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Evaluation of Antibacterial Activity of Different Honey samples on *Staphylococcus aureus*, *Klebsilla pneumoniae* and *Escherichia coli* Isolated from Wounds

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***Abstract*—** *Infections and other health related problems have been of great concern to human being; and chemotherapy is the main approach in the treatment of such conditions. Among the possible alternatives, the use of natural substances has been reconsidered. Honey which is historically known as a non-toxic and very efficient antimicrobe with a broad spectrum action has been among such natural substances. The aim of this research is to determine the antimicrobial activities of honey from three states in Nigeria (Adamawa in the North, Enugu in the East and Taraba in the North) on three (3) species of bacteria – Staphylococcus aureus, Klebsiella pneumoniae and Escherichia coli isolated from different wounds of patients at Specialist Hospital Jimeta Yola, Adamawa state. The antibacterial activity of honey assayed was determined using disc diffusion method. Noticeable variations in the antibacterial activity of the three honey samples on the test organisms were observed. Staphylococcus aureus was most sensitive to the honey samples; followed by Klebsiella pneumonia and Escherichia coli at 100% and 80% honey concentration. The highest antibacterial activity was observed in honey sample from Taraba with* average zone of inhibition of *20.6mm followed by sample from Enugu with 20.2mm as the average zone of inhibition on Staphylococcus aureus at 100% concentration.*

***Keywords*—** *Adamawa; Antibacterial activity; Disc diffusion; Enugu; Honey; Isolates, Taraba, Wounds.*

I. INTRODUCTION

Investigation into the microbial flora of wound began in the late 19th century and since then improvements in techniques have facilitated the recovery, identification and enumeration of a wide variety of microbial species. Most wounds support relatively stable polymicrobial communities often without signs of clinical infection [1]. However, potential pathogens may be present and the delicate balance between colonized wound and an infected wound depends on the interplay of complex host and microbial influences [2]. The development of wound infection has deleterious effect on patients by causing increased pain, discomfort, inconveniences and can lead to life threatening conditions or even death. In recent years, medical authorities reported increased infections and emergence of bacterial strains resistant to certain antibacterial compounds mainly due to the misuse of these substances. Among the possible alternatives, the use of natural substances has been reconsidered [3]. This renewed interest is mainly due to the growing clinical problem of antibiotic-resistant bacteria and the combined difficulties for medical practitioners in managing chronic wound types that may become infected [4].

Honey is a sweet syrupy substance produced by honeybees from the nectar of flowers and used by humans as a sweetener and a spread. Honey is comprised of 17-20% water, 76-80% glucose, and fructose, pollen, wax, and mineral salts. Its composition and colour is dependent upon the type of flower that supplies the nectar [5].

Honey contains a wide variety of vitamins, minerals, amino acids and antioxidants. The vitamins found in honey include niacin, riboflavin and pantothenic acid; minerals present include calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc. In addition honey contains a variety of flavonoids and phenolic acids which act as antioxidants, scavenging and eliminating free radicals. Honey has had a long history in human consumption, and is used in various foods and beverages as a sweetener and flavouring. It also has a role in religions and symbolism. Medicinal importance of honey has been documented in the world's oldest medical literatures, and since the ancient times, it has been known to possess antimicrobial property as well as wound-healing activity [5]. More than 1,400 years ago, honey is described as a source of healing in the Quran: "And your Lord inspired the bees saying: Take your habitations in the mountains and in the trees and in what they erect. Then, eat of all fruits and follow the ways of your Lord made easy (for you)'. There comes forth from their bellies a drink of varying colors wherein is healing for men. Verily, in this is indeed a sign for people who think" [6].

Infectious diseases are known to be treated with herbal remedies throughout the history of mankind; and natural substances still play a major role in primary health care as therapeutic remedies in many developing countries [7]. This study aimed to determining the antimicrobial activities of honey from three States of Nigeria (Adamawa, Enugu and Taraba) on three (3) strains of bacteria – *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli* isolated from different wounds of patients at Specialist Hospital Jimeta Yola, Adamawa state.

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II. Material and Methods

A. Source of Honey

The honey sample used was obtained from local commercial producers in Enugu (Nsukka), Taraba (Jalingo) and Adamawa state (Mayo-belwa).

B. Source of Test organisms

The organisms used in this work was obtained from microbiology laboratory of Specialist Hospital Jimeta Yola, Adamawa state, and were isolated from different wounds such as burns, accidents, infectious disease lesions, and caesarean sections.

C. Identification of test Organisms

Identification of the organisms was done microscopically via Gram staining and morphology, as well as biochemically.

Gram staining

A smear of the isolate were made on a clean grease free slide, air dried and heat fixed. The slides were flooded with 0.5 % solution of crystal violet and were allowed for 30 seconds. The stains were washed off with water and flooded again with iodine solution (mordant) and allowed for 10 seconds after which they were washed off. The slides were counter stained with Safranin for 30 seconds, rinsed with water and air dried. The stained slides were viewed under the microscope using immersion oil under x100 objective lens [8].

Indole Test

The test organism (isolate) was inoculated in a test tube containing 3ml of sterile tryptone water. Incubation was done at 37 °C for 24hours 0.5 ml of Kovac's reagent was added and shacked gently. The examination for a red ring like colour on the surface of the layer within 10 minute indicates the presence of *Escherichia coli* [9].

Catalase Test

This test is primarily used to distinguish among gram – positive cocci, members of genus *Staphylococcus* which are catalase -positive and *Streptococcus* which are catalase-negative. This was performed by dropping a loopful of hydrogen peroxide on a clean grease free slide followed by the mixing of the loopful of isolate with the hydrogen peroxide on the slide. The production of gas bubbles from the mixture which occurred almost immediately is a positive reaction, indicating the presence of *Staphylococcus species* [10].

Coagulate Test

A loop full of the test isolate was smeared on a slide, mixed with normal saline and treated with a drop of serum which is then mixed together. Agglutination or clumping occurs within 5-10 second which shows positive reaction for *Staphylococcus aureus* [11]. This test is used to differentiate *Staphylococcus aureus* from other *Staphylococcus* species. It is known as clumping factor, it cross-link the α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result individual coccus stick to each other and a clump is observed.

Methyl red and Voges-Proskauer test

About 5ml of glucose broth was inoculated and incubated for 48 hours at 37^{o}C, after the period of incubation, 1ml of the broth was transferred to small serological test tube, and 2 drops of methyl red was added. Following addition of the indicator, a red colour precipitate signifies a positive methyl red test. While a yellow colour precipitate signifies negative test. A heavy inoculum of the test organism was inoculated into Voges-Proskauer medium contained in different test tubes. The tubes were incubated at 37^{o}C for 48 hours. After which 0.5ml of alpha-naphthol was added followed by 0.5ml of 40% KOH. It was then agitated and allowed to stand for 30 minutes; a red color signifies a positive test Voges-Proskauer [8].

D. Antibacterial Sensitivity Test

Disc diffusion method

Disc of 5mm in diameter was punched out from a disc Whatman Number 1 filter paper. The disc was sterilized at 160°C for an hour in a dry oven and was allowed to cool to the room temperature after which 1ml of the different concentration of the honey extracts from the different dilution was pipette into different Petri dishes containing the disc and was placed 15mm apart from the edges of the plates to prevent overlapping of inhibition zones. The disc was allowed to dry for 15minutes following placing of the Petri dishes in an incubator for 24hours at about 37°C[8]. They were examined and the diameter of the zone of inhibition was measured in mm. In the determination of antibacterial activity, Augmentin antibiotic disc (30µg) was used as a positive control.

Determination of Minimum Inhibitory Concentration (MIC)

Honey batches were investigated for their MIC against the isolates where 1ml of honey sample was used in bifold dilution method as described by Quinn *et al.* 2004, with series of 5 tubes containing 1ml Mueller Hinton broth (Accumix – Verna, India) to achieve final dilutions of 100, 80, 60, 40, 20% v/v. Standard bacterial inoculums (5 x 10^{5}) of the test organisms was inoculated into all the 5 dilutions post thorough honey mix. The inoculated tubes were left overnight incubated at 37˚C. The dilution of the sample honey to inhibit growth (no turbidity in the tube) was considered as the MIC [12].

Determination of Minimum Bactericidal Concentration (MBC)

From, all tubes with no visible signs of growth (turbidity) MIC tube, loopful was inoculated onto sterile Mueller Hinton agar (Accumix – Verna, India) using streak plate method. The plates were incubated overnight at 37˚C. The least concentration that did not show any growth of tested organism was considered as the Minimum Bactericidal Concentration (MBC) [12].

III. Results and Discussion

The morphological and biochemical characteristics of the isolates are represented in Table 1. The microbiological and biochemical characteristics of the isolates conform to that described by Sarbojoy *et al.,* (2018) [13]. The result showed that the zone of inhibition (Z.I) followed a

Similar trend by decreasing with decreased honey concentration. Figure 1 shows the average zones of inhibition of honey sample from Adamawa on the test organisms. *Staphylococcus aureus* (13.6mm) has the highest average zone of inhibition compared to *Klebsiella pneumoniae* (12.8mm)

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and *Escherichia coli* (12.4mm) at 100%. There was decrease in average zone of inhibition in response to decrease in honey concentration where *Staphylococcus aureus* 80% (10.8mm) and 60% (6.4mm) and *Klebsiella pneumoniae* 80% (9.0mm) and 60% (4.6mm); and *Escherichia coli* 80% (9.4mm) and 60% (5.4mm). The control maintained 25.8mm average zone of inhibition on the test organisms. The average zone of inhibition of honey sample from Enugu is presented in Figure 2. The result showed that the average zone of inhibition on *Klebsiella pneumoniae* at 100% was 16.2mm; *Escherichia coli (E. coli),* however, showed lower average zone of inhibition (0 – 18.2mm) than *S. aureus* (0 – 20.0mm) and *Klebsiella pneumoniae* (0 – 19.0mm).

TABLE 1: Morphological and biochemical characteristics of isolated Bacteria

S/N	Colour	Elev	Shape	Arrang	Gram	Cat	Coa	Ind	Vp	Mr	Probable isolates
1	Yellow	Convex	Coccus	Irregular clustered	+	+	+	_	+	_	*Staphylococcus aureus*
2	Pink	Raised/Smooth	Rod	Single	_	+	_	_	+	_	*K. pneumoniae*
3	Pink	Raised/Flat	Short Rod	Single	_	+	_	+	_	+	*Escherichia coli*

Elev: Elevation; Arrang-Arrangement; Gram: Grams' Staining; Cat: Catalase, Coa: Coagulase; Ind: Indole, Mr: Methyl red; Vp: Voges-Proskauer; (+): Positive, (–): Negative

The antibacterial effect of honey sample on the test organism decreased as honey concentration decreases; and this was observed in all the isolates. This conforms to the finding by Badawy *et al.,* (2004) [14] that concentration of honey has an impact on antibacterial activity; the higher the concentration of honey the greater its usefulness as an antibacterial agent. The highest zone of inhibition at 100% concentration is expected. Murugan (2012) [15] conducted similar research and found out that honey inhibits bacterial growth more at its natural and undiluted state with highest inhibition zone of about 20 – 24mm for *Staphylococcus aureus* and *Escherichia coli*; and 18 – 20mm for *Klebsiella pneumoniae*. The average zone of inhibition of honey sample from Taraba is presented in Figure 3. The honey sample showed better antibacterial activity on the test organisms when compared to the honey sample obtained from the other two states (Figure 1 and 2).

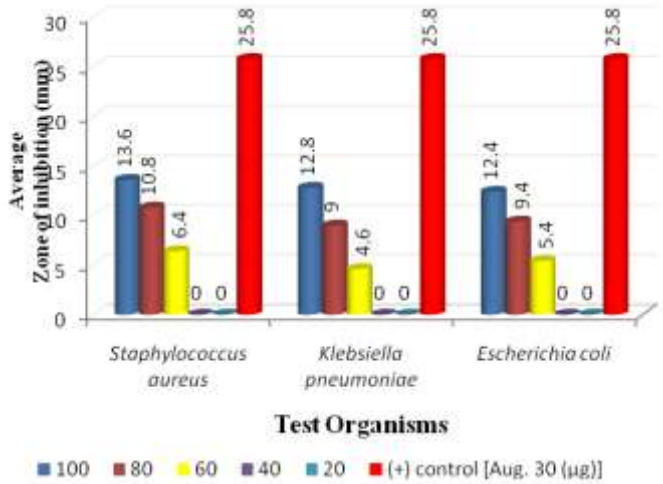

Fig. 1. Average Zone of inhibition of Honey sample from Adamawa State against the isolates at different concentration

The average zone of inhibition of honey samples from Taraba on the selected organisms were 20.0mm and 19.0mm for *Staphylococcus aureus* and *Klebsiella pneumoniae* compared to honey sample from Adamawa (*Staphylococcus aureus* 13.6mm; *Klebsiella pneumoniae* 12.8mm) and Enugu (*Staphylococcus aureus* 16.2mm; *Klebsiella pneumoniae* 15.4mm) at 100%. The average inhibitory effect of honey sample obtained from Taraba on *Escherichia coli* was similar to that obtained from Enugu. The honey sample from Taraba has the highest average inhibitory effects with zones of inhibition for *Staphylococcus aureus; Klebsiella pneumoniae*; and *Escherichia coli* ranging from 3.4 – 20.0mm; 2.4 – 19.0mm; and 4.4 – 18.2mm respectively.

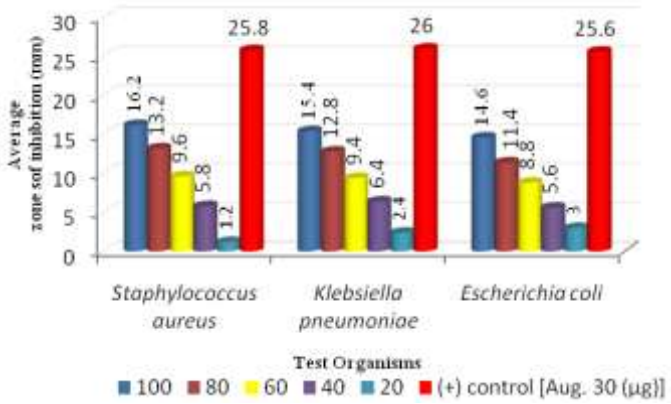

Fig. 2. Average Zone of inhibition of Honey sample from Enugu State against the isolates at different concentrations

The honey samples used in this study showed higher antibacterial activity on *Staphylococcus aureus* than *Klebsiella pneumoniae* and *Escherichia coli*. The reason for this attribute was not clear but agrees with other findings of Rahmanian *et al.,* (1970) [16]. Jeddar *et al.,* (1985) [17] previously found that honey inhibited the growth of bacteria at 40% concentration; and this is in conformity with the present finding on *Staphylococcus aureus*; *Klebsiella pneumoniae;* and *Escherichia coli* except for the honey sample from Adamawa where there was no visible action at 40% concentration. Any zone of diameter less than 7mm shows that the organism is resistant to the honey sample; but if the zone of inhibition is greater than 11mm, it suggests that the microorganism is sensitive to the honey sample [18]. At 20% concentration, none of the honey samples inhibited the microorganism. At 40% concentration, honey sample from Enugu and Taraba achieved highest average zone diameter of 5.8mm and 9.8mm on *Escherichia coli* respectively; followed by 5.8mm and 8.4mm on *Staphylococcus aureus*. This is almost similar to 8.3mm observed by Astrada *et al*., (2005)

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[19] for *Staphylococcus aureus*; but differs from results of Molan *et al.,* (1992) [20] whose findings revealed higher degree of sensitivity.

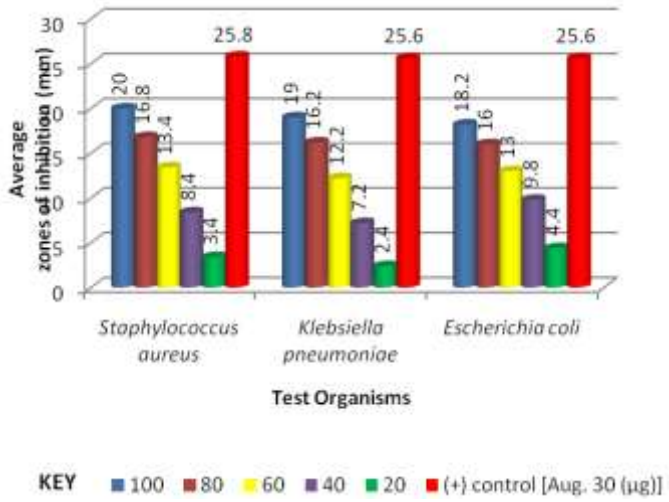

Fig. 3. Average Zone of inhibition Honey sample from Taraba State against the isolates at different concentrations

The minimum inhibitory concentration (Figure 4) is consistent for honey samples from Enugu and Taraba states at 20%; but higher MIC (60%) was observed for sample from Adamawa state. The MBC (Figure 5) indicated that two of the honey samples (from Adamawa and Enugu) have 100% as MBC with that from Taraba having 80% and this may be due to high sensitivity of the test organisms to the honey samples at that concentrations. At 20% concentration, there was heavy growth (+ + +) which signifies that the test organism was resistant to the honey sample.

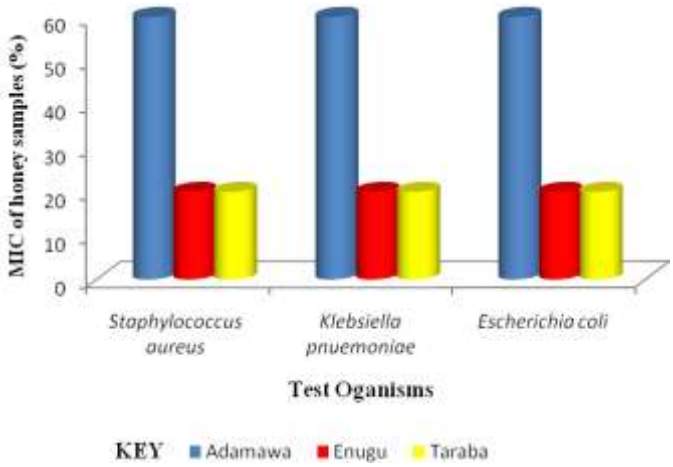

Fig. 4. Minimum Inhibitory Concentration (MIC) of Honey samples against isolates

The differences in sensitivity may be related to variation in the type of vegetation found in the three different locations with respect to geographical zones. The source of the nectar used in the production of the honey may have caused the differences in the antibacterial activities of honeys from the different sources as reported by (NHB, 1984) [21]. The difference in sensitivity can also be due to differences in growth rate of microorganism and also their nutritional requirements; this is also reported by Gail, and Jon, 1995 [19]. The honey sample used in this study exhibited different antibacterial activities on the test organisms. The result obtained from the study showed that honey sample from Taraba has highest antibacterial action followed by sample from Enugu state. This suggests that geographical factors can influence the inhibitory capacity of the honey tested.

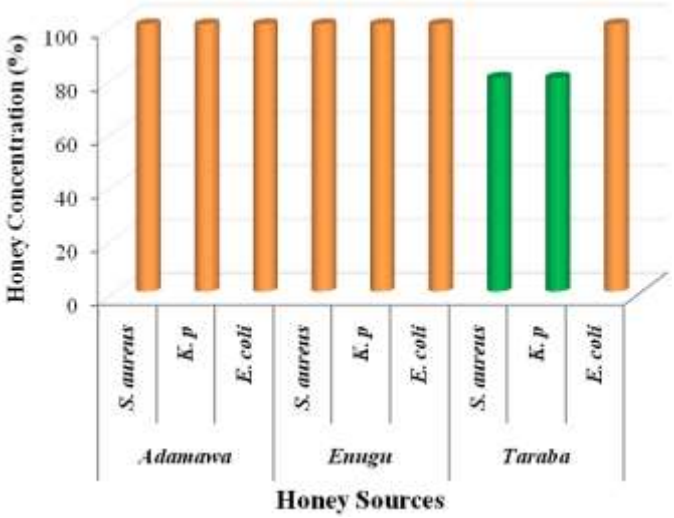

Fig. 5. Minimum Bactericidal Concentrations (MBC) of the three different honey samples against isolates

IV. Conclusion and Recommendations

The honey samples from Adamawa, Enugu and Taraba State exhibits different level of inhibitory activity on the test organisms and the maximum antibacterial activity was observed at 100% concentration. Therefore, honey have an antibacterial action; and can be used in treatment of wounds. The study recommends that 100% honey as a natural, nontoxic and less expensive antibacteria should be used in dressing as well as treatment of wounds. Hospitals and clinics should be stocked with pure and undiluted natural honey to be used in treatment of burns or wounds. The old practice of using honey to treat burns/wound at low cost and as an excellent alternative antimicrobial agent should be encouraged. Further work should be carried out on extraction of honey and their antibiogram.

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