THE INHIBITION POTENTIALS OF DIFFERENT HONEY AGAINST STAPHYLOCOCCUS AUREUS, ESCHERICHIA COLI AND BACILLUS SPECIES ISOLATED FROM CLINICAL SOURCE

Abstract

As a result of the increased prevalence of antibiotic resistance among different bacteria, different plants and other natural products have been studied and found to be highly effective against pathogenic bacteria. Honey, over the years has been used as an antibacterial agent to treat certain infections caused by bacteria and is believed to be effective especially in rural areas. This study was thus aimed at comparing the effect of different honey samples against some pathogenic bacteria (Escherichia coli, Staphylococcus aureus, and Bacillus cereus) isolated from clinical source. This study was carried out in the microbiology laboratory, department of microbiology Rivers State University Nigeria from January 2018 to August 2019.The antibacterial sensitivity test was carried out using agar well diffusion method while the Minimum inhibitory concentration and Minimum bactericidal concentration were determined using broth tube micro dilution technique in two fold dilution. The inhibition efficiency of the honey samples on the test organisms increased with increase in concentration from 20 to 100% as 100% concentration had the highest zone of inhibition. Staphylococcus aureus (6.33mm – 26.33mm) was the most sensitive to the honey samples while Bacillus cereus (0.00 – 19.67mm) was less sensitive. At concentrations of 20 – 80%, raw and Rowse honey were more effective on E. coli compared to Princenic Global honey, while at 100%, Princenic Global honey was more effective on Staphylococcus aureus. Raw and Rowse honey were more effective at 20 -60% concentrations followed by Princenic Global honey; whereas at 80 -100% concentrations, Raw and Princenic Global honey were more effective. Bacillus cereus was resistancet to the honey samples at 20 –

60% but sensitive at 80 – 100% concentrations to Rowse, Raw and Princenic Global honey. The inhibition efficiency of the honey samples on the growth of the tested organisms was found to be dependent on the concentration and type of honey used, as well as they type of organism tested. The result of the minimum inhibitory and minimum bactericidal concentration showed that Staphylococcus aureus was inhibited most at a lower concentration of 25% compared to other bacterial isolates. All honey samples tested did not show any bactericidal effect but was bacteriostatic to some of the tested organisms. Pharmacological standardization and clinical evaluation on the effect of honey is essential before honey can be used as a preventive and curative measure to common diseases related to the tested bacterial species.

Keywords: Honey, antibiotic resistance, antimicrobial agents, minimum inhibitory concentration,

INTRODUCTION

European Union Council Directive defined honey as the natural sweet substance produced by Apis mellifera bees from the nectar of plants or from the secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants which bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honey combs to ripen and mature[1]. Bogdanov in their research stated that honey is the only food sweetener that can be used industrially without processing. Honey can be classified according to its origin (such as nectar or honey dews), mode of production and preservation [2].

Honey as a concentrated aqueous solution composed of a mixture of glucose and fructose but also contains at least 22 other complex carbohydrates, various amino and organic acids, proteins, enzymes, phenol antioxidants, vitamins, minerals, pigments, waxes and pollen grains [3]. In nature honey is very viscous and acidic ranging between 3.2 and 4.5. in pH. Over the years

around the world, honey has been effectively used as medicine most especially as traditional remedy in so many countries. Most of the ancient countries that have used honey as a traditional remedy include Egyptians, Assyrians, Chinese, Greeks and Romans. This countries employed honey for wounds and diseases of the gut [4]. Over the years, many researchers have reported the antibacterial activity of honey and found that natural unheated honey has broad-spectrum antibacterial activity when tested against some pathogenic and oral bacteria [5].In some developed countries, honey is used for the treatment of ulcers, bed sores and other skin infections resulting from burns and wounds [6].

Lusby in their research indicated that the healing properties of honey can be attributed to the fact that it offers antibacterial activity, keeps the wound environment moist which promotes fast healing and has a glueyness which helps to provide a defensive barrier to prevent infection [7]. Many researchers have shown that the properties present in honey that is responsible for the antibacterial activity is the phytochemical properties such as high content of reducing sugar, high viscosity, high osmotic pressure, low pH, low water activity, low protein content and presence of hydrogen peroxide[8]. Again Alnimat stated that the main antibacterial agent in honey is hydrogen peroxide, which is produced by glucose-oxidase action[9]. The level of hydrogen peroxide in honey is determined also by the presence of catalase, which originates from the pollen of plants. Light, temperature and oxygen affect the amount of hydrogen peroxide which shows a discrepancy according to the processing and storage conditions of the honey. Research has revealed a positive correlation between the endogenous hydrogen peroxide concentration and the inhibitory activity of bacterial growth by honey[10]. Indeed honey with a high concentration of hydrogen peroxide has s higher antibacterial activity.

Libonatti and his group reported that the antibacterial activity of honey is due entirely to the non-peroxide components such as acidity, osmolarity, flavonoids, phenolic compounds and lysozyme[11]. Different studies have claimed that honey contains bioactive components such as lysozyme, a well-known antibacterial agent[12]

Abd-El Aal when comparing the inhibitory activity of honey and some commonly used antimicrobial agents on some gram-negative bacteria (Pseudomonas aeruginosa, Enterobacter spp, and Klebsiella spp.) showed that honey had a pronounced inhibitory activity of 85.7%. A 100% inhibition was observed in the case of gram positive methicillin resistant Staphylococcus aureus in comparison to the use of antibiotics alone[13].

Kwakman and Zaat reported that the sugar content of honey is sufficient to retain antibacterial activity when diluted to approximately 20-40%. Based on extensive research on the medicinal uses of honey, antimicrobial action of honey on Staphylococcus aureus, Escherichia coli and Bacillus cereus, isolated from open wound were investigated[14].

MATERIALS AND METHODS

MATERIALS

Collection and preparation of samples.

A total of three honey samples (Princenic Global, Rowse and Raw) were used in this study. Princenic Global (PG) and Rowse honey were bought from supermarket in Port- Harcourt metropolis and Raw honey was bought from local bee keepers in Etche local Government Rivers state, Nigeria. The samples were stored in sterile bottles at temperature of 20 – 21^{o}C in a dark

place before analyses. These honey samples were selected because they are widely sold in the supermarkets around the area and they are regularly consumed by people around the area.

Collection and confirmation of bacteria isolates

Bacterial isolates used in this study were wound associated bacteria including Staphylococcus aureus, Escherichia coli and Bacillus cereus. The isolates were obtained from Optimal Diagnostic Center, Mgbuoba, Port-Harcourt. The isolates were collected in sterile Bijou bottles containing nutrient broth and immediately incubated at a temperature of 37^{o}C for 24 hours. The isolates were identified microscopically and biochemically using tests such as Grams Stain, catalase, simmon Citrate utilization, indole, motility, methyl Red-Voges proskauer, oxidase, sugar fermentation, starch hydrolysis, coagulated, hemolysis and spore stain.

Methods

Antibiotics sensitivity test: This test was performed using disc diffusion method[15]. The test organisms were first standardized. Five colonies from fresh 24 hours culture were aseptically transferred to a 4 ml sterile normal saline, the suspension was compared with 0.5 McFarland prepared by adding 0.05ml of 1% Barium Chloride (BaCl) to 9.95ml of 1% sulfuric acid. The standardized isolates were then streaked on the surface of a large Mueller-Hinton agar plate and allowed to dry for 5 min. Eight (8) commercially-prepared, fixed concentrations, Abtek paper antibiotic disks were placed on the inoculated agar surface using sterile forceps. The antibiotics used include; Ceftazidime (30µg), Cefuroxime (30µg), Gentamicin (10µg), Ceftriaxone (30µg), Erythromycin (5µg), Cloxacillin (5µg), Ofloxacin (5µg), Augmentin (30µg), Cefixime (5µg), Nitrotrantion (300µg), Ciprofloxacin (5µg). Plates were incubated at 37^{o}C for 24 hours .After incubation the zones of growth inhibition around each of the antibiotic disks was measured to the

nearest millimeter. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute[15]

Antibacterial Sensitivity Test of Honey: the antibacterial activity of honey samples was tested invitro against three pathogenic bacteria (Staphylococcus aureus, Escherichia coli and Bacillus cereus) using agar well diffusion method[16][17]. The honey samples were prepared by diluting each in sterilized distilled water at different dilutions (concentration), 20%, 40%, 60%, 80% and also net honey (100%). Concentrations were achieved by adding 80 ml of distilled water to 20ml of honey sample (20%), 60ml of distilled water to 40ml of honey, 40ml of distilled water to 60ml of honey, 20ml of distilled water to 80ml of honey, and lastly 100% was pure undiluted honey.

Mueller-Hinton agar plates were prepared and each plate was properly inoculated with each test organism using a sterile swab stick dipped into the inoculum suspension and spread over the surface of the medium. Wells were made using a sterile cork borer and each well was filled with different concentrations of the honey. A distance was maintained from the edges of the plates to prevent overlapping of the inhibition zones. The plates were incubated for 24 hrs at 37°C. After incubation the plates were examined and the diameter of the inhibition zones was measured in triplicate for each isolate.

Minimum Inhibitory Concentration (MIC): the minimum inhibitory concentration of the honeys was determined using broth tube micro dilution method[18]. The purpose of this test was to determine the minimum concentration of honey that can inhibit growth of the test organisms. Eight (7) sterile test tubes were placed in a rack and labeled 1 to 5. Honey control tube (HC) and growth control tube (GC) were used as quality controls. One ml (1 ml) of freshly prepared nutrient broth was added to each tube, sterilized and cooled. Then 1 ml of undiluted honey

solution (100 %) was added to test tube number 1 and honey control with a sterile micropipette and tips. Then serial twofold dilution was performed by transferring 1 ml undiluted honey into the second tube with separate sterile micropipette. After a thorough mixing, 1 ml of the honey sample was transferred with another sterile micropipette from tube 2 and tube 3. This procedure continued until six tubes with a dilution of 1:125 was reached and finally 1 ml was taken and discarded from tube 5. The growth control tube received no honey was served as a growth control while the HC tube received no bacterial inocula served as honey control.

Except the honey control tube, each tube was inoculated with 1 ml of the culture of respective prepared organism. The procedure was repeated for all the organisms tested to each of the honeys. Tubes were then incubated at 37 °C for 24 hours and observed by visual inspections for the presence and absence of growth (turbidity).

Minimum Bactericidal Concentration (MBC): the test was done to determine the minimum concentration of honey that can kill the test isolates. To determine the MBC, incubated tubes showing no visible sign of growth/turbidity in MIC, were sub-cultured onto sterile nutrient agar plates by streak plate method and incubated at 37 °C for 24 hours aerobically. The least concentration of honey that did not show growth of test organisms was considered as the MBC [18]. Then inoculated plates were scored as bactericidal if no growth; bacteriostatic if there is light to moderate growth and no antibacterial activity if there is heavy growth[18].

Statistical analysis: Results obtained were expressed as mean ± standard deviations and differences between means were analyzed statistically using analysis of variance (ANOVA) on the SPSS version 22.0; ddifferences were considered significant when $p<0.05$ and where differences occurred, Tukey method was used to separate the means.

RESULTS AND DISCUSSION

Table (1) showed the antibiotics sensitivity of the bacteria isolates. Ofloxacine showed the highest zone of clearing on Staphylococcus while Cloxacillin and ceftazidime did not have any effect on Staphylococcus aureus, this means that the organism was resistant to Cloxacillin and ceftazidime. Again for Bacillus cereus, Ofloxacin also showed the highest zone of inhibition of 25mm while Cloxacillin and ceftazidime showed no zone of clearing meaning the organism was resistant to these antibiotics. Escherichia coli were resistant to Augmentin and Cefuroxime while Ciprofloxacin was more effective with inhibition zone of 24.7mm.

Table 1. Antibiotics Sensitivity Pattern Exhibited by Test Bacterial Isolates

Isolates	Ofloxacin(5µg)	Augmentin(30 µg)	Nitrotrantion(30 µg)	Ciprofloxacin(5 µg)	Ceftazidime(30 µg)	Cefuroxime(30 µg)	Gentamycin(10 µg)	Cefixime(5 µg)	Ceftaxone(30 µg)	Erythromycin(5 µg)	Cloxacillin(5 µg)
Escherichia. coli	S	R	S	S	I	R	S	S	-	-	-
Staphylococcus Sp	S	S	-	-	R	S	S	-	S	S	R
Bacillus Sp	S	I	-	-	R	I	S	-	S	S	R

Keys;

R = resistance (≤13)

S = sensitive (≥17)

I = intermediate (≥14-16)

Figure (1) showed the effects of different concentrations of honey samples on the growth of Escherichia coli. From this figure, PG honey at concentrations of 20, 40, 60, 80 and 100% gave 0.00, 3.67, 12.00, 20.33 and 31.00 mm zones of inhibition, respectively. Also raw honey at the same concentrations gave 0.00, 6.67, 17.67, 22.67 and 29.33 mm zones of inhibition, respectively while Rowse honey at similar concentration on Escherichia coli gave 1.33, 8.33, 15.33, 21.67 and 29.67 mm zones of inhibition, respectively.

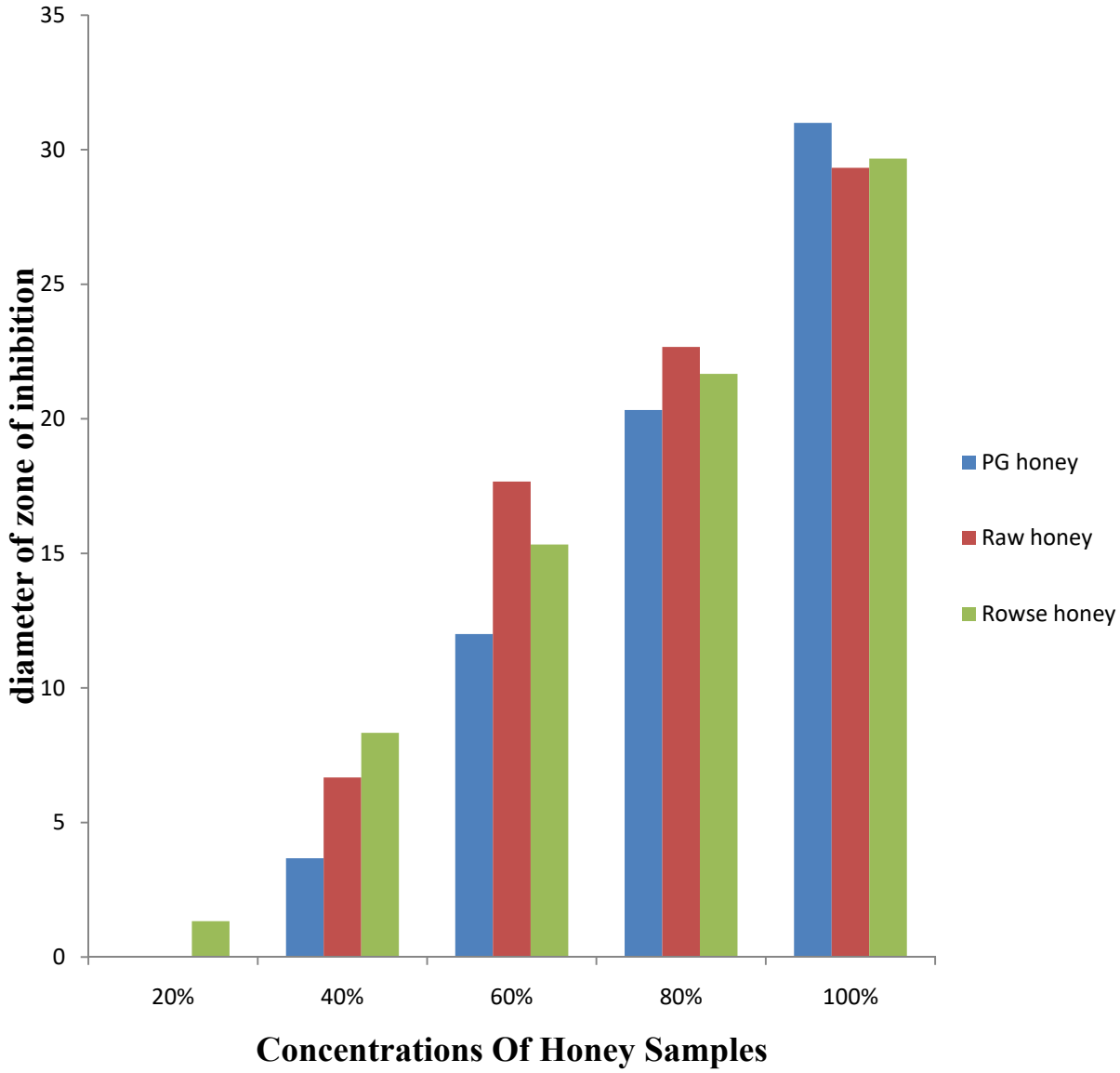

Fig 1: Effects of different concentrations of honey samples on the sensitivity pattern of Escherichia coli as shown by the diameter of the zones of inhibition.

Figure (2) showed the effect of different concentrations of honey samples on the growth of S. aureus. It showed that PG honey gave zones of inhibition of 6.33, 10.33, 15.67, 21.00 and 28.67 mm at concentrations of 20, 40, 60, 80 and 100%, respectively while on Raw honey at similar concentration gave zones of inhibition of 8.00, 13.00, 18.67, 23.67 and 29.67 mm, respectively. Furthermore, the effect of Rowse honey on the growth of S. aureus at concentrations of 20, 40, 60, 80 and 100% gave 7.67, 10.00, 15.33, 18.33 and 26.33 mm zones of inhibition, respectively.

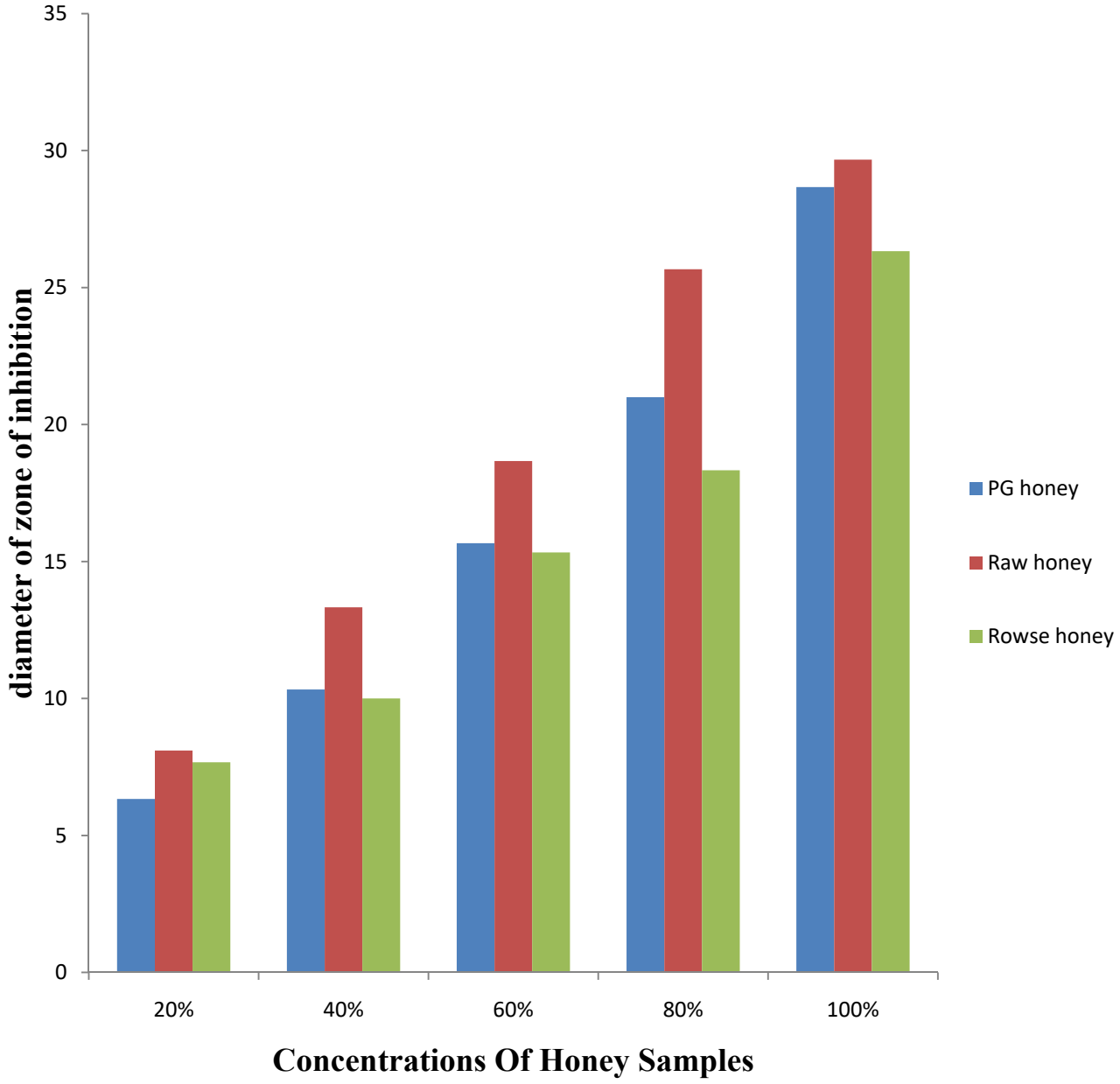

Fig 2: Effects of different concentrations of honey samples on the sensitivity pattern of Staphylococcus aureus as shown by the diameter of the zones of inhibition.

Figure (3) showed that only 80 and 100% concentrations of the honey samples were effective on B. cereus. PG honey was effective on B. cereus at a concentration of 100% with zone of inhibition of 1.33 mm, while Raw honey inhibited its growth at 80 and 100% concentrations with zones of inhibition of 2.33 and 7.00 mm, respectively. Also, Rowse honey inhibited at 80 and 100% concentrations with inhibition zones of 9.67 and 14.67mm, respectively.

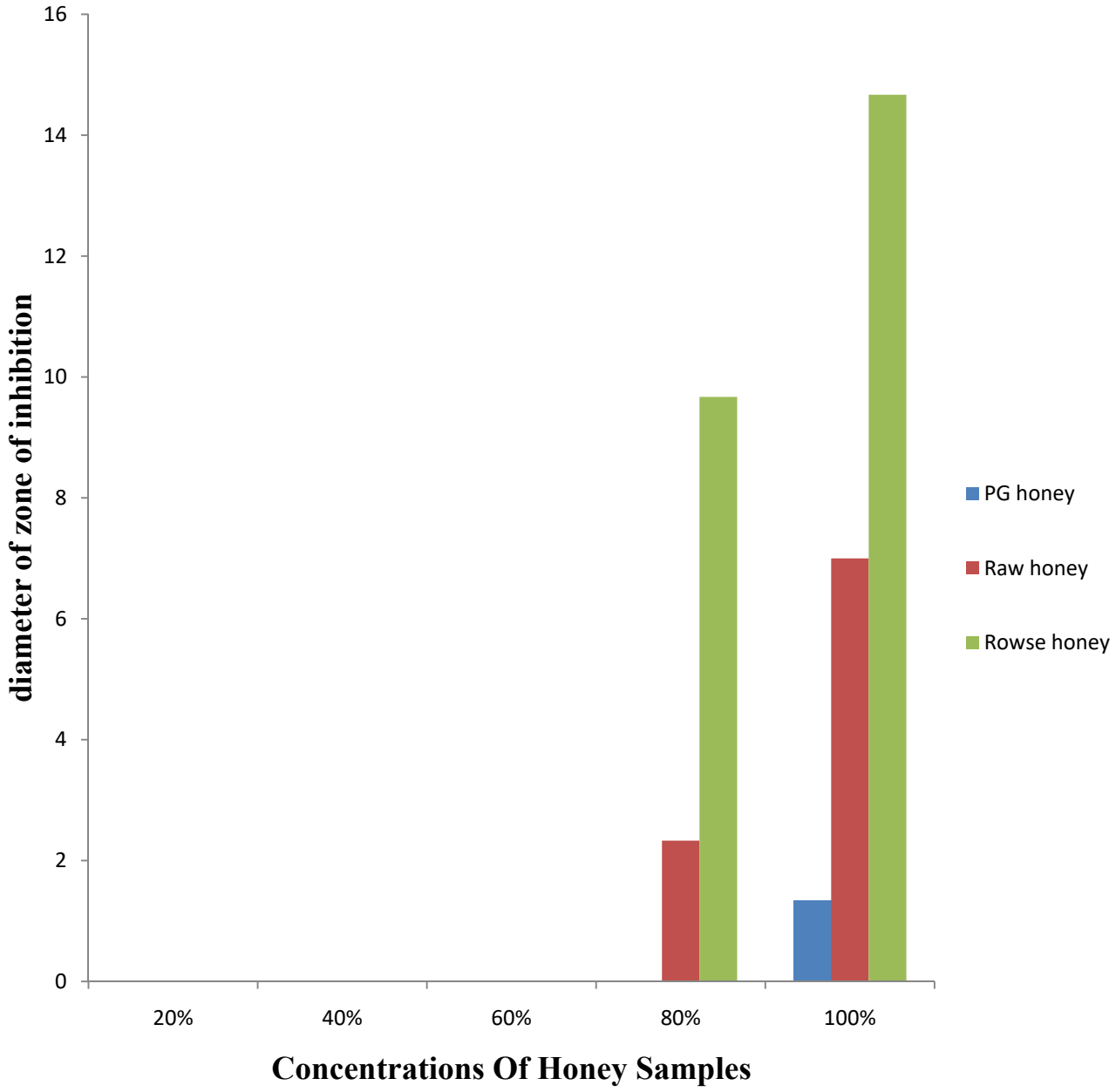

Fig 3: Effects of different concentrations of honey samples on the sensitivity pattern of Bacillus cereus as shown by the diameter of the zones of inhibition.

Table (2) showed that all the three honey types inhibited the growth of the bacterial isolates at a minimum concentration of 50%. This indicated that the bacterial isolates were sensitive to the honey samples at 50% concentration while resistant to honey samples at the rest concentrations.

Table 2. The Minimum Inhibitory Concentration of Honey Samples at Different Concentrations against bacterial isolates

	Concentrations of honey (%)														
Honey samples	E.coli					S. aureus					Bacillus				
	50	25	12.5	6.5	3.1	50	25	12.5	6.5	3.1	50	25	12.5	6.5	3.1
PG	S	R	R	R	R	S	S	R	R	R	S	R	R	R	R
Rowse	S	R	R	R	R	S	S	R	R	R	S	R	R	R	R
Raw	S	R	R	R	R	S	S	R	R	R	S	R	R	R	R

KEYS; S = Sensitive, R = Resistant

Table (3) showed that all the honey samples were not bactericidal to the bacterial isolates except PG honey which was bacteriostatic to Staphylococcus aureus at 50%.

Table 3; The Minimum Bactericidal Concentration of Honey Samples against Bacteria Isolates at Different Concentration.

	Escherichia coli		Staphylococcus aureus		Bacillus	
Honey samples	50(%)	25(%)	50(%)	25(%)	50(%)	25(%)
PG	++		+	++	++	
Rowse	++		++	++	++	
Raw	++		++	++	++	

Keys; NS= Non-sterilized; ++= not bactericidal (heavy growth), += bacteriostatic (light growth)

Discussion

Results of this study showed that Rowse honey inhibited the growth of Escherichia coli at the lowest concentration compared to other honey types. At 100% PG honey had higher inhibition zone on Escherichia coli with zone in diameter of 31.00mm, whereas Staphylococcus aureus at 20% had inhibition zone of 6.33mm by PG honey, while Bacillus was not inhibited at 20%. Comparison of this honey types showed that Staphylococcus aureus was most inhibited by the honey types at lowest concentration followed by Escherichia coli and Bacillus. Again Rowse honey was most effective at the lowest concentrations against Escherichia coli and Bacillus while Raw honey was most effective against Staphylococcus aureus at lowest concentration. The wide-ranging inhibition level of the honey samples is due to the fact that different honey types possess different efficacies against the same type of bacterium and different bacteria[19]. Reports have shown that the ability of honey to inhibit microbial growth is not only due to osmolality, viscosity, presence of hydrogen peroxide and low protein contents but also due to other factors that affect the composition of honey[19]. Such factors depend on a great extent on the bee's source, the location of the flowers and related weather conditions, the storage time and conditions and the method of preservative treatment[20]

The results of this study was in agreement with the study performed by Al-Haj who used Malaysian honey on both methicillin sensitive Staphylococcus aureus and methicillin resistant Staphylococcus aureus. They concluded that honey completely inhibited the growth of the two bacteria[21]. Also, the reports of this study is in consonance with the study by Taormina, where they investigated the antibacterial activity of honey from six floral sources against Escherichia coli, Salmonella thyphimurium, Shigella sonnei, Staphylococcus aureus and Bacillus cereus using

disc diffusion method. Their results showed that the development of inhibition zones depended on the concentration of the honey used as well as the test pathogen; their result showes that B. cereus was least inhibited while S. aureus was most inhibited by the different honey samples[22].

Conclusion

The results of this study showed an increase in concentrations of honey samples increased their inhibitory effects on the test isolates. Also, among the three studied pathogenic bacteria, E. coli was the most inhibited with 29.33, 29.67 and 31.00 mm zones of inhibition by Raw, Rowse and PG honey samples, respectively while B. cereus was the least inhibited with 1.33, 7.00 and 14.67 mm zones of inhibition by PG, raw and Rowse honey samples, respectively. Comparison of the results of the figures showed that PG honey was most effective on Escherichia coli with zone a inhibition of 31.00 mm while on S. aureus Raw honey was the most effective with a diameter of 29.67 mm. Also, Rowse honey showed higher efficiency on B. cereus with inhibition diameter of 14.67mm. Although, the three honey samples exhibited varied inhibitory effects on the same bacterium and the different bacteria, all three samples were found to have antibacterial effects against the isolates. This further proves that honey is a potent antibacterial agent and could be used in place of synthetic antibiotics if properly standardized especially with the rising occurrence of antibiotic resistance among synthetic drugs.

Competing Interest

Authors declare that there is no competing interest.

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