 1 through 8. Honey control tubes (HC), broth control tube (BC), and growth control tube (GC) were used as quality controls. One milliliter of freshly prepared nutrient broth was added to each tubes, sterilized, and cooled. Then one milliliter of undiluted honey solution 100% was added to test tube number 1 and HC with a sterile micropipette and tips. Then, twofold serial dilution was performed by transferring 1ml undiluted honey into the second tube with separate sterile micropipette and tips and vortexed for homogenization. After a thorough mixing, 1ml was transferred with another sterile micropipette from tube 2 and tube 3. These procedures continued until the eighth tube with a dilution of 1:128 was reached, and finally 1ml was taken and discarded from tube 8. The GC tube that received no honey and BC that received no bacterial inoculums served as growth control while the HC tube that received no bacterial inoculums served as a honey control. Except for the HC tube, each tube was inoculated with 1ml of the culture of the respective prepared organism. The whole procedures were repeated for all the organisms tested to each of the honey. Tubes were then incubated at 37^oC for 24h and observed by visual inspections for the presence and absence of growth (turbidity). MIC was recorded as the lowest concentration of honey that inhibited bacterial growth (no visible growth or turbidity).

Determination of Minimum Bactericidal Concentration (MBC)

From the result of MIC, the test tubes that did not show visible growth were used for MBC determination. About 0.1ml was aseptically transferred onto the surface of Mueller Hinton agar plates. The plates were incubated at 37^0C for 24hours. The MBC of the honey mixture was recorded as the lowest concentration of the honey mixture that had less than 99% growth on Mueller Hinton agar plates (Weston, 2000).

Physicochemical analysis of honey

The crude honey obtained from Eha-alumona in Nsukka L.G.A was sent to National soil, plant, fertilizer and water laboratories opposite timber modern market Umuahia for Analysis of Physicochemical component of the honey. The following parameters were analysed using variable methods.

P^H of Honey using Spectrophotometer

The P^H of honey is measured by AOAC method. P^H is measured in solution, according to the 943.02 method (Lane 1995). A total of 10g of honey were weighed and placed in an Erlenmeyer flask, added with 100ml of distilled water at 25^0C after the water was added, the content was mixed in an electronic agitator, for 30minutes the contents were placed in a beaker, left to rest for 10minutes, and P^H was measured. To measure P^H, an electrode from a digimed DM 22P^H meter, adjusted for P^H Solutions with P^H 3.01 and 9.18, and an accuracy of 0.01% P^H, was used.

Alkalinity of Honey by spectronic method

Alkalinity is a measure of the negatively charged ions in solution of the honey sample. The alkalinity was measured using the spectronic method. About 5mls of the honey sample were diluted with 100ml of distilled water and filtered with a No 42 whatman fitter paper. About 10mls of the filtrate were measured into the cuvette of the SMART 3 colorimeter and the concentration of the alkalinity of the sample recorded as mgl¯1 as the value obtained for the OH of the sample.

Other Physicochemical Compositions of Honey

The turbidity, colour, sulphate (SO_4) phosphate, Nitrates and Ammonium nitrogen were all determined automatically with the SMART 3 colorimeter. The moisture content (MC) and specific gravity (SG) were determined by gravimetric oven drying method.

Statistical Analysis

The data obtained from this study was analyzed using analysis of variance (ANOVA). P-values <0.05 was considered to be significant. Mean and standard deviation were also determined.

RESULTS

Table 1: Gram Stain and Biochemical Test of wound pathogens collected from patients attending clinic at FMC, Owerri

Test Bacteria	Gram Stain	Catalase	Oxidase	Motility	Indole	Coagulase
Staphylococcus aureus	+	+	_	_	_	+
Escherichia coli	_	+	_	+	+	_
Pseudomonas Aeruginosa	_	+	+	+	_	_

Key + = Positive, - = Negative

Table 2: Antibacterial activity of honey sample from Eha-alumona in Nsukka L.G.A against the isolated pathogenic bacteria from wound

Test bacteria	No of Sample N=80	Zone of inhibition (mm) at different honey concentration 25%	50%	75%	100%	Control (Ciprofloxacin)
Staphylococcus aureus	31	7.69± 0.09	15.17± 0.10	19.25± 0.20	22.17± 0.20	30.00± 0.34
Escherichia coli	22	3.09± 0.05	9.05± 0.05	14.01± 0.02	17.33± 0.17	27.00± 0.70
Pseudomonas aeruginosa	27	0.028± 0.02	1.03± 0.04	4.07± 0.05	9.28± 0.03	37.01± 0.04

Table 3: Minimum Bactericidal Concentration (MBC) and Minimum inhibitory concentration of Raw Honey from Eha-Alumona

Test bacteria	MIC (% V/V)	MBC (% V/V)
Staphylococcus aureus	25	50
Escherichia coli	75	100
Pseudomonas aeruginosa	50	75

Table4: Physicochemical Analysis of Raw honey obtained from Eha-alumona in Nsukka L.G.A.

Physicochemical Components of Raw Honey from Eha-alumona in Nsukka	Concentration of the Physicochemical Analytes from Honey
PH	3.8 mg/L
Alkalinity	178.08 mg/L

Specific gravity (SG)	1.872 mg/L
Turbidity	1577.94 mg/L
Colour intensity	17095.815 mg/L
Ammonium Nitrogen (NH_4N)	19.03 mg/L
Nitrate Nitrogen ($N0_3N$)	3.745 mg/L
Sulphate (SO_4)	559.94 mg/L
Moisture Content (MC)	5.35 mg/L
Phosphate (PO_4)	297.04 mg/L

Discussion

In this study, the natural honey at various concentrations of 25%, 50%, 75% and 100% showed certain degree of Antibacterial activity among all test bacteria isolated from wound. Among the isolated pathogens, *Staphylococcus aureus* showed high level of susceptibility when compared with *Escherichia coli* and *Pseudomonas aeruginosa.* This was the same with the work of Molan[17].

Staphylococcus aureus degree of susceptibility to honey was traced to hydrogen peroxide content, which represent the inhibition factor by its ability to cleave on the bacteria DNA causing oxidative DNA damage and bacterial growth inhibition. Other factor includes low PH of honey and osmotic effect [17].

The antibacterial activity of the undiluted honey (100% concentration) was effective on all wound pathogens than when diluted. It can be observed in table 4.2 that the zone of inhibition increased with the concentration of the honey. These correspond with the work done by Gambo *et al*. [18]. Although, the effect of undiluted honey on *Pseudomonas aeruginosa* was not that remarkable as can be seen on *Staphylococcus aureus* and *Escherichia coli.* This is due to emerging resistivity of *Pseudomonas aeruginosa* [19].

The result of this study is contrary to the work of Omojasola [20] who reported that the antibacterial activity of the undiluted honey (100% concentration) was low when compared to the diluted honey. Moreso, *Staphylococcus aureus* showed no inhibition zone in the work of Omojasola [20] while in this study, *Staphylococcus aureus* showed the highest level of susceptibility to undiluted honey.

The experimented natural honey was found to have a low P^H value of 3.8. Omojasola 2021 reported that local natural honey has P^H values ranging from 3.6 to 4.8. Low P^H value obtained from the experimented honey is part of the antibacterial properties of honey. This was in line with what Yatsunami and Echigo [21] said that P^H of honey, in addition to the high osmolarity, where responsible for the antibacterial activity of honey.

The experimented honey exhibited high colour intensity. The intensity of the colour measures up to 17095.815mg/l which corresponds with the work of Sohaimy *et al* [22] who reported that high colour intensity of honey is indicative of the presence of high amount of phenolic compounds and flavinoids. This could be the reason why the natural honey appeared dark in colour. Pereira, 2009 disclosed that phenolic acids possess the highest antibacterial activity by its ability to inhibit bacterial virulence factors, enzymes and toxins, interact with cytoplasmic membrane, suppress biofilm formation and exert a synergistic effect with antibiotics.

The result obtained in the Physicochemical analysis of the honey is in line with the antibacterial activities observed in table 4.2 and correspond to the work of Moumbe *et al* [23] who reported that minor ingredient present in honey are responsible for medical and biological activities of honey in the treatment of infections, burns, wounds and ulcers.

Conclusion

The result of this study shows that raw honey obtained from Eha-Alumona in Nsukka has inhibitory effect on the test organisms isolated from wound. The degree of inhibition is dependent on the organism

in question and concentration of honey used. However, this study has clearly demonstrated that honey might not adequately proffer a total solution to the current problems facing bacterial chemotherapy. Users therefore need to be enlightened that honey, being a natural product with very few side effects may not necessarily be superior to conventional therapies in tackling the problem of bacterial resistance.

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