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# Physiochemical Parameters of Stored Honey Samples and Their Combined Effect with Antibiotics on Clinical Isolates

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author OEA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BE and ISW managed the analyses of the study. Authors OEA, BE and ISW managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The objective of the study is to determine the effect of storage on the physicochemical parameters of honey samples and their combined effect with antibiotics on clinical isolates from wound swab.
**Study Design:** It is a retrospective study.
**Place and Duration of Study:** The microbiological aspect of study was carried out in the medical microbiology laboratory while the physicochemical parameters was carried out in plant science and biotechnology laboratory all of the University of Port Harcourt, Rivers State, Nigeria from 2014 to 2016.
**Methodology:** Three brands of honey sourced from Abia state (Sample A), Delta state (Sample B) and Enugu state (Sample C) of Nigeria screen for their antibacterial activity against *Escherichia coli*

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and *Pseudomonas aeruginosa* (sourced from different wound swabs) using Agar well diffusion method at various concentration ranging from 100%, 80% and 60%. The result indicated that each of their effects was concentration dependent as all the three honey samples exerted a full inhibition of bacterial growth at the highest concentration tested (100%). More so, the inhibitory effect was clearer with concentration of 80% than 60% and this was most evident in the case of sample C and Sample A as compared to sample B. Again, the single effect of the antibiotic: Rifampicin tested using Disc diffusion method recorded no sensitivity among all the *E.coli* and *P.aeruginosa* isolates and are thus rifampicin resistant. Synergistic testing of rifampicin and honey was done using the disc diffusion method. Though, considerable zones of inhibition were observed but when the combined effect of Rifampicin and Honey was evaluated with the single effect of honey, the former inhibitory values were virtually lower than the latter, thus indicating antagonism instead of synergism. These findings require further study to be done in order to spotlight the underlying mechanism of this antagonist interaction in respect to Nigeria and compare with other antibiotics.

*Keywords: Antibiotics; honey; physicochemical parameters; rifampicin.*

## 1. INTRODUCTION

Infectious diseases impact on human health and mortality. Effective treatment of these diseases is becoming more difficult as a result of emerging antimicrobial resistance and the lack of development of new antimicrobial drugs poses a threat to healthcare. These problems can be combated by combination therapy of natural products such as honey with antibiotics. Since ancient times, honey has been used for its medicinal properties to treat a wide variety of ailments. It may be used alone or in conjunction with other substances and administered orally or topically for the eradication of certain ailments. However, misuse of antibiotics, the emergence of resistant bacteria, high cost and unavailability of some conventional drugs and increasing interest in therapeutic honey have provided an opportunity for honey to be used as a broad-spectrum antibacterial agent. Combination therapy can be used to expand the antibacterial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antibacterial activity. Honey has potent activity against both antibiotic sensitive and -resistant bacteria, and is an interesting agent for topical antibacterial application to chronic wound infections not responding to antibiotic therapy [1]. Honey is a natural product that is produced by honey bees from the nectar of plants, based on its unique nutritional and medicinal properties it is widely sought for [2,3]. Honey serves as a natural antioxidant and a rich source of minerals, carbohydrates, proteins, and vitamins with therapeutic and probiotic properties [4,5]. The beneficial role of honey is due to its antimicrobial, anti-inflammatory and anti-oxidant activities as well as boosting of the immune system [6]. In honey glucose oxidase produces hydrogen peroxide alongside gluconic acid from glucose in the presence of water and also the non-peroxide antimicrobial components also known as phytochemicals composed [7,8,9,10, 11]. Medihoney (a blend of Manuka and jelly bush honey) has been one of the first medically certified honeys licensed as medical product for professional wound care in Europe, America and Australia [12,13]. Manuka honey therefore offers a promising alternative for topical use, both as a single multi-component agent in its own right as well as in combination with antibiotics. Synergistic interactions between manuka honey and antibiotics, including oxacillin has been studied.

The broad-spectrum antibiotic rifampicin is commonly used in the treatment of skin-associated infections, including chronic wounds [14]. The chemical structure of rifampicin allows it to penetrate well into tissues and abscesses, which are poorly penetrated by most other anti-staphylococcal agents. Rifampicin when prescribed is often combined with other antibiotics as bacteria can develop rifampicin-resistance during a single passage [15]. The development of resistance to rifampicin in bacteria is typically due to a single, but variable, point mutation in its target, the β subunit of bacterial RNA polymerase [16].

Combination therapy can be used to expand the antibacterial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antibacterial activity [17]. Given the difficulty in treating infected chronic wounds due to multi-resistant bacteria, honey is increasingly being used as a topical treatment for these wounds [18,19]. Honey has potent activity against both antibiotic sensitive and antibiotic

resistant bacteria and is a potent agent for topical antibacterial application to chronic wound infection not responding to antibiotic therapy [20]. A study carried out showed that bacteria are unable to develop resistance to Manuka honey, even when sub-inhibitory concentrations are used. This cannot be said of antibiotics where resistance is readily induced with sub-inhibitory exposure [21,22,23]. The inability of bacteria in honey to develop resistance may be due to the multiple antibacterial properties of honey that overwhelm bacterial stress responses [24,25]. A broad spectrum of wounds is being treated all over the world with natural unprocessed honeys from different sources [26]. Honey is equally found as an active ingredient in products such as ointments for the treatment of minor burns and cuts in Nigeria [27]. The study is aimed at investigating the physicochemical parameters and synergistic activity of stored honey and rifampicin against *E. coli* and *P. aeruginosa* isolated from wound swab.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

A total of seven (7) honey samples collected in duplicates were used for the study, the samples were harvested from different locations in Nigeria. Five of the honey samples were harvested in 2014 and stored for two years at room temperature in the dark, the remaining 2 samples (ODS and RCRIU) were harvested in 2016. The first set of honey samples were used to determine the physicochemical parameters of the honey samples. Three honey samples were selected to determine the combined effect of honey and antibiotics. Selected honey samples were prepared in different concentrations of 60%, 80% and 100% using sterile distilled water.

#### 2.1.1 Test isolates and source of rifampicin disc

Ten test isolates (*E. coli* and *Pseudomonas aeruginosa*) were obtained from microbiology laboratory University of Port Harcourt Teaching Hospital. The isolates were further confirmed by carrying out standard microbiological and biochemical technique. Identified isolates were stored in slants for further analysis. The single foreign rifampicin discs used for the study were obtained from Oxoid Pharmaceutical Company Nig. Ltd. Lagos.

### 2.2 Determination of Physicochemical Parameters

Physiochemical analysis was carried out on the honey samples to determine the effect of storage on honey physiochemical parameters namely: pH, moisture content, ash content, glucose, sucrose and fructose. Moisture and carbohydrate content were determined using the methods of AOAC [25]. Ash content was determined using the methods of AOAC [27]. The pH of honey samples were determined by measuring out 10 mL of each honey sample into a clean beaker and its pH was determined using a pH meter (Equip –Tronics, Digital pH meter model EQ-610) which has been calibrated with buffer solutions of pH 4 and 7.

### 2.3 Antibacterial Activity of Honey Samples

The inhibitory effect of selected honey samples was determined by agar well diffusion technique [28]. In brief, clinical isolates were cultured on Mueller Hinton broth (Oxoid) at 37℃ for 4 hrs. Each isolate was used at a concentration of 1.5 x $10^6$ cells/ ml (adjusted to 0.5 McFarland turbidity standards) which was inoculated onto the surface of Mueller Hinton agar plates using swabs. The plates were allowed to dry at room temperature; the medium was punched with six millimeters diameter wells. The different concentration ( 60%, 80% & 100%) of the honey samples were determined by introducing 50 µl into each well and allowed to diffuse at room temperature at 37℃ for 24 hrs. and the inhibition zone diameter was measured (mm) using a ruler.

### 2.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility test was determined on clinical isolates using the methods of Betoni et al. and Ahmed et al. [29,30]. In brief clinical isolates at a concentration of 1.5 x $10^6$ cells/ ml (adjusted to 0.5 McFarland turbidity standards) were inoculated on the Mueller Hinton agar plates using swabs. Rifampicin antibiotics disc were carefully placed on the surface of each inoculated plate and they were saturated with 10 µl of various honey concentration and without honey. The plates were incubated at 24 hrs. at 37℃. Inhibition zone diameters were recorded and compared with that antibiotic alone. Base on the diameters obtained, the zone sizes were compared to the following Oxoid standard antimicrobial susceptibility chart for rifampicin.

Resistance (at most 16 mm), Intermediate (ranging from 17-19), sensitive (at least 20) and no zone of inhibition as 0.

## 3. RESULTS AND DISCUSSION

### 3.1 Samples

Seven honey samples were used for the study, five of which were stored for two years (2014-2016). Two of the honey (ODS and RCRIU) samples were harvested fresh in 2016. Physicochemical parameters were carried out on the 7 honey samples, results obtained are detailed in Figs. 1 and 2. The pH of the honey samples ranged from 4.58 - 5.13, moisture content (24.00 – 45. 05 %), ash content (0.233 – 0.946%), glucose (14.79 -29.00 %), sucrose (14.44 – 27.98 %), fructose (15.71- 30.76) and conductivity (0.170 – 1.220$\mu/cm$). The highest moisture content (36.15 %) was observed in honey sample UEA, while ADS had the highest value of glucose, sucrose and fructose. The lowest glucose, sucrose and fructose level was observed in honey sample from TS. The parameters showed varying results which were significant in few cases. Conductivity result is detailed in Fig. 2. The moisture content in all the honey samples was not all high with the least in ADS and the highest in UEA.

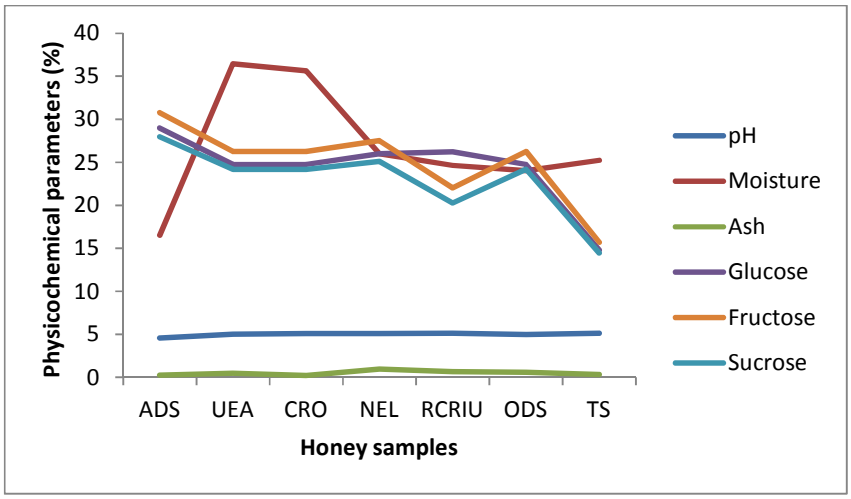


**Fig. 1. Physicochemical parameters of honey samples**

*Key: ADS: Agbor Delta State; UEA: Udi Enugu Amokwe; CRO: Cross Rivers Obudu; NEL: Nsukka Ezike LGA RCRIU: Root Crop Research Institute Umudike; ODS: Ozoro Delta State; TS: Taraba State*

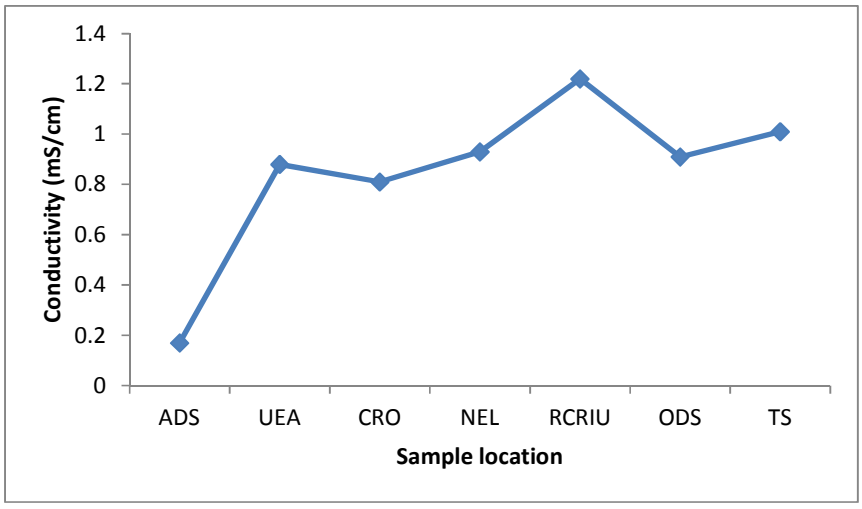


**Fig. 2. Conductivity of honey samples**

### 3.2 Antibacterial Activity of Honey and in Combination with Rifampicin

The antibacterial activity of honey was evaluated according to the criteria stated by Ahmed [31, 32], where zone of inhibition range >18 mm showed significant activity, 16-18 mm good activity, 13-15 low activity, 9-12 mm non-significant activity, and <8 mm no activity. Table 1 shows the means standard deviation results of the zone of inhibition of the various honey samples and rifampicin on the test isolates. No inhibition was observed in all the isolates when rifampicin was used alone, while the honey samples showed various zone of inhibition on the test isolates. Higher zone of inhibition was observed when the honey samples were used undiluted on all the isolates. The result obtained for honey sample showed that honey sample A at 100% was able to inhibit *E. coli* with zone of inhibition of 24.6+2.966 mm and *P.* sp 20.4+2.302 mm. Results showed increase in the zone of inhibition as a result of increase in honey concentration. Good activity was observed at 60% against *E. coli* (17.2 + 3.563 mm) and low activity against *P.* sp (8.6 +2.702 mm) at the same concentration. Honey sample B showed significant activity (22.6 +3.435 mm) against *E. coli* and good activity (18 +1.581mm) against *P.* sp. At 80% it showed significant activity of 19.4 +3.209 mm and low activity 13.8 +1.483 mm against *P.* sp. Honey sample C at the net state (100% concentration) showed the greatest activity (25.2 +1.924 and 22.6 +2.408 mm). When Rifampicin was combined with honey resulted in some level of inhibition but than when rifampicin was used alone. Honey sample A at 100 % concentration had zone of inhibition for *E. coli* as 16.4 + 1.140 and 13.64 +2.408 mm for *P.* sp. Honey sample A (12.6 +0.894 and 8.0 + 2.000 mm) against *E. coli* and *P.* sp. Similarly, sample B at 100% concentration yielded (15.4 + 3.209 and 11.4 + 1.140 mm) for *E. coli* and *P.* sp respectively. Honey sample C at 60% concentration yielded lower activity (8.0 + 1.871 and 5.6 +1. 817 mm).

The growing need of antibiotic resistance has made it necessary to address the problem of antimicrobial resistance. Our study was carried out to evaluate the antimicrobial activity of combination of stored and fresh Nigerian honey with rifampicin. Synergism is a positive interaction created when two agents combined and exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects. Antagonism results if the combination provides an effect less than the effect of either agent alone or less than the sum of the effects of the individual agents [20]. Recently, synergistic action between honey and curcuma starch was reported for *E. coli* and *P. sp* [32]; and additively among of rifampicin, tetracycline and colistin and Manuka honey for *P.* sp [5]. Al-Jabri related that Omani honey had a marked synergistic effect on the antibacterial activity of gentamicin towards *Staphylococcus aureus* [33]. Also, Jenkins and Cooper observed a synergistic effect between manuka honey and oxacillin against methicillin-resistant *Staphylococcus aureus* [1].

Physicochemical properties studies are usually carried out for quality purposes and also to verify the authenticity of the product. The significance of pH at acidic range in foods cannot be overemphasized, it prevents the honey samples from constant infection by various species of microorganisms and this helps to ensure constant shelf life for honey [31,34]. The pH of 2 of the honey samples were within the range of 3.2 and 4.5 according to EU [35]. The remaining 5 samples had pH that was above the stipulated range. This indicates that the pH level of the honey samples were affected by storage, this effect is not significant based on the initial pH of the honey samples. The pH of honey is very important during the extraction and storage of honey. It has the ability to influence the texture, stability and shelf- life. Our present study shows that some of the pH results are similar to other findings [34]. This is also in accordance with the study carried out by Agbagwa on fresh honey samples in Nigeria where some of the pH exceeded the range required for honey [36]. This explains that the pH of freshly harvested honey has an influence on the honey when it is stored. The ash content required for honey is 0.04 - 0.09% while ash content obtained for the present study ranged from 0.233 -.0.946% which was within the acceptable range required for honey [34]. The ash content of the present study differs significantly from those carried out on fresh honey samples by Agbagwa with ash content of 0.357 – 4.187% [36]. The variability observed in the physicochemical properties of the present study maybe due to the floral origin, plant type, season of harvesting and processing technique. A study carried out by Boussaid et al. [37] obtained moisture content of 17.27 -19.80%, which were within the range that required (≤ 20%) according to the International Regulations of Quality [38,39]. This is in disagreement with the present study with moisture content ranging from 24.00 – 45.05% which is above the

**Table 1. Diameter (mm) of zone of inhibition of honey and rifampicin at various concentrations against test organisms**

| S/N | | Honey A | | | Honey B | | | Honey C | | | Rifam. Disc |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 60% | 80% | 100% | 60% | 80% | 100% | 60% | 80% | 100% | |
| 1 | *E. coli* | 17.2+3.563 | 21.0+2.236 | 24.6+2.966 | 15.8+2.775 | 19.4+3.209 | 22.6+3.435 | 12.4+2.408 | 20.8+2.588 | 25.2+1.924 | NZ |
| 2 | *P. aeruginosa* | 8.6+2.702 | 15.2+1.924 | 20.4+2.302 | 9.4+2.302 | 13.8+1.483 | 18.0+1.581 | 10.6+2.074 | 18.0+2.236 | 22.6+2.408 | NZ |

*Mean+Standard deviation; NZ: No zone of inhibition*
*Honey A= ADS; Honey B= UEA; Honey C= RCRIU*

**Table 2. Antimicrobial susceptibility test of rifampicin disks and honey on test isolates**

| S/N | Organism | Honey A + Rifam | | | Honey B +Rifam | | | Honey C +Rifam | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 60% | 80% | 100% | 60% | 80% | 100% | 60% | 80% | 100% |
| 1 | *E. coli* | 9.4+2.074 | 12.6+0.894 | 16.4+1.140 | 9.4+2.702 | 11.6+2.302 | 15.4+3.209 | 8.0+1.871 | 13.2+1.483 | 16.4+2.074 |
| 2 | *P. aeruginosa* | 4.4+1.140 | 8.0+2.000 | 13.6+2.408 | 3.0+0.707 | 8.0+1.581 | 11.4+1.140 | 5.6+1.817 | 11.2+1.304 | 15.0+1.871 |

*Mean+Standard deviation*

stipulated range [38]. The moisture content of the present study were higher in some than those obtained by Agbagwa which ranged from 16.12 - 34.67%. This confirms the high moisture content of some Nigerian honey even before storage [36]. Some of the honey samples became watery and lost the viscosity property associated with honey. When these honey samples are stored they tend to increase in moisture content. The higher moisture content in honey may result in undesirable honey fermentation during storage and formation of acetic acid. Electrical conductivity of honey is closely related to the concentration of minerals, total ash, salts, organic acids and proteins [37]. The sugars in honey are quality criteria which are influenced by honey storage and the initial sugar content after harvesting.

Individual effects were concentration dependent as all the three honey samples exerted a full inhibition of bacterial growth at the highest concentration tested. This was in agreement with previous studies [8]. The inhibitory effect was most evident in the case of sample C and Sample A as compared to Sample B. The antibiotic rifampicin recorded no zone of inhibition when used alone among all the *E. coli* and *P. aeruginosa* isolates and are thus rifampicin resistant. The result obtained in respect to antibiotics in this study was in agreement with findings from the study carried out by Firdaus [40]. The honey samples were combined with rifampicin, results obtained showed some level of inhibition but the inhibitory effect obtained from the synergistic effect of rifampicin and honey were lower than the single inhibitory values of honey which is in contrast with studies carried out on honey and rifampicin against *Staphylococcus aureus* [31,39]. Synergism is a positive interaction created when two agents combined and exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects. Antagonism results if the combination provides an effect less than the sum of the effects of the individual agent [41,42,43]. Combined effect of honey and rifampicin in our study exhibited antagonistic effect which might be due to the fact that rifampicin can develop resistance in bacteria which is due to a single, but variable point mutation in its target, the beta subunit of bacterial RNA polymerase. Combined therapy has been demonstrated to be effective although resistance can still emerge [44]. A study carried out by Patrick on combination of rifampicin and honey maintained rifampicin susceptibility in *S. aureus* which was lost in the presence of rifampicin alone. Chronic wound infections have become urgent health problem and bacterial infection have a significant role they play in the inability of these wounds to heal. These chronic wound infections are often treated with combination of antibiotics in an effort to increase efficacy and reduce antibiotic resistance [45]. Certified medical grade honey could also be combined with antibiotics to increase effective healing of wound infections.

Our findings in this study support the use of Nigeria honey in clinical treatments against resistant *E. coli* and *P.* sp. The present study also confirms that honey that is properly stored will not have any effect on the antimicrobial potential of the honey. This further suggests that honey can be used as adjuvants for antibiotics in combined therapy for antibiotic resistant organisms that are associated with wound infections. The use of honey as adjuvants for antibiotics may not be applicable to all microbial strains.

## 4. CONCLUSION

Consequent upon the above, the results contained in our analysis can be concluded that due to a higher significant antimicrobial activity of the single effect of honey to the combined effect of rifampicin and honey. In addition, within the ambit of our study, honey can be used as an alternative therapy for healing wound incriminated by *E. coli* and *P. aeruginosa* as rifampicin has no clinical activity against *E. coli* and *P. aeruginosa*. Further research should be carried out.

## ETHICAL APPROVAL

All authors declare that this study was approved by the appropriate authority. We wrote a letter to the Microbiology department of the University of Port Harcourt Teaching Hospital and we were given the consent to collect the isolates.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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