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Research Article
Antibacterial Activity of Raw and Branded Honey Infused with Moringa, Ginger and Garlic on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Abstract
Background and Objective: Honey is a natural product known to have antibacterial activity associated with the composition of honey. The study was carried out to compare the antibacterial activity of raw honey and branded commercial honey infused with moringa, ginger and garlic on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. **Materials and Methods:** Ten honey samples were subjected to physicochemical analysis and further screened for the antibacterial activity by agar well diffusion method at various concentrations ranging from 100, 75 and 50%.The samples were first investigated on their ability to inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from wound infections. The activity of the honey samples after the neutralization of hydrogen peroxide with the enzyme Proteinase-k was determined. **Results:** Physicochemical results were in accordance to international standards. Significant antibacterial activity 14-20 mm zone of inhibition was observed from branded honey samples with infused with moringa, ginger and garlic, respectively. Weaker antibacterial activity was observed in branded honey samples without infusions ranging 6-12 mm for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. After treatment with Proteinase-k, antibacterial activity reduced ranging from 6-10 mm for some unbranded honey samples, while branded honey samples with infusions of ginger, garlic and moringa had a zone of inhibition from 10-14 mm. Antibacterial activity was not observed in three honey samples after treatment. **Conclusion:** The study concludes that the antibacterial activity of honey increase when it is combined with moringa, ginger or garlic. The active components in these natural products can be combined in manufacturing drugs and wound healing for effective therapy.

Key words: Antibacterial, garlic, ginger, honey, moringa, peroxide, physicochemical, protinase-k

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Antibiotics resistance has become a major challenge worldwide, which has led to an increase in the resistance of organisms to conventional antibiotics. From ancient times honey has been used for traditional medicine to treat wound infections and other health related issues. Thus the need for further studies on honey in combination with other natural products such as moringa, ginger and garlic as an alternative antimicrobial therapy for wound infections and purposely use the active components to manufacture drugs that can be used on resistant organisms with less side effect[1-3].

Honey is a natural product that is produced by honeybees from the nectar of plants. It has been consumed for many years now because of its nutritive and therapeutic potential. The unique composition of honey is responsible for its nutritive and therapeutic abilities[2-3]. Previous studies have shown the broad spectrum activity of honey which was attributed to the acidity (low pH), osmotic effect, high sugar concentration, presence of bacteriostatic and bactericidal factors (hydrogen peroxide, antioxidants, lysozyme, polyphenols, phenolic acids, flavonoids, methylglyoxal and bee peptides) and increase in cytokine release and to immune-modulating and anti-inflammatory properties of honey as reported by Carina *et al.*[4]. The main antibacterial factor in honey is hydrogen peroxide which is produced by the glucose-oxidase action[5]. Honey has shown to have a positive effect on humans based on its antioxidants, bacteriostatic, anti-inflammatory wound-healing effects and antimicrobial potential[6,7]. Despite the fact that it's said that no other product should be added to honey, Alemseged *et al.*[8] and Mathai *et al.*[9] studied the antibacterial activity of the mixture of honey and other natural products such as ginger garlic and lemon extracts. Garlic had the highest inhibitory effect and increased the efficacy of honey, when the honey was used alone which was not effective on *Streptococcus mutans* that causes dental infection Manuka honey which is certified medical-grade honey is applied on burns and wounds and its effective on methicillin-resistant organisms. It has high potency even when it's not combined with any other product. The floral source of manuka honey is responsible for its high level of activity.

The use of conventional antibiotics to treat infections can result in antibiotic resistance of some organisms which is due to the excessive and abuse of antibiotics. To this effect natural products such as honey, ginger, garlic and moringa with known antibacterial potential can be used to produce therapeutics that are effective on resistant organisms with minimized side effects. The study aimed to determine the non-peroxidase activity of Nigerian honey and also to compare the physicochemical parameters and antibacterial activity of raw honey and honey infused with moringa, ginger and garlic on *Staphylococcus aureus and Pseudomonas aeruginosa* from the wound.

MATERIALS AND METHODS

Study area: The study was carried out in the Medical Microbiology Laboratory of the University of Port Harcourt from March-November, 2019.

Sample collection: Ten honey samples were used for this study, six of which were branded and 4 were unbranded from Calabar, Enugu and Ibadan, respectively. Three (3) of the honey samples were infused with ginger, garlic and moringa, respectively. The honey samples were stored in a closed cupboard and retrieved when needed for the study.

Sources of isolates used: This study was carried out in the Medical Microbiology Laboratory, University of Port Harcourt. Stock isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from the wound was obtained from the Medical Microbiology and Parasitology Laboratory, University of Port Harcourt Teaching Hospital, Rivers State Nigeria. The organisms were re-identified morphologically and biochemically by standard microbiological techniques.

Determination of physicochemical parameters: Selected physicochemical parameters of the honey samples such as moisture content, total sugar, flavonoid content and pH were determined by the methods adopted by Agbagwa *et al.*[10].

Microbial population and preparation of honey concentrations: Honey samples were prepared in 75, 50 and 25% concentration by diluting in sterile distilled water. Microbial population of honey samples was determined by the spread plated method. The plates were incubated at 37°C for 24 hrs.

Detection of antibacterial activity of honey: Susceptibility testing was performed by Kirby-Bauer disk diffusion technique adopted from the study of Allen *et al.*[11]. The inoculums were prepared in sterile normal saline and compared with opacity standard on 0.5 McFarland standards. 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 (6 mm diameter) wells was made in the agar medium. 50 μL of honey with the various concentration of was added to the wells in the plate. The plates were incubated at 37°C for 24 hrs. The mean diameters of inhibition zones were measured in mm.

Bacteriostatic and bactericidal effect of honey samples: The bacteriostatic and bactericidal concentrations of the selected honey samples were determined for each isolate by the tube dilution method as described by Aamer *et al.*[12] and Gambo *et al.*[13]. The MIC of the honey was determined using broth dilution technique.

Detection of non-peroxide activity of honey: The honey samples with remarkable inhibiting activity were treated with an enzyme, proteinase-K to investigate the significance of a possible bioactive polypeptide and H_2O_2. The 500 μL of each honey sample (75% honey) was pipetted into two Eppendorf tubes. One sample was added 20 μL of a 30 mg mL^{-1} proteinase-K solution K (CAS No. 39450-01-6, SIGMA-ALDRICH) for a 1 mg mL^{-1} solution. All samples were incubated at 37°C for 2 hrs before being filled in the wells and tested as described previously. The experiment was carried out on the isolates that showed remarkable inhibiting activity.

RESULTS

Physiochemical parameters of selected Honey samples: The honey samples used were analysed for its physiochemical properties and all honey samples fell within the acceptable ranges as shown in Table 1. The moisture content ranged from 11.15-24.85%, pH (acidity content) ranged from 3.50-4.05, fructose level ranged from 31.67-45.01% and Total sugar had values of 43.61-99.10 % and Flavonoid concentration 57.07-73.83%.

Microbial count of honey samples: Microbiological analysis of the honey samples were done with the two-fold serial dilution. Bacteria analysis for the Colony Forming Units (CFU) count resulted in 1.20×10^2 to 2.52×10^3. A comprehensive result of bacterial count is shown in Table 2.

Antibacterial activity of honey: Figure 1 and 2 shows the antibacterial potential of branded and unbranded honey samples on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The branded honey samples (A_1, A_2, A_3) infused with moringa, ginger and garlic had the highest antibacterial activity compared with other unbranded honey without moringa, ginger and garlic. The honey samples were less active on *Pseudomonas aeruginosa* especially with concentrations of 50 and 75%. For both organisms the honey samples were more effective when used undiluted. The unbranded honey samples were not effective on *Pseudomonas aeruginosa* except for sample C_2 at 100% as shown in Fig. 2.

Non-peroxide inhibitory potential of honey samples: The contribution of protein content in the honey samples were evaluated against *Staphylococcus aureus* which was more susceptible to the honey samples. All the honey samples showed varying inhibitory effects when hydrogen peroxide was neutralized. Honey samples infused with moringa, garlic and ginger (A_1-A_3) had significantly reduced activity after Proteinase-k treatment (Fig. 3).

The different honey samples were used to ascertain the bactericidal (MBC) and bacteriostatic (MIC) effect of the most sensitive strain of *Staphylococcus aureus.* Bacteriostatic activity was highly effective at 50v/v, showing light bacterial growth while at 3.125 v/v, it showed ineffectiveness against the inhibition of the bacterial growth exhibiting heavy growth. The highest bacteriostatic results were noted in sample A_1-A_3 and C_4, while the lowest bacteriostatic effect were recorded in B_3, C_1, C_2 and C_3, None of the honey samples used in this research exhibited bactericidal activity.

Table 1: Mean values of physicochemical parameters of selected honey samples

Honey samples	Moisture content (%)	pH	Fructose (%)	Total sugar (%)	Flavonoid (%)
A_1	20.45	4.00	36.67	71.27	68.44
A_2	16.75	3.80	38.34	76.00	66.47
A_3	24.85	3.80	35.00	59.01	67.18
B_1	16.49	3.97	36.67	94.28	57.07
B_2	16.05	3.65	40.00	43.61	67.79
B_3	16.24	3.85	40.00	79.65	64.22
C_1	13.14	4.00	45.01	72.36	67.44
C_2	15.15	4.05	41.67	99.10	67.49
C_3	12.19	3.50	37.50	88.73	73.83
C_4	11.15	3.65	31.69	96.63	73.53

A_1: Honey infused with Moringa, A_2: Honey infused with garlic, A_3: Honey infused with ginger, B_1: Natural Honey, B_2: Divine Honey, B_3: PG Honey, C_1: Enugu 1, C_2: Enugu 2, C_3: Ibadan, C_4: Calabar

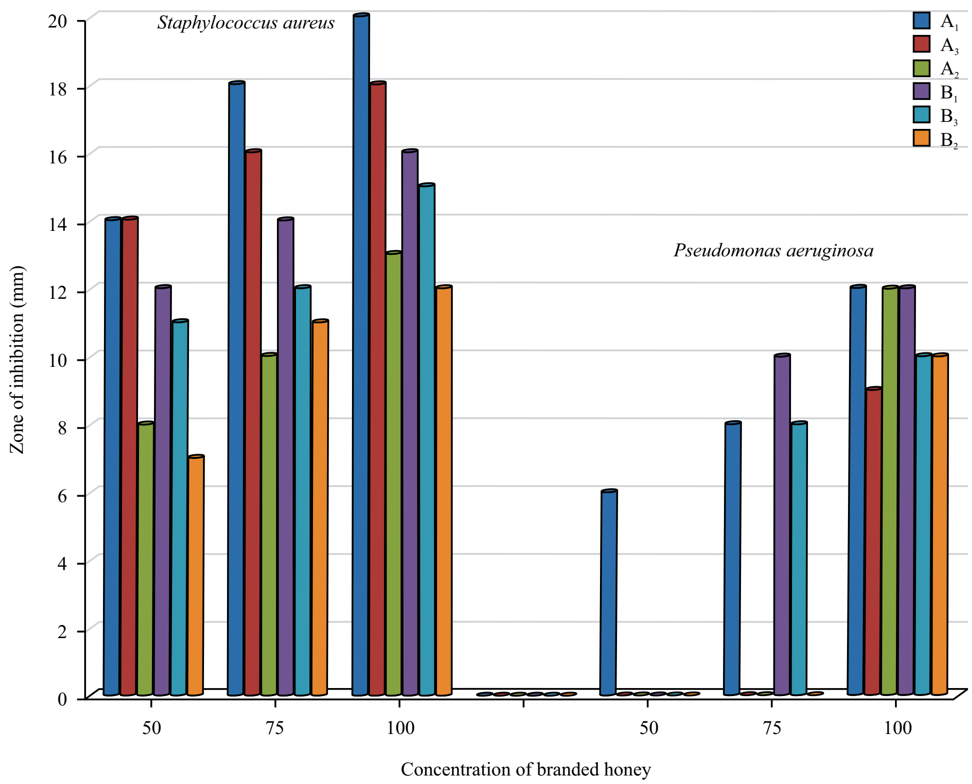

Fig. 1: Antibacterial activity of branded honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

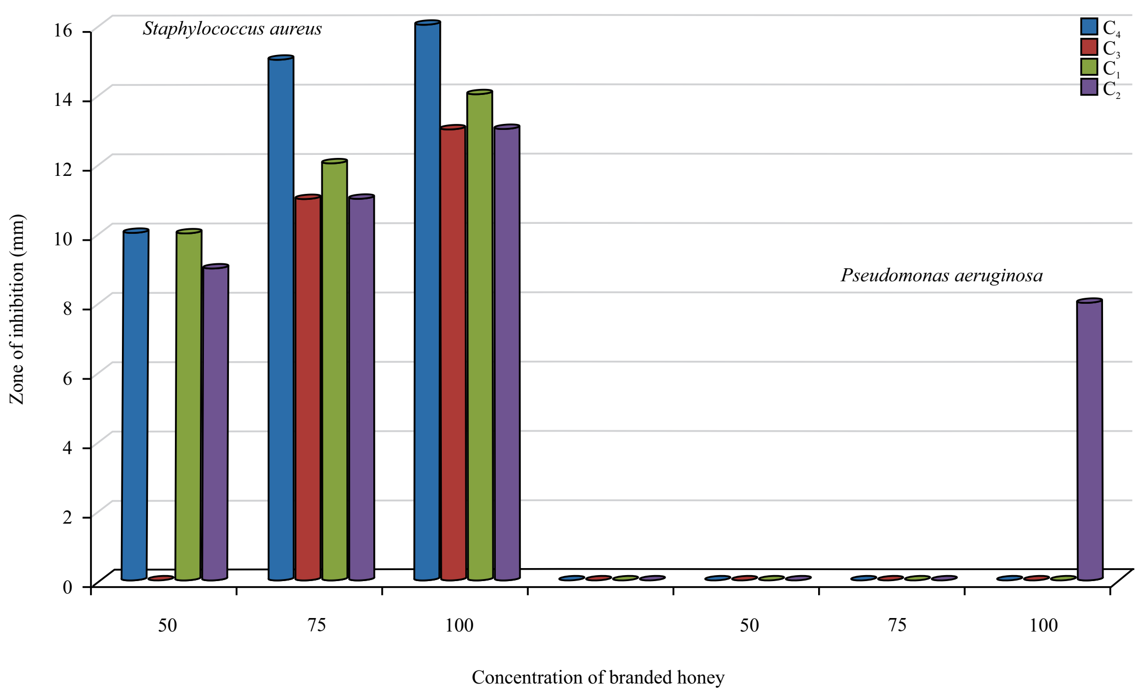

Fig. 2: Antibacterial activity of unbranded honey on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

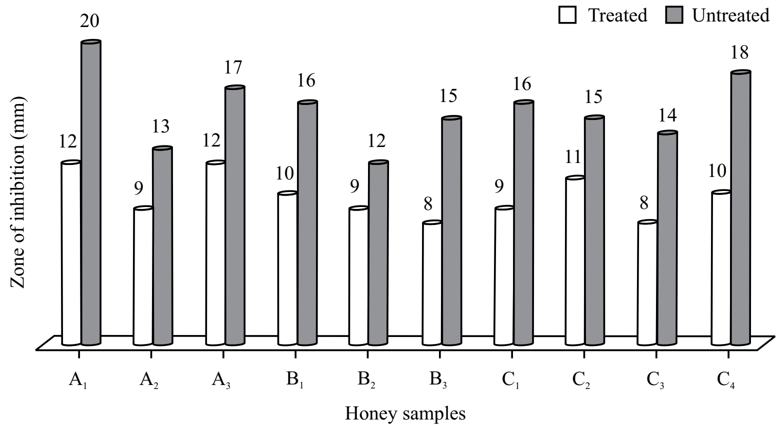

Fig. 3: Non-peroxide potential of honey samples

Table 2: Bacterial count of honey samples

Honey sample	10^{-1} Cfu mL^{-1}	10^{-2} Cfu mL^{-1}
A_1	1.20×10^2	0.00×10
A_2	2.52×10^3	1.59×10^4
A_3	1.44×10^3	1.90×10^2
B_1	1.84×10^4	1.20×10^4
B_2	2.42×10^5	1.60×10^4
B_3	1.40×10^4	1.00×10^4
C_1	1.40×10^4	2.20×10^3
C_2	2.22×10^4	1.30×10^3
C_3	2.34×10^5	1.8×10^4
C_4	1.46×10^2	0.0×10

A_1: Honey infused with Moringa, A_2: Honey infused with garlic, A_3: Honey infused with ginger, B_1: Natural Honey, B_2: Divine Honey, B_3: PG Honey, C_1: Enugu 1, C_2: Enugu 2, C_3: Ibadan, C_4: Calabar

DISCUSSION

Honey contains at least 200 substances mainly carbohydrates and water. It also contains minerals, proteins, free amino acids, enzymes, vitamins, organic acids, flavonoids, phenolic acids and other phytochemicals. The quality of honey is determined by its sensory, physicochemical and microbiological characteristics. The composition of honey varies from one sample to another, due to geographical location, floral source, seasonal and environmental factors[14,15]. Ascertaining the physiochemical properties of honey is necessary to know the quality level of the honey. Previous studies carried out showed that, the standard moisture content of honey is between 13.21-26.50[16,17] which is in contrast to current findings in this present work, with lower level of moisture content ranging from (11.15-24.85) and also the research carried out by Agbagwa *et al.*[10] having moisture content of 34.7 g/100 g. The lower moisture content obtained in this research maybe attributed to several factors such as source of honey, seasonal influence and harvesting period of the honey. The lowest moisture content was observed in the honey sample obtained from calabar and the highest was observed in the honey sample infused with ginger. Honey infused with other natural product increased the moisture content of the honey. Moisture content is an important parameter as it affects storage life and processing characteristics. The result of pH obtained in this study was within the approved range (3.5-5.5) and is also in conformity with the research carried out by other researcher[15,18-21]. The pH is low enough to prevent the growth of bacteria. Results obtained in this study for fructose was within the acceptable range of 37.07-42.65%, which implies that the honey samples used for this study were stored properly. This also shows that the incorporation of garlic, ginger and moringa extracts separately into the honey samples during manufacturing did not alter the quality of the honey rather it increased the effectiveness of the honey samples and slight increase in the moisture.

This present study showed susceptibility of the test organisms to the branded honey (infused with ginger, garlic and moringa respectively) and unbranded honey samples. The concentration of honey has an impact on the antibacterial activity, the higher the concentration of honey the greater the inhibitory effect[21]. Thus for optimum antibacterial activity, honey should be stored in a cool, dark place and be consumed when fresh. This study showed remarkable inhibiting activity of the honey samples against *S. aureus*, this in correlation with a study on the effect of raw commercial honeys by Agbagwa and Frank-Peterside[22] with the observation *S. aureus* was the most sensitive to the honey samples tested for and reduced inhibitory activity to *Pseudomonas aeruginosa*.

The color of honey ranges from light yellow, through to amber to a nearly black color. The honey samples used for this study were mostly dark colored, although two branded honey samples was light colored which had low levels of antibacterial activity. Research findings show that dark honey has a high level of phenolic compounds and this has shown to have good correlation with its higher antibacterial activity[23,24].

Microorganisms in honey might come from several and different sources. Primary sources include pollen, digestive tracts of honey bees, dust, air, soil and nectar. Secondary sources are due to honey handlers and processing and are easy to control by the application of Good Manufacturing Practices (GMP). In this study, ten honey samples were analyzed for their microbial counts, of which the unbranded honey samples had higher microbial loads compared to the branded honey samples. This could have been from contamination of the honey during processing. The morphology of the bacterial count done on the honey samples implicated *Bacillus* sp. A study by Agbagwa *et al.*[25] revealed that certain *Bacillus* sp. from honey contains certain antimicrobial components and can be used to inhibit pathogenic organisms. From the results of this research it was observed that honey samples with greater amount of microorganism had reduced bactericidal and bacteriostatic activity on the organism, which is in line with other findings[17] which stated that the microbiological analysis of honey detects contamination and the presence of microbes or their spores in honey can cause its deterioration and result in enzymatic changes. The quality of honey is significantly affected by microorganism present in it.

In the present study, all tested honey samples showed antibacterial effect on the clinical isolates during the well in agar sensitivity test. The inhibitory concentrations of honey against the isolates in current study were between 50 and 100%. The type of honey and the concentration affected the sensitivity result towards the organism. Three factors where considered which were, the honey sample, the concentration and the strain of microorganism. All honey samples however showed a higher antimicrobial effect at 100% v/v concentration generally having zones of inhibition ranged from 0-20 mm. For the branded honey samples, *Staphylococcus aureus* and *Pseudomonas aeruginosa* had zones of inhibition at 100%v/v ranging from (0-20) mm and (0-12) mm, respectively. For the unbranded honey samples, *Staphylococcus aureus* and *Pseudomonas aeruginosa* had zone of inhibition at 100%v/v ranging from (0-18) mm, (0-15) mm. The various zones of inhibition suggest the varying degree of efficacy and different phytoconstituents of the different honey samples. The zones of inhibition obtained from this research was higher than that obtained in the research[22] who examined different honey samples and compared their abilities to inhibit the growth of *S. aureus*, *P. aeruginosa*, *E. coli* and *Proteus mirabilis* with average zones of inhibition 5.3-11.6, 1.4-15.4, 4.4-13.5 and 9.1-17 mm, respectively and with honey concentrations of 80-100%. The values obtained was lower than that of the research carried out by Gambo[13], who examined different honey samples against *Staphylococcus* spp. and *Pseudomonas* spp. with zones of inhibition ranged from 15-22 and 17-19 mm, respectively at 100%. Many studies have demonstrated that not all honey samples have the same degree of antibacterial activity. The sensitivity of the organisms hence cannot be concluded using the results from different studies as the honey used in this study may have had widely differing antimicrobial activities. In the present study, the highest antibacterial activity was observed in Honey infused with moringa and the least antibacterial activity was observed in Divine honey.

The bactericidal and bacteriostatic determination of the honey samples was done using the isolate that showed the highest sensitivity to the honey samples. The tested honey samples were seen to exhibit only bacteriostatic effect on the tested isolates. The MIC and MBC results in this study indicated that the test honey samples had more potential bacteriostatic activities against the organisms tested. This implied that not all honey samples have bactericidal activities. From the results of this present study, honey infused with moringa exhibited the best bacteriostatic effect against the isolate tested. This was followed by honey infused with ginger, honey infused with garlic, honey gotten from calabar. The other honey samples showed antibacterial activity within the minimum range. The high bacteriostatic effect of honey infused with moringa can be attributed to *Moringa oleifera* having an impressive range of antimicrobial action, anti-inflammatory properties and antioxidant properties[26]. The results showed that the synergistic antimicrobial activity of the honey sample infused with moringa had the highest bacteriostatic activity which is in line with the research carried out by Oluyemi *et al.*[27] where all the organisms carried out in the research were successfully inhibited by honey and moringa leaf extract.

Ginger in the same light is valued for its antibacterial properties. The constituents in ginger have potent antioxidant and anti-inflammatory activities[28]. Combining these constituents of ginger with that possessed by honey brings about the high bacteriostatic effect compared to the plain honey samples. The results from this research showed that the synergistic activity of ginger and honey had great effect on the organism tested which is in line with the research carried out by Adderrahim *et al.*[29], who concluded that the zones of inhibition increased tremendously due to the synergistic effect of honey and ginger. The synergic antimicrobial effect of garlic and honey against the isolate was found to also be higher compared to the plain honey samples. The cumulative effect of allicin in garlic and high osmolarity, acidity, content of H_2O_2 as well as the presence of phytochemical components in honey may play a great role in the inhibitory activity against the isolates tested[30].

Further characterization on the mechanisms behind the antibacterial effect observed in the respective honey samples, the involvement of bioactive peptides was investigated by adding proteinase k to the different honey samples. Proteinase k was expected to cleave the proteins and thereby inactivate bioactive peptides such bee defensin-1[31]. Bee defensin-1 is produced in the bee salivary gland and is incorporated during primary honey processing. Like other antimicrobial peptides it is a broad acting protein and is thought to be a constituent of the bee immune system. Defensins are antibacterial peptides created to protect the host cells from invasion and infection by pathogens[32]. This study showed the reduced effect on bacterial growth after adding proteolytic enzyme and this is indicative of involvement of a biologic active peptide but amongst all the honey samples, honey infused with moringa, ginger and garlic had the highest zones of inhibition, this would have occurred due to the synergistic effect of honey and other antibacterial infusions even after neutralization of bioactive peptides. A study of honey derived from Danish flora indicated reduced activity of the honey samples after treatment with Proteinase-K on *S. aureus* but for Coagulase Negative Staphylococci, comparable results were obtained[33,34]. further studies are necessary to elucidate if Nigerian honey contains bioactive bee-defensin-1.

CONCLUSION

The findings of this study confirm the increase of inhibitory activity of honey when mixed with other natural extracts. Decreased inhibitory activity was observed when hydrogen peroxide in the honey was neutralized. This suggests that hydrogen peroxide is responsible for most of the inhibitory activity of Nigeria honey. Despite the decreased inhibition, the credit to the small zones suggests that the additional activity in this honey is exerted by components other than hydrogen peroxide. For future studies, the inhibitory components in honey, moringa, ginger and garlic should be extracted and identified. The active component can also be used to manufacture drugs that will be effective on organisms that are resistant to conventional antibiotics.

SIGNIFICANCE STATEMENT

This study discovered the non-peroxide and increased efficacy of honey in combination with other natural products that can be beneficial for nutrition, wound healing and pharmaceutical industries. This study will help the researchers to uncover the active components of honey and the natural products and determine their effect on drug-resistant genes. Thus a medical-grade maybe certified in Nigeria.

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